

# Hepatic STAMP2 decreases hepatitis B virus X protein-associated metabolic deregulation

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Abbreviations: COX-2, cyclooxygenase-2; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; IR, insulin receptor; IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SREBP1, sterol regulatory element-binding protein 1; STAMP2, six transmembrane protein of prostate 2; STEAP4, six transmembrane epithelial antigen of prostate 4

## Abstract

**Six transmembrane protein of prostate 2 (STAMP2) plays a key role in linking inflammatory and diet-derived signals to systemic metabolism. STAMP2 is induced by nutrients/feeding as well as by cytokines such as TNF $\alpha$ , IL-1 $\beta$ , and IL-6. Here, we demonstrated that STAMP2 protein physically interacts with and decreases the stability of hepatitis B virus X protein (HBx), thereby counteracting HBx-induced hepatic lipid accumulation and insulin resistance. STAMP2 suppressed the HBx-mediated transcription of lipogenic and adipogenic genes. Furthermore, STAMP2 prevented HBx-induced degradation of IRS1 protein, which mediates hepatic insulin signaling, as well as restored insulin-mediated inhibition of gluconeogenic enzyme expression, which are gluconeogenic genes.**

**We also demonstrated reciprocal expression of HBx and STAMP2 in HBx transgenic mice. These results suggest that hepatic STAMP2 antagonizes HBx-mediated hepatocyte dysfunction, thereby protecting hepatocytes from HBV gene expression.**

**Keywords:** fatty liver; gluconeogenesis; hepatitis B virus X protein; insulin resistance; liver; STEAP4 protein, human

## Introduction

The novel gene six transmembrane protein of prostate 2 (STAMP2) was cloned due to its high sequence similarity to STAMP1, which is highly enriched in the prostate and overexpressed in prostate cancer (Korkmaz *et al.*, 2005). STAMP2, also known as TNF-induced adipose-related protein (TIARP) or six transmembrane epithelial antigen of prostate 4 (STEAP4), belongs to the family of six transmembrane proteins (STEAP family) (Moldes *et al.*, 2001; Korkmaz *et al.*, 2005; Ohgami *et al.*, 2006). In humans, STEAP4 is expressed in the prostate, placenta, lung, heart, bone marrow, adipose tissue, and liver (Korkmaz *et al.*, 2005; Arner *et al.*, 2008; Zhang *et al.*, 2008). STAMP2 was identified as a factor linking inflammatory and diet-derived signals to adipocyte function and systemic metabolism (Waki and Tontonoz 2007; Wellen *et al.*, 2007). STAMP2 is induced by nutrients/feeding as well as by cytokines such as TNF $\alpha$ , IL-1 $\beta$ , and IL-6 (Moldes *et al.*, 2001; Fasshauer *et al.*, 2004; Kralisch *et al.*, 2009). Recently, Wellen *et al.* (2007) demonstrated that STAMP2 plays a role in preventing insulin resistance in mice, as mice lacking *stamp2* become insulin-resistant and exhibit impaired insulin signaling in visceral WAT and the liver. Especially, Ramadoss *et al.* (2010) suggested that increased hepatic expression of *stamp2* plays a protective role in maintaining hepatic insulin signaling in the presence of inflammation and obesity.

Among the four proteins that originate from the hepatitis B virus (HBV) genome, including polymerase, surface, core, and HBx, HBx has been reported to be associated with HBV-related pathogenesis.

Previous reports have demonstrated that HBx protein induces the expression of lipid synthesis-related genes as well as inflammation in transgenic mice (Kim *et al.*, 2007). Generally, hepatic steatosis, which involves the accumulation of lipids in hepatocytes, has negative effects on liver function as the result of inflammation. Recently, we also showed that HBx expression induces lipid accumulation in hepatic cells through the induction of sterol regulatory element-binding protein 1 (SREBP1), a key regulator of lipogenic gene expression in the liver (Kim *et al.*, 2007). Furthermore, another study confirmed that LXR $\alpha$  plays a key role within HBx-induced lipogenic pathways, suggesting a molecular mechanism through which HBV infection can stimulate SREBP1-mediated control of hepatic lipid accumulation (Kim *et al.*, 2008.). In addition, patients with chronic hepatitis display impaired glucose metabolism with hyperinsulinemia and insulin resistance (Gavrilova *et al.*, 2003). Finally, another report demonstrated a high frequency of HBV infection in diabetes patients.

Based on previous studies, we hypothesized that HBx-induced lipid accumulation and inflammation in the liver can disturb hepatic insulin signaling. Actually, we previously had reported that HBx interferes with the activation of insulin signaling, thereby inhibiting the activities of insulin such as gluconeogenic gene expression (Kim *et al.*, 2010). These reports indicate that HBV or HBx protein performs a crucial function in the development of various types of liver failure resulting from disrupted hepatic metabolism. Here, we observed that STAMP2 protein antagonized HBx function, resulting in hepatic metabolic dysregulation. In addition, HBx protein stability was decreased by STAMP2 expression in HBx-expressing cells and transgenic mouse liver tissues.

## Results

### STAMP2 inhibits hepatic lipid accumulation by HBx

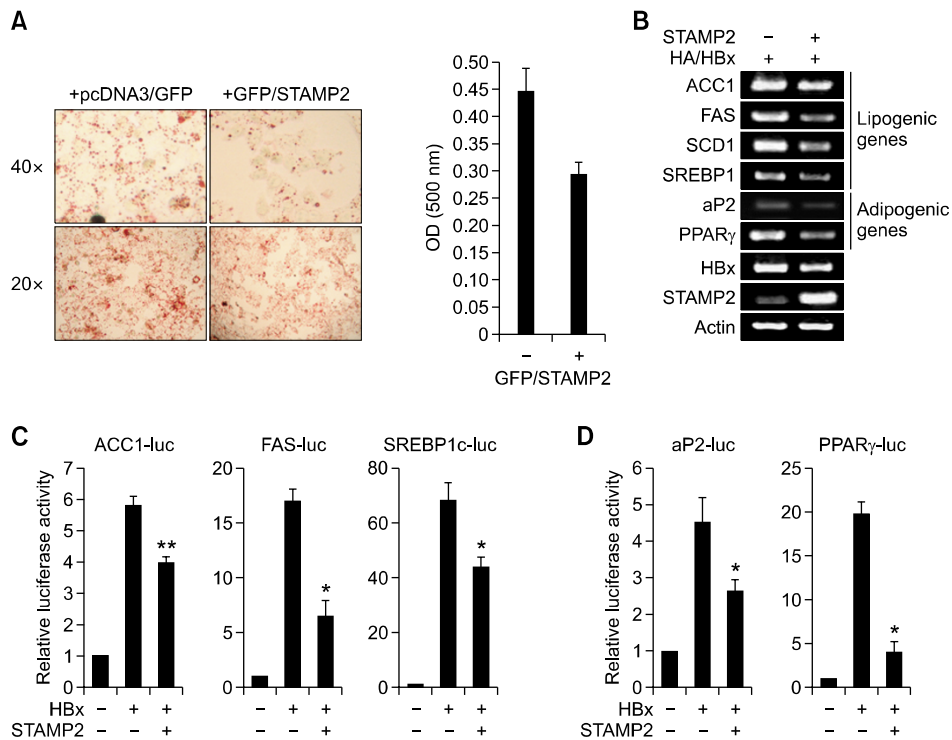
HBx protein has been implicated in abnormal lipid metabolism in HBV-associated hepatic steatosis (Kim *et al.*, 2007, 2008; Na *et al.*, 2009). We have previously reported that HBx protein induces the expression of lipid synthesis-related genes in transgenic mice (Kim *et al.*, 2007, 2008). On the other hand, STAMP2 deficiency is sufficient to spontaneously recapitulate many cardinal features of metabolic syndrome, including inflammation, insulin resistance, glucose intolerance, mild hyperglycemia, dyslipidemia, and fatty infiltration of liver, as well as markedly exacerbate metabolic abnormalities in an ob/ob model of severe obesity (Wellen *et al.*, 2007; Chen *et al.*, 2010; Ramadoss *et al.*, 2010).

Consequently, we have hypothesized that STAMP2 may reverse HBx-mediated metabolic impairment in the liver. To investigate the effects of STAMP2 protein on HBx-induced hepatic lipid accumulation, the hepatic lipid content was examined in HepG2-HBx stable cell lines using Oil-Red O staining. The percentage of Oil-Red O-positive cells among HepG2 cells co-expressing HBx and STAMP2 was significantly lower compared to HBx-expressing cells without STAMP2 co-transfection (Figure 1A). This indicates that STAMP2 inhibits the HBx-induced lipid accumulation. Next, we investigated whether STAMP2 also inhibits the lipogenic and adipogenic gene induction by HBx.

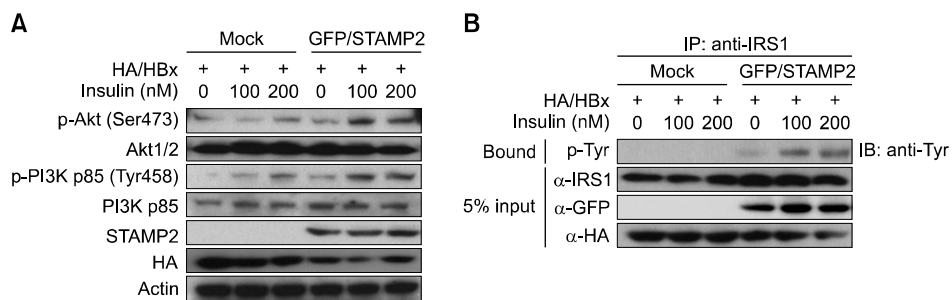
Recent studies have suggested that HBx increases the levels of SREBP1 and PPAR $\gamma$ , resulting in hepatic lipid accumulation through upregulation of adipogenic and lipogenic gene expression (Kim *et al.*, 2007). As shown in Figure 1B, mRNA expression levels of lipogenic (ACC1, FAS, SCD1, and SREBP1) and adipogenic genes (aP2 and PPAR $\gamma$ ) decreased upon STAMP2 overexpression in HepG2-HBx stable cells. The effect of STAMP2 on HBx-mediated transactivation of these genes was confirmed by examining promoter-based luciferase activities of target genes (Figures 1C and 1D). Taken together, these results indicate that STAMP2 acts as a negative modulator of HBx-induced hepatic lipid deregulation.

### STAMP2 counteracts HBx-mediated hepatic insulin resistance

In our previous report, we presented direct experimental evidence that HBx protein contributes to impairment of insulin signaling (Kim *et al.*, 2010). In addition, Wellen *et al.* (2007) demonstrated that STAMP2 plays a role in preventing insulin resistance in mice, as mice lacking *stamp2* become insulin-resistant and exhibit impaired insulin signaling in visceral WAT and the liver. Based on previous studies, we hypothesized that STAMP2 can prevent HBx-mediated insulin resistance in the liver. In response to insulin, insulin receptor (IR) phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the activation of two main signaling pathways (Taniguchi *et al.*, 2006), the phosphatidylinositol 3-kinase (PI3K)-Akt/protein kinase B (PKB) pathway, which is responsible for most of the metabolic actions of insulin, and the Ras-mitogen-activated protein kinase (MAPK) pathway, which regulates the expression of some genes and cooperates with the PI3K pathway to control cell growth and differentiation. As shown in Figure 2A, insulin-induced phosphorylation of both the p85 subunit of PI3K (Tyr458) and Akt (Ser473)



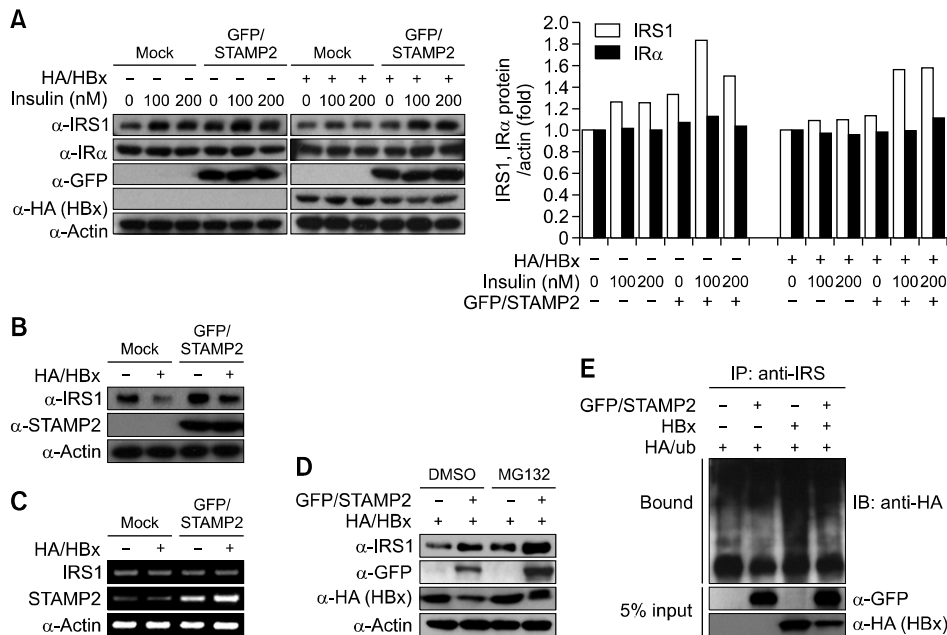
**Figure 1.** STAMP2 inhibits HBx-mediated hepatic lipid accumulation. (A) HepG2-HBx cells were transiently transfected with empty vector or mammalian STAMP2 expression vector. Oil-Red O staining was performed with spectrophotometric quantification of lipid staining at 500 nm. (B) HepG2-HBx cells were transiently transfected with empty vector or mammalian STAMP2 expression vector. Total RNA was prepared from cells, and the indicated mRNA levels were assessed by RT-PCR. (C) HepG2 cells were transiently transfected with the indicated lipogenic gene promoter constructs (ACC1-luc, FAS-luc, and SREBP1c-luc) and co-transfected with empty vector or mammalian HBx and/or STAMP2 expression vectors. Cells were harvested and analyzed for luciferase activity. (D) HepG2 cells were transiently transfected with the indicated adipogenic gene promoter constructs (aP2-luc and PPAR $\gamma$ -luc) and co-transfected with empty vector or mammalian HBx and/or STAMP2 expression vectors. Cells were harvested and analyzed for luciferase activity. Values are expressed as the means  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$  and \*\* $P < 0.01$  compared with mock transfectants.



**Figure 2.** STAMP2 counteracts HBx-mediated hepatic insulin resistance. (A) HepG2-HBx cells were transiently transfected with empty vector or mammalian STAMP2 expression vector, followed by incubation with different concentrations of insulin for 1 h. Whole cell lysates were subjected to immunoblotting for PI3K-p85, phospho-PI3K-p85 (Tyr458), Akt1/2, and phospho-Akt (Ser473). (B) HepG2-HBx cells were transiently transfected with empty vector or mammalian STAMP2 expression vector, followed by incubation with different concentrations of insulin for 1 h. IRS1 protein was immunoprecipitated (IP) from total cell extracts using anti-IRS1 antibody, followed by immunoblotting with anti-phosphotyrosine antibody.

was upregulated by STAMP2. In general, insulin-induced tyrosine phosphorylation of IRS1 positively regulates insulin signaling, in opposition to phosphorylation of Ser307. As expected, tyrosine phosphorylation of IRS1 was higher in STAMP2-expressing HepG2-HBx cells treated with insulin,

compared to control vector-transfected cells (Figure 2B). Collectively, these results demonstrate that STAMP2 counteracts the impairment of insulin signaling by HBx.



**Figure 3.** STAMP2 prevents degradation of IRS1 by HBx. (A) HepG2 and HepG2-HBx cells were transfected with empty vector or GFP/STAMP2 expression plasmid, followed by treatment with insulin at concentrations from 0 to 200 nM for 1 h. Whole cell lysates were subjected to immunoblotting for IRS1 and IR $\alpha$  (left), followed by image analysis of IRS1 and IR $\alpha$  protein expression by densitometry (right). Western blotting bands were quantified using ImageJ version 1.35d (National Institutes of Health Image software). (B, C) HepG2 cells were transiently transfected with mammalian HBx and/or GFP/STAMP2 expression vectors. Whole cell lysates were subjected to immunoblotting for IRS1 and STAMP2. Total RNA was prepared from cells, after which mRNA levels of IRS1, STAMP2, and  $\beta$ -actin were assessed by RT-PCR. (D) HepG2 cells were transiently transfected with mammalian HBx and/or GFP/STAMP2 expression vectors. Whole cell lysates were subjected to Western blotting with anti-IRS1, anti-STAMP2, and anti-actin antibodies. (E) HepG2 cells were transiently transfected with mammalian HBx, HA/ub, and/or GFP/STAMP2 expression vectors. Whole cell lysates were immunoprecipitated with anti-IRS1 antibody, followed by immunoblotting with anti-HA antibody.

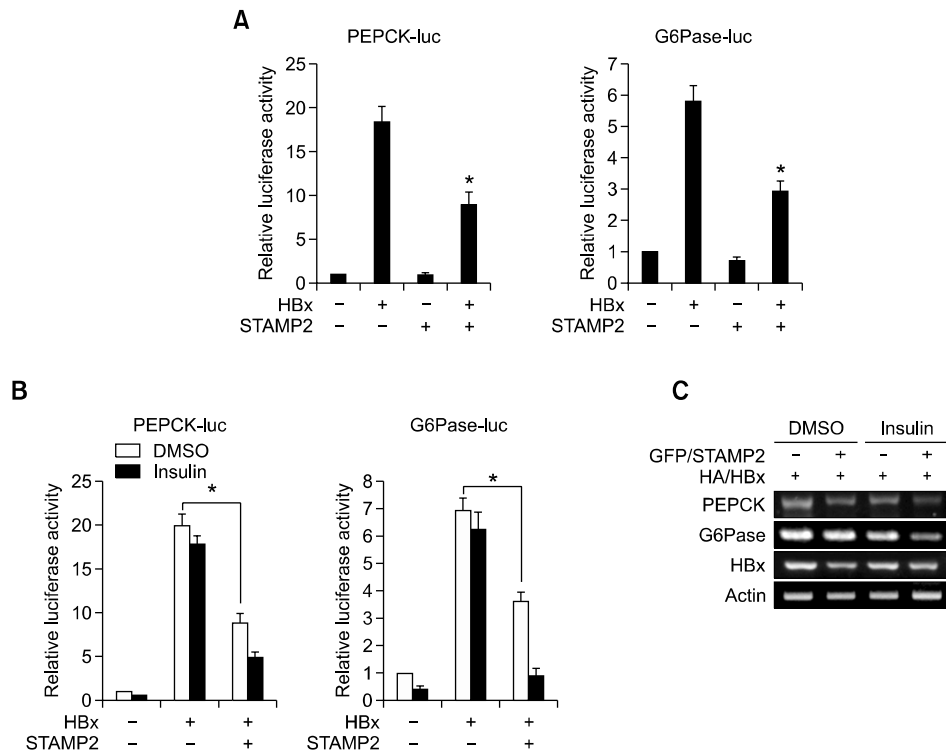
### STAMP2 prevents degradation of IRS1 by HBx

The first critical node in the insulin-signaling network is IR and associated IRS proteins (Taniguchi *et al.*, 2006). To define the inhibitory mechanism of STAMP2 on HBx-induced insulin resistance in the liver, expression levels of IR and IRS1 were analyzed in HBx-constitutively expressing HepG2 cells upon insulin treatment in the presence or absence of STAMP2 overexpression. As shown in Figure 3A, insulin-induced expression of IRS1, but not IR protein, was upregulated by STAMP2. Next, differences in the mRNA and protein expression of IRS1 were measured between HBx- and/or STAMP2-expressing HepG2 cells. As shown in Figure 3B, the protein level of IRS1 was higher in STAMP2-transfected cells compared to empty vector-transfected cells. The protein level of IRS1 was also upregulated by STAMP2 in HBx-expressing HepG2 cells. In contrast, the mRNA level of IRS1 remained unchanged (Figure 3C). This indicates that the increased protein level of IRS1 is not dependent on the transcriptional regulation. However, the proteasome inhibitor MG132 elevated IRS1 protein expression (Figure 3D), suggesting that STAMP2 regulates the IRS1 level at the post-

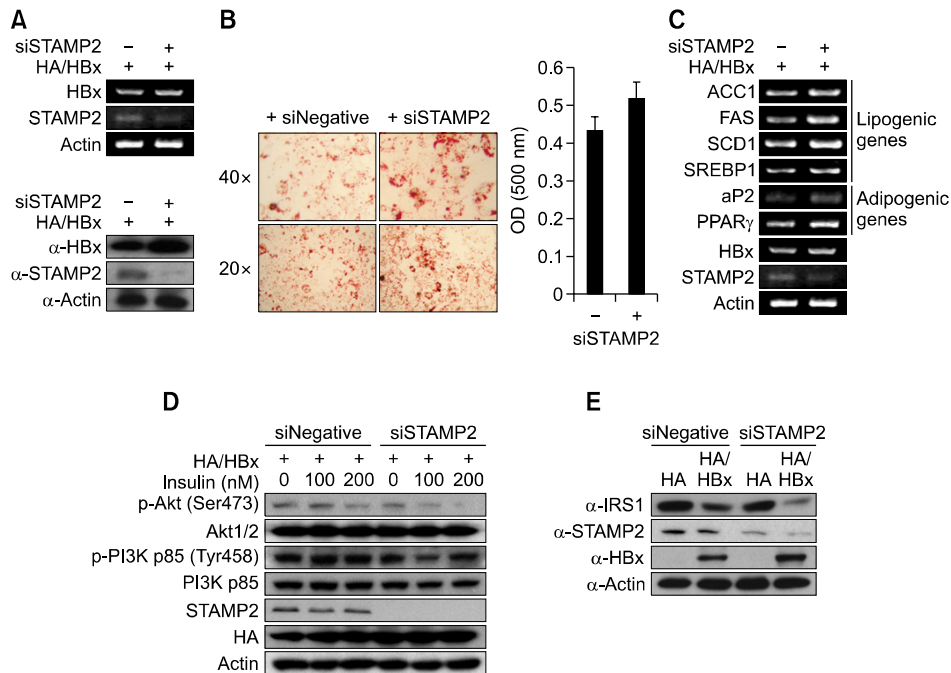
translational control. We further confirmed whether or not STAMP2 protects IRS1 degradation by ubiquitination in HBx-transfected cells (Figure 3E). These results suggest that STAMP2 prevents HBx-induced degradation of IRS1 protein *via* the ubiquitin-proteasome pathway.

### STAMP2 restores insulin-mediated inhibition of gluconeogenic gene expression abrogated by HBx

Insulin reduces gluconeogenesis *via* specific inhibition of PEPCK and G6Pase transcription (O'Brien and Granner, 1996). In our previous study, we found that overexpression of HBx and SOCS3 enhances expression of gluconeogenic PEPCK and G6Pase (Kim *et al.*, 2008). To determine whether or not STAMP2 restores insulin activity impaired by HBx, we examined the transcriptional activity and expression of PEPCK and G6Pase after co-expression of HBx and STAMP2. As expected, STAMP2 expression significantly decreased HBx-induced transactivation of PEPCK and G6Pase (Figure 4A). Furthermore, STAMP2 restored insulin-mediated inhibition of PEPCK and G6Pase transactivation abrogated by HBx (Figure 4B). To further confirm the promoter



**Figure 4.** STAMP2 restores insulin-mediated inhibition of gluconeogenic gene expression abrogated by HBx. (A) HepG2 cells were transfected with PEPCK (left) and G6Pase (right) promoter constructs as well as HBx and/or STAMP2 expression vectors. (B) HepG2 cells were transiently transfected with PEPCK (left) or G6Pase (right) promoter constructs as well as HBx and/or STAMP2 expression vectors in the presence or absence of insulin (100 nM). Luciferase assay was conducted to assess the promoter activities of PEPCK and G6Pase, and error bars represent the standard deviation. \* $P < 0.05$  compared with the indicated control. (C) HepG2-HBx cells were transiently transfected with empty vector or GFP/STAMP2 expression plasmid in the presence or absence of insulin (100 nM). Total RNA was isolated and subjected to RT-PCR.



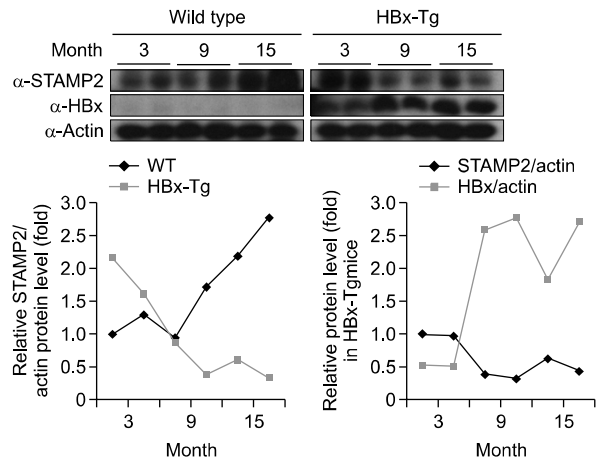
**Figure 5.** Knockdown of STAMP2 accelerates HBx-mediated metabolic phenotypes. (A) HepG2-HBx cells were co-transfected with siNegative control or siSTAMP2. Total RNA was prepared from cells, and mRNA levels were assessed by RT-PCR (upper) and Western blotting (bottom). (B) HepG2-HBx cells were transiently transfected with siNegative control or siSTAMP2. Oil-Red O staining was performed with spectrophotometric quantification of lipid staining at 500 nm. (C) HepG2-HBx cells were transiently transfected with siNegative control or siSTAMP2. The mRNA levels of lipogenic (ACC1, FAS, SCD1, and SREBP1) and adipogenic (aP2 and PPAR $\gamma$ ) genes were assessed by RT-PCR. (D) HepG2-HBx cells were transiently transfected with siNegative control or siSTAMP2, followed by incubation with different concentrations of insulin for 1 h. Whole cell lysates were subjected to immunoblotting for PI3K-p85, phospho-PI3K-p85 (Tyr458), Akt1/2, and phospho-Akt (Ser473). (E) HepG2 cells were transiently transfected with HA or HA/HBx in the presence or absence of siSTAMP2. Western blotting was performed using anti-IRS1, anti-STAMP2, anti-HBx, and anti-actin antibodies.

regulation by STAMP2 in the transcriptional level, we examined the mRNA expression level of both genes in the ectopic expression of HBx and STAMP2. The mRNA levels of these genes by using RT-PCR decreased by STAMP2 expression (Figure 4C). Collectively, these results demonstrate that STAMP2 antagonizes the molecular function of HBx, thereby neutralizing HBx-disrupted insulin signaling.

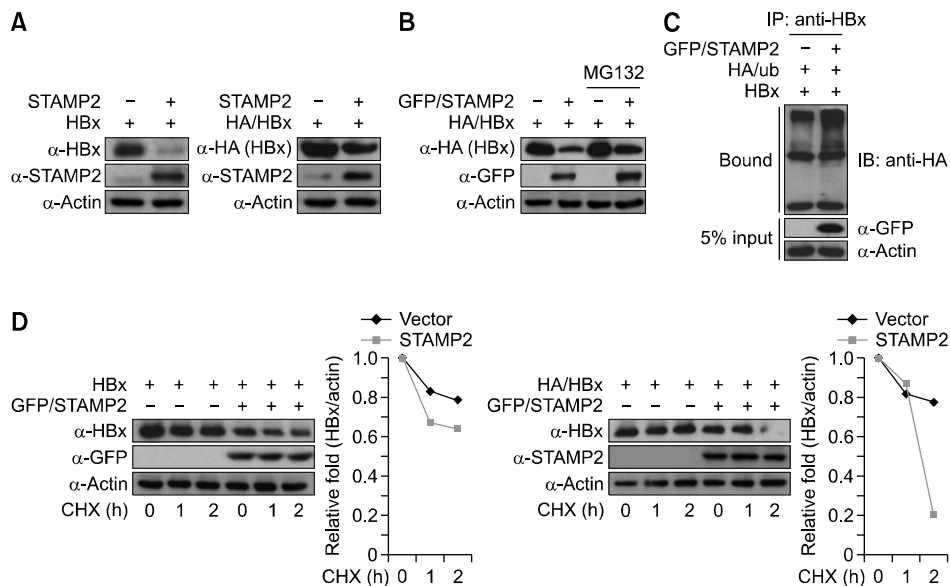
**Knockdown of STAMP2 accelerates HBx-mediated metabolic disorders**

To further confirm whether or not STAMP2 neutralizes HBx-induced hepatic metabolic disorders, RNAi against STAMP2 was expressed and its effects on HBx-mediated metabolic phenotypes, hepatic lipid accumulation, and insulin resistance examined. Specifically, HepG2-HBx cells were co-transfected with negative control (siNegative) or STAMP2-specific siRNA (siSTAMP2) (Figure 5A). We confirmed that siSTAMP2 transfection dramatically abolished the endogenous expression of STAMP2. As shown in Figure 5B, siSTAMP2-transfected HepG2-HBx cells displayed increased hepatic lipid accumulation compared to siNegative-transfected HepG2-HBx cells. Furthermore, mRNA expression levels of lipogenic (ACC1, FAS, SCD1, and SREBP1) and

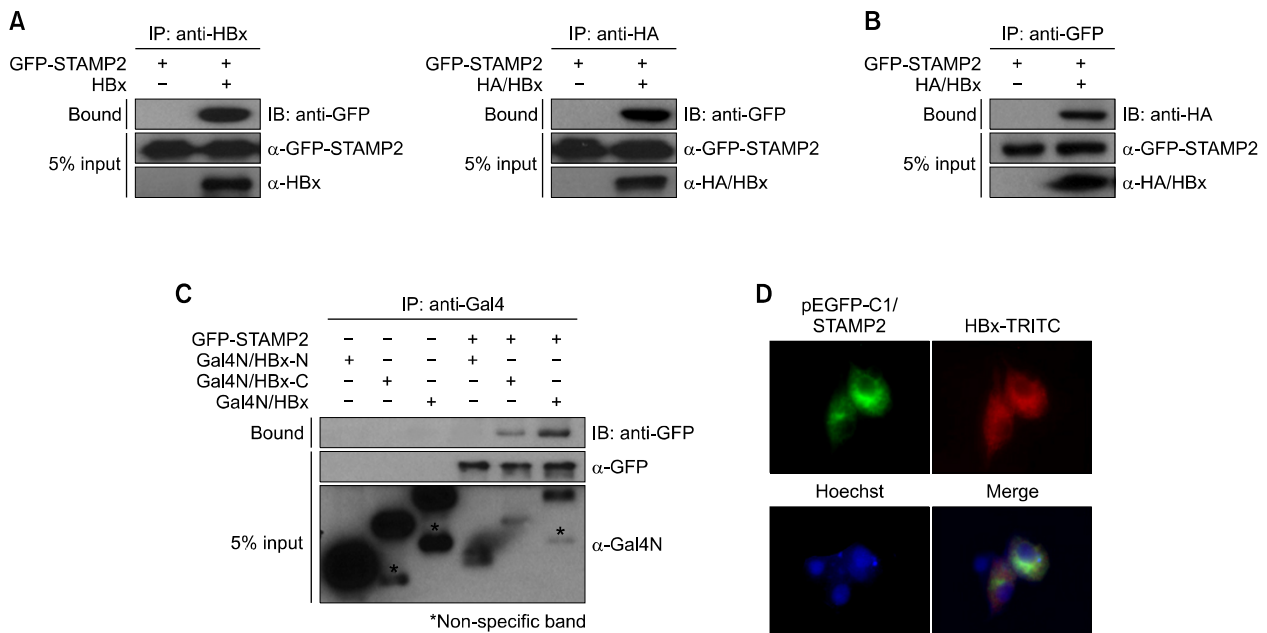
adipogenic (aP2 and PPAR $\gamma$ ) genes were higher in siSTAMP2-transfected HepG2-HBx cells (Figure 5C). Based on our previous results that STAMP2 reversed HBx-induced hepatic insulin resistance,



**Figure 6.** STAMP2 and HBx show antagonistic expression patterns in HBx transgenic mice. Proteins in liver extracts from control and HBx transgenic mice were immunoblotted using anti-STAMP2, anti-HBx, and anti-actin antibodies. The bands were quantified and normalized relative to the anti-actin control band using ImageJ version 1.35d (National Institutes of Health Image software).



**Figure 7.** STAMP2 stimulates degradation of HBx protein. (A) HepG2 cells were transiently (left) or stably (right) transfected with mammalian HBx expression vectors (pSG5-HBx or HA/HBx). Western blotting was performed using anti-HBx, anti-HA, anti-STAMP2, and anti-actin antibodies. (B) HepG2 cells were transiently transfected with HA/HBx and/or GFP/STAMP2 in the presence or absence of MG132. Western blotting was performed using anti-HA, anti-GFP, and anti-actin antibodies. (C) HepG2 cells were transiently transfected with mammalian HBx, HA/ub, and/or GFP/STAMP2 expression vectors. Whole cell lysates were immunoprecipitated with anti-HBx antibody, followed by immunoblotting with anti-HA antibody. (D) HepG2 cells were transiently transfected with HBx and/or GFP/STAMP2 expression vectors. After 24 h of transfection, HBx-transiently (left) and HA/HBx-stably (right) transfected HepG2 cells were treated with 10  $\mu$ M CHX for the indicated time. Western blotting was performed using anti-HBx, anti-STAMP2, anti-GFP, and anti-actin antibodies. The bands were quantified and normalized relative to the anti-actin control band using ImageJ version 1.35d (National Institutes of Health Image software).



**Figure 8.** STAMP2 interacts with HBx protein. (A) HepG2 cells were transiently transfected with mammalian HA/HBx and/or GFP/STAMP2 expression vectors. Cell lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-GFP antibody. (B) Cell lysates were immunoprecipitated with anti-GFP antibody, followed by immunoblotting with anti-HA antibody. (C) HepG2 cells were transiently transfected with HBx and/or GFP/STAMP2 expression vectors. Cell lysates were immunoprecipitated with anti-Gal4 antibody, followed by immunoblotting with anti-GFP antibody. (D) Huh7 cells were transfected with different combinations of GFP-STAMP2 and pSG5-HBx expression vectors. HBx expression was confirmed by staining with anti-HBx monoclonal antibody and TRITC-conjugated anti-mouse antibody. After 24 h of transfection, cells were counterstained with Hoechst in order to label nuclei, and cell imaging was assessed by fluorescence microscopy.

insulin-induced phosphorylation of both the p85 subunit of PI3K (Tyr458) and Akt (Ser473) were inhibited by STAMP2 knockdown (Figure 5D). Moreover, HBx-mediated degradation of IRS1 protein was accelerated by siSTAMP2 (Figure 5F). These results indicate that inhibition of STAMP2 expression accelerates HBx-induced hepatic lipid accumulation and insulin resistance.

### STAMP2 and HBx show antagonistic expression patterns in HBx transgenic mice

To assess the relationship between STAMP2 and HBx expression in liver tissue *in vivo*, we measured the protein expression of STAMP2 in HBx transgenic mice by immunoblotting as well as quantified the expression levels of normal and HBx transgenic mice relative to the anti-actin control band using ImageJ version 1.35d. Protein expression of STAMP2 significantly decreased in liver tissues of 9- and 15-month-old HBx transgenic mice (Figure 6). These results suggest that hepatic STAMP2 and viral HBx protein display antagonistic expression patterns in HBx transgenic mice.

### STAMP2 stimulates degradation of HBx protein

Based on the antagonistic expression patterns of HBx and STAMP2 in HBx transgenic mice, we examined whether or not STAMP2 expression regulates HBx protein stability. As shown in Figure 7A, ectopic STAMP2 expression significantly reduced the protein level of HBx in both transiently and stably transfected HA/HBx cells. However, treatment with MG132, a proteasome inhibitor, repressed STAMP2-mediated downregulation of HBx (Figure 7B), which implies that STAMP2 expression controls the protein level of HBx through post-translational regulation. As shown in Figure 7C, GFP/STAMP2 protein expression increased the level of ubiquitinated-HBx, indicating that STAMP2 induces HBx protein instability through protein ubiquitination. To further confirm this, we measured the half-life of HBx protein following STAMP2 expression. As shown in Figure 7D, the half-life of HBx was about 2 h in both transiently and stably transfected cells. However, co-expression of STAMP2 reduced the half-life of HBx protein, indicating that STAMP2 stimulates degradation of HBx.



### STAMP2 interacts with HBx protein

Based on the downregulation of HBx protein by STAMP2 expression, we examined the protein-protein interaction between HBx and STAMP2. As shown in Figures 8A and 8B, co-immunoprecipitation assay showed that HA/HBx and GFP/STAMP2 formed a complex in HepG2 cells. Previous use of HBx deletion constructs has demonstrated the presence of a transactivation domain in the C-terminus of HBx (51-154 aa), whereas the N-terminus of HBx (1-50 aa) has the ability to repress transactivation (Weisberg *et al.*, 2003; Taniguchi *et al.*, 2006; Waki and Tontonoz, 2007). As shown in Figure 8C, the C-terminus of HBx, not the N-terminal domain, interacted with STAMP2. In addition, to confirm the physical interaction between HBx and STAMP2 in HBx-expressing cells, we examined their subcellular localizations by fluorescence microscopy. As shown in Figure 8D, HBx co-localized with STAMP2 in the cytosol. Taken together, these results indicate that HBx co-localizes with STAMP2 in the cytosol.

### Discussion

The transmembrane protein STAMP2 links inflammatory and diet-derived signals to systemic metabolism. In STAMP2 knockout mice, STAMP2 deficiency mimics many aspects of metabolic syndrome in humans. At the systemic level, STAMP2 deficiency results in impaired insulin sensitivity as well as dyslipidemia. Mice lacking STAMP2 display markedly higher plasma glucose, insulin, triglyceride, and cholesterol levels, as well as hepatic steatosis (fatty liver) (Waki and Tontonoz, 2007). The cytokine TNF $\alpha$  is produced in obese animals and contributes to the development of insulin resistance. A number of other cytokines and signaling molecules, including IL-6, JNK, IKK $\beta$ , and SOCS proteins, have subsequently been linked to insulin resistance induced by obesity (Wellen and Hotamisligil, 2005; Hotamisligil, 2006; Shoelson *et al.*, 2006). A common theme emerging from many of these studies is that inflammatory pathways such as those activated by JNK and NF- $\kappa$ B directly antagonize the actions of insulin in metabolic tissues such as the liver, fat, and skeletal muscle.

Recent studies have also highlighted a potentially important role for tissue macrophages in the production of inflammatory mediators and the development of insulin resistance in obese mice (Weisberg *et al.*, 2003). Loss of STAMP2 is associated with suppressed insulin responses, including reduced phosphorylation of insulin signaling molecules such as IR and Akt, as well as inhibition of glucose transport. In line with these *in vitro* results, loss of

STAMP2 expression *in vivo* is sufficient to cause inflammation. Further, STAMP2 knockout mice exhibit increased expression of inflammatory mediators such as TNF $\alpha$ , IL-6, MCP-1, SOCS-3, and haptoglobin, as well as increased numbers of macrophages in tissues under basal conditions. Consistent with upregulated cytokine production in cultured cells, induction of IL-6 and SOCS-3 expression in response to glucose and lipid administration was shown to increase in STAMP2-deficient mice (Ramadoss *et al.*, 2010).

The hypothesis that inflammation in metabolic tissues contributes to the development of insulin resistance is an intense area of research. At the systemic level, STAMP2 deficiency results in impaired insulin sensitivity and dyslipidemia. Mice lacking STAMP2 display markedly higher plasma glucose, insulin, triglyceride, and cholesterol levels, as well as hepatic steatosis. These mice also display impaired glucose metabolism in key metabolic tissues (liver, skeletal muscle, and adipose). At the molecular level, such physiological defects are associated with impaired insulin receptor signaling in liver and adipose tissues (Wellen *et al.*, 2007). Further, STAMP2 deficiency results in aberrant inflammatory responses to both nutrients and acute inflammatory stimuli. Similarly, in whole animals, visceral adipose tissue from STAMP2<sup>-/-</sup> mice exhibits overt inflammation, and these mice develop spontaneous metabolic disease even on a regular diet, including insulin resistance, glucose intolerance, mild hyperglycemia, dyslipidemia, and fatty liver disease (Waki and Tontonoz, 2007; Wellen *et al.*, 2007). In humans as well as mice, previous research has demonstrated that STAMP2 associates with metabolic disorders, including obesity and insulin resistance (Arner *et al.*, 2008; Chen *et al.*, 2010). However, the cellular mechanism through which STAMP2 exerts its inflammatory and metabolic effects is unresolved.

As it is estimated that approximately 53% of hepatocellular carcinoma (HCC) cases worldwide are associated with HBV, research into HBV infection has focused primarily on the pathogenesis of HCC. However, it is also possible that the deregulation of metabolic components in HBV-infected livers contributes to the pathogenesis of HCC as well as other advanced liver diseases. Previous reports have demonstrated that HBx can induce fatty liver disease by altering the expression of lipid synthesis-related genes in transgenic mice and hepatic cells, and hepatic inflammation is observed frequently in HBx-transgenic mice. Other reports have shown that patients infected with HBV display an association between steatosis and insulin resistance, clinically manifested as obesity,



hyperglycemia, and hypertriglyceridemia.

HBV has been reported to exert synergistic effects along with fatty acids on chronic inflammation. Pro-inflammatory proteins, including TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , appear to participate in the induction and maintenance of the subacute inflammatory state associated with fat accumulation. Here, we showed that the mRNA levels of TNF- $\alpha$ , IL-6, IL-1 $\beta$ , cyclooxygenase-2 (COX-2), C-reactive protein (CRP), and matrix metalloproteinase-9 (MMP-9) increased in the presence of HBx (data not shown). Presumably, HBx-mediated fat accumulation may be associated with increased production of cytokines in response to viral hepatic infection or inflammation. Therefore, further research should focus on determining the relationships between chronic HBV infection, inflammation, and inflammation-related liver dysfunction, such as disruption of insulin signaling.

## Methods

### Plasmids, reagents and antibodies

pEGFP-C1/STAMP2 and 2 kb pGL2B/STAMP2 Luc were kindly provided by Dr. Fahri Saatcioglu (Department of Molecular Biosciences, University of Oslo, Norway). Insulin was purchased from Sigma (St. Louis, MO). The transfection reagents PolyFect, SuperFect and JetPEI were purchased from Qiagen and Polyplus Transfection. Antibodies against IRS1 (sc-559), Akt1/2 (sc-8312), GFP (sc-9996) and Gal4 (DBD) (sc-577) were purchased from Santa Cruz Biotechnology and antibodies against actin (A2066), HBx (MAB8419), STAMP2 (MAB4626), and HA (1 867 423) were obtained from Sigma, BD Biosciences, Abcam, Chemicon, Roche, ABGENT and Bethyl Laboratories respectively. Phospho-Akt (Ser473) (#9271), PI3K-p85 (#4292), phospho-PI3K-p85 (Tyr458) (#4228), phospho-Tyr (#9411), phospho-IRS1 (Ser307) (07-247) and Insulin receptor (07-724) were obtained from Cell Signaling or Upstate.

### Transgenic animals

The liver tissues of wild-type and HBx-transgenic mice derived from the HBV adr subtype genome were kindly provided from Dr. Dae-Yeul Tu (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea). The production of HBx-transgenic mice has already been reported (Yu *et al.*, 1999; Kim *et al.*, 2007, 2008). The wide-type mice are derived from littermates between HBx heterozygous transgenic male mice and female mice, with a mixed genetic background of 2 strains (C57BL/6 and CBA). Mice were housed in a specific pathogen-free environment. Mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Korea Research Institute of Bioscience and Biotechnology.

### Cell culture and stable transfection

HepG2 and Huh7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin (PS) at 37°C in a humid atmosphere of 5% CO<sub>2</sub>. For establishment of HBx-stable cell lines, HepG2 cells were seeded in 6-well culture plates for 24 h prior to transfection. Cells were transfected with 2  $\mu$ g of pcDNA3-HA or pcDNA3-HA-HBx plasmids using Fugene6 reagent (Roche) according to the manufacturer's instructions. After 48 h, cells were trypsinized and plated in a medium containing 800  $\mu$ g/ml of G418. Following selection for 2 weeks, total populations of G418-resistant cells were pooled and single-cell sorted into 96-well plates with a growth medium containing 800  $\mu$ g/ml of G418. Sorted single cells were grown under selection for an additional 2 weeks and expanded into stable cell lines. HepG2/HA and HepG2/HA/HBx stable transfectants were maintained in DMEM-10% FBS containing 200  $\mu$ g/ml of G418.

### Luciferase assay

Cells were plated in a 24-well culture plate and transfected with reporter vector (0.2  $\mu$ g) and  $\beta$ -galactosidase expression plasmid (0.2  $\mu$ g), together with each indicated expression plasmid using PolyFect (QIAGEN), SuperFect (QIAGEN) or JetPEI (Polyplus-transfection) reagent. The pcDNA3.1/HisC empty vector was added to the transfections to achieve the same total amount of plasmid DNA per transfection. After 36 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega, Madison, WI) followed by measuring the luciferase activity. Luciferase activity was normalized for transfection efficiency using the corresponding  $\beta$ -galactosidase activity. All assays were performed at least in triplicate.

### RNA isolation and RT-PCR

Total RNA from HepG2 cells were prepared using TRIzol (Invitrogen) in accordance with the manufacturer's recommendations. The cDNA was synthesized from 3  $\mu$ g of total RNA with MMLV reverse transcriptase (Promega) using a random hexamer (Cosmo, Korea) at 37°C for 1 h. The PCR primers for STAMP2 gene amplification were: 5'-CGA AAC TTC CCT CTA CCC G-3' (sense), 5'-ACA CAA ACA CCT GCC GAC TT-3' (antisense); for HBx gene amplification were: 5'-ATG GCT GCT AGG CTG TGC TGC-3' (sense), 5'-ACG GTG GTC TCC ATG CGA CG-3' (antisense); for  $\beta$ -actin gene amplification: 5'-GAC TAC CTC ATG AAG ATC-3' (sense), 5'-GAT CCA CAT CTG CTG GAA-3' (antisense). The cDNAs were amplified by PCR under the following conditions: 30 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s in a thermal cycler.

### RNA interference and transfection

For the siRNA-mediated down-regulation of STAMP2, genes-specific siRNA and negative control siRNA were purchased from Bioneer (Daejeon, Korea). Cells were transfected with either the siRNA molecule specific for STAMP2

or with a negative control siRNA using HiPerFect (QIAGEN) or JetPEI (Polyplus-transfection) transfection reagent.

### Immunostaining

Cells were seeded on a cover glass and transiently transfected with GFP-STAMP2 and HBx. After fixation with ice-cold 100% methanol, the glass was incubated with blocking buffer (PBS, 0.01% bovine serum albumin, and 0.05% Triton X-100) for 1 h. After washing with PBST, the cells were reacted with the anti-HBx antibody (1:100), and subsequently with the TRITC-conjugated mouse antibody (1:500).

### Fluorescence microscopy in living cells

Fluorescence microscopy was conducted on Huh7 cells transfected with pcDNA3/RFP/HBx and pEGFP-C1/STAMP2 constructs. Following transfection, the cells were incubated for 48 h. Before imaging, the cells were counterstained with Hoechst dye for 10 min at 37°C to stain the nuclei, and were visualized with a Zeiss Axiovert 200M fluorescence microscope.

### Co-immunoprecipitation

The cells were lysed *via* the addition of RIPA buffer and were incubated for 10 min on ice, then scraped into micro-centrifuge tubes. After 15 min of high-speed centrifugation, an aliquot of the lysates was removed for Western blotting, and the remainder was immunoprecipitated overnight with 1.5 µg of anti-HA, anti-HBx, anti-GFP and anti-IRS1 antibody and 40 µl of Protein A or G-Sepharose (50% suspension). The lysates and immuno-precipitates were then separated *via* SDS/PAGE (12% gels) and transferred on to PVDF membranes for blotting. The proteins were detected using horseradish-peroxidase-conjugated secondary antibodies and visualized *via* ECL.

### Oil Red O staining

Cells were washed twice in PBS and fixed for 1 h with 10% (w/v) formaldehyde in PBS. After two washes in 60% isopropyl alcohol, the cells were stained overnight in freshly diluted Oil Red O solution. The stain was then removed, and the cells were washed twice in water. Absorbance of eluted Oil Red O by adding 100% propan-2-ol at 500 nm was then measured in a spectrophotometer.

### Statistical analysis

Statistical analyses were conducted *via* unpaired or paired Student's *t* tests as appropriate. All data are expressed as means ± S.D. *P* < 0.05 was considered to be significant.

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