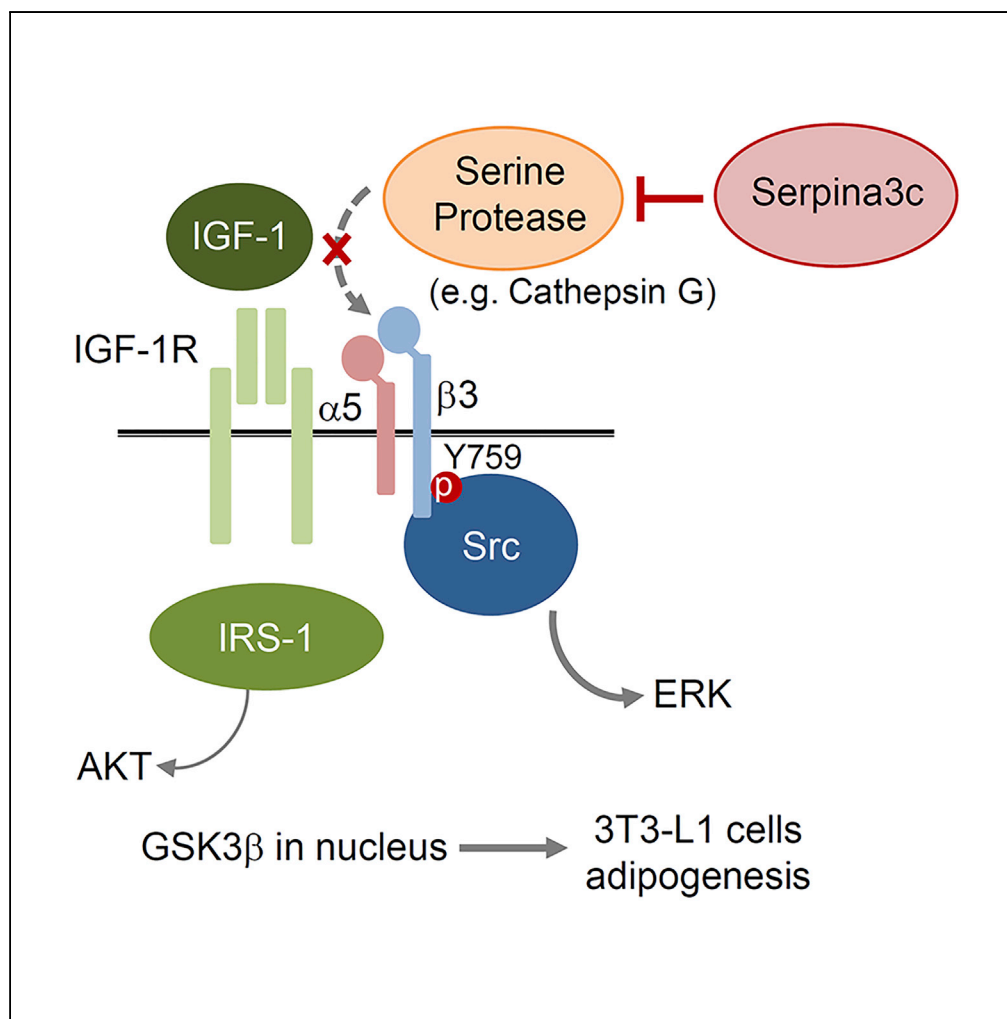


Article

Serpina3c Regulates Adipogenesis by Modulating Insulin Growth Factor 1 and Integrin Signaling



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HIGHLIGHTS

RNA-seq revealed Serpina3c as a critical factor regulating adipogenesis

Knockdown of Serpina3c attenuated the mitotic clonal expansion of 3T3-L1 cells

Knockdown of Serpina3c leads to the degradation of integrin $\alpha 5$

Serpina3c regulates integrin-mediated IGF-1 signaling and ERK/AKT activation

DATA AND CODE

AVAILABILITY

GSE144130

Choi et al., iScience 23, 100961
March 27, 2020 © 2020 The Author(s).
<https://doi.org/10.1016/j.isci.2020.100961>

Article

Serpina3c Regulates Adipogenesis by Modulating Insulin Growth Factor 1 and Integrin Signaling

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SUMMARY

Preadipocyte differentiation can be induced upon a hormonal treatment, and various factors secreted by the cells may contribute to adipogenesis. In this study, RNA-seq revealed Serpina3c as a critical factor regulating the signaling network during adipogenesis. Serpina3c is a secretory protein and is highly expressed in fat tissues. Knockdown of Serpina3c decreased adipogenesis by attenuating the mitotic clonal expansion of 3T3-L1 cells. These cells exhibited decreases in integrin $\alpha 5$, which abolished the phosphorylation of integrin $\beta 3$. We found that Serpina3c inhibits a serine protease that regulates integrin $\alpha 5$ degradation. Knockdown of Serpina3c disrupted integrin-mediated insulin growth factor 1 (IGF-1) signaling and ERK activation. Serpina3c-mediated regulation of integrin-IGF-1 signaling is also associated with AKT activation, which affects the nuclear translocation of GSK3 β . Altogether, our results indicate that Serpina3c secreted from differentiating adipocytes inhibits serine proteases to modulate integrin/IGF-1-mediated ERK and AKT signaling and thus is a critical factor contributing to adipogenesis.

INTRODUCTION

Obesity is a global disease associated with metabolic disorders such as diabetes, hypertension, and cardiovascular disease (Kopelman, 2000). Studies of the complex process by which adipocytes differentiate from preadipocyte precursors (i.e., adipogenesis) are important for understanding the underlying mechanisms of this disease (Gregoire et al., 1998). The 3T3-L1 preadipocyte cell line is an *in vitro* model widely used to study the molecular mechanisms of adipocyte differentiation (MacDougald and Lane, 1995). When proliferating 3T3-L1 preadipocytes become confluent in culture dishes, they enter G₁ growth-arrest phase (Tang et al., 2003b). Upon treatment with hormonal cocktail (a mixture of 3-isobutyl-1-methylxanthine, dexamethasone, and insulin, hereafter called MDI) and fetal bovine serum (FBS), growth-arrested preadipocytes reenter the cell cycle and initiate differentiation via serial expression of adipogenic genes such as those encoding CCAAT/enhancer binding protein (C/EBP) β , C/EBP α , and peroxisome proliferator-activated receptor γ (PPAR γ) (Lin and Lane, 1994; Tang et al., 2003a). This resumed cell cycle is called mitotic clonal expansion, and the process sustains during the first 2–3 days of differentiation and is essential for 3T3-L1 differentiation because an inhibition of this step completely blocks adipogenesis (Tang et al., 2003b).

In our previous studies to identify important differentiation factors secreted by clonally expanding cells, we observed that the differentiation program was accelerated in adipocytes treated with conditioned medium (CM) relative to those treated with MDI (Choi et al., 2014). Moreover, the CM-treated cells expressed adipocyte-specific genes before undergoing clonal expansion (Choi et al., 2014). Thus, the aim of the present study was to reveal the factors in CM responsible for promoting adipogenesis. To this end, we began by screening gene expression changes in cells treated with CM. We subsequently identified the serine protease Serpina3c as a protein regulating 3T3-L1 preadipocyte differentiation. Serpina3c is a member of the Serpin superfamily, and some Serpin family proteins have been implicated in adipocyte differentiation and obesity in the proteomic study (Zvonic et al., 2007); however, their precise physiological roles are unknown. Serpina3c is classified in the serpin A clade, which comprises 11 genes (*SERPINA1* and *SERPINA3* to *-12*) and 2 pseudogenes (*SERPINA2* and *SERPINA13P*) in humans and 9 genes (*Serpina3a*, *-b*, *-c*, and *-fto* *-n*) in mice (Heit et al., 2013). Serpina3 is a known inhibitor of chymotrypsin and cathepsin G (Law et al., 2006) and is reported to be expressed in the blood, liver, kidney, and lung (Heit et al., 2013). We reveal here that Serpina3c is highly expressed in adipose tissue of mice and has an important role in adipocyte

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<https://doi.org/10.1016/j.isci.2020.100961>



differentiation. This effect was closely associated with ERK and AKT activation via insulin-like growth factor 1 (IGF-1) signaling, which involves a complex formed by the IGF-1 receptor (IGF-1R) and the integrin $\alpha 5$ - $\beta 3$ heterodimer (Tahimic et al., 2013). We further demonstrate that *Serpina3c* inhibits a serine protease that degrades integrin $\alpha 5$. Thus, *Serpina3c* is a critical factor involved in the early signaling pathway of adipogenesis, and inhibition of *Serpina3c* may offer beneficial effects in the treatment of obesity.

RESULTS

Serpina3c Is Expressed and Secreted by Differentiating Adipocytes

3T3-L1 preadipocytes were cultured to confluence in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Two days later, the cells were treated with MDI and FBS to induce cell-cycle reentry and preadipocyte differentiation. On differentiation day 2, the medium (i.e., CM) was collected and used to treat 3T3-L1 preadipocytes. Cells were then harvested 0, 4, or 24 h after treatment with CM (or MDI as a control) for RNA sequencing (Figure 1A). As previously observed (Choi et al., 2014), the terminal differentiation of CM-treated 3T3-L1 adipocytes was accelerated as evidenced by Oil Red O staining (Figure 1B). To identify genes associated with this acceleration, we focused on genes (1) up-regulated >2-fold by MDI and FBS after 24 h of differentiation, (2) induced immediately (at 4 h of differentiation) by CM, and (3) with a CM/MDI ratio >2-fold at 4 h of differentiation (Figure 1C). We thought that these criteria would meet a critical condition to find factors that are up-regulated by CM, that is, by the factors secreted by differentiating adipocytes. This enables us to identify ~20 candidate genes (Figure 1D; the total gene expression dataset is available in Data S1). Of these, *Serpina3c* and *Serpina3n* have attracted immediate attention, because these are predicted as secreting molecules whose roles are not clarified in adipogenesis yet.

We further examined the expression of *Serpina3c* and *Serpina3n* during the course of 3T3-L1 differentiation. In cells treated with MDI, *Serpina3c* expression gradually increased, peaking at 24–48 h, whereas the expression of *Serpina3n* did not change markedly (Figures 2A and 2B). By contrast, the expression of *Serpina3c* rapidly reached the highest level soon after CM treatment and maintained this level during the whole differentiation process (Figures 2A and 2B). During the late stage of adipogenesis, the expression levels of adipokines such as leptin and adiponectin were not affected by *Serpina3c* (Figure 2C). *Serpina3c* mRNA expression was high in white adipose tissues and liver but much lower in other mouse tissues (Figure 2D). These data are in accordance with a role for *Serpina3c* in adipocyte differentiation and the rapid progression of adipogenesis induced by CM.

Serpina3c (also known as alpha 1 proteinase inhibitor antitrypsin [Heit et al., 2013; Law et al., 2006]) is predicted to be a secretory protein with a signal peptide on its N terminus (Figure 2E). To confirm that it is a secretory protein, we generated *Serpina3c* and *Serpina3n* overexpression vectors with FLAG tags and transfected them into HEK293T cells. *Serpina3c* and -3n proteins were detected in the medium from transfected cells (Figure 2F), indicating that they are indeed secreted from cells. In addition, the secretion of *Serpina3c* protein was paralleled with intracellular expression of *Serpina3c* (Figure 2G).

Knockdown of *Serpina3c* Inhibits Mitotic Clonal Expansion and Adipocyte Differentiation

To clarify the role of *Serpina3c* or *Serpina3n* in adipogenesis, we knocked down expression via RNA interference. Although *Serpina3c* and *Serpina3n* are located on the same chromosome and encode proteins with similar amino acid sequences, only knockdown of *Serpina3c* affected 3T3-L1 adipogenesis (Figures 3A and 3B). Interestingly, the effect of *Serpina3c* knockdown was observed in cells treated with MDI but not with CM, indicating that CM may already contain *Serpina3c* secreted by differentiating cells. In other words, differentiating 3T3-L1 cells with MDI secrete *Serpina3c* into the medium (i.e., CM), then CM-treated 3T3-L1 cells no longer require *Serpina3c* expression.

During mitotic clonal expansion, cell numbers normally increase 2- to 3-fold (Lane et al., 1999; Lee et al., 2009). However, this was not observed in cells with *Serpina3c* knockdown after MDI induction (Figure 3C). These results were confirmed by fluorescence-activated cell sorting analysis. The G₁-S₂ transition was inhibited in cells with *Serpina3c* knockdown, whereas more control cells reentered the cell cycle after MDI treatment (Figure 3D). Consistent with a role for *Serpina3c* in adipogenesis, knockdown of *Serpina3c* resulted in reduced expression of PPAR γ and C/EBP α (Figure 3E). This result suggests that *Serpina3c* is associated with mitotic clonal expansion in adipogenesis.

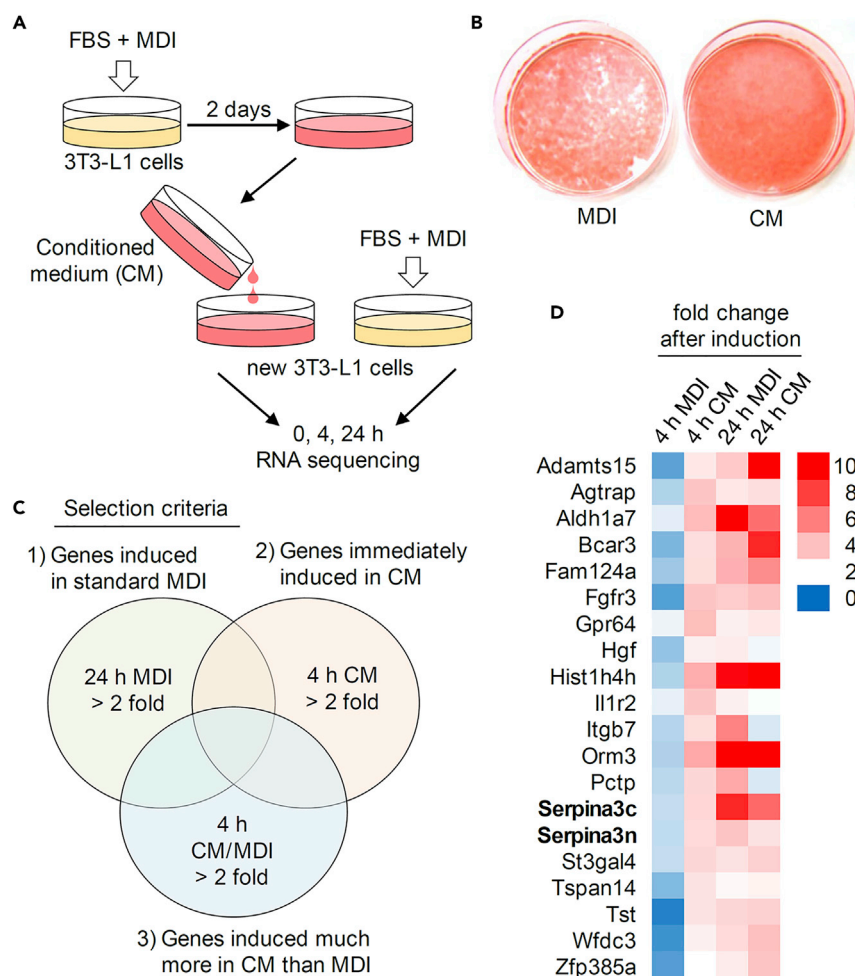


Figure 1. Gene Expression Profile in Differentiating 3T3-L1 Cells Treated with CM

(A) A schematic model of CM preparation using a 3T3-L1 preadipocyte differentiation system. Confluent growth-arrested 3T3-L1 preadipocytes were induced to differentiate with MDI and FBS. After 2 days, cell-exposed medium was collected to treat other preadipocytes. Differentiating cells were harvested after 0, 4, and 24 h for RNA sequencing. MDI + FBS-treated cells were used as a control group.

(B) CM accelerated 3T3-L1 adipocyte differentiation as shown by Oil Red O staining at day 5 of induction.

(C) Strategy for the identification of CM-containing factors via RNA sequencing data.

(D) Heatmap showing relative expression of 20 genes identified from RNA sequencing data. Red, high expression; blue, low expression. Fold changes of the total genes are presented in [Data S1](#). These RNA sequencing data using MDI and CM are publicly available from Gene Expression Omnibus (GEO: GSE144130).

Serpina3c Is Involved in Adipogenesis via the Integrin-Mediated IGF-1 Pathway

As human SERPINA3 is reported as an inhibitor of cathepsin G (Heit et al., 2013; Law et al., 2006), a type of serine protease known to modulate integrin clustering in neutrophils and induce integrin cleavage in platelets (Raptis et al., 2005; Si-Tahar et al., 1997), we speculated that integrin regulation may be altered in cells with *Serpina3c* knockdown. Western blotting revealed that integrin $\alpha 5$ but not integrin $\alpha 6$ was decreased by *Serpina3c* knockdown (Figure 4A). Integrin $\alpha 5$ and $\beta 3$ heterodimerize and interact with IGF-1R (Sekimoto et al., 2005), and in the presence of IGF-1, Tyr759 of integrin $\beta 3$ is phosphorylated and binds Src (Cowan et al., 2000; Fagerholm et al., 2004). As shown in Figure 4A, integrin $\beta 3$ phosphorylation was diminished in cells with *Serpina3c* knockdown, suggesting that integrin-IGF-1R signaling is altered. Accordingly, phosphorylation of the downstream signaling enzyme ERK, which is required for C/EBP β phosphorylation and mitotic clonal expansion in adipogenesis (Kim et al., 2007; Tang et al., 2003a), was decreased by *Serpina3c* knockdown. Notably, the expression of integrin $\alpha 5$ mRNA was increased by *Serpina3c* knockdown

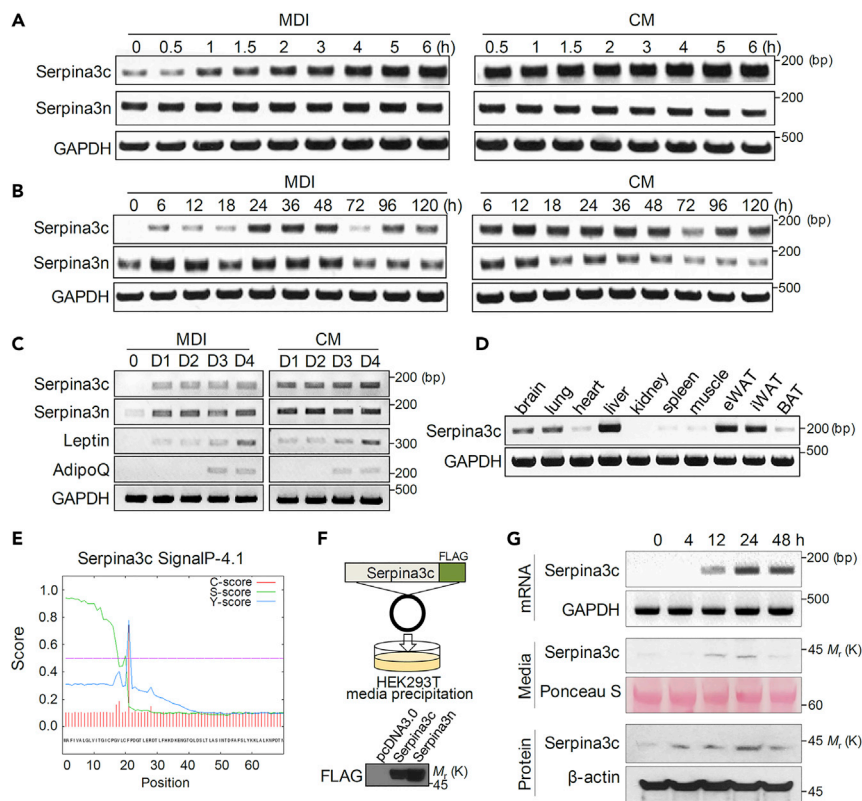


Figure 2. General Characterization of Serpina3c in Adipocytes

(A–C) *Serpina3c* expression patterns induced by MDI or CM treatment in 3T3-L1 adipocytes. Cell lysate were prepared at various times, (A) 0~6 h or (B) 0~120 h after treatment for RT-PCR analysis of *Serpina3c*, *Serpina3n*, and *Gapdh*. (C) Also, the expression of adipokines, *Leptin* and *AdipoQ*, was compared with those of *Serpina3c* and *Serpina3n*.

(D) Tissue distribution of *Serpina3c* by RT-PCR in C57BL/6 male mice.

(E) Amino acid sequence of *Serpina3c* was analyzed by SignalP-4.1 prediction (www.cbs.dtu.dk/services/SignalP-4.1/) showing the N-terminal signal peptide for secretory proteins.

(F) Overexpression vector was generated by inserting mouse *Serpina3c* (or *Serpina3n*) encoding a C-terminal FLAG tag into pcDNA3.0. HEK293T cells were transfected with this construct, and 2 days later, the medium was collected, precipitated by cold acetone, and subjected to western blot analysis to detect the FLAG-tagged protein secreted from cells. pcDNA3.0 was used as a negative control.

(G) *Serpina3c* expression and secretion patterns induced by MDI in 3T3 L1 adipocytes. RT-PCR was performed to detect expression of *Serpina3c* mRNA after treatment. Western blot analysis was performed to investigate expression and secretion of *Serpina3c* protein in precipitated media or cell lysate after treatment.

(Figure 4B), indicating that the effect of *Serpina3c* is not at the level of transcription but rather at the level of protein translation or degradation. Nevertheless, knockdown of integrin $\alpha 5$ mRNA inhibited adipocyte differentiation with MDI. With CM treatment, adipogenesis of si-*Serpina3c* cells was slightly rescued owing to the intracellular *Serpina3c* protein in CM. In contrast, knockdown of integrin $\alpha 5$, a downstream molecule of *Serpina3c*, showed no change between MDI and CM treatment (Figure 4C). Besides transducing signals from the extracellular matrix to the cell, integrin also serves to attach the cell to the extracellular matrix (Gao et al., 2014). Our experiment indicates that knockdown of integrin $\alpha 5$, without altering cellular structure, blocks adipogenesis by inhibiting ERK signaling (Figure 4D). Confocal microscopy also has shown that the cellular structure of integrin $\alpha 5$ knockdown cells was not changed during differentiation (Figure S1).

How does *Serpina3c* contribute to the maintenance of integrin $\alpha 5$ protein? To this end, we measured cathepsin G activity. During adipogenesis, cathepsin G activity was significantly increased during adipocyte differentiation in cells with *Serpina3c* knockdown (Figure 4E). Moreover, administration of a synthetic cathepsin G inhibitor during adipogenesis increased PPAR γ and C/EBP α expression, suggesting that the inhibition of cathepsin G promotes adipogenesis (Figure 4F). Altogether, these data show that *Serpina3c* leads to the inhibition of cathepsin G, thereby protecting integrin $\alpha 5$ from degradation.

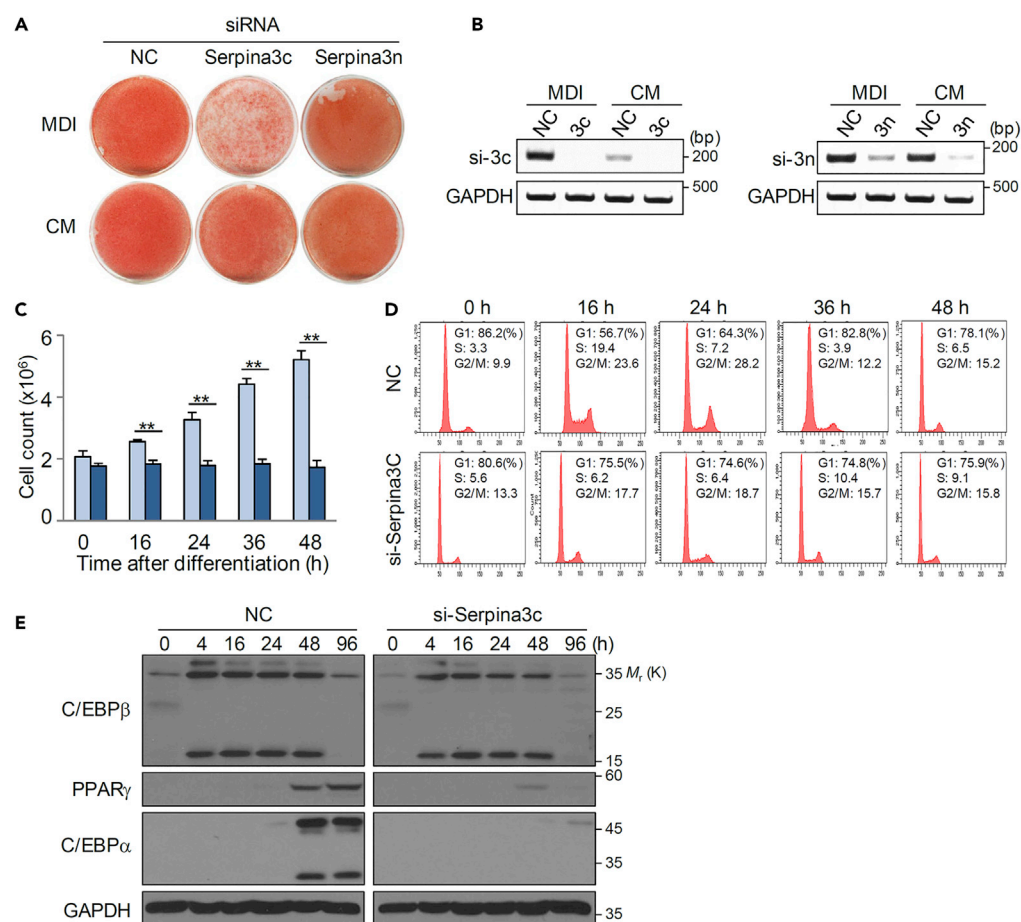


Figure 3. Serpina3c Is Required for Mitotic Clonal Expansion during Adipogenesis

(A and B) 3T3-L1 cells were transfected with negative control (NC), *Serpina3c*, or *Serpina3n* siRNAs. Differentiation of transfected cells was induced by MDI or CM. *Serpina3c* depletion abolishes 3T3-L1 differentiation in MDI-treated cells. Oil Red O staining was conducted on day 8 (A) and knockdown of *Serpina3c* was assessed by RT-PCR (B). (C) Cell proliferation determined by cell counting at 0, 16, 24, 36, and 48 h after MDI treatment. Data are represented as mean \pm SE. *p* Values less than 0.05 were considered significant, with **p* < 0.05, ***p* < 0.01 as determined by Student's *t* test. (D) Cell-cycle analysis by flow cytometry at the indicated time points. (E) Western blot analysis was performed for differentiation markers of adipocytes in control cells and those with *Serpina3c* knockdown (si-*Serpina3c*).

Serpina3c Knockdown Attenuates Nuclear Translocation of GSK3β via Prolonged AKT Activation

We next investigated AKT activation, which is also induced by IGF-1 signaling (Kim et al., 2016). Knockdown of *Serpina3c* prolonged the increase in AKT phosphorylation during differentiation (Figure 5A). Accordingly, phosphorylation of the downstream target glycogen synthase kinase 3β (GSK3β) was also prolonged (Figure 5A). Phosphorylated GSK3β does not translocate to the nucleus (Tang et al., 2005). Consistent with this, no phosphorylated GSK3β was detected in the nuclear fraction of 3T3-L1 adipocytes, and a decreased amount of nuclear GSK3β was observed in cells with *Serpina3c* knockdown (Figure 5B), indicating that nuclear translocation was suppressed. The inhibition of GSK3β nuclear translocation by knockdown of *Serpina3c* was confirmed by immunocytochemistry (Figure 5C). Thus, *Serpina3c* promotes GSK3β translocation to the nucleus, which is required for the phosphorylation of Ser184 and Thr179 of C/EBPβ that is necessary for adipogenesis (Tang et al., 2005).

Serpina3c Increases Adipogenesis by 3T3-L1 Preadipocytes and Is Increased in Animals and Humans with Obesity

To verify the role of *Serpina3c* in adipogenesis, the differentiation of adipocytes was examined in cells overexpressing *Serpina3c*. The expression of C/EBPα and PPARγ during differentiation was

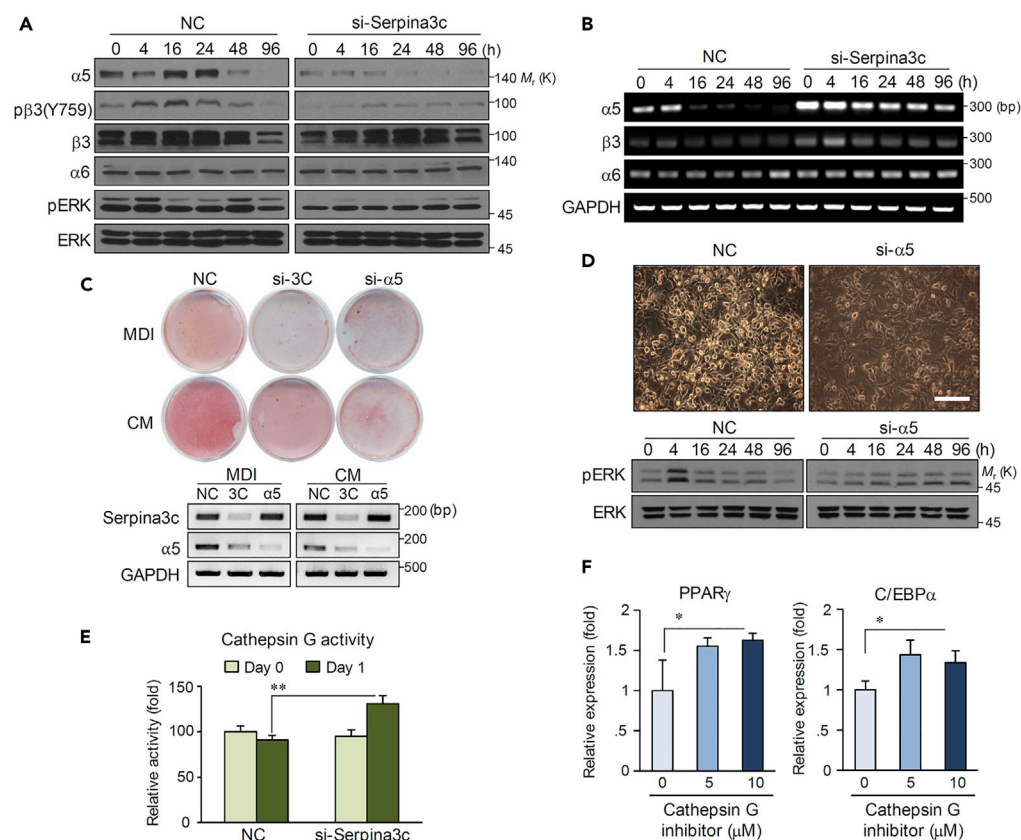


Figure 4. Knockdown of *Serpina3c* Results in Integrin $\alpha 5$ -mediated ERK Inactivation

(A) 3T3-L1 cells were transfected with either negative control siRNA (NC) or si-*Serpina3c* and then differentiated using MDI. Integrin $\alpha 5$, $\beta 3$, and $\alpha 6$ and phosphorylated integrin $\beta 3$ and ERK were detected by western blotting at the indicated time points.

(B) RT-PCR was performed to assess the expression of integrin $\alpha 5$, $\beta 3$, and $\alpha 6$. 3T3-L1 cells were transfected with NC or *Serpina3c* siRNA, and then differentiation was induced by MDI.

(C) Cells treated with NC, si-*Serpina3c* (si-3C), or si-Integrin $\alpha 5$ (si- $\alpha 5$) were stained with Oil Red O on day 6, and depletion of *Serpina3c* or integrin $\alpha 5$ was assessed by RT-PCR.

(D) Microscopy images of cells 2 days after transfections with NC and si- $\alpha 5$. Cell structure was not changed. Western blot analysis was performed for ERK and phosphorylated ERK. Scale bar, 50 μ m.

(E) Cathepsin G activity was measured in 3T3-L1 cells transfected with NC or si-*Serpina3c* on day 0 or day 1 after differentiation. Data are represented as mean \pm SD. p Values less than 0.05 were considered significant, with *p < 0.05, **p < 0.01 as determined by Student's t test.

(F) Gene expression changes of PPAR γ and C/EBP α during differentiation after treatment with cathepsin G inhibitor. Data are represented as mean \pm SD. p Values less than 0.05 were considered significant, with *p < 0.05, **p < 0.01 as determined by Student's t test.

increased in 3T3-L1 cells transfected with pcDNA3.0 encoding FLAG-tagged *Serpina3c* (Figure 6A). Oil Red O staining revealed that the differentiation of these cells was accelerated (Figure 6B). Consistent with an increase of adipogenesis by *Serpina3c*, we found that the expression of this factor was higher in epididymal white adipose tissues of mice fed a high-fat diet than in those fed a chow diet (Figure 6C). When comparing the expression of *Serpina3c* by the duration of high-fat diet, the *Serpina3c* expression in high-fat diet for 8 weeks was slightly higher than that of 16 weeks but was not statistically significant. We also accessed a public repository (NCBI, Gene Expression Omnibus GDS5056 and GDS1480) to obtain expression data from adipose stem cells from morbidly obese and non-obese individuals. The expression levels of *SERPINA3*, a human ortholog gene of *SERPINA3C*, was higher in obese individuals than in lean individuals (p < 0.05; Figure 6D). These data show that SerpinA3 family proteins, including *Serpina3c*, play a critical role in adipocyte differentiation and obesity.

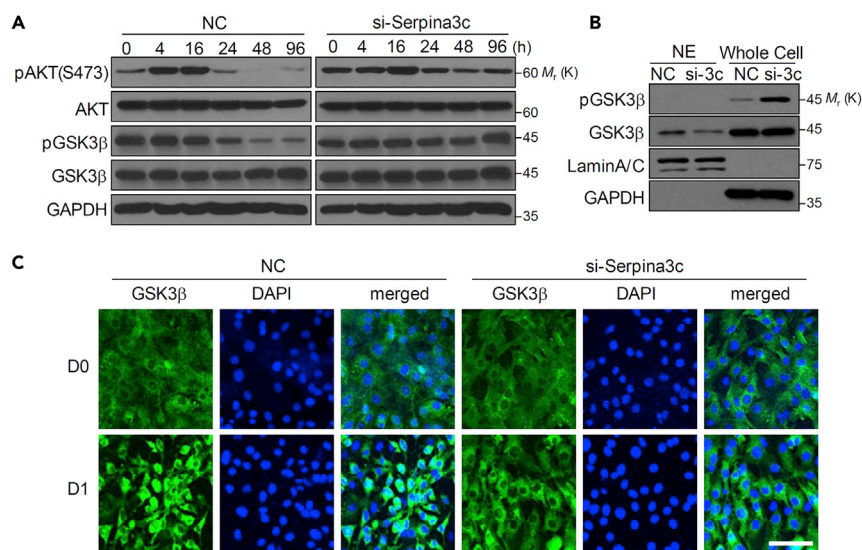


Figure 5. *Serpina3c* knockdown induces prolonged AKT phosphorylation resulting in inhibited GSK3 β Translocation

3T3-L1 cells were transfected with NC or si-Serpina3c and then differentiated using MDI.

(A) Protein levels in transfected cells were measured by western blotting.

(B) Nuclear extracts (NE) and whole-cell lysates were prepared 24 h after MDI treatment and phosphorylated (p) GSK3 β and GSK3 β levels were measured by western blotting. LaminA/C and GAPDH were used as nuclear and cytosol protein marker, respectively.

(C) Cells transfected with NC or si-Serpina3c were induced to differentiate with MDI and fixed for immunocytochemistry against GSK3 β 24 h later. Scale bar, 50 μ m.

DISCUSSION

The mechanisms of 3T3-L1 differentiation have been investigated since these cells were first cloned by Howard Green (Green and Meuth, 1974). These efforts have identified the roles of transcription factors C/EBP β , C/EBP α , and PPAR γ in adipogenesis (Gregoire et al., 1998; Lin and Lane, 1994), but the complex molecular processes regulating adipocyte differentiation have yet to be fully defined. In the present study, we found that *Serpina3c* is a regulator of adipogenesis and was responsible for the accelerated differentiation of adipocytes induced by CM (Choi et al., 2014). We further show that its regulation is via modulation of integrin-IGF-1 signaling.

From the RNA sequencing data, the gene expression profile was compared between CM-induced adipocytes and MDI-induced adipocytes, elucidating *Serpina3c* as a gene expected to be critically involved in adipogenesis. Serpins are the largest and most broadly distributed superfamily of protease inhibitors (Bartlett et al., 2001; Irving et al., 2000; Law et al., 2006), nearly ubiquitous in higher eukaryotes and with highly diverse functions. However, the role of serpins in metabolism has not been investigated fully. SerpinB1 was recently found to promote pancreatic β -cell proliferation and contribute to insulin sensitivity (El Ouaamari et al., 2016). With regard to adipocytes, *Serpina12* (vaspin [visceral adipose tissue-derived serine protease inhibitor]) is the most well-known serpin, functioning as an insulin-sensitizing adipokine in obesity (Hida et al., 2005). Our data here reveal that *Serpina3c* stimulates adipocyte differentiation.

Serpina3c is a secretory protein that can affect extracellular matrix proteins. We show here that it is a serine protease inhibitor, with one target protein being cathepsin G for integrin α 5 degradation (Figure 4A). The knockdown of *Serpina3c* interfered with IGF-1 signaling, disrupting the activation of ERK and AKT. Figure 7 shows a possible mechanism by which *Serpina3c* controls adipogenesis. IGF-1 receptors form a complex with integrin α 5- β 3 heterodimers required for activation of the IGF-1 pathway in 3T3-L1 cells. The binding of IGF-1 to its receptor induces the phosphorylation of integrin β 3 to bind Src and activate downstream targets, such as ERK. Serine proteases (e.g., cathepsin G) degrade integrin α 5 and abolish Src-mediated ERK activation. In the absence of the integrin complex, IGF-1 signals through insulin response substrate 1, which leads to a sustained AKT signaling and the phosphorylation and nuclear export of GSK3 β . Thus,

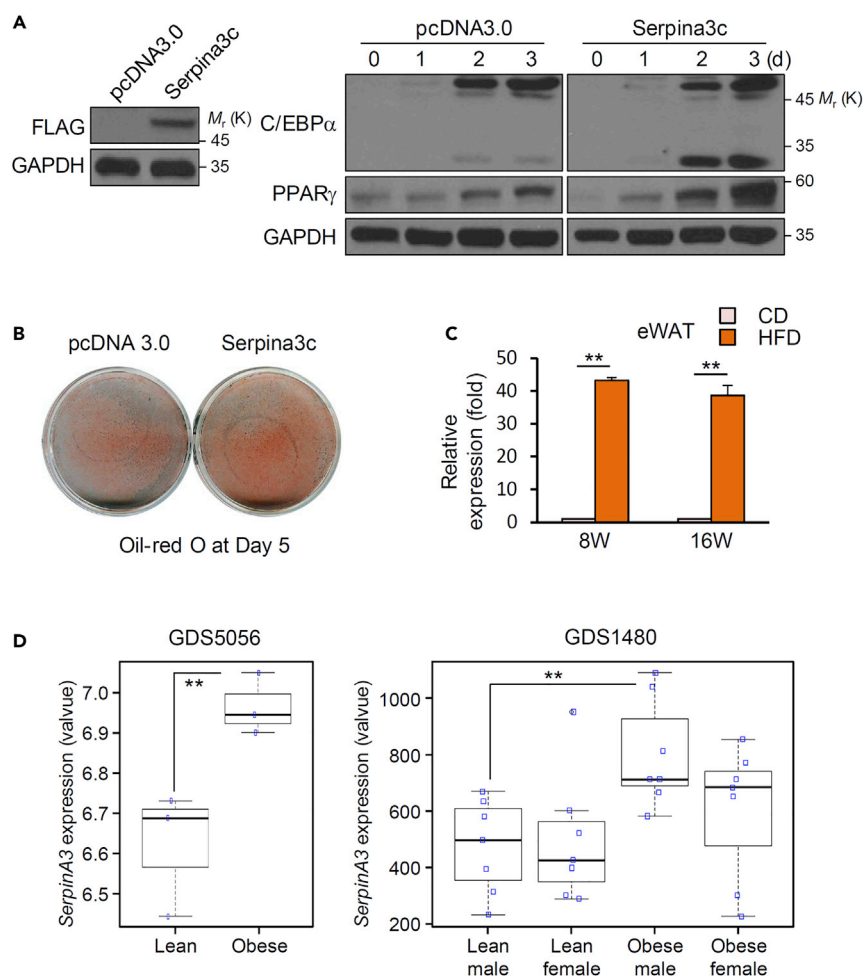


Figure 6. *Serpina3c* Overexpression Increases Adipocyte Differentiation

3T3-L1 cells were transfected with a control (pcDNA 3.0) or *Serpina3c* overexpression vector and induced to differentiate.

(A) Overexpressed *Serpina3c* was confirmed 2 days after transfection by detection of the FLAG tag from whole-cell lysates. Proteins collected at the indicated times after induction were used to assay the levels of C/EBP α and PPAR γ by western blot (GAPDH was used as loading control).

(B) Oil Red O staining on day 5.

(C) *Serpina3c* expression by quantitative real-time PCR in epididymal WAT tissues from C57BL/6 male mice fed a chow diet (CD) or a high-fat diet (HFD) for 8 or 16 weeks. WAT, white adipose tissue. Data are represented as mean \pm SD. p Values less than 0.05 were considered significant, with *p < 0.05, **p < 0.01 as determined by Student's t test.

(D) *SERPINA3* gene expression levels in adipose stem cells or preadipocytes from obese and lean individuals, using publicly available repository data (Gene Expression Omnibus accessions GDS5056 and GDS1480). Data are represented as mean \pm SD. p Values less than 0.05 were considered significant, with *p < 0.05, **p < 0.01 as determined by Student's t test.

Serpina3c is required for activation of ERK and nuclear translocation of GSK3 β , which are both are necessary for C/EBP β phosphorylation and adipogenesis.

In summary, the results from this study provide important advances in understanding the mechanism of adipogenesis. We show that CM induces the transcription of several genes, including *Serpina3c*, which stimulates adipocyte differentiation without mitotic clonal expansion. Furthermore, we show that integrin in the extracellular matrix contributes to adipocyte differentiation. Specifically, *Serpina3c* inhibits serine proteases that degrade integrin $\alpha 5$, thereby shifting IGF-1 signaling away from AKT activation toward ERK signaling via the IGF-1-integrin complex. Thus, *Serpina3c* plays an important role in the transition from mitotic clonal expansion to terminal differentiation and is critical for adipocyte differentiation and adipogenesis.

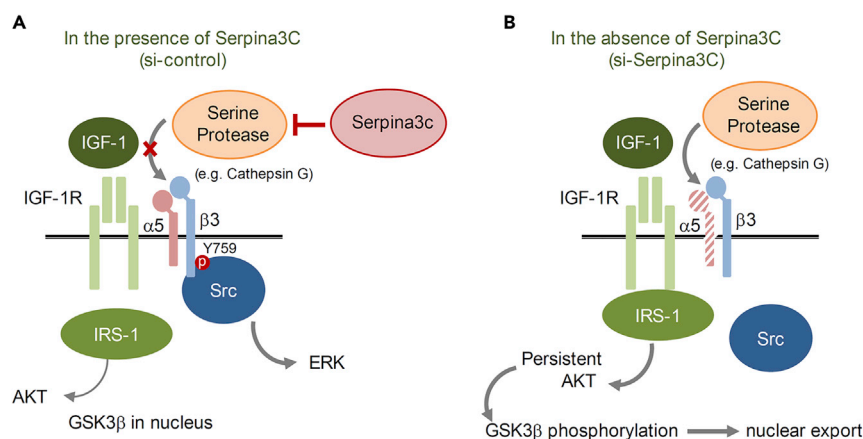


Figure 7. Schematic Illustration of How Serpina3c Regulates Adipogenesis

(A) IGF-1 receptors form a complex with the integrin $\alpha 5$ - $\beta 3$ heterodimer that is required to activate ERK. With ligand binding, integrin $\beta 3$ is phosphorylated at Tyr759, leading to Src binding and ERK activation. In this situation, Serpina3c blocks any serine proteases, which can degrade integrin $\alpha 5$. Insulin receptor substrate 1 (IRS-1) may transiently induce AKT signaling.

(B) In the absence of Serpina3c, serine proteases degrade integrin $\alpha 5$, and the integrin-IGF-1 receptor complex is not formed, resulting in persistent activation of AKT by IRS-1 and phosphorylation of GSK3 β , thereby attenuating adipose differentiation.

Limitations of the Study

In this study, we suggested cathepsin G as a candidate serine protease that is inhibited by Serpina3c. However, there is a possibility that other proteases under the inhibition of Serpina3c may exist in addition to cathepsin G. Another limitation is that, in humans, Serpina3 clade is consisted of single gene, *SERPINA3*, so it is necessary to confirm whether *SERPINA3* functions in the same mechanism as *SERPINA3C* in mice. In addition, further study using *SERPINA3C* knockout mice should be performed to determine how Serpina3c works *in vivo*. Finally, further purification of Serpina3c protein is needed to fully clarify the effect of Serpina3c overexpression.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

DATA AND CODE AVAILABILITY

The accession numbers for the 3T3-L1 RNA-seq using MDI and CM is GEO: GSE144130. Expression levels of *SERPINA3* in human adipose tissue, which is a human ortholog gene of *SERPINA3C*, were obtained from a public repository (GEO: GDS5056 and GDS 1480). Expression levels of *SERPINA3* were compared among groups by Student's t tests using R version 3.6.0 software (R Development Core Team, Vienna, Austria).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100961>.

ACKNOWLEDGMENTS

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government, Ministry of Science and ICT (MSIT) (NRF-2018R1A5A2025079).

AUTHOR CONTRIBUTIONS

Y.C., H.J.K., and J.-w.K. designed research; Y.C., H.C., B.K.Y., H.L., J.W.S., H.J.K., and J.-w.K. performed research; Y.C., H.J.K., and J.-w.K. analyzed data; and Y.C. and J.-w.K. wrote the paper.

DECLARATION OF INTEREST

The authors declare no conflict of interest.

Received: July 17, 2019

Revised: November 19, 2019

Accepted: February 28, 2020

Published: March 27, 2020

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iScience, Volume 23

Supplemental Information

Serpina3c Regulates Adipogenesis

by Modulating Insulin Growth Factor 1

and Integrin Signaling

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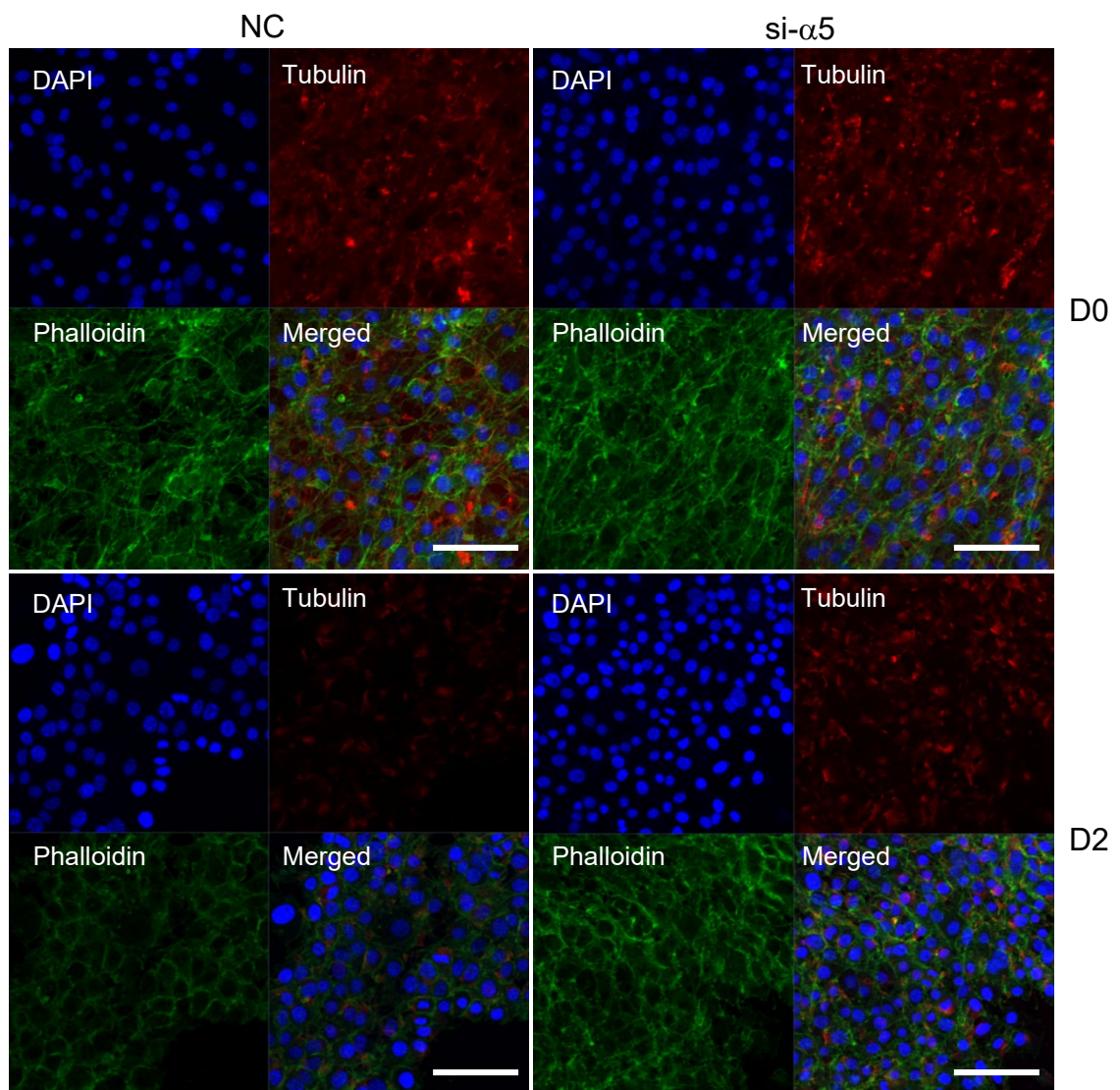


Figure S1. Integrin $\alpha 5$ knockdown does not change the cellular structure during early adipogenesis. Cells transfected with NC or si- $\alpha 5$ were induced to differentiate with MDI and fixed for immunocytochemistry against phalloidin or tubulin 48 h later. Scale bar = 50 μm .

Transparent Methods

Animals. C57BL/6J male mice were maintained under a 12 h light/12 h dark cycle. A five mouse per cage maximum was followed. Five-wk-old mice were fed a high fat diet (Research Diets) or a normal chow diet (Dyets) for up to 8 or 16 wk. The composition of the HFD we used was 60 kcal% fat. All procedures were approved by the Committee on Animal Investigations of Yonsei University. All animal protocols were performed according to the National Institutes of Health guidelines and approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Cell culture and *in vitro* differentiation. 3T3-L1 preadipocytes were maintained at 37°C in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 8 µg/ml biotin, and 10% heat-inactivated calf serum at 37 °C in an atmosphere of 90% air and 10% CO₂. To induce differentiation, 2-day postconfluent 3T3-L1 cells (designated day 0) were incubated in MDI medium, which consists of DMEM with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, and 1 µg/ml of insulin, for 2 days. Cells were then cultured in DMEM containing 10% FBS and insulin for another 2 days, after which they were grown in DMEM containing 10% FBS. For preparation of CM, 2-day postconfluent 3T3-L1 cells were induced differentiation by MDI for 48 h, after which the used culture medium was harvested (Choi et al. 2014).

Oil Red O staining. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% formalin in PBS for 5 min. After rinsing with distilled water, the cells were stained for 1 h in a 60% filtered solution (vol/vol in distilled water) of Oil Red O stain (0.5 g Oil Red O/100 ml isopropanol) and rinsed twice with distilled water.

PCR analysis. Five micrograms of total RNA isolated with Trizol reagent was reverse transcribed with SuperScript II reverse transcriptase using random hexamer primers. The

primers (forward, reverse) used for PCR were as follows: *Serpina3n*, 5'-CACTG TGGTG GAGCT GAAGT-3', 5'-TGTGG ACCAC CTGAG AGACT-3'; *Serpina3c*, 5'-TGGCC TCCAT CAACA CTGAC-3', 5'-ATGGC TGAGC CTCTG TAGGA-3'; *Leptin*, 5'-TGG GGA GTT TTG TTC CAG TG-3', 5'- AGG GAC ATG AGC CTC TGA TT-3'; *AdipoQ*, 5'- AGA TGT GAA GGT GAG CCT CT -3', 5'- GGC TAT GGG TAG TTG CAG TC-3'; *Itga5* (integrin α 5), 5'-ACGTC CTCCA GGATG TTTCTC-3', 5'-TGGGA CTTAA ACTCC AGTGG G-3'; *Itgab3* (integrin β 3), 5'-GACAA CTCTG GGCCG CTC-3', 5'-CCTTC AGGTT ACATC GGGGT-3'; *Itga6* (integrin α 6), 5'-GAGTG ACGGT GTTTC CCTCA-3', 5'-CCTTG TGATA GGTGG CATCG T-3'; *Gapdh*, 5'-ACCAC AGTCC ATGCC ATCAC-3', 5'-TCCAC CACCC TGTTG CTGTA-3'. Real-time quantitative PCR to detect *Serpina3c* from mouse tissues was performed using SYBR green master mix with an ABI PRISM 7300 RT-PCR system and the following primers (forward, reverse): *Serpina3c*, 5'-TCACA GCAGA CTTCC AGCAG-3', 5'-GTCAC GGGGA TTAAA GGGCA-3'; 18s rRNA (for normalization), 5'-GCAGG TGTTT GACAA CGGCA G-3', 5'-GATGA TGGAG TGTGG CACCG A-3'.

RNA sequencing. RNA was isolated as described above from confluent 3T3-L1 preadipocytes treated with MDI or CM harvested after 4 h and 24 h. RNA sequencing was performed by DNA Link (Seoul, South Korea).

Western blot analysis. Cells were washed with cold PBS and lysed in 1% sodium dodecyl sulfate and 60 mM Tris-HCl (pH 6.8). The cell lysates were heated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Western blotting. Primary antibodies against C/EBP β (Lee et al. 2009), C/EBP α (Lee et al. 2009), PPAR γ , integrins (α 5, α 6, and phosphorylated β 3) were from Santa Cruz Biotechnology (Dallas, TX), the FLAG antibody was from Sigma-Aldrich (St. Louis, MO), and antibodies against integrin β 3, GAPDH, and total and phosphorylated forms of ERK, AKT, and GSK β were from Cell Signaling (Danvers, MA). The *Serpina3c* antibody was from Antibodies-online (Aachen, Germany). Ponceau S staining was

used as a loading control alternative to GAPDH in precipitated media Sigma-Aldrich (St. Louis, MO). Targeted proteins were visualized via enhanced chemiluminescence.

Fluorescence-activated cell sorting. 3T3-L1 cells were trypsinized, centrifuged, washed with PBS, and fixed with 90% cold methanol. After washing with PBS, the cells were stained for 30 min in the dark with 50 µg/ml propidium iodide (in PBS with 100 µg/ml RNase A) prior to cell sorting with a FACS caliber flow cytometry system (BD Biosciences, Franklin Lakes, NJ) and analysis via ModeFit software.

Cell count assay. 3T3-L1 cells cultured in 12-well plates (1.5×10^5 cells/well), incubated for 24 h and synchronized to quiescence by serum starvation for 12 h. At indicated time points, the cells were trypsinized and counted with an ADAM automated cell counter (NanoEnTek, Seoul, South Korea) according to the manufacturer's instructions (Kim et al. 2016).

RNA interference. 3T3-L1 preadipocytes were transfected with validated double-stranded stealth mouse short interfering RNA (siRNA) oligonucleotides from Santa Cruz Biotechnology 18–24 h after they were plated in 35-mm dishes: mouse *Serpina3c*, 60 nM sc-153358 (set of 2 siRNAs); *Serpina3n*, 60 nM sc-153364 (set of 3 siRNAs); *Itga5*, 100 nM sc-35687 (set of 3 siRNAs). Control cells were transfected with oligonucleotides with comparable GC contents (60 nM SN-1013; Bioneer, Daejeon, South Korea). Transfections were performed in OPTI-MEM medium using Lipofectamine RNAiMAX (Invitrogen of Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. The medium was replaced the next day with fresh Dulbecco's modified Eagle's medium containing 10% calf serum. Differentiation was induced 24 h later.

Preparation of nuclear extracts. Cells were washed twice with cold PBS, centrifuged, and lysed in hypotonic lysis buffer (20 mM Tris-Cl [pH 8.0], 10 mM NaCl, 3 mM MgCl₂, 1 mM

dithiothreitol, 1 mM sodium orthovanadate, 30 mM β -glycerophosphate) containing 10% NP-40 (IGEPAL CA-630) with a Dounce homogenizer. The lysates were centrifuged and the nuclear pellet was resuspended in nuclear storage buffer (40% glycerol, 50 mM Tris-Cl [pH 8.0], 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β -glycerophosphate). The nuclear lysate was centrifuged and the pellet and cell nuclei were resuspended in NUN buffer (0.3 M NaCl, 1 M urea, 1% NP-40, 25 mM HEPES [pH 7.6], 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β -glycerophosphate) before a final centrifugation. The supernatants were transferred to new tubes with 10% glycerol (final concentration).

Immunocytochemistry. 3T3-L1 cells were transfected with siRNAs and differentiated on coverslips in 6-well plates. After 24 h, the cells were washed with PBS, fixed for 10 min in 3.7% formaldehyde, and incubated for 1 h in blocking solution (PBS containing 5% bovine serum albumin). Cells were then incubated in blocking solution with GSK3 β antibody (1:100; Cell Signaling) for 1 h and then with anti-rabbit IgG-fluorescein isothiocyanate for 2 h. 4',6-Diamidino-2-phenylindole was used to stain nuclei, and the cells were imaged with a confocal laser scanning microscope (Olympus FV1000; Olympus Corp, Tokyo, Japan).

Serpina3c overexpression. Cells were transiently transfected with 0.5 μ g pcDNA3.0-Serpina3c-FLAG encoding full-length Serpina3c with a C-terminal FLAG tag via electroporation with a OneDrop MicroPorator MP-100 (Digital Bio, Seoul, South Korea) to maximize the transfection efficiency. The cells were trypsinized, washed with 1 \times PBS, and resuspended in 10 μ l of resuspension buffer R with 0.5 μ g of plasmid at a concentration of 200,000 cells per pipette. The cells were then microporated at 1,300 V, with two consecutive 20-ms pulses. Following microporation, the cells were seeded in 35-mm cell culture dishes and placed at 37 $^{\circ}$ C in a 10% CO₂-humidified atmosphere (Kim et al. 2016). The cells were then plated in 35-mm cell culture dishes.

Cathepsin G activity assays. 3T3-L1 cells transfected with siRNAs were harvested from 60-mm dishes 24 h after differentiation was induced. Cathepsin G activity was measured by a Colorimetric Cathepsin G Activity Assay kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol. For cathepsin G inhibitor treatment, confluent cultures of 3T3-L1 cells seeded (2×10^4 cells) in 60-mm dishes were treated with MDI and 0, 5, or 10 μ M cathepsin G inhibitor (Abcam). After 48 h, total RNA was isolated as described above for real-time PCR analysis.

Statistical Analysis. All results are expressed as means \pm standard deviations. Groups were compared by using unpaired Student's *t* tests.

Data and Software Availability. The accession numbers for the 3T3-L1 RNA-seq using MDI and CM is GEO: GSE144130. Expression levels of *SERPINA3* in human adipose tissue, which is a human ortholog gene of *SERPINA3C*, were obtained from a public repository (GEO: GDS5056 and GDS 1480). Expression levels of *SERPINA3* were compared among groups by Student's *t* tests using R version 3.6.0 software (R Development Core Team, Vienna, Austria).

Supplemental Reference

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