Brief communication

MOLECULAR METABOLISM

A novel crosstalk between Alk7 and cGMP signaling differentially regulates brown adipocyte function

Aileen Balkow [1], Johanna Jagow [1], Bodo Haas [1,2], Franziska Siegel [1], Ana Kilić [1], Alexander Pfeifer [1,*]

ABSTRACT

Objective: Obesity is an enormous burden for patients and health systems world-wide. Brown adipose tissue dissipates energy in response to cold and has been shown to be metabolically active in human adults. The type I transforming growth factor β (TGFβ) receptor Activin receptor-like kinase 7 (Alk7) is highly expressed in adipose tissues and is down-regulated in obese patients. Here, we studied the function of Alk7 in brown adipocytes.

Methods: Using pharmacological and genetic tools, Alk7 signaling pathway and its effects were studied in murine brown adipocytes. Brown adipocyte differentiation and activation was analyzed.

Results: Alk7 is highly upregulated during differentiation of brown adipocytes. Interestingly, Alk7 expression is increased by cGMP/protein kinase G (PKG) signaling, which enhances brown adipocyte differentiation. Activin AB effectively activates Alk7 and SMAD3 signaling. Activation of Alk7 in brown preadipocytes suppresses the master adipogenic transcription factor PPARγ and differentiation. Stimulation of Alk7 during late differentiation of brown adipocytes reduces lipid content and adipogenic marker expression but enhances UCP1 expression.

Conclusions: We found a so far unknown crosstalk between cGMP and Alk7 signaling pathways. Tight regulation of Alk7 is required for efficient differentiation of brown adipocytes. Alk7 has differential effects on adipogenic differentiation and the development of the thermogenic program in brown adipocytes.

Keywords Alk7; cGMP; Brown adipocytes; UCP1; Activin

1. INTRODUCTION

Obesity is not only an esthetic, but a major health issue with a steadily growing, global prevalence. Health consequences of overweight and obesity include diabetes, cardiovascular diseases and some types of cancer. Presently, there are only few drugs that can be used to treat obesity. Brown adipose tissue (BAT) has emerged as a potential target for the development of novel anti-obesity drugs. BAT dissipates energy in the form of heat upon cold exposure or β-adrenergic stimulation [1–4]. β-adrenergic signaling induces break-down of triglycerides to free fatty acids (FFA) and glycerol. The FFA serve as fuel for the mitochondrial uncoupling protein 1 (UCP1), which disrupts the proton gradient through the inner mitochondrial membrane, thereby funneling energy to produce heat instead of ATP in brown adipocytes (BA). Taken together, activation of BAT leads to increased energy expenditure, which has positive effects on whole-body metabolic homeostasis.

The delineation of regulatory pathways would be an important basis for development of novel BAT-centered therapies. Recently, cyclic guanosine monophosphate (cGMP) was identified as a major factor that controls adipogenic and thermogenic differentiation of brown adipocytes [5–7]. The effects of cGMP in BAT are mediated by protein kinase G (PKG) [7,8].

The transforming growth factor-β (TGF-β) superfamily has been implicated in different biological processes including tumor growth and white adipose tissue inflammation amongst others [9,10]. The type I TGFβ receptor Activin receptor-like kinase 7 (Alk7) is highly expressed in adipose tissues of rodents and humans [11,12]. Interestingly, Alk7 expression is reduced in obese patients and negatively correlates with clinical parameters of metabolic disease [11], indicating that Alk7 is relevant for the maintenance of a healthy lean state. Moreover, global Alk7 knockout mice are partially resistant to diet-induced obesity in comparison to their wildtype (wt) littermates [13] but develop insulin resistance and liver steatosis [14].

The TGFβ receptor family is heterogenous and its receptors can be activated by a plethora of ligands. Known ligands for Alk7 are Nodal [15], GDF11 [16], GDF3 [13] and Activin AB and B [17]. After ligand binding, SMAD2 and 3 are phosphorylated by Alk7 as shown in rat PC12 pheochromocytoma cell line [18], murine MIN6 insulinoma cells

[1]Institute of Pharmacology and Toxicology, University Hospital Bonn, University of Bonn, 53127 Bonn, Germany [2]Division of Genetic and Reproductive Toxicology, Federal Institute for Drugs and Medical Devices, Kurt-Georg-Kiesinger-Allee 3, 53175 Bonn, Germany

*Corresponding author. Institute of Pharmacology and Toxicology, University of Bonn, 53127 Bonn, Germany. E-mail: alexander.pfeifer@uni-bonn.de (A. Pfeifer).

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[19] and the murine white adipocyte cell line 3T3-L1 [20]. Phosphorylated SMADs form complexes with the cofactor SMAD4 and regulate gene expression together with additional transcription factors [21]. In addition to the canonincal SMAD2/3 pathway, SMAD-independent pathways of Alk7 signaling include MAPK, RhoA/ROCK, AKT/PI3K and Wnt/β-Catenin pathways [22].

So far, studies of Alk7 focused on its role in white adipose tissue. Here, we investigated its role in brown adipocytes and a possible interplay of cGMP with Alk7 signaling. We found that Alk7 expression is regulated by cGMP/PKG pathway. Alk7 activation differentially regulates adipogenic and thermogenic differentiation of brown adipocytes.

2. MATERIAL AND METHODS

2.1. Adipogenic differentiation

Stromal vascular fraction (SVF) cells isolated from BAT of wt or $PKGI^{-/-}$ mice were immortalized and differentiated into mature brown adipocytes as described previously [7,8,23,24]. In short, immortalized SVF cells were seeded and cultured in growth medium [DMEM supplemented with 5% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S)]. Two days after seeding (day −2) the medium was exchanged to differentiation medium (growth medium supplemented with 20 nM insulin and 1 nM triiodothyronine). Differentiation was induced two days later (day 0) by replacing the medium with induction medium (differentiation medium supplemented with 0.5 mM isobutylmethylxanthine and 1 μM dexamethasone) for 48 h. Until day 7 post induction the medium was replenished with differentiation medium every second day. Treatment with either 200 μM 8-Br-cGMP or 8-pCPT-cGMP started on day −2. Chronic Activin AB (both 10 ng/ml) treatment started on day −2 or day 4 of differentiation, as indicated in the respective experiments. For SMAD3 phosphorylation experiments cells were acutely treated with Activin AB or Activin B (10 ng/ml) for 60 min on day 0 or day 7 of differentiation.

2.2. Lentiviral plasmids and transduction of brown adipocytes

Lentiviral vectors were obtained either by cloning wt (LV-Alk7) or constitutively active (LV-caAlk7; kindly provided by Chun Peng) human Alk7 into the Bam HI and Sal I sites of the vector p156rrlsinPPTCMV, which carries a cytomegalovirus promoter. The control vector (p156rrlsinPPT) contained neither promoter nor transgene (LV-ctrl). The production of lentiviruses and infection of cells were performed as previously described [7,8,23]. In brief, cells were seeded on six-well plates. After 8 h, the medium was changed to medium containing amounts of lentivirus corresponding to 50 ng of viral reverse transcriptase per six-well plate, and incubated overnight. Adipogenic differentiation was performed as described above.

2.3. Measurement of lipolysis

Glycerol release was measured on day 7 in BA that were differentiated in the absence or presence of Activin AB. Cells were washed with lipolysis medium [DMEM without phenol red (Invitrogen)] and incubated with lipolysis medium supplemented with 2% essential fatty acid–free BSA at 37 °C and 5% CO_2 with or without addition of Activin AB (10 ng/ml) or norepinephrine [NE; 1 μM (Sigma–Aldrich)] in the respective samples. After 2 h media were collected and glycerol concentration was determined by addition of free glycerol reagent (Sigma–Aldrich). After an incubation of 5 min at 37 °C, absorption was measured at 540 nm against lipolysis medium, which was not incubated with cells, and a glycerol standard. Glycerol release was calculated and normalized to the protein content of the wells.

2.4. RNA isolation and qPCR analysis

Total RNA was isolated from cells using InnuSOLV (Analytik Jena, Germany) reagent. 500 ng of RNA was reverse transcribed using the Transcriptor First Strand Synthesis Kit (Roche). qPCR was performed with SYBR Green (Roche) or Power SYBR Green (ABI) PCR master mix using the qPCR instruments HT7900 or ViiA7 (both Applied Biosystems). Primers are listed in the Supplementary Table 1. Fold changes were calculated using relative quantification methods with mHPRT serving as internal control.

2.5. Oil Red O staining

Cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the cells were incubated with Oil Red O (Sigma–Aldrich) solution (3 mg/ml in 60% isopropyl alcohol) for 1 h at room temperature, washed with distilled water and visualized.

2.6. Western blot analysis

Protein lysates were prepared as previously described [7,8] with radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail Complete (Roche), 1 mM Na_3VO_4, and 10 mM NaF. Protein contents were determined by the Bradford method. Western blotting was performed as described previously [7,8]. The following antibodies were used: antibodies against aP2, and PPARγ from Santa Cruz Biotechnology; antibodies against PSMAD3, SMAD3, HSL, ATGL from Cell Signaling Technology; antibody against UCP1 from Sigma–Aldrich and antibodies against Tubulin (Dianova). Secondary horse radish peroxidase–linked antibodies against goat (Pierce), mouse (Dianova), and rabbit (Cell Signaling) were used. All bands were quantified by densitometric analysis with Image J software.

2.7. Luciferase reporter assays

HlB1B cells were transiently cotransfected with firefly and Renilla luciferase expression vectors using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24 h after transfection cells were treated with Activin AB or B (10 ng/ml) for another 18 h. Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega) according to the assay protocol. Cell lysates were prepared following the manufacturer's instructions. The activity of the firefly luciferase was normalized to the corresponding Renilla activity value for each sample. The 2000bp Alk7 promoter was divided into three subunits (0–1000bp = Alk7A; 500–1500bp = Alk7B; 1000–2000bp = Alk7C) and each subunit was cloned into the pGL3-basic luciferase vector. The UCP1 (pGL3-basic) promoter luciferase construct was kindly obtained by Dr. Stephan Herzig, Deutsches Krebsforschungszentrum, Heidelberg. Vectors without promoter were used as a negative control. The internal control was pRL-TK vector (Promega) expressing Renilla luciferase under the control of the herpes simplex virus thymidine kinase (TK) promoter.

2.8. Statistical analysis

Values are presented as means ± SEM. Statistical differences among multiple groups were determined using oneway analysis of variance (ANOVA) with Newman–Keuls Multiple Comparison Test, unless otherwise indicated. Unpaired, two-tailed student's *t*-tests were used for single-comparisons. GraphPad Prism 5 was used to calculate P-values.

3. RESULTS

3.1. Alk7 expression increases during brown adipocyte differentiation and is modulated by cGMP/PKGI

To study Alk7 expression during BA differentiation, we used preadipocytes isolated from newborn mice and differentiated them to mature BA (Suppl. 1). Alk7 expression was significantly upregulated between day 4 and day 7 of differentiation reaching a 17- $\pm$ 2.5-fold increase at day 6 of differentiation in comparison to undifferentiated preadipocytes (day -2) (Figure 1A). Mature BA (day 7) showed an 8.3- $\pm$ 1.3-fold higher expression of Alk7 than preadipocytes (Figure 1A). In comparison to Alk7, the expression level of PPARγ significantly increased already at day 2 of differentiation and exhibited no significant further upregulation until the end of differentiation (Suppl. 1B).

To study whether enhanced differentiation is correlated with Alk7 expression, we treated the cells with cGMP, which enhances differentiation of BA [7]. cGMP treatment increased Alk7 mRNA expression 2.1- $\pm$ 1.2-fold and 50.2- $\pm$ 17.6-fold in preadipocytes and in mature BA, respectively, compared to the untreated control (Figure 1B).

Interestingly, Alk7 mRNA expression was reduced by 95 $\pm$ 3.9% in mature BA deficient for PKGI ($PKGI^{-/-}$) as compared to wt BA (Figure 1C) indicating that cGMP signaling controls also basal Alk7 levels in BA. To further examine the mechanism of cGMP/PKGI-dependent regulation of Alk7 expression, the Alk7 promoter was divided into three different parts and cloned into a luciferase reporter backbone (Figure 1D). Luciferase assays were performed in the BA cell-line HIB1B in the presence and absence of cGMP. The 3′ part of the Alk7 promoter (Alk7C) exhibited the highest luciferase activity under basal conditions and cGMP treatment significantly enhanced Alk7C promoter activity by 36%, whereas the other (5′ and middle part) elements of the promoter did not respond to cGMP (Figure 1E).

These data show that Alk7 expression increases during differentiation and that Alk7 expression is regulated by the cGMP/PKGI signaling pathway at the transcriptional level.

3.2. Expression of Alk7 ligands and activation of the Alk7 signaling pathway by Activins

Next, we analyzed the expression of the endogenous Alk7 ligands GDF11, Nodal, GDF3 and Activins in brown preadipocytes and mature BA. Activins are homo- or heterodimers composed of the subunits Inhibin βA and βB. The mRNA of all ligands analyzed was detected in BA and preadipocytes (Figure 2A). Expression of the individual ligands was not significantly changed during BA differentiation (Figure 2A). Nevertheless, we observed major differences in expression levels of the potential Alk7 ligands (Inhibin βA $>$ GDF11 $>$ Inhibin βB $>>$ GDF3 $>$ Nodal) (Figure 2A). Inhibin βA showed the highest expression levels (ca. 300-fold higher in comparison to Nodal), followed by GDF11 ($>$130-fold in comparison to Nodal) and Inhibin βB (more than 50-fold in comparison to Nodal) (Figure 2A). GDF11 is a weak ligand for Alk7 and has previously been shown to predominantly signal via Alk4 and Alk5 [11]. Therefore, we focused on Activin AB (Inhibin βA/Inhibin βB) and B (Inhibin βB) for further experiments.

Treatment of mature BA with Activin AB and B activated the canonical Alk7 downstream signaling pathway resulting in SMAD3 phosphorylation (PSMAD3) (Figure 2B, C). The level of PSMAD3 was significantly higher after treatment with Activin AB than with Activin B. Activin AB-induced PSMAD3 did not increase in adipocytes overexpressing Alk7 (LV-Alk7, Suppl. 2A, 2B) in comparison to control cells (Figure 2B, C) presumably due to already high endogenous expression of Alk7 in mature BA. Since Activins can also signal through Alk4 and Alk5, we studied the effect of a constitutive active mutant of Alk7 (LV-caAlk7, Suppl. 2A, 2B) in BA. LV-caAlk7 also induced PSMAD3 showing that active Alk7 stimulates SMAD3 signaling. Thus, Activin AB effectively activates Alk7 and canonical SMAD signaling in BA.

3.3. Early activation of Alk7 diminishes adipogenic differentiation

Next, we studied the effect of early activation of Alk7 with Activin AB starting at the preadipocyte stage (day -2 to day 7). Treatment with both Activin AB or B induced phosphorylation of SMAD3 in ctrl and LV-

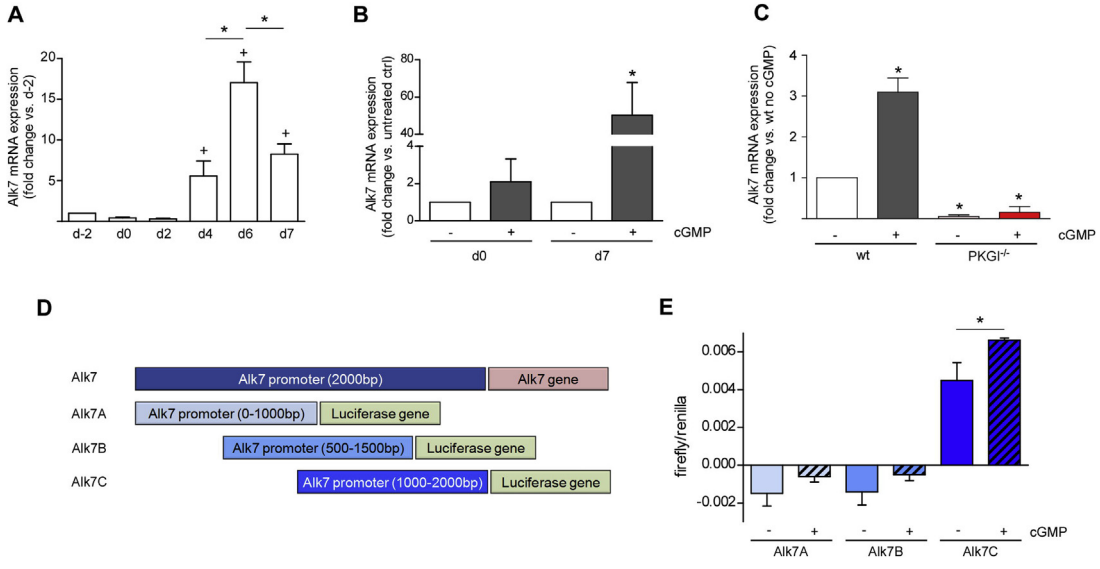

Figure 1: Alk7 expression is upregulated in mature adipocytes and modulated by the cGMP/PKGI pathway. **(A)** Alk7 mRNA expression in brown adipocytes at different time points during differentiation. **(B)** Alk7 mRNA expression after chronic 8-pCPT-cGMP (200 μM) treatment at day 0 and day 7, normalized to untreated controls. **(C)** Alk7 mRNA expression in differentiated brown wt or $PKGI^{-/-}$ adipocytes with and without chronic 8-pCPT-cGMP (200 μM) treatment. **(D)** Scheme of the endogenous Alk7 promoter divided into three different parts (light blue = Alk7A 0–1000 bp; blue = Alk7B 500–1500 bp; dark blue = Alk7C 1000–2000 bp) and cloned into a luciferase-reporter vector. **(E)** Analysis of the Alk7 promoter activity with and without cGMP treatment. Data are presented as mean $\pm$ SEM from 3 independent experiments. *($p < 0.05$; ANOVA) significant difference vs. no cGMP treatment or as indicated; $^{+}$($p < 0.05$; ANOVA) significant difference vs. d-2, d0 and d2.

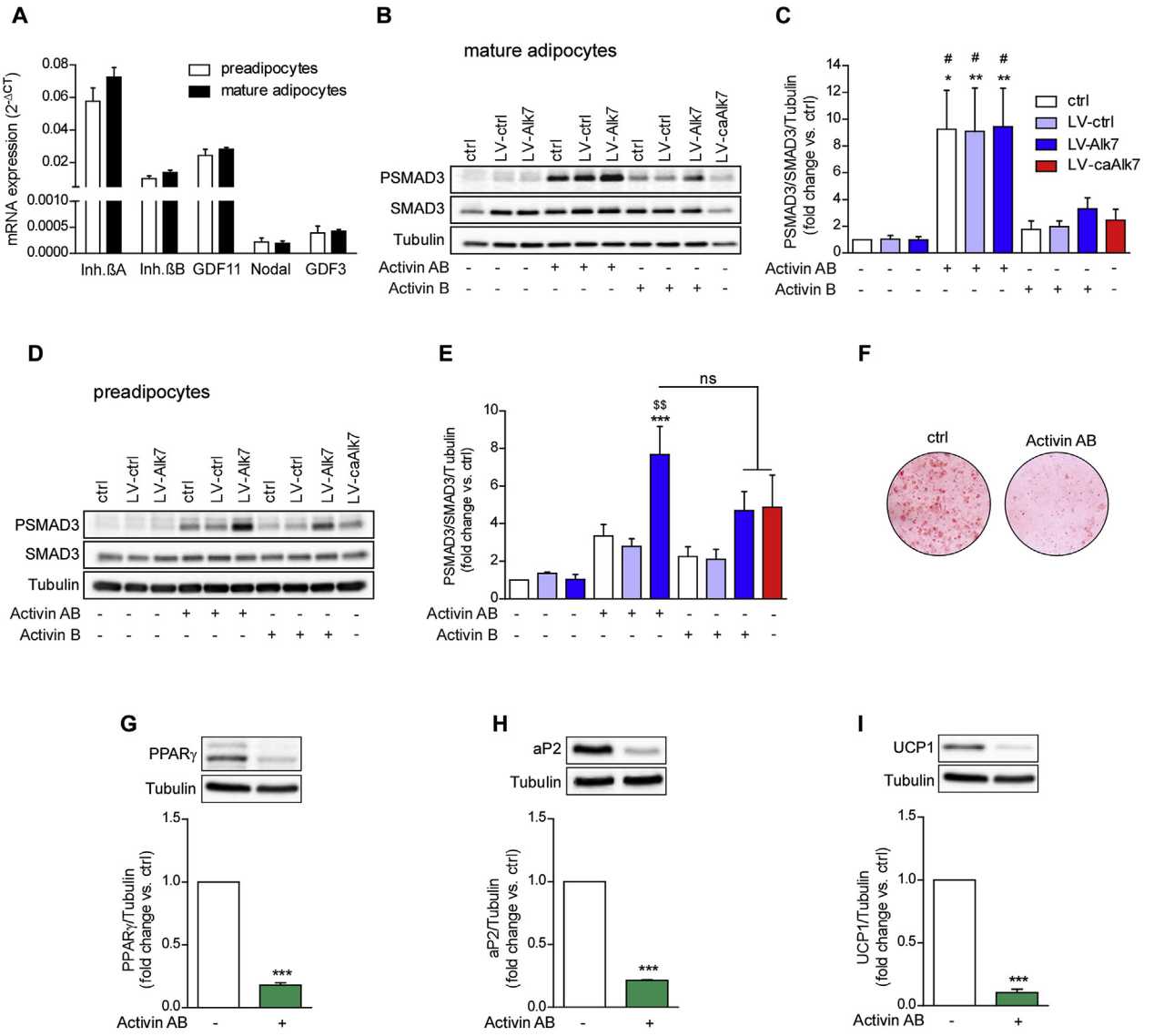

Figure 2: Alk7 signaling is activated by Activins and early activation of Alk7 diminishes adipogenic differentiation. **(A)** mRNA expression of Inhibin βA (Inh.βA), Inhibin βB (Inh.βB), GDF11, Nodal and GDF3 in preadipocytes and mature adipocytes. **(B–E)** Western blot analysis of SMAD3 phosphorylation (PSMAD3) in mature brown adipocytes (day 7) or preadipocytes overexpressing Alk7 with Activin AB or B treatment for 60 min. **(F–I)** Analysis of brown adipocytes chronically treated with Activin AB (day −2 to day 7). **(F)** Representative Oil Red O staining. **(G–I)** Western blot analysis of PPARγ, aP2 and UCP1 expression in comparison to Tubulin. Data are presented as mean ± SEM from 3 to 5 independent experiments. *($p < 0.05$; ANOVA), **($p < 0.01$; ANOVA),***($p < 0.001$; ANOVA) significant difference vs. untreated cells; $^{\$\$}$($p < 0.01$; ANOVA) significant difference vs. Activin AB and Activin B treated ctrl and LV-ctrl cells; $^{\#}$($p < 0.05$; ANOVA) significant difference vs. Activin B treatment and LV-caAlk7 cells.

ctrl preadipocytes albeit not significantly (Figure 2D, E). Preadipocytes overexpressing LV-Alk7 responded to Activin AB with a significant increase in PSMAD3 (Figure 2D, E).

Notably, early Activin treatment suppressed adipogenic differentiation as seen in Oil Red O stainings (Figure 2F). This was confirmed by analysis of PPARγ, aP2 and UCP1 protein levels. PPARγ and aP2 were reduced by 82 ± 1.9% and 79 ± 0.5%, respectively (Figure 2G, H). UCP1 was reduced by 90 ± 2.7% in comparison to untreated cells (Figure 2I). Transduction of preadipocytes with LV-caAlk7 suppressed differentiation with a significant reduction of PPARγ expression by 70 ± 17.2% (Suppl. 2C). These data show that basal levels of SMAD3/Alk7 signaling are low in preadipocytes and that early activation of Alk7 or expression of a constitutively active Alk7 suppresses expression of PPARγ and adipogenic differentiation.

3.4. Differential effects of Alk7 on adipogenic and thermogenic programs in mature BA

To study the role of Alk7 signaling in mature BA, we activated Alk7 during the last three days of differentiation (day 4 to day 7) using Activin AB. Activin AB treatment of mature BA reduced intracytoplasmic lipid content as shown by reduced Oil Red O staining (Figure 3A). Furthermore, Activin AB treatment significantly reduced protein levels of the adipogenic markers PPARγ and aP2 (Figure 3B, C), as well as of the lipases HSL and ATGL (Figure 3D, E). Lipolysis is an important parameter of BA function, because it liberates FFA that activate UCP1 and serve as fuel for thermogenesis. We found a significantly reduced lipolysis in Activin AB treated BA under basal conditions (Figure 3F) as well as after norepinephrine stimulation (Suppl. 2D).

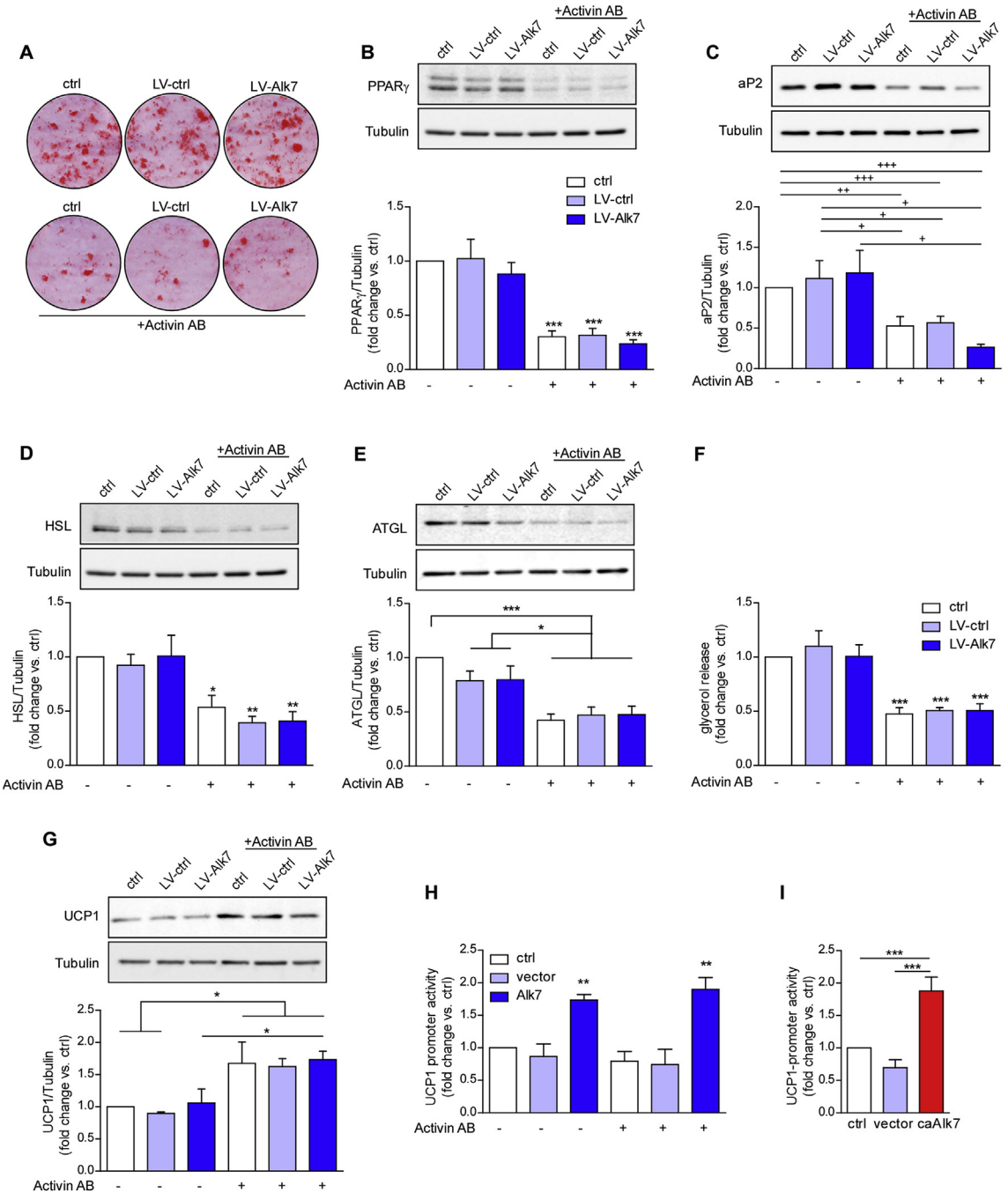

Figure 3: Activation of Alk7 in mature brown adipocytes reduces adipogenic differentiation but enhances UCP1 expression. **(A–G)** Analysis of adipocytes transduced with LV-ctrl or LV-Alk7 virus or untransduced cells treated with and without Activin AB (day 4 to day 7). **(A)** Representative Oil Red O stainining. **(B–E)** Western blot analysis of PPARγ, aP2, HSL and ATGL; Tubulin was used as loading control. **(F)** Analysis of lipolysis by measuring glycerol release of brown adipocytes treated with and without Activin AB (day 4 to day 7). **(G)** Western blot analysis of UCP1. **(H)** UCP1 promoter activity in HIB1B control (ctrl) cells or cells transfected with empty vector or LV-Alk7 with and without Activin AB treatment. **(I)** UCP1 promoter activity in HIB1B control (ctrl) cells or transfected with empty vector or LV-caAlk7. Data are presented as mean ± SEM from 3 to 6 independent experiments. *($p < 0.05$; ANOVA); **($p < 0.01$; ANOVA), ***($p < 0.001$; ANOVA) significant difference vs. all untreated cells or as indicated. $^{+}$($p < 0.05$; t-test), $^{++}$($p < 0.01$; t-test), $^{+++}$($p < 0.001$; t-test) significant difference as indicated.

Unexpectedly, activation of Alk7 increased the protein levels of the thermogenic marker UCP1 more than 1.6-fold (Figure 3G). To further analyze this effect of Alk7 on UCP1 levels, we studied UCP1 promoter activity in HIB1B cells. Cells were transfected with vectors carrying Alk7 or caAlk7 or a control vector and treated with Activin AB. Overexpression of Alk7 alone induced a significant upregulation of the UCP1 promoter activity by 1.7- ± 0.1-fold. Activin AB treatment caused a 1.9- ± 0.2-fold increase as compared to untreated control (Figure 3H). caAlk7 transfection also resulted in a 1.9- ± 0.2-fold increase in UCP1 promoter activity, comparably to Alk7

overexpression (Figure 3I). These data demonstrate that Alk7 negatively regulates the adipogenic program but enhances the thermogenic program by activation of UCP1 transcription in BA.

3.5. Effect of Alk7 on cGMP-mediated regulation of adipogenic and thermogenic programs

cGMP facilitates adipogenic and thermogenic differentiation of BA [7]. Simultaneous activation of Alk7 by Activin AB and cGMP signaling from day 4 to day 7 reduced cGMP effects on adipogenesis as seen in Oil Red O stainings (Figure 4A). Furthermore, Activin AB treatment significantly reduced the effects of cGMP on PPARγ and aP2 expression by 44% and 36.5% respectively (Figure 4B, C). Alk7 signaling had a similar inhibitory effect on the cGMP-induced increase of HSL and ATGL expression (Figure 4D, E). Strikingly, Activin AB and cGMP had additive effects on UCP1 protein expression. cGMP treatment alone lead to a 3.8- ± 0.6-fold increase in UCP1 expression as compared to untreated control cells. Combination of Activin AB and cGMP treatment increased UCP1 protein levels to 8.1- ± 1.7-fold in comparison to untreated control (Figure 4F). In conclusion, cGMP effects on the adipogenic program are counteracted by Alk7 activation, whereas UCP1 expression is positively enhanced.

4. DISCUSSION

4.1. Alk7 expression is modulated by cGMP/PKGI

Only a few years ago, metabolically active BAT was found to be present in human adults [25]. It is estimated that BAT activity is responsible for ca. 5% of daily basal metabolic rate in humans. If activated this would correspond to the dissipation of about 4 kg of white fat per year [26,27]. Therefore, BAT attracted a lot of attention as a way to increase energy expenditure in order to fight the rising prevalence of obesity. The second messenger cGMP acts through PKGI to facilitate adipogenic and thermogenic differentiation of BAT [7]. cGMP regulates BA differentiation through different pathways: (i) cGMP inhibits Rho/ROCK activity, thereby enhancing insulin signaling in BA [7]. (ii) cGMP increases UCP1 and PPARγ transcription [7]. (iii) Here, we show that the TGFβ type I receptor Alk7 is regulated by the cGMP/PKGI signaling pathway, thus, identifying a so far unknown interaction of the cGMP/PKGI with the TGFβ pathway in BA.

Alk7 is expressed at very low levels in brown preadipocytes and expression is significantly upregulated 4 days after induction of differentiation. Treatment with cGMP further enhances Alk7 expression in BA. This effect is blunted in PKGI$^{-/-}$ adipocytes and caused by an

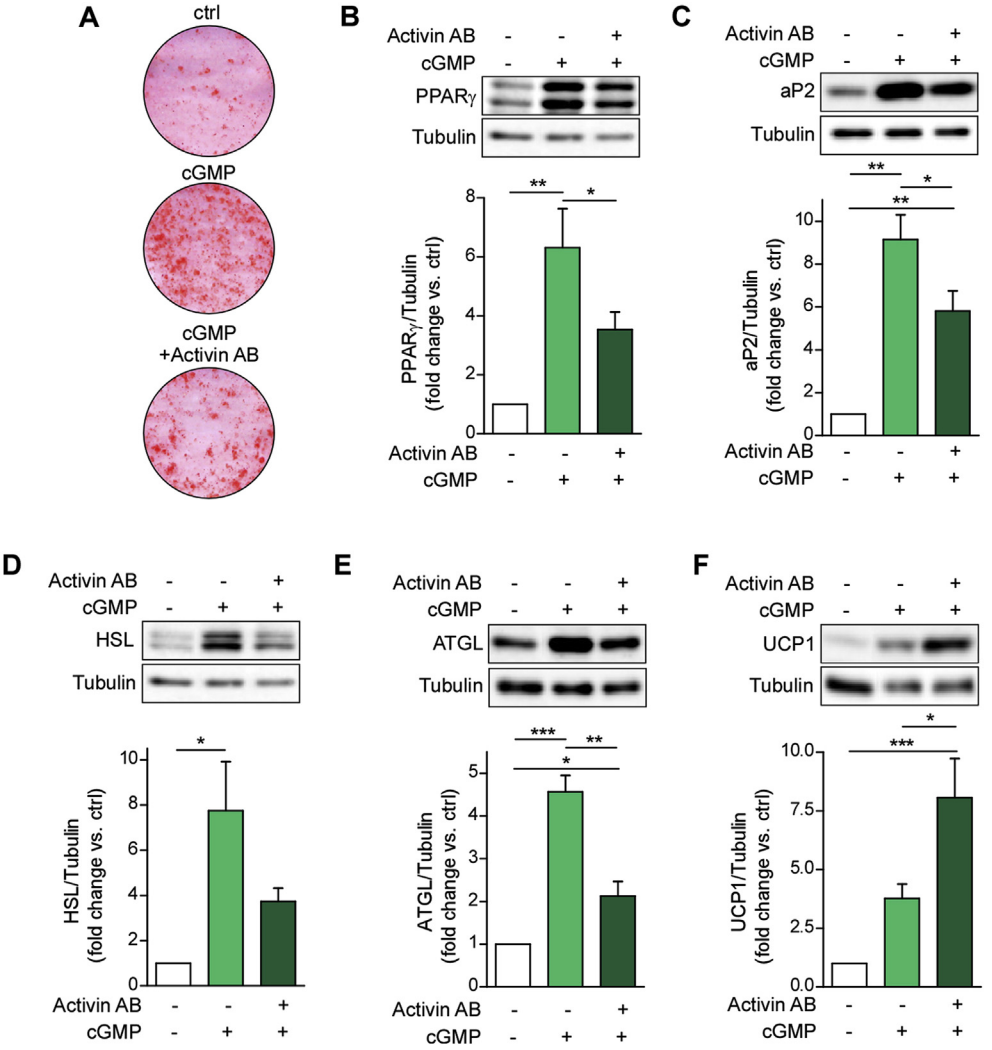

Figure 4: Differential regulation of cGMP effects by Alk7 activation. Analysis of BA chronically treated with 200 μM 8-Br-cGMP (day −2 to day 7) and additionally with Activin AB (day 4 to day 7). **(A)** Representative Oil Red O stainings. **(B–F)** Western blot analysis of PPARγ, aP2, HSL, ATGL and UCP1 (Tubulin loading control). Data are presented as mean ± SEM from 3 to 6 independent experiments. *($p < 0.05$; ANOVA); **($p < 0.01$; ANOVA), ***($p < 0.001$; ANOVA) significant difference as indicated.

increase in Alk7 promoter activity. Moreover, basal Alk7 levels in PKGI$^{-/-}$ BA are reduced to ~5% of the levels observed in wt cells. The type of cGMP-dependent Alk7 regulation is different from the previously reported direct interaction of PKGI with the TGFβ family member bone morphogenetic protein (BMP) receptor [28].

4.2. Alk7 acts as a potential brake for cGMP effects on adipogenic differentiation

Several ligands for Alk7 have been described including Nodal [15], GDF11 [16], GDF3 [13] and Activin AB and B [17]. We found, that the subunits of Activins, i.e. Inhibin βA and Inhibin βB, are most highly expressed in BA. Activin AB induced SMAD3 phosphorlyation most efficiently demonstrating that Alk7 signaling occurs through the canonical pathway in BA. Alk7-mediated activation of SMAD3 significantly reduced abundance of the master adipogenic transcription factor PPARγ in BA, which is induced in early adipogenesis. Stimulation of Alk7 during early differentiation had a deleterious effect and abrogated BA differentiation due to the significantly reduced expression of PPARγ. Interestingly, reduced PPARγ expression was only seen after activation of Alk7 by Activins, whereas sole overexpression of Alk7 did not change PPARγ levels indicating that Alk7 expression and activation needs to be tightly regulated during BAT differentiation.

It was shown previously that activated SMAD3 suppresses PPARγ expression in the 3T3-L1 white adipocyte cell line. The underlying mechanism for SMAD3-dependent regulation of PPARγ is the interaction of SMAD3 with C/EBP and the subsequent repression of C/EBP-mediated transcription of PPARγ promoter [29]. Moreover, Yogosawa et al. have shown that SMAD3 disrupts the positive feedback loop between the adipogenic master regulators PPARγ and C/EBPα in white adipocytes [20].

Alk7 activation during the end/late stage of differentiation (last 3 days of adipogenesis) resulted in differential effects on the adipogenic and thermogenic programs of BA. Abundance of adipogenic markers, lipid content and lipolysis was significantly reduced in Activin AB treated cells. In line with these findings, Guo et al. recently published that Activin B treatment of mouse embryonic fibroblast (MEF) during differentiation to white adipocytes results in reduced levels of PPARγ and HSL, as well as reduction of lipolysis [30]. Unexpectedly, we found that protein expression of UCP1 is upregulated after Alk7 activation in BA, by enhancing UCP1 promoter activity through SMAD activation.

The differential effects of Alk7 in mature BA were especially pronounced after cGMP treatment. On one hand, we propose that Alk7 acts as a potential safety mechanism to ensure that overstimulation of the adipogenic program by cGMP is avoided. On the other hand, Alk7 activation together with cGMP treatment had an additive effect on UCP1 expression and doubled the amount of UCP1 protein as compared to cGMP treatment alone. In obese patients with decreased Alk7 expression [11], it could be of clinical interest to enhance cGMP signaling in BAT to endogenously increase Alk7 expression and boost UCP1 activity.

5. CONCLUSION

The type 1 TGFβ receptor Alk7 is highly expressed in BA. We found that Alk7 and cGMP/PKGI signaling are tightly connected in BA to ensure a balance between the adipogenic and thermogenic program — favoring the thermogenic capabilities of BA if Alk7 is activated together with cGMP. Understanding the link between the cGMP/PKGI and Alk7 signaling pathway could proof beneficial when looking for new targets for anti-obesity drug development.

AUTHOR CONTRIBUTIONS

A.B. designed and performed most of the experiments, analyzed the data and wrote the manuscript; J.J. designed and performed; F.S. designed, performed and analysed Alk7-promoter experiments; B.H. performed Alk7 gene expression in PKGI$^{-/-}$ BA, cloned LV-Alk7 constructs; A.K. designed experiments; A.P. supervised all experiments and wrote the manuscript.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.06.003

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