Metabolic role of dipeptidyl peptidase 4 (DPP4) in primary human (pre)adipocytes

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Dipeptidyl peptidase 4 (DPP4) is the target of the gliptins, a recent class of oral antidiabetics. DPP4 (also called CD26) was previously characterized in immune cells but also has important metabolic functions which are not yet fully understood. Thus, we investigated the function of DPP4 in human white preadipocytes and adipocytes. We found that both cell types express DPP4 in high amounts; DPP4 release markedly increased during differentiation. In preadipocytes, lentiviral DPP4 knockdown caused significant changes in gene expression as determined by whole-genome DNA-array analysis. Metabolic genes were increased, e.g. PDK4 18-fold and PPARγC1α (=PGC1α) 6-fold, and proliferation-related genes were decreased (e.g. FGF7 5-fold). These effects, contributing to differentiation, were not inhibited by the PPARγ antagonist T0070907. Vice versa, the PPARγ agonist pioglitazone induced a different set of genes (mainly FABP4). DPP4 knockdown also affected growth factor signaling and, accordingly, retarded preadipocyte proliferation. In particular, basal and insulin-induced ERK activation (but not Akt activation) was markedly diminished (by around 60%). This indicates that DPP4 knockdown contributes to adipocyte maturation by mimicking growth factor withdrawal, an early step in fat cell differentiation. In mature adipocytes, DPP4 becomes liberated so that adipose tissue may constitute a relevant source of circulating DPP4.

Diabetes mellitus is a major health problem in the 21st century world-wide and hence, there is a growing line of antidiabetic drugs and therapies¹₂. Gliptins, small molecular inhibitors of the peptidase DPP4 (dipeptidyl peptidase 4), were developed recently as a new class of antidiabetic drugs. DPP4 degrades incretin peptides (e.g. GLP1) and is widely known for its regulatory effect in glucose metabolism³⁴. Findings in DPP4 knockout mice revealed that absence of this enzyme improves glycemic control and leads to reduced fat mass⁵⁶. This made DPP4 inhibitors promising candidates for treating human Type 2 diabetes. Inhibition of the enzymatic activity of DPP4 prevents the rapid cleavage of incretins and as a consequence, higher endogenous incretin levels are obtained which enhance the glucose-induced insulin secretion. This so-called incretin-like effect is responsible for approximately 60% of postprandial insulin secretion⁷. DPP4 is a ubiquitous glycoprotein and occurs as a cell membrane bound protein as well as in a soluble, extracellular form⁸⁹. It is well established that DPP4 has multiple functions⁸¹⁰, which raises concerns of unexpected off-target effects of gliptins. Changes in the peptide activity or enzyme concentration were described to be associated among others with obesity, diabetes and neurologic and inflammatory diseases⁸¹¹,¹². Beside of cleaving several substrates (e.g. chemokines, neuropeptides, growth factors⁹¹¹,¹³), there is evidence of non-enzymatic functions by interaction with ADA (adenosine deaminase) and other matrix proteins or by immunological mechanisms⁸¹⁴,¹⁵. In addition, DPP4 was originally identified as CD26 antigen on lymphocytes so that an immunological role of this gene product was assumed¹⁶. Therefore, concerns about potential unintended immunosuppressive effects were expressed. Meanwhile, DPP4 is discussed as a potential link between obesity and the metabolic syndrome or insulin resistance¹⁵⁻¹⁷. DPP4 was suggested to be a new adipokine, involving the adipose tissue as a possible source for the circulating DPP4 activity in the plasma, which is relevant for GLP1 degradation, suggesting that DPP4/CD26 could play a major role in metabolism.

The adipose tissue as a major endocrine and energy storage organ plays an important role in metabolic systems and insulin action¹⁸⁻²² and is already a target for another class of antidiabetics, the glitazones. The latter, being PPARγ-agonists, lead to enhanced insulin sensitivity, accompanied by an increased differentiation and fat

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accumulation. However, the role of DPP4 in human adipose tissue is currently unclear. Furthermore, recent animal studies with DPP4 inhibitors support the notion that DPP4 may play a functional role within adipose tissue, because DPP4 inhibition has been seen to prevent adipose tissue inflammation and development of glucose intolerance in high fat diet induced obesity in mice. Based on these considerations, we aimed to gain more insight in the role of DPP4 in primary cultured human white preadipocytes and adipocytes.

Results
Expression and release of DPP4 in human white (pre)adipocytes. In order to examine the role of DPP4 in the human adipose tissue, we first measured the expression of this enzyme in primary human white adipocytes at mRNA and protein level during differentiation. Mature adipocytes were obtained by in-vitro differentiation of primary cultured preadipocytes, following the protocol described in the Methods section. DPP4 mRNA was expressed in preadipocytes (i.e. Day 0 of differentiation) and mature adipocytes (up to Day 21 of differentiation) at comparable levels (Fig. 1A). There was a brief dip in DPP4 expression at Days 3 to 9 of differentiation (Day 0, switch to differentiation medium) but there were no major long-term changes.

Comparison of DPP4 expression level in preadipocytes and adipocytes with various other human tissues (Fig. 1B) revealed that preadipocytes and mature adipocytes express 4th and 6th highest level of DPP4, respectively (highlighted in Fig. 1B).

DPP4 protein weakly increased with differentiation as revealed by Western blotting and consecutive densitometric quantification (Fig. 1C), but statistical significance was not reached.

In order to test whether (pre)adipocytes could be a source of soluble DPP4, we analyzed cell culture supernatants, collected at different time points of differentiation, by ELISA (Fig. 1D). Preadipocytes liberated DPP4 to a low extent only (around 1 ng/ml), but DPP4 release markedly increased during differentiation. Mature adipocytes released up to 11-times more DPP4 than non-differentiated cells. A linear regression analysis revealed statistical significance. It should be noted that DPP4 is not stored in vesicles before release (instead, the extracellular part of this membrane protein is cleaved off) so that cellular DPP4 content does not need to increase when liberation of DPP4 increases.

In a next step, we questioned which events could influence DPP4 release. Cellular triglyceride content was a candidate because triglyceride storage and liberation is a prominent function of adipocytes. In order to change the triglyceride content of the cultured adipocytes, we induced lipolysis by treatment with the cAMP-mimetics forskolin (10 μM) or DBcAMP (100 μM) for 24 h (Fig. 1E). Triglyceride degradation was verified by measuring the increase of glycerol content in the cell culture supernatants. DPP4 release was measured by ELISA 24 h after induction of lipolysis. Leptin secretion was also determined for comparison. It turned out that leptin secretion was significantly reduced following lipolysis whereas no influence on DPP4 release was detected (Fig. 1E).

For further characterization, we determined the intracellular localization of DPP4 protein in preadipocytes. Western blotting following cell fractionation revealed a strong DPP4 signal in the membrane fraction (Fig. 1F). The cytosolic fraction showed several weak bands, probably due to unspecific binding of the antibody. No signal could be detected in the nucleus and in the cytoskeleton. In accordance with this observation, detection of DPP4 protein in preadipocytes by immunofluorescence with z-stack analysis (Fig. 1G) revealed DPP4 (green signal) localization primarily in the outer cell regions, i.e. in a typical appearance of membrane proteins.

Taken together, DPP4 is expressed in preadipocytes and adipocytes, is located primarily in the cell membrane from which it becomes increasingly released during maturation.

Gene expression profile after DPP4 knockdown. As described above, DPP4 was highly expressed in preadipocytes but was hardly released from these cells. This implies a different function of DPP4 in preadipocytes as compared to mature adipocytes. For closer investigation, we produced preadipocytes with a stable knockdown of DPP4 expression, achieved by lentiviral shRNA (see Methods section). Successful lentiviral transduction was verified with a GFP construct (Supplementary Figure S1A). Unspecific effects of the lentiviral vector were excluded by using a non-targeting shRNA (“sh-control” or “SHc”) as negative control in each experiment.

Knockdown of DPP4 expression, achieved by lentiviral shRNA (see Methods section). Successful lentiviral transduction, collected at different time points of differentiation, by ELISA (Fig. 1D). Preadipocytes liberated DPP4 to a low extent only (around 1 ng/ml), but DPP4 release markedly increased during differentiation. Mature adipocytes released up to 11-times more DPP4 than non-differentiated cells. A linear regression analysis revealed statistical significance. It should be noted that DPP4 is not stored in vesicles before release (instead, the extracellular part of this membrane protein is cleaved off) so that cellular DPP4 content does not need to increase when liberation of DPP4 increases.

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No clear hints were available what the function of DPP4 in preadipocytes could be so that we started our study with a screen for changes in gene expression using a whole genome oligo microarray (Agilent). A heat map of four replicate experiments visualizing genes that were altered at least 5-fold is shown in Fig. 2C. Salient genes, altered at least two-fold and forming functional clusters are listed in Table 1. The most pronounced changes were found in genes involved in lipid metabolism (with an up-regulation in most cases) and in some proliferation related genes (down-regulated in most cases) as summarized in Table 1. For example, a 5- to 24-fold up-regulation of FABP4, PDK4 and PPAR-γ1 was observed.

The transcription factor C/EBPα, a relative of C/EBPα and C/EBPβ, was induced 20-fold. C/EBPα and C/EBPβ are known to be involved in adipocyte differentiation. C/EBPα was initially detected in lymphoid and myeloid cells based on its structural similarity to C/EBPα and C/EBPβ, and is assumed to regulate their differentiation. The function of C/EBPβ in preadipocytes is unknown but, due to its structural similarity, C/EBPβ could mimic the effects of C/EBPα and/or C/EBPβ. Other transcription factors were also increased, such as members of the KLF family (e.g. KLF15, −5, −2). Among the genes induced or suppressed at least twofold, four functional clusters became obvious (Table 1), namely lipid metabolism, proliferation, structural genes including cell-cell contact and cell migration.

Representative metabolic and proliferative genes were selected for further investigation by quantitative PCR. We studied the time course of expression and the interaction with other agents known to be involved in adipocyte differentiation. The time course of PPAR-γ1 and PDK4 expression after knockdown is shown in Fig. 2D. A
Figure 1. DPP4 expression, release and localization in human white (pre)adipocytes. Part (A) Time course of DPP4 expression was measured by RT PCR during differentiation. CP values were normalized to the housekeeping gene GAPDH, and are presented relative to Day 0. The left y-axis shows the original ΔCP values, and the calculated fold change vs. Day 0 is displayed on the right y-axis. Data are displayed as mean values ± SEM, n = 4. Part (B) DPP4 expression was measured by Real Time PCR in different human tissues (RNA Panel from Clontech, Mountain View, CA, USA), normalized to the housekeeping gene GAPDH, and is presented as mean fold change values ± SEM, n ≥ 2, in a logarithmic scale. Part (C) DPP4 protein levels during differentiation were analyzed by Western blotting followed by densitometric quantification. The upper panel shows a representative blot, which was cropped for clarity. The full gel is shown in Supplementary Figure S2A. For quantification, at least three blots per time point were used. Densitometric data were normalized to α-actinin and are displayed relative to Day 0 in the lower panel as mean values ± SEM. Part (D) DPP4 release during differentiation was assessed by ELISA. Data are represented relative to Day 0 as mean values ± SEM, n = 5. Statistical evaluation was done by regression analysis. **p < 0.01. The regression line is shown dashed. Part (E) Effect of lipolysis, induced by forskolin (10 μM, “F”) or DBcAMP (100 μM, “cAMP”) on the liberation of DPP4 and leptin into the cell culture supernatants over 24 hours. Data are presented relative to untreated control as mean values ± SEM, n ≥ 4. Statistical analysis was done by one sample t test; **p < 0.01 vs. control. Part (F) Presence of DPP4 in different cell fractions as indicated was analyzed by Western blotting after cell fractionation; the blot was cropped for clarity; the whole gel is shown in Supplementary Figure S2B. Part (G) Microscopic images (40x) showing immunofluorescence staining of DPP4 (green) to determine its subcellular localization. The cytoskeleton (F-actin) is labeled in red.
Figure 2. Lentiviral knockdown of DPP4 in human preadipocytes. Human preadipocytes were stably transduced with shRNA constructs directed against DPP4 mRNA by lentiviral vectors. Unspecific, non-target shRNA was used as a negative control (labeled “SHc” in the figure). Knockdown (KD) of DPP4 expression was confirmed on mRNA Part (A) and protein Part (B) level by quantitative Real Time PCR and Western blotting, respectively. The picture of the Western blot was cropped for clarity. The entire lanes are shown in Supplementary Figure S2C. PCR data are presented as mean ΔCP values (normalized to GAPDH, relative to SHc) ± SEM (left y-axis) and calculated fold change values vs. control (right y-axis), n ≥ 5. Statistical analysis was done by one-way ANOVA with Dunnett post-test; **p < 0.01 vs. negative control. Part (C) At least 5-fold changes in gene expression resulting from DPP4 knockdown, measured by whole genome DNA array hybridization, are visualized in a heat plot. Hybridization was performed in two-color mode; each line represents the difference between a DPP4 knockdown and sh-control sample. The four lines represent four biological replicates. Up-regulated genes in DPP4 knockdown compared to control are marked in green, down-regulated genes in red. The color intensity indicates the expression level of the respective gene. Part (D) Changes in the expression of two representative genes (PPARγ1C1α and PDK4) over time after infection were followed by quantitative PCR. Data represent mean ΔCP values (vs. GAPDH) ± SEM (left y-axis) and calculated fold change values vs. control (SHc at Day 0) on the right y-axis, n ≥ 3. Statistical analysis was done by t test; *p < 0.05; **p < 0.01 vs. control. Part (E) shows a Western blot confirming the up-regulation of PPARγ1C1α on protein level when DPP4 is suppressed. The blot was cropped for clarity. The entire lanes are shown in Supplementary Figure S2D. The results for DPP4, PPARγ1C1α and the loading control α-actinin from cells infected with sh-control vector (“SHc”) and sh-DPP4 vector (“DPP4 KD”), respectively, are shown as indicated in the figure.
| Abbreviation, accession | Gene | log(ratio) mean, SEM | Mean fold change | Description |
|-------------------------|------|----------------------|-----------------|-------------|
| **A. Lipid metabolism** |      |                      |                 |             |
| PLIN5 NM_001013706      | Homo sapiens lipid storage droplet protein 5 | 1.41 ± 0.06 | 25.43 | coats lipid droplets |
| PDK4 NM_002612          | Homo sapiens pyruvate dehydrogenase kinase | 1.37 ± 0.08 | 23.48 | involved in glucose metabolism |
| CEBPE NM_001805         | Homo sapiens CCAAT/enhancer binding protein | 1.31 ± 0.33 | 20.25 | transcription factor |
| PLIN NM_002666          | Homo sapiens perilipin | 1.15 ± 0.21 | 14.20 | coats lipid droplets |
| FABP4 NM_001442         | fatty acid binding protein 4 | 1.15 ± 0.12 | 14.19 | maybe involved in fatty acid metabolism |
| ACSM5 NM_017888         | Homo sapiens acyl-CoA synthetase medium-chain family member 5 | 1.11 ± 0.02 | 12.85 | involved in fatty acid metabolism |
| APOE NM_000041          | Homo sapiens apolipoprotein E | 1.10 ± 0.07 | 12.66 | involved in fatty acid metabolism |
| KLF15 NM_0014079        | Homo sapiens Kruppel-like factor 15 | 0.75 ± 0.09 | 5.65 | transcription factor |
| PPARC1A NM_0013261      | Homo sapiens peroxisome proliferator-activated receptor gamma | 0.70 ± 0.09 | 4.99 | involved in energy metabolism |
| KLF2 NM_016270          | Homo sapiens Kruppel-like factor 2 | −0.70 ± 0.04 | −5.01 | transcription factor |
| FADS1 NM_013402         | fatty acid desaturase 1 | −1.03 ± 0.02 | −10.83 | involved in fatty acid metabolism |
| FADS2 NM_004265         | fatty acid desaturase 2 | −1.14 ± 0.05 | −13.87 | involved in fatty acid metabolism |
| **B. Proliferation**    |      |                      |                 |             |
| FGFR1 NM_023110         | Homo sapiens fibroblast growth factor receptor 1 | −0.31 ± 0.06 | −2.06 | growth factor receptor |
| TIMP1 NM_003254         | Homo sapiens TIMP metalloepitidase inhibitor 1 | −0.57 ± 0.03 | −3.70 | involved in cell proliferation, inhibitors of the matrix metalloproteinases |
| CAPNS1 NM_001749        | Homo sapiens calpain, small subunit 1 | −0.59 ± 0.04 | −3.91 | involved in proliferation and adhesion |
| FGFI7 NM_002009         | fibroblast growth factor 7 (keratinocytic growth factor) | −0.78 ± 0.07 | −6.02 | growth factor |
| PDGFRL NM_006207        | Homo sapiens platelet-derived growth factor receptor-like | −0.94 ± 0.02 | −8.66 | growth factor receptor-like |
| SULF1 NM_015170         | Homo sapiens sulfatase 1 | −1.67 ± 0.02 | −47.18 | coreceptor for growth factors and cytokines |
| **C. Structural genes / cell-cell contact** | | | | |
| HPSE NM_006665          | Homo sapiens heparanase | 1.35 ± 0.02 | 22.32 | component of the membrane and extracellular matrix |
| HAS1 NM_001523          | Homo sapiens hyaluronan synthase 1 | 1.22 ± 0.11 | 16.57 | component of the extracellular matrix |
| ITGB3 NM_000212         | Homo sapiens integrin, beta 3 | 0.36 ± 0.04 | 2.30 | involved in cell adhesion |
| COL4A1 NM_001845        | Homo sapiens collagen, type IV, alpha 1 | 0.32 ± 0.08 | 2.07 | component of the membrane |
| COL13A1 NM_008081       | Homo sapiens collagen, type XIII, alpha 1 | −0.37 ± 0.10 | −2.36 | component of the membrane |
| COL1A1 NM_000088        | Homo sapiens collagen, type I, alpha 1 | −0.45 ± 0.10 | −2.82 | component of connective tissue |
| RAC1 NM_018890          | Homo sapiens ras-related C3 botulinum toxin substrate 1 | −0.61 ± 0.04 | −4.11 | maybe involved in cell growth, cytoskeletal reorganization |
| KRT19 NM_002276         | Homo sapiens keratin 19 | −0.72 ± 0.03 | −5.21 | involved in structural integrity |
| SPARC NM_003118         | Homo sapiens secreted protein | −1.11 ± 0.06 | −12.92 | involved in extracellular matrix synthesis |
| **D. Cell migration**   |      |                      |                 |             |
| RARRES2 NM_002889       | Homo sapiens retinoic acid receptor responder 2 | 1.44 ± 0.07 | 27.39 | chemotactic protein |
| ANGPT1 NM_001146        | Homo sapiens angiopoietin 1 | 0.42 ± 0.06 | 2.65 | involved in vascular development and angiogenesis |
| ILK NM_00101479         | Homo sapiens integrin-linked kinase (ILK) | −0.53 ± 0.05 | −3.37 | involved in integrin-mediated signal transduction |
| VEGFR NM_003377         | Homo sapiens vascular endothelial growth factor B | −0.54 ± 0.06 | −3.49 | vascular endothelial growth factor |
| PDGFRA NM_006206        | Homo sapiens platelet-derived growth factor receptor, alpha polypeptide | −0.98 ± 0.03 | −9.57 | cell surface receptor |
| ACVRL1 NM_000020        | Homo sapiens activin A receptor type II-like 1 | −1.37 ± 0.01 | −23.27 | cell-surface receptor |
Effect of DPP4 knockdown in later stages of differentiation. DPP4 knockdown elicited changes in gene expression in preadipocytes. Thus, we investigated the effect of DPP4 knockdown also in later stages of adipocyte maturation. Preadipocytes stably transduced with DPP4 shRNA were differentiated according to the standard protocol (see Methods section), and the expression of the genes of interest was studied by RT-PCR at Days 0, 6 and 12 of differentiation (Fig. 4). For comparison, the differentiation protocol was also performed with cells expressing non-target control shRNA.

Compared to Day 0 of differentiation, the effect of DPP4 knockdown diminished during differentiation despite DPP4 mRNA remained suppressed. At Day 0 of differentiation (i.e. before switching the cells to differentiation medium), the expression rates of the genes shown were higher (or lower in case of FGF7) in DPP4 knockdown strongly and consistently induced PPARγ1 and APOE which were hardly affected by pioglitazone (Fig. 3A). Furthermore, expression of the growth factor FGF7 was suppressed by DPP4 knockdown but was not affected by pioglitazone. For further confirmation of independent actions of DPP4 and PPARγ, we employed the PPARγ1 antagonist T0070907 (10 μM). The changes in gene expression were similar with DPP4 knockdown alone and with DPP4 knockdown plus T0070907 (Fig. 3A). Thus, T0070907 did not alter the gene expression induced by DPP4 knockdown.

A further difference between the action of DPP4 knockdown and PPARγ activation was revealed by determining lipid accumulation. Lipid droplets (marked by arrows in the figure) were observed after pioglitazone stimulation but not after DPP4 knockdown (Fig. 3B).

T0070907 alone had no major effects on the DPP4 knockdown expression profile except for some up-regulation of PDK4 (Fig. 3C, right group of bars). In order to confirm that T0070907 had no unspecific effects (i.e. actions beyond PPARγ inhibition) we compared the effect of T0070907 on gene expression with the effect of PPARγ knockdown (Fig. 3C, mid group of bars). Overall, the pattern was similar except for an up-regulation of PLIN1 by PPARγ knockdown but not by T0070907 treatment.

Effect of DPP4 knockdown on intracellular signaling. For closer investigation of the mechanisms by which DPP4 knockdown exerts the described effects on gene expression, we studied the activation of protein kinase signaling pathways (Fig. 5). Growth factor withdrawal is the first step of adipocyte differentiation in vitro, and it was reported that in vivo an autocrine EGF-related growth factor, Pref-1, prevents differentiation via activation of ERK28,29. In cultured preadipocytes we observed a basal activity of the ERK pathway, measured as phosphorylated ERK (pERK) by Western blotting. Preadipocytes express insulin receptors (Fig. 5A); insulin receptor expression was not influenced by DPP4 knockdown. ERK phosphorylation was markedly enhanced by stimulation with insulin (100 nM for 10 min). After knockdown of DPP4, insulin-induced ERK phosphorylation was significantly weaker (Fig. 5A; densitometric quantification in Fig. 5B). In contrast, activation of the pAkt...
pathway by insulin was not diminished after DPP4 knockdown (Fig. 5A,B). Thus, DPP4 knockdown selectively attenuated the growth factor-like signaling of insulin.

In line with the described effects on growth factor signaling, DPP4 knockdown prevented further proliferation of the preadipocytes as measured by cell counting over time (Fig. 5C).

Taken together, DPP4 knockdown in preadipocytes diminished the ability of insulin and probably other growth factors to activate the ERK signaling pathway. This mimics growth factor withdrawal, leads to growth arrest and could thereby contribute to initiate the first step of differentiation.

Discussion

The various functions of DPP4 have been widely discussed, among others in the fields of immunology, (neuro-)endocrinology and glucose homeostasis. However, the role of DPP4 in human adipose tissue is still unclear. Our results now revealed a strong expression of this gene in human white preadipocytes and adipocytes and
revealed a possible contribution of DPP4 to the adipocyte differentiation process. Furthermore, mature adipocytes were identified as a potential source of circulating DPP4.

Adipocyte maturation is a complex process and involves several different mediators and signaling pathways. Among these are the two master regulators PPARγ and the C/EBP family. Our knockdown experiments revealed changes in the expression of functional gene clusters indicative for adipocyte differentiation. Investigation of signaling pathways identified a potential mechanism by which DPP4 knockdown could contribute to differentiation. It became obvious that basal and insulin-induced ERK phosphorylation was attenuated. In contrast, activation of the Akt pathway by insulin was not affected, arguing for a selective action of DPP4 on growth factor signaling via ERK.

It should be noted that insulin probably has a dual role in respect to adipocyte differentiation. On one hand, insulin promotes differentiation and is a component of the differentiation medium. For this effect activation of the pAkt signaling pathway appears to be relevant. On the other hand, by activation of ERK insulin behaves like a growth factor and may thereby counteract the onset of differentiation. The role of ERK in adipocyte differentiation is not fully clear, but in the case of the EGF-related growth factor Pref-1, which acts on preadipocytes in an autocrine way, it was clearly shown that ERK activation by Pref-1 prevents differentiation.

The effects of DPP4 knockdown were not influenced by inhibition of PPARγ, an important player in adipocyte maturation but acting at a later stage of this process. Accordingly, the set of genes induced by DPP4 knockdown differed from the set induced by PPARγ. The action of DPP4 at an early step in the differentiation process also explains why DPP4 knockdown, in contrast to PPARγ activation, did not promote triglyceride accumulation because the latter most likely is a late event in adipocyte maturation.

Beside of metabolic genes, genes encoding extracellular matrix proteins and proteins being involved in cell-cell interaction and migration were altered by DPP4 knockdown. A link between the extracellular matrix
composition and the differentiation competence involving the action of matrix metalloproteinases (MMPs) was described already\textsuperscript{33}. Moreover, a role for DPP4 in the modulation of the extracellular matrix could be shown in different studies\textsuperscript{35}.

In accordance with our findings, a recent publication by Han \textit{et al.}\textsuperscript{36} identified other members of the DPP4 family, DPP8 and DPP9, as players in adipocyte differentiation. This group used a permanent mouse cell line (3T3-L1), and in contrast to the effects of DPP4 observed in our work, blocking or knockdown of DPP8 and DPP9 inhibited differentiation with involvement of PPAR\textgamma.

One strongly induced gene in response to DPP4 knockdown was PPAR\textgamma C1\alpha. This gene, also known as PGC1\alpha, is related to mitochondrial function and it is necessary for brown fat development\textsuperscript{26,37}. It acts as a master regulator of mitochondrial biogenesis in mammals and therefore participates in energy balance. However, other genes characteristic for brown or “beige” fat cells, such as UCP1\textsuperscript{37,38}, were not increased.

In line with previous studies, we detected increased DPP4 protein expression and release in a maturation-dependent way\textsuperscript{18,39}. This was also observed during monocyte differentiation to dendritic cells\textsuperscript{44}. However, in our study adipocyte maturation predominantly affected DPP4 liberation; DPP4 expression changed only to a minor extent. This is in contrast to findings by Das \textit{et al.}\textsuperscript{39} in the mouse cell line 3T3-L1 where a strong increase in DPP4 protein expression during adipocyte differentiation was observed. We could hardly measure a DPP4 release in the supernatants from preadipocytes but observed a marked increase during adipogenesis, pointing to DPP4 release as an important function of mature adipocytes. This is in line with the recently recognized role of the adipose tissue as an endocrine organ\textsuperscript{21}. The mechanism underlying increasing DPP4 release during maturation is not clear, and as it is not yet established which factors trigger DPP4 release. Another prominent adipokine, leptin, becomes released in dependence of the adipocyte triglyceride content. No such dependency was observed for DPP4.

Figure 5. Effect of DPP4 knockdown on ERK and Akt activity. Human preadipocytes were transduced by lentiviral shRNA directed against DPP4 (labeled “DPP4-KD” in the figure) or, as control, by non-targeting shRNA (labeled “SHc” in the figure. Part (A) Activation of signaling pathways was analyzed by Western blotting with antibodies directed against the phosphorylated (active) form of the respective signaling protein, pERK (phospho-Extracellular-signal Regulated Kinase) or pAkt (phospho-Akt1). The effect of DPP4 knockdown on insulin (ins) signaling via the pAkt and the pERK pathway is shown. The insulin concentration used was 100 nM, incubation time was 10 min. Detection of \(\alpha\)-actinin served as loading control. Insulin receptor expression was also detected in the preadipocytes (lower panel of Part A) and was not affected by treatment. The blots were cropped for clarity. Uncropped pictures are shown in Supplementary Figure S3. The densitometric quantification of the phosphoproteins is shown in Part (B). Statistical analysis was done by one-way ANOVA with Dunnett post-test; *\(p<0.05\); **\(p<0.01\) vs. insulin-treated sh-control (SHc). Part (C) Proliferation of the preadipocytes after DPP4 knockdown vs. SHc was assessed by cell counting at various time points as indicated.
Table 2. Nucleotide sequences of the PCR primers used.

| Accession number | Upstream primer | Downstream primer |
|------------------|-----------------|-------------------|
| NM_002046        | TCTCGTT CGACAG TGCGACAGT | TGAAGA CCGCA GTGGACTCCACG |
| NM_001935        | CTCCAGA AGACAC A CTTGACATACGAA | TCACTCATC ATCCTGACAGT CAGTTTTGAG |
| NM_001142        | TCATACCTGGGCAGA AGATTTTGACAGA | ATCGGAA CTCGT CAGTCCACGTCACG |
| NM_017888        | TGGAGACTTGGAGGACGCTGGGAAG | CCGGGTGTT TCCGGTGGTAAATAGTA |
| NM_000041        | CAAGCCAGAAAGATTGAA AGTCTCTGACG | GCCGCA CCGGCGTCA GAGTT |
| NM_013261        | AGGAGCAGTCTTGTGGCCTTTCGTG | AGGGCCTTGAGCAGTCCA GACACC |
| NM_002612        | TCACAGACAGAAACCAC CAAGCCACA | CCGTAA CAAACACCGGCAAGGAG |
| NM_002666        | GCCCTGGGACAGCTTGAAGGTT | CCTCTCCTCGTCGTCGTCGTCG |
| NM_015170        | AGGCTGGAGGACGCTAA GAAGCFCACA | TCCAAA AAGCAGTAGCA AACCTGCACGA |
| NM_006207        | AAGGTCTGGCTGCTGCTTGGTCTTC | ACCTGTGTCGTCGAGT |
| NM_016207        | AAGGTCTGGCTGCTGCTTGGTCTTC | ACCTGTGTCGTCGAGT |
| NM_002009        | AACTGTGCCAGCCTGAGCGACAC | GCAACAAACATTTCCTCCCTTCGTT |

Taken together, DPP4 was found to be highly expressed in adipose cells, and its knockdown contributes to differentiation of human preadipocytes, obviously at an early stage and in a PPARγ-independent way. In preadipocytes DPP4 appears to play a different role than in mature adipocytes where it becomes released and thereby can influence glucose metabolism via incretin (e.g. GLP1) cleavage.

Methods

Cell culture. Primary human preadipocytes from subcutaneous adipose tissue were commercially obtained from PromoCell (Heidelberg, Germany) and Zen-Bio (Durham, NC, USA). They were cultured according to the manufacturer’s protocol and using media obtained from PromoCell. Differentiation was induced at confluent stages (Day 0) by a medium containing d-biotin (8 μg/ml), dexamethasone (400 ng/ml), 3-isobutyl-1-methylxanthin (44 μg/ml), insulin (0.5 μg/ml), L-thyroxine (9 ng/ml) and pioglitazone (4 μg/ml) for 3 days, followed by cultivation in d-biotin (8 μg/ml), dexamethasone (400 ng/ml), insulin (0.5 μg/ml), 3% FCS and pioglitazone (4 μg/ml) until termination of the experiment. Cells from 5 female subjects (aged 28–58) were used for the experiments, with at least two different donors for key experiments.

Lentiviral knockdown. Stable knockdown (KD) of the DPP4 or PPARγ expression was achieved by lentiviral shRNA constructs directed against DPP4 or PPARγ. Lentiviral plasmid vectors containing the shRNA constructs (pLKO.1-U6-sh-NM935-PGK-Puro, pLKO.1-U6-sh-NM712-PGK-Puro and the controls pLKO.1-U6-sh-ctl-PGK-Puro and pLKO.1-CMV-GFP-PGK-Puro) were obtained from Sigma Life Science (St. Louis, MO, USA). Generation of lentiviral particles was performed as described previously40. In brief, human embryonic kidney (HEK293T, ATCC, Wesel, Germany) packaging cells were seeded in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Darmstadt, Germany) on Poly-L-lysine coated 150 mm² dishes.

For transduction, human primary preadipocytes were seeded with a confluence of 30–40%. Within 24 h, cell medium was renewed containing polybrene (3 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and 150 ng (per 6 well) or 20–35 ng reverse transcriptase (per 24 well) virus particles. The GFP expression vector pLKO.1-CMV-GFP-PGK-Puro was used to optimize the transduction protocol, and pLKO.1-U6-sh-ctl-PGK-Puro (“sh-control” or “Shc” for short) was included in each experiment to correct for effects due to the infection process itself. In order to avoid overgrowth by residual untransduced cells in longer-term experiments (> 4 days), a selection step with 5 μg/ml puromycin for 72 hours (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed starting 3 days after infection.

RNA Extraction and Real-Time PCR. Cells were harvested with lysis buffer from the RNeasy Total RNA Extraction Kit (Qiagen, Hilden, Germany), and RNA was isolated according to the manufacturer’s protocol. Complementary DNA was synthesized using Reverse Transcriptase Kit (Roche, Mannheim, Germany). Real-Time PCR reactions were performed with the LightCycler SYBR Green Master mix (Roche, Mannheim, Germany). Complementary DNA was synthesized using Reverse Transcriptase Kit (Roche, Mannheim, Germany). Real-Time PCR reactions were performed with the LightCycler SYBR Green Master mix (Roche, Mannheim, Germany). Real-Time PCR reactions were performed with the LightCycler SYBR Green Master mix (Roche, Mannheim, Germany).

DNA Array Hybridization. Cells were harvested with lysis buffer as described above to extract total RNA. Quality of RNA was controlled by the Agilent 2100 Bioanalyzer platform (Agilent Technologies, Santa Clara, CA, USA); RNA Integrity Number (RIN) was between 9.8 and 10. Amplification and labelling (Cy3 and Cy5) were performed using the Low RNA Input Linear Amp Kit (Agilent Technologies, Santa Clara, CA, USA). Hybridization was conducted on Agilent Whole Genome Oligo (60-mer) 4 × 44K microarrays with the Agilent Gene Expression Hybridization Kit. Agilent’s feature extraction Software was used to determine spot intensities and Cy5/Cy3 ratios after background subtraction with ratios displaying the expression level in KD compared to control in a logarithmic scale. Mean fold changes were calculated from four replicate measurements. The heat map was created by Gene Spring Software (Agilent Technologies, Santa Clara, CA, USA).
Western blotting. Cells were cultured in a 6-well dish and harvested for the experiments or, for signal-
images, incubated in a serum-free basal medium for 24 h. For analyzing signaling pathways, the lat-
ter were activated by treatment with 100 nM insulin (Sigma-Aldrich, Steinheim, Germany) for 10 min. After
washing the cells with ice-cold PBS, cellular proteins were extracted with either RIPA lysis buffer (Cell Signaling
Technology, Danvers, MA, USA) containing Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and
PMSF (Sigma-Aldrich, Steinheim, Germany) for standard Western blotting and signaling experiments or with
lysis buffer from Qiagen (Hilden, Germany) for protein fractionation. The latter was performed according to
the Qproteome Cell Compartment KIT protocol. For SDS-PAGE, 10–30 μg protein were loaded on a gradient 4–15%
polyacrylamide gel in a reducing buffer (Roth, Karlsruhe, Germany). Separated proteins were transferred to a
PVDF membrane (Bio-Rad, Munich, Germany) in a tank blotting apparatus (Bio-Rad, Munich, Germany). Blots
were blocked in tris-buffered saline containing 0.1% Tween and 5% dried milk protein and incubated over night
with anti-DPP4 (1:1000; Abnova, Taipei, Taiwan), anti-PGC1α (1:1000; Cell Signaling Technology, Danvers, MA,
USA) or an antibody against the insulin receptor β subunit (CT-3, 1:300, Santa Cruz Biotechnology, Santa Cruz,
CA, USA) followed by anti-α-actinin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody over-
night at 4 °C. HRP-coupled secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA);
chemiluminescence signals were detected by chemiluminescent reagent (ECL; Thermo Scientific, Rockford,
IL, USA). For signaling experiments, the phosphoproteins were detected by anti-pAkt (Ser473; 1:1000; Cell
Signaling Technology, Danvers, MA, USA) or by anti-pERK (Thr202/Tyr204; 1:500; Cell Signaling Technology,
Danvers, MA, USA) and secondary anti-mouse antibodies (Dianova, Hamburg, Germany). Furthermore, anti-
bodies against total Akt (1:1000; Cell Signaling Technology, Danvers, MA, USA) or ERK (1:1000; Cell Signaling
Technology, Danvers, MA, USA) with secondary anti-rabbit IgG antibodies were used. Quantification of the
ECL-developed bands was performed by densitometric analysis with ImageJ software. The optical density was
normalized to the loading control.

Elisa. Release of DPP4 or leptin from human primary adipocytes were detected in the cell culture supernatants
by ELISA (R&D Systems, Wiesbaden, Germany). Cells were cultured in either growth or differentiation media.
After renewal of media, cells were further incubated for 24 h and finally, cell supernatants were collected for anal-
ysis. The assays were performed according to the manufacturer's protocol and measured in a microplate reader
(Dynex, Chantilly, VA, USA) against a standard curve.

Oil Red O Staining. Preadipocytes were treated with either 10 μM pioglitazone for 10 days or lentiviral par-
ticles according to our standard protocol (see respective subsection). For Oil Red O staining, cells were washed
in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at 4 °C for 1 h. Subsequently, cells were
washed again and incubated with 3 mg/ml Oil Red O solution (Sigma, Steinheim, Germany) for 1 h at room tem-
perature. Finally, the staining solution was replaced by aqua dest., and evaluation of the lipid droplets was done by
light microscopy (Leica Microsystems, Wetzlar, Germany).

Immunofluorescence. For detection of DPP4 by immunofluorescence, human preadipocytes were seeded on
poly-L-lysine coated cover slips. Cells were washed in PBS and fixed with 4% paraformaldehyde at 4 °C for 1 h.
Blocking was done in a 0.1% BSA solution (Sigma-Aldrich, Steinheim, Germany) for 1 h. The incubation with
primary antibody anti-DPP4 (Abnova, Taipei, Taiwan) was performed in a 1:50 dilution overnight. Secondary
antibody (Life Technologies, Darmstadt, Germany) was incubated in a 1:200 dilution for 90 min. in the dark. Cells
were counterstained with Texas Red Phallolidin (Life Technologies, Darmstadt, Germany) and the fluorescense
signals were visualized on a laserscanning microscope (Zeiss, Jena, Germany) in a z-stack analysis.

Lipolysis. Mature adipocytes were washed in PBS and incubated in serum free medium for 24 h. Then
medium containing 10 μM forskolin (Sigma-Aldrich, Steinheim, Germany) or vehicle DMSO was added. After
24 h, cell culture supernatants were collected for analysis of leptin and DPP4 by ELISA. For confirmation of
lipolysis, free glycerol was determined in the supernatant. To this end, free glycerol reagent (Sigma-Aldrich,
Steinheim, Germany) was added to the supernatants and incubated for 3 h at room temperature. Finally, the solution
was replaced by aqua dest., and evaluation of the lipid droplets was done by

Statistical analysis. All data are represented as means ± SEM. Determination of significant differences was
done by Student t test, linear regression analysis or between groups by ANOVA followed by Dunnett post-test.
Samples were normalized against controls by inclusion of a baseline correction. Statistical analyses were per-
formed by Graph Pad Prism Software (Graph Pad Software, La Jolla, CA, USA).

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Author Contributions
P.Z., P.M. and K.R. conceived the experiments, P.Z. and J.C. conducted the experiments, A.K. constructed the lentiviral particles, P.Z. and P.M. analyzed the results. All authors reviewed the manuscript.

Additional Information
Accession code: Gene chip data shown in Fig. 2C were submitted to GEO database; the accession number is GSE75328.

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