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Bi-directional Regulation Between Adiponectin and Plasminogen Activator-inhibitor-1 in 3T3-L1 Cells

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Abstract. *Background: Adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1) are adipocytokines, and low levels of serum APN and high levels of PAI-1 are observed in obese patients. Moreover, both APN and Pai-1 are known to be involved in colorectal carcinogenesis. Recently, we demonstrated that serum Pai-1 levels are elevated in APN-deficient mice. We hypothesized that Pai-1 expression levels could be depressed by APN. Thus, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. Materials and Methods: We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-γ on APN and Pai-1 expression at early and late differentiation stages. Results: In the early phase of differentiation, Pai-1 expression increased and APN slightly decreased. Reduction of Pai-1 or activation of PPARγ resulted in elevation of APN, and supplementation of APN with activation of AMPK resulted in reduction of Pai-1. In the late phase of differentiation, APN increased its expression and Pai-1 decreased. Supplementation of Pai-1 resulted in a slight reduction of APN. Conclusion: It is suggested that APN and Pai-1 expressions are inversely-regulated. Understanding of the regulatory system between APN and Pai-1 may lead to finding novel methods for colorectal cancer prevention.*

Adiponectin (APN; 30 kDa protein) is one of the adipocytokines discovered in adipose tissue (1), and abundant amounts of APN are detected in plasma (3-30 μg/ml). A decrease of APN levels is associated with insulin-resistant type-2 diabetes, coronary artery disease, and the development of cancer, including colorectal cancer (2-6). There are two APN receptors, AdipoR1 and AdipoR2 (7). The physiological function of APN is evoked by binding to these receptors. It is known that AdipoR1 activates AMP-activated protein kinase (AMPK) and AdipoR2 activates peroxisome proliferator-activated receptor-α (PPARα) (8, 9).

We have been studying the involvement of APN in colorectal cancer risk. *Adenomatous Polyposis Coli* (*Apc*)-deficient Min mice ($Apc^{Min/+}$), a model of familial adenomatous polyposis (FAP), with APN deficiency were used to investigate the effects of *APN* knockout on intestinal polyp development. APN-deficient *Min* mice show a 2- or 3-fold increase in the total number of intestinal polyps developed compared with APN wild-type *Min* mice, regardless of gender (10). APN-deficient C57BL/6J mice treated with azoxymethane (AOM) demonstrated increased incidence and multiplicity of colorectal tumors, including adenomas and adenocarcinomas. *Min* mice exhibited an increase in serum plasminogen activator inhibitor-1 (Pai-1) levels with decreasing expression levels of *APN*. In addition, the tendency for elevation of serum Pai-1 levels was observed with APN-deficiency in C57BL/6J mice at the age of 55 weeks (10).

Pai-1 is one of the adipocytokines whose levels increase with obesity. Pai-1 directly inhibits tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). tPA and uPA activate plasminogen to produce plasmin through serine protease activity, and physiologically breakdown blood clots. Pai-1 is also reported to possess/exhibit multifunctional factors. Although the molecular mechanisms are not fully-established, Pai-1 was found to modulate cell proliferation and stimulate angiogenesis (11, 12). PAI-1 is known to be induced by triglyceride (TG), very low-density lipoprotein (TG-rich lipoprotein), transforming growth factor-β (TGFβ), various

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growth factors, tumor suppressor p53, nuclear factor kappa B (NFκB) and Wnt signaling (13-17), all of which are plausibly involved in carcinogenesis.

Adipocytokines can affect each other. Among them, APN is known to act as a major regulator of other adipocytokines. For instance, APN stimulates AMPK in the hypothalamus to promote food intake under starvation conditions and inhibit leptin activation (18). In peripheral tissues, especially in skeletal muscle, APN activates AMPK, insulin receptor substrate-1 and fatty acid transport protein-1, to stimulate fatty acid combustion and glucose intake. It is interesting that these types of activation can be inhibited by tumor necrosis factor α (TNFα), another adipocytokine. Thus, it is assumed that APN deficiency affects the action elicited by other adipocytokines or the production of other adipocytokines, such as Pai-1. Therefore, we hypothesized that Pai-1 expression levels might also be depressed by APN.

In the present study, we aimed to clarify the bi-directional regulatory mechanisms between APN and Pai-1. We investigated the expression levels of APN and Pai-1 during 3T3-L1 pre-adipocyte differentiation, and examined the role of AMPK and PPARγ on APN and Pai-1 expression at the early and late differentiation stage. We demonstrated that APN can suppress Pai-1 expression through activation of AMPK, and Pai-1 can suppress APN expression through inhibition of a transcription factor, PPARγ.

Materials and Methods

Cell culture and induction of adipocyte maturation in the 3T3-L1 cell line. 3T3-L1 cells (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone Laboratories Inc., Logan, UT, USA) (basal medium). Induction of differentiation into adipocyte phenotypes was performed by treating confluent cells with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich Co., St. Louis, MO, USA), 1 μM dexamethasone (Sigma-Aldrich) and 1.6 μM insulin (Life Technologies, Co., Carlsbad, CA, USA) in basal medium for two days (Figure 1A). After the treatment, the medium was replaced by basal medium, and the cells were incubated for three days (indicated as day 5 in Figure 1A) and 16 days (indicated as day 18 in Figure 1A).

Mouse recombinant adiponectin (R&D Systems Inc., Minneapolis, MN, USA), metformin (Wako Pure Chemical Industnes, Osaka, Japan), troglitazone (Sigma-Aldrich) and PNU74654 (Wnt-I; Sigma-Aldrich) were applied to adipocyte cells on day 2 and the cells were incubated until day 5. Mouse recombinant Pai-1 (Merck, Darmstadt, Germany) was applied to adipocyte cells on day 15 and the cells were incubated until day 18.

Mouse fat tissue samples. Five abdominal fat tissue samples from 15-week-old male APN-deficient mice and APN wild-type mice were obtained from our previous experiment reported elsewhere (10).

Western blot analysis. Protein expression was analyzed by western blot. Cells (2×10^5) were seeded in 24-well plates. After treatment, cells were lysed in 100 μl lysis buffer [0.0625 M Tris-HCl (pH 6.8), 20% 2-mercaptoethanol, 10% glycerol, 5% sodium dodecyl sulfate] and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) added. Equal amounts of protein were separated in 5-20% gradient polyacrylamide gel electrophoresis-sodium dodecyl sulfate gels and transferred onto polyvinylidene difluoride membranes (Merck-Millipore, Billpore, MA, USA). Antibodies against p-AMPK and AMPK (Cell Signaling Technology, Danvers, MA, USA) were used at a 1:1,000 and 1:2,000 dilution, respectively. Blots were developed with enhanced chemiluminescence western blotting detection reagents (GE Healthcare, Buckingham Shire, UK).

Quantification of mRNA expression by quantitative real-time Polymerase Chain Reaction (qRT-PCR). Total RNA was isolated from cultured adipocyte and tissue samples using TRIzol Reagent (Invitrogen, Grand Island, NY, USA). One-microgram aliquots in a final volume of 20 μl were used for synthesis of cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using a CFX96™ (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with FastStart Universal SYBR Green MIX (×2) (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Primers for mouse *APN* (5' AGGATGCTA CTGTTGCAAGCTCTC, 5' CAGTCAGTTGG TATCATGGTAGAG), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; 5' TTGTCTC CTGCGACTTCA, 5' CACCACCCTGT TGCTGTA), Pai-1 (5' ACAGCCTTTGTCATCTCAGCC, 5' AGGG TTGCACTAAACAT GTCAG) were employed. The data were normalized by *GAPDH*. To assess the specificity of each primer set, amplicons generated from the PCR reaction were analyzed for melting curves.

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Differences were considered to be statistically significant at $p<0.05$.

Results

Difference in APN and Pai-1 expression pattern during 3T3-L1 pre-adipocyte differentiation. 3T3-L1 is a suitable cell line to examine differences in molecular change during pre-adipocyte differentiation. Thus, expression levels of APN and Pai-1 were examined in 3T3-L1 cells at day 5 after the initiation of differentiation (early phase) and at day 18 after the initiation of differentiation (late phase). In the early phase, Pai-1 mRNA levels were significantly higher compared to those of undifferentiated 3T3-L1 cells (Figure 1B). In the late phase, *Pai-1* mRNA levels were significantly lower than those of undifferentiated 3T3-L1 cells. Comparing the expression levels of Pai-1 in early and late phases, an obvious reduction was observed in differentiated 3T3-L1 cells, while a slight induction of Pai-1 was observed in undifferentiated 3T3-L1 cells (Figure 1B). Comparing day 5 and day18, induction of APN was observed in both undifferentiated and differentiated 3T3-L1 cells in the late phase (Figure 1C). *APN* mRNA levels in differentiated cells tended to be lower compared to those of undifferentiated 3T3-L1 cells in the early phase, and higher when cells were in the late phase (Figure 1C and D).

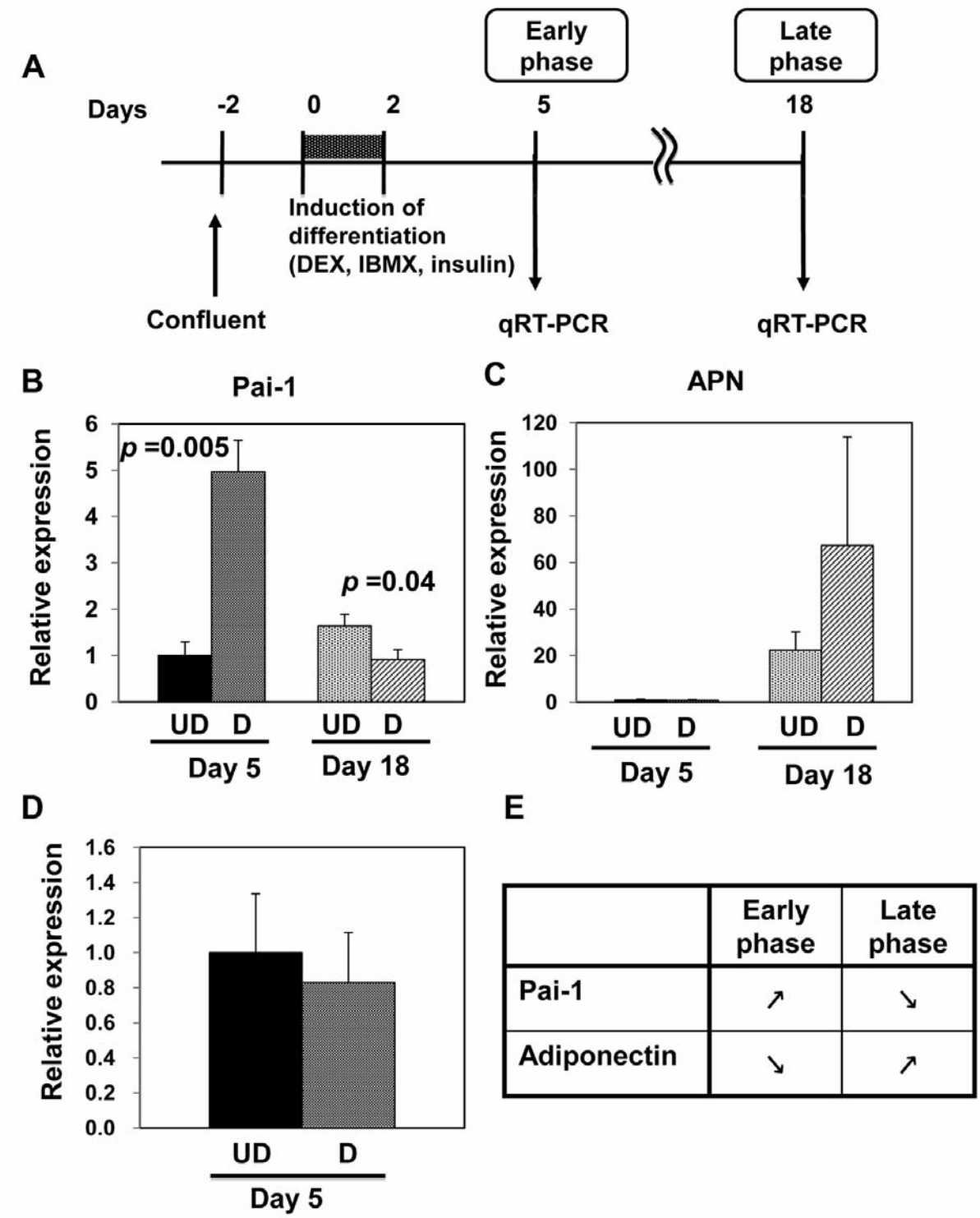

Figure 1. *Adiponecti n(APN) and plasminogen activator inhibitor-1(Pai-1) expression levels in 3T3-L1 pre-adipocytes. A: Illustration of differentiation protocol for 3T3-L1 pre-adipocytes is shown. Day 5 after the initiation of differentiation is defined as the 'early phase' and day 18 is defined as the 'late phase'. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (B) and APN (C) was performed at day 5 and day 18. The data are normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture values. Data are means±SD (n=3). Similar results were obtained from more than three separate experiments. D: The data focused on low relative expression levels of Figure 1C. E: Summary of APN and Pai-1 expression patterns during 3T3-L1 pre-adipocyte differentiation. DEX, Dexamethasone; D, differentiated; IBMX, 3-isobutyl-1-methylxanthine; UD, undifferentiated.*

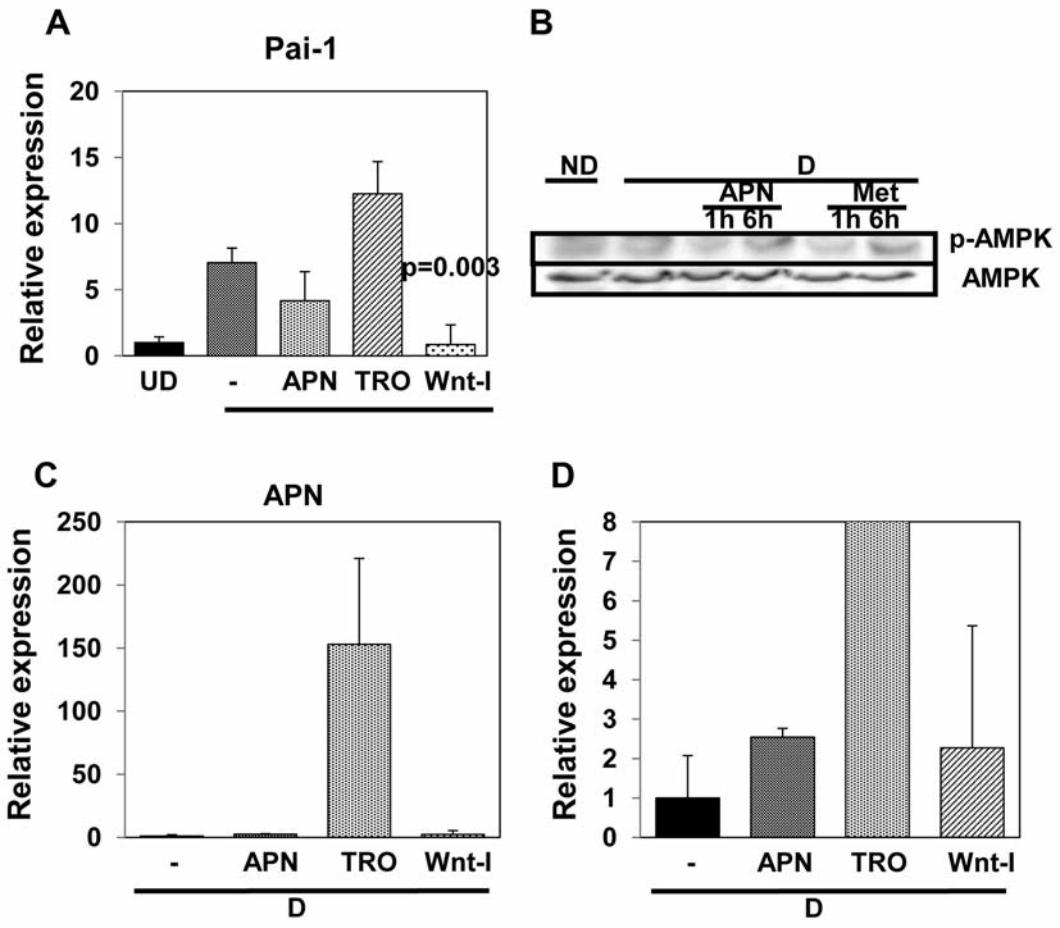

Figure 2. *Effects of adiponectin (APN), peroxisome proliferator-activated receptor (PPAR)γ ligand and Wnt inhibitor on 3T3-L1 cells at the early stage of differentiation. At day 2 after the initiation of differentiation, 3T3-L1 cells were treated with mouse recombinant protein APN (10 μg/ml), troglitazone (TRO), PPARγ ligand (10 μM) and Wnt inhibitor PNU74654 (20 μM) for three days. Quantitative real time-polymerase chain reaction (qRT-PCR) for Pai-1 (A) and APN (C and D) was performed. Relative Pai-1 and APN mRNA expression levels are plotted as the ratio of the untreated and undifferentiated control culture value. Data are means±SD (n=3). Similar results were obtained from three separate experiments. B: 3T3-L1 cells were treated with APN (10 μg/ml) and metformin (MET) an AMPK activator (5 mM) for 1 or 6 h, and AMPK and phosphorylated AMPK were examined by western blot. D: The data focused on low relative expression levels of Figure 1C. D, differentiated; UD, undifferentiated.*

Correlation between adiponectin and Pai-1 in the early phase. To clarify the relation between APN and Pai-1, 3T3-L1 cells were treated with APN at a dose of 10 μg/ml on day 2, after the initiation of differentiation. At the early-phase time point (day 5), high *Pai-1* mRNA expression levels were observed and it was found that APN could slightly reduce Pai-1 expression levels compared to those differentiated cells not treated with APN (Figure 2A). Phosphorylation of AMPK was confirmed by western blotting after six hours treatment with 10 μg/ml APN and 5 mM metformin, used as a positive control, at day 2 after the initiation of differentiation (Figure 2B). In addition, we tried to induce an increase in APN expression by treatment with troglitazone, a PPARγ ligand. As expected, treatment with 10 μM troglitazone markedly induced APN as shown in Figure 2C. However, treatment with troglitazone did not suppress but rather increased Pai-1 expression. On the other hand, we tried to reduce the high levels of Pai-1 by inhibiting Wnt/β-catenin signaling. PNU74654, a Wnt inhibitor, at a dose of 20 μM successfully suppressed Pai-1 expression levels (Figure 2A). APN expression levels under this treatment were examined, and almost a 2-fold elevation was observed (Figure 2C and D).

Correlation between adiponectin and Pai-1 in the late phase. To clarify the relation between APN and Pai-1 in the late phase, Pai-1 at a dose of 1 μg/ml was added to the medium at 15 days after the initiation of differentiation. At the late-phase time point (day 18), APN expression was slightly reduced by Pai-1 treatment compared to those of untreated

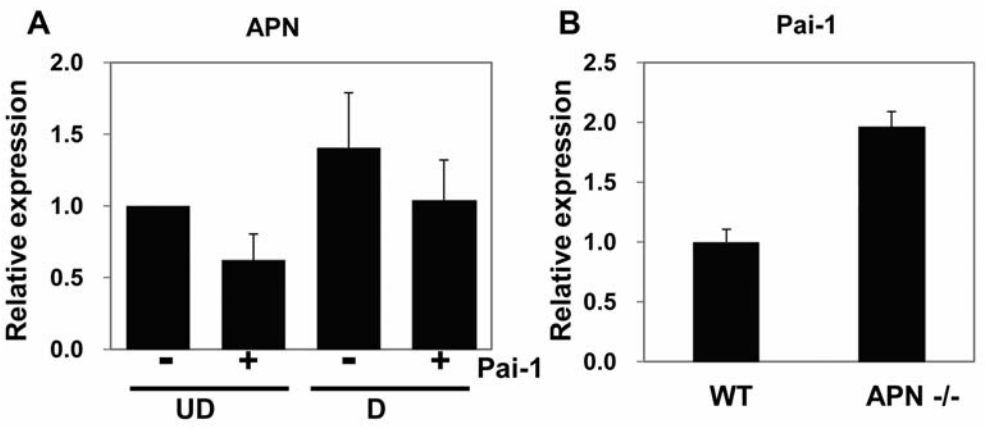

Figure 3. *Effects of plasminogen activator inhibitor-1 (Pai-1) on adiponectin (APN) mRNA levels and expression of Pai-1 in adiponectin-deficient mice. A: 3T3-L1 pre-adipocytes were treated with 1 μg/ml mouse recombinant Pai-1 at day 15 after the initiation of differentiation, and cells were collected for Quantitative real time-polymerase chain reaction (qRT-PCR) analysis at day 18. Relative APN mRNA expression levels are plotted as the ratio of the unstimulated-control culture value. Data are means±SD (n=3). Similar results were obtained from more than two separate experiments. B: qRT-PCR was performed on abdominal fat tissue from 12-week-old male adiponectin homozygous knockout mice (C57BL/6J background; n=5) and its control wild-type (WT) mice (n=5) as described in the Materials and Methods. Relative Pai-1 mRNA expression levels are plotted as the ratio of the value for the wild-type control fat tissue. Data are means±SD. APN(-/-), Homozygous adiponectin knockout mice; D, differentiated; UD, undifferentiated.*

differentiated and undifferentiated cells (Figure 3A). Furthermore, we confirmed an almost doubling of Pai-1 levels in the abdominal adipose tissue in *APN* homozygous knock-out mice compared to those of *APN* wild-type mice (Figure 3B).

Discussion

The present study demonstrated seesaw patterns of APN and Pai-1 expression in the different stages of pre-adipocyte differentiation. Moreover, bi-directional regulation observed between APN and Pai-1 may be through activation of AMPK and PPARγ (Figure 4).

In the early phase of 3T3-L1 cell differentiation, Pai-1 expression increased and APN slightly decreased. Besides, the late phase of differentiation showed low Pai-1 and high APN (Figure 1E). PPARγ, sterol regulatory element-binding protein-1c (SREBP-1c) and CCAAT/enhancer-binding proteins (C/EBP) are known to be involved in the early changes during pre-adipocyte differentiation (19). In the late phase of pre-adipocyte differentiation, the canonical Wnt signaling pathway reduces adipogenesis (19). These signalings might affect expression patterns observed for APN and Pai-1.

Indeed, a PPARγ ligand remarkably induced APN expression in this study, especially in the early phase (Figure 4A). Another PPARγ ligand, pioglitazone was also found to induce APN expression (20). Of note, Pai-1 is reported to suppress PPARγ expression (21). In this study, APN induction did not effectively lower Pai-1, but addition of 10 μg/ml APN to the culture medium did reduce Pai-1 expression. Concentrations of APN

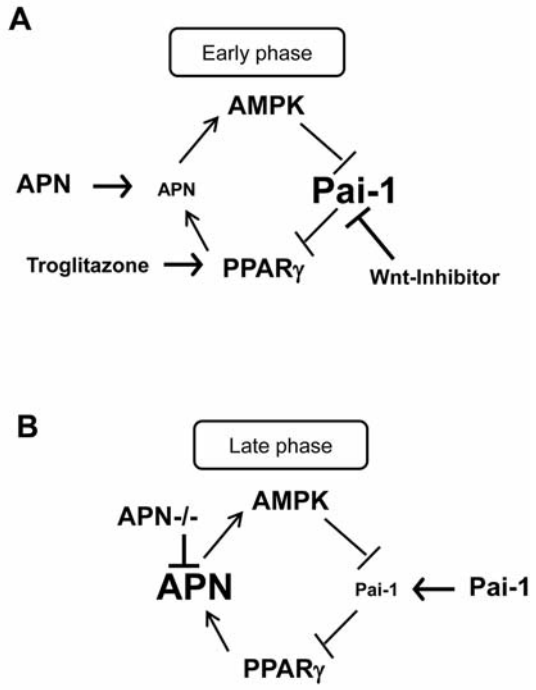

Figure 4. *Proposed mechanism for the seesaw regulation between adiponectin (APN) and plasminogen activator inhibitor-1 (Pai-1). A: Low APN and high Pai-1 expression levels in the early differentiation phase of 3T3-L1 cells may be regulated by AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)γ. Wnt inhibitor was used for suppression of Pai-1. Troglitazone was used for activation of PPARγ. APN was used for activation of AMPK. B: High APN and low Pai-1 expression levels in the late differentiation phase of 3T3-L1 cells may also be regulated by AMPK and PPARγ. Pai-1 was used for suppression of PPARγ. Abdominal fat tissue from APN-deficient mice (APN–/–) was used to determine the effects of APN deficiency on Pai-1 expression levels (→induction; —| suppression/inhibition).*

detected in plasma range from 3-30 μg/ml. Thus, a biologically appropriate dose might be used in this study. It has been reported that the activation of AMPK leads to the inhibition of adipogenesis (22). AMPK activation by APN resulted in suppression of Pai-1 expression. Similar findings were obtained in our recent experiment (10), in which APN-deficiency evoked hepatic Pai-1 induction. These findings suggest that in addition to the Pai-1-suppressive function of TNFα, APN-induced AMPK activation acts as a more direct physiological suppressor of Pai-1. Moreover, Wnt signal inhibitors lowered Pai-1 expression in the early phase. Pai-1 is reported to be a downstream target of Wnt/β-catenin signaling (17), and this might be the reason why APN is induced by a Wnt inhibitor. Summarizing effects in the early phase in 3T3-L1 cells, reduction of Pai-1 resulted in elevation of APN, and supplementation of APN resulted in reduction of Pai-1 (Figure 4A).

In the later phase of pre-adipocyte differentiation, low expression of Pai-1 and high expression of APN were observed. Addition of 1 μg/ml Pai-1 in the culture medium slightly reduced APN expression. Generally, the concentration of PAI-1 detected in human plasma is lower than 50 ng/ml. This dose may not be a biologically-appropriate dose, but may partly explain Pai-1 functions on specific occasions, such as in a localized area in the late phase of differentiation. We also examined the effect of APN-knockout conditions on Pai-1 expression using abdominal fat tissue samples from APN homozygous knock-out mice. Pai-1 expression was observed at a high level compared to that of fat tissue from APN wild-type mice. In the late phase of 3T3-L1 cells, supplementation of Pai-1 resulted in a slight reduction of APN (Figure 4B).

Both APN and Pai-1 are known to be involved in colorectal carcinogenesis. An absence of APN results in an increase of intestinal polyp development in *Min* mice (10), while a PAI-1 inhibitor was reported to reduce intestinal polyp development in *Min* mice (23). It is assumed that both induction of APN and inhibition/suppression of Pai-1 may be a useful approach in colorectal cancer prevention. Here, we have demonstrated a seesaw pattern of regulation between APN and Pai-1. Further studies are required to identify more direct regulatory mechanisms between APN and Pai-1, and the molecular targets identified might be utilized as novel chemopreventive targets.

Conflicts of Interest

None.

Acknowledgements

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