Alk7 signaling and direct lentivirus injection as novel approaches to investigate brown fat development and function

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Abbreviations

AAV	adeno-associated virus
Alk7	activin receptor-like kinase 7
aP2	fatty acid binding protein 4
ATGL	adipose-triglyceride lipase
ATP	adenosine triphosphate
BAT	brown adipose tissue
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte
BMI	body mass index
BMP	bone morphogenic protein
caAlk7	constitutively active Alk7
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
C/EBPα	CCAAT/enhancer-binding protein alpha
DIO	diet-induced obesity
DNA-MI	DNA-microinjection
FGF21	fibroblast growth factor 21
GDF	growth and differentiation factor
GFP	green fluorescent protein
GLP1	glucagon-like peptide 1
GqQL	constitutively active Gq protein
GTP	guanosine triphosphate
HFD	high-fat diet
HSL	hormone-sensitive lipase
LV	lentiviral vectors
NE	norepinephrine
NO	nitric oxide
NP	natriuretic peptides
NST	non-shivering thermogenesis
PDE	phosphodiesterase
pGC	particulate guanylyl cyclase
PGC1α	PPARγ coactivator 1 alpha

PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
PPARγ	peroxisome proliferator-activated receptor gamma
sGC	soluble guanylyl cyclase
TGFβ	transforming growth factor beta
T_3	triiodthyronine
UCP1	uncoupling protein 1
WAT(i/g)	white adipose tissue (inguinal/gonadal)
WHO	world health organization

1 Introduction

1.1 How to treat the worlds obesity pandemic

Over the past decades the population of the world has gained weight (Ng et al., 2014). Weight gain is the result of an imbalance between energy intake and consumption. The most recent global health observatory data from the world health organization (WHO) on overweight and obesity has shown that in 2014, around 39% of adults aged 18 and older were overweight (body mass index (BMI) ≥25 kg/m²) and 13% were obese (BMI ≥30 kg/m²) (World Health Organization, 2016). Consequences of overweight and obesity are detrimental not only for the health of affected people but also for the economy, e.g. rising direct medical costs, productivity costs, transportation costs and human capital costs (Hammond and Levine, 2010). Particularly direct medical costs arise from diseases associated with overweight and obesity, i.e. the metabolic syndrome. Obese patients suffering from the metabolic syndrome usually fulfil at least two of the following criteria: excessive abdominal fat, hypertension, dyslipidemia and insulin resistance, type 2 diabetes, non-alcoholic fatty liver disease, cardiovascular complications and certain types of cancer (Grundy et al., 2004). It is estimated that around 2.8 million adults die every year as a result of being overweight or obese (World Health Organization, 2016). A systematic review and meta-analysis has associated obesity with a significantly higher all-cause mortality (Flegal et al., 2013). Until today, therapeutic options to treat overweight and obesity are rare. The basic therapy for the treatment of obesity includes dietetic treatment, exercise therapy and behavior therapy. Further therapeutic options like bariatric surgery and pharmacological interventions are only indicated if basic therapies do not lead to desired weight loss effects (Deutsche Adipositas-Gesellschaft (DAG) et al., 2014). In Germany, only two

pharmacological drugs have been approved for the treatment of obesity by the Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM): Orlistat und Liraglutide (as of October 2016). The inhibitor of pancreatic and gastrointestinal lipases Orlistat blocks absorption of 30% of ingested fat when eating a balanced diet (30% fat diet) (Zhi et al., 1994), leading to a reduction in energy intake. Liraglutide is a glucagon-like peptide 1 (GLP1) receptor agonist that stimulates insulin secretion and has originally been approved for the treatment of type 2 diabetes (Bray et al., 2016). Because it delays emptying of the stomach and increases the feeling of satiety it has received additional approval for the treatment of adipositas in March 2015. Even though these drugs have proven to be useful in weight loss, they also have several drawbacks. Orlistat causes severe gastrointestinal side-effects (mainly steatorrhea), which are a strong burden for patients. Liraglutide needs to be injected subcutaneously and is therefore rather impractical for long-term application. All in all, there is an urgent need to find novel therapeutics against the ever growing pandemic of obesity. Importantly, so far we are lacking a treatment that directly targets adipose tissues, leading to enhanced "burning" of fat and circumventing major side effects that arise by targeting broadly expressed factors/receptors.
In this thesis, a novel crosstalk of two signaling pathways in adipocytes (Publication 1) as well as a new way to deliver genes into adipose tissues (Publication 2) is investigated. The presented findings could be useful for the development of new therapeutic approaches for the treatment of obesity.

1.2 Three shades of fat

Basically, mammals have two ways to store energy: 1. "short-term" energy storage in the form of glycogen in liver and muscles and 2. "long-term" energy storage in the form of triglycerides in adipose tissues. The polysaccharide glycogen functions as the primary short-term energy source, which is broken-down by enzymes in the liver and muscle upon fasting and exercise for fast energy release (Adeva-Andany et al., 2016). When glycogen storages are emptied, the body switches to fat catabolism for further energy supply. The advantage of triglycerides over glycogen as energy storing molecule is that

triglycerides do not need to be stabilised with water and are therefore very energy dense (Berg et al., 2002). The main tissue to store triglycerides is white adipose tissue (WAT). In times of high caloric intake, WAT is able to accumulate excessive energy in the form of fat in unilocular lipid droplets in white adipocytes (Cinti et al., 1985). Up to a certain extent, fat accumulation merely leads to an enlargement of adipocyte size (hypertrophy), whereas further energy administration also increases adipocyte number (hyperplasia) (Krotkiewski et al., 1983). Next to storing energy as triglycerides (lipogenesis) and releasing glycerol and fatty acids as energy source (lipolysis), WAT also functions as an endocrine organ which secretes a plethora of adipokines, cytokines and chemokines, thereby regulating whole-body metabolism (Galic et al., 2009; Vazquez-Vela et al., 2008). Two very prominent examples of such are adiponectin and leptin. Both adipokines mediate positive effects on metabolically active organs, like the adipose tissue itself, pancreas and liver (Stern et al., 2016), thereby positively influencing metabolism.

Besides WAT, mammals posses another type of adipose tissue with discrete functions: brown adipose tissue (BAT) (Gesta et al., 2007). This type of adipose tissue was first described to be involved in heat production in hibernating mammals in the 1960's (Hull and Segall, 1965; Smith, 1961). The function of BAT is to utilize stored chemical energy for the production of heat in a process called non-shivering thermogenesis (NST) (Foster and Frydman, 1978). The protein responsible for NST is the uncoupling protein 1 (UCP1). Brown adipocytes feature a high abundance of mitochondria, in which UCP1 is spanning the inner membrane to disrupt the proton gradient (Cinti et al., 1989; Heaton et al., 1978; Ricquier and Kader, 1976). Consequently, the excessive energy is no longer transformed to adenosine triphosphate (ATP) but is dissipated as heat (Nicholls et al., 1978). One major stimulus to activate BAT is cold. BAT is highly innervated by the sympathetic nervous system, which releases norepinephrine (NE) upon cold stimulation (Hull and Segall, 1965). NE induces the production of the second messenger cyclic adenosine monophosphate (cAMP) especially through β_3-adrenoceptors in brown adipocytes (Rubio et al., 1995), which in turn activates cAMP-dependent protein kinase (PKA). PKA phosphorylates lipases like hormone-sensitive lipase (HSL) and adipose-triglyceride lipase (ATGL), resulting in the hydrolysis of triglycerides to glycerol and free fatty acids (Garton et al., 1989; Kim et al., 2016), which finally serve as fuel for UCP1 dependent proton

leakage and thermogenesis (Fedorenko et al., 2012). In addition, PKA induces UCP1 expression in specific adipose depots to increase energy expenditure and improve metabolic health (Dickson et al., 2016).

BAT is essential for the maintenance of a normal body temperature in human infants as their muscles are thus far not able to sustain euthermic conditions (Cannon and Nedergaard, 2004). The interest in BAT has dramatically increased, since it was found to be metabolically active in human adults (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). It is estimated that BAT activity can account for about 5% of the basal metabolic rate (van Marken Lichtenbelt and Schrauwen, 2011), corresponding to an amount of energy equivalent to consuming approximately 4.1 kg of fat within one year (Virtanen et al., 2009). However, BAT activity depends on sex (female > male), age (young > old), BMI (low > high) and ambient temperature (cold > warm) (Enerbäck, 2010). Especially in terms of reduced BAT activity in obese patients, it still remains elusive whether an increase in BMI leads to a loss of BAT function or vice versa.

In addition to BAT, inducible brown adipocytes called brite (brown-in-white) or beige adipocytes also exist in WAT. These inducible brown cells are causative for a "browning" effect in WAT upon cold acclimatisation and are likewise dissipating energy as heat (Harms and Seale, 2013). Their abundance varies significantly between adipose depots, with the highest numbers found in inguinal and retroperitoneal fat and much lower numbers seen in perigonadal fat (Frontini and Cinti, 2010). Beige adipocytes share major characteristics with classical brown adipocytes, including multilocular fat droplets, a high mitochondrial content and expression of a brown-like gene program (Pfeifer and Hoffmann, 2014). On the other hand, they originate from two different precursor cell lines. Lineage tracing studies have revealed that classical brown adipocytes as well as skeletal muscle cells derive from a Pax7+/Myf5+ lineage, whereas white and beige adipocytes derive from Pax7-/Myf5- cells (Seale et al., 2008). It is still under debate, whether mature white adipocytes have the ability to transdifferentiate into beige adipocytes (Frontini et al., 2013; Vitali et al., 2012) or whether beige cells derive from a separate precursor cell line, which shares the same origin as white adipocytes (Lee et al., 2012; Vegiopoulos et al., 2010). This might be different for varying adipose depots. A multitude of ligands to induce browning and positive effects on whole-body metabolism have been described already,

amongst those are fibroblast growth factor 21 (FGF21), bone morphogenic protein 7 (BMP7), orexin, adenosine, GLP1, irisin, triiodothyronine (T_3) and natriuretic peptides amongst others (Forest et al., 2016). Consequently, it is clear that activiating BAT as well as browning of WAT results in positive properties for metabolism and obesity-linked comorbidities (Kim and Plutzky, 2016).

1.3 Two signaling pathways involved in adipocyte development and function

Development and function of white and brown adipocytes depends on a multitude of different signaling pathways, which have only partially been identified so far. One aspect of this work was the identification of a novel crosstalk between two very important pathways in brown adipocytes, i.e. the cyclic guanosine monophosphate (cGMP) and the activin receptor-like kinase 7 (Alk7) pathways, which will be introduced in the following sections.

1.3.1 The cGMP signaling pathway in adipocytes

The second messenger cGMP is an important effector molecule, regulating diverse functions in many cells and tissues, e.g. phototransduction in photoreceptor cells of the eye, or vasodilation and bronchodilation by smooth muscle cells (Hamad et al., 2003; Sauzeau et al., 2000). Soluble (sGC) or particulate guanylyl cyclases (pGC) - activated by NO and natriuretic peptides, respectively - convert guanosine triphosphate (GTP) into cGMP (Potter, 2011). Downstream effectors of cGMP are cGMP-gated ion channels (mostly present in photoreceptors of the eye), cGMP-converting phosphodiesterases (PDEs) and most importantly cGMP-dependent protein kinases (PKG) (Francis et al., 2010). Activated PKG phosphorylates serine/threonine residues, thereby modulating several downstream proteins like RhoA (Haas et al., 2009; Sauzeau et al., 2000), RGS2 (Tang et al., 2003) and others, finally leading to transcriptional regulation of many different genes (Pilz and Casteel, 2003). Importantly, it has been

presented by our working group that PKGI is crucial for the induction of the master adipogenic factor peroxisome proliferator–activated receptor γ (PPARγ) as well as proteins of the thermogenic program (i.e. UCP1 and PPARγ-coactivator 1α (PGC1α)) in brown and white adipocytes (Haas et al., 2009; Mitschke et al., 2013). In addition, major components of the cGMP/PKGI pathway are expressed in brown and white adipocytes as well as adipose tissues (Haas et al., 2009; Mitschke et al., 2013; Nisoli et al., 2003). A graphical overview of the cGMP singaling pathway in brown adipocytes is demonstrated in Figure 1.

Analysis of mouse models for various up- and downstream effectors of cGMP have demonstrated an involvement of the cGMP/PKGI signaling pathway in the regulation of metabolism. Global $sGC\beta_1$ knockout mice have a severe intestinal phenotype, leading to survival of mice only with special diets (Friebe and Koesling, 2009). Nevertheless, ablation of $sGC\beta_1$ reduces BAT mass, BAT-dependent body surface temperature and UCP1 gene expression in newborn mice, indicating a crucial role of sGC in BAT differentiation and function (Hoffmann et al., 2015). Alterations in the natriuretic peptide/pGC pathway similarly verify a positive influence of cGMP on resistance to body weight gain, fat accumulation and insulin sensitivity (Bordicchia et al., 2012; Inuzuka et al., 2010; Miyashita et al., 2009; Tamura et al., 2004). Correspondingly to $sGC\beta_1$ knockout mice, PKGI knockout mice suffer from intestinal dysfunction leading to premature death (Pfeifer et al., 1998). Interestingly, newborn mice display reduced BAT mass and function (Haas et al., 2009), indicating that PKGI is essential for triggering cGMP effects in BAT. Furthermore, endogenous increase of cGMP levels via inhibition of PDE5 using sildenafil induces browning of inguinal WAT (WATi) in C57Bl/6 mice after short-term treatment (7 days) (Mitschke et al., 2013) as well as reducing body weight and improving energy balance after long-term (i.e. 12 weeks) HFD feeding (Ayala et al., 2007). These studies demonstrate a beneficial role of the cGMP/PKGI signaling pathway on whole-body metabolism and especially on brown fat function as well as browning of WAT.

cGMP is involved in the regulation of many genes in diverse tissues and cell types (Pilz and Casteel, 2003). cGMP-regulated transcription factors include the cAMP-response element binding protein CREB, the serum response factor SRF, and the nuclear factor of activated T-cells NF/AT. Furthermore, it has been shown in several different cell lines, including the white adipocyte cell line 3T3-L1 and primary brown adipocytes, that NO induces mitochondrial biogenesis (Nisoli et

al., 2003). The proposed molecular mechanism underlying this beneficial effect is a crosstalk of the cGMP/PKGI signaling pathway with the RhoA and insulin pathway in brown fat cells, which induces mitochondrial biogenesis and adipogenic differentiation (Haas et al., 2009). Publication 1 reveals the gene encoding for Alk7 as a novel gene regulated by cGMP as well as a crosstalk between the cGMP and Alk7 signaling pathways in brown adipocytes.

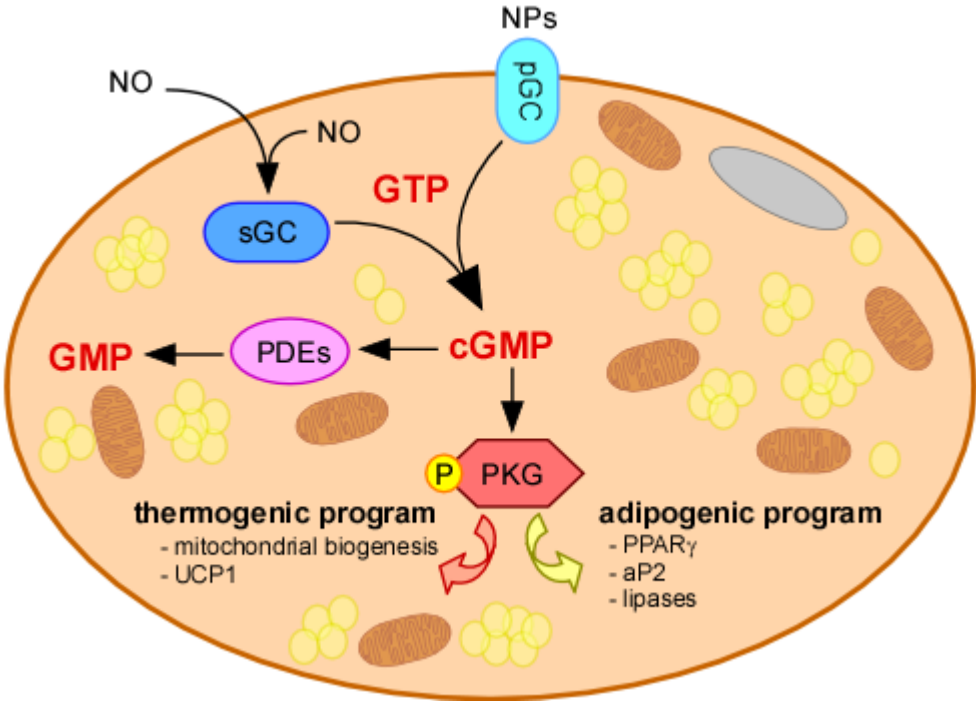

Figure 1: Graphical demonstration of the cGMP-signaling pathway in brown adipocytes. For details see main text.

1.3.2 The TGFβ signaling pathway

Alk7 is a membrane-bound type I receptor of the superfamily of TGFβ receptors. Signal transduction of this pathway occurs via activation of two types of receptors: type I and type II serine/threonine kinase receptors, which are generally present in the membrane as receptor dimers. Five type II and seven type I receptors (Alk1-7) have been identified. Upon ligand binding, a type II receptor dimer recruits a type I receptor dimer and phosphorylates it, generating a hetero-tetrameric receptor complex (Derynck and Zhang, 2003). Depending on the ligand bound, specific combinations of type I and type II receptors are formed. Type I receptor phosphorylation in turn leads to phosphorylation and thus activation of receptor-regulated Smads (R-Smads). There exist five different

R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8, which are activated by different receptor subtypes (Feng and Derynck, 2005). Activated R-Smads form a heteromeric complex with the co-factor Smad4. This complex in turn translocates into the nucleus and acts as transcription factor inducing or repressing gene transcription (Shi and Massague, 2003). One can differentiate between two major subgroups in the TGFβ signaling pathway, depending on the Smads intracellularly activated (see table 1). Smads 1, 5 and 8 are mainly activated by BMPs, which signal for the most part through BMP type I and II receptors. The classical TGFβ signaling pathway results in activation of Smad2/3. Ligands to activate these are TGFβ itself, activins, growth and differentiation factor (GDF) 3 and 11 and nodal amongst others (Feng and Derynck, 2005). The binding of each of these ligands is specific for a certain complex of type I and type II receptors (ten Dijke and Hill, 2004).

Table 1: List of R-Smads with corresponding type I and type II receptors as well as their main ligands. Alternative names are presented in brackets. Further details are given in the main text. Abbreveations: AcvRIa/b/c = Activin receptor type I a/b/c; AcvRIIa/b = Activin receptor type II a/b; AcvRLI = Activin receptor ligand type I; AMH = Anti-Müllerin hormone; AMHRII = AMH receptor type II; BMPRIa/b = BMP receptor type I a/b; BMPRII = BMP receptor type II; TβRI/II = TGFβ receptor type I/II.

R-Smad	Type I receptor	Type II receptor	Ligands
Smad1	Alk1 (AcvRLI)	BMPRII	BMP2/4
Smad5	Alk2 (AcvRIa)	AMHRII	BMP6
Smad8	Alk3 (BMPRIa)		BMP7
	Alk6 (BMPRIa)		AMH
Smad2	Alk4 (AcvRIb)	AcvRIIa	TGFβ1/2/3
Smad3	Alk5 (TβRI)	AcvRIIb	Activin A/AB/B
	Alk7 (AcvRIc)	TβRII	Nodal
			GDF1/3/11/15

1.3.3 Alk7 and its role in metabolism

The activin-receptor type Ic (AcvR1c, Alk7) has first been discovered as an orphan receptor in the rat brain in 1996 (Ryden et al., 1996; Tsuchida et al., 1996). In the same year it has also been isolated from a rat prostate cDNA library as well as adipose tissue (Kang and Reddi, 1996). Analysis of Alk7 expression in different human tissues has discovered Alk7 abundance in brain, pancreas, heart, colon, small intestine and ovary (Bondestam et al., 2001). Adipose tissue has been revealed to be the major expression site of Alk7 in humans and mice years later (Carlsson et al., 2009; Murakami et al., 2012). The intracellular domain of Alk7 features a strong convergence to other TGFβ type I receptors (accordance in up to 78% of the amino acid sequences compared to the receptors Alk4 and Alk5), but a rather distinct extracellular domain (Ryden et al., 1996). Due to this convergence, Alk4, Alk5 and Alk7 signal through the same canonical downstream pathway (i.e. Smad2/3) (Watanabe et al., 1999). Alk7 has been deorphanized in 2001 by demonstrating that Nodal acts through this receptor and that it collaborates with the activin receptor type II b (ActRIIB) to confer responsiveness to Nodal (Reissmann et al., 2001). In addition, Activin AB and B (Tsuchida, 2004), GDF11 (Andersson et al., 2006) and GDF3 (Andersson et al., 2008) have been postulated to be functional ligands for Alk7. Investigation of efficacious responses of Alk7 to several TGFβ ligands has detected the strongest responsiveness of Alk7 to Activin B, a maximal efficacy of 26% and 31% to GDF11 and Myostatin, respectively, and no effect to Activin A (Khalil et al., 2016). As many ligands for Alk7 are key players during vertebrate development as well as in the male and female reproductive system, studies on the function of Alk7 have focused especially on these tissues (Bernard et al., 2006; Miles et al., 2013; Munir et al., 2004; Roberts et al., 2003; Sandoval-Guzman et al., 2012; Wang et al., 2006). Other tissues investigated for their role of Alk7 are brain (Jornvall et al., 2001) and heart (Ying et al., 2016), whereas the interest in its function in metabolism (particularly in the pancreas and adipose tissue) has accelerated over the years.

Alk7 serves as marker for adipocyte differentiation, as its expression levels are upregulated during differentiation of the white adipocyte cell line 3T3-L1 (Kogame et al., 2006). Furthermore, Alk7 and Activin B expression are correlated to factors implicated in metabolic disease in humans (Carlsson et al., 2009;

Sjoholm et al., 2006). Although global Alk7 knockout (Alk7$^{-/-}$) mice do not differ in body weight in comparison to their wildtype littermates on a chow diet, they develop age-dependent hyperinsulinemia, reduced insulin sensitivity, impaired glucose tolerance as well as liver steatosis (Bertolino et al., 2008). On a HFD, Alk7$^{-/-}$ mice gain less body weight in comparison to their wildtype littermates accompanied by reduced weight of epididymal fat pads and total fat content. On the other hand, these mice are not healthier than their wildtype littermates on a HFD, as they also develop hyperinsulinemia, reduced insulin sensitivity and liver steatosis in the course of HFD feeding (Andersson et al., 2008). Moreover, mice with a nonsense mutation of the *Acvr1c* gene encoding for Alk7 have been analysed. This gene mutation results in a COOH terminal deletion of the Alk7 kinase domain and subsequently in the global expression of a non-functional form of Alk7 (Yogosawa et al., 2012). In contrast to global Alk7$^{-/-}$ mice, these mice are not only resistant to diet-induced obesity (DIO) but also display improved obesity-induced glucose tolerance and insulin sensitivity *in vivo* (Yogosawa et al., 2012). The underlying mechanism has been described as a suppression of lipolysis by Alk7 through a Smad3-dependent downregulation of the master adipogenic transcription factor PPARγ and CCAAT/enhancer-binding protein alpha (C/EBPα), resulting in reduced lipase expression and lipolysis. The non-functional form of Alk7 in turn increases adipose lipase abundancy, which leads to a net decrease in fat accumulation (Yogosawa and Izumi, 2013; Yogosawa et al., 2012). Adipocyte-specific Alk7 knockout induces a likewise positive influence on metabolism (Guo et al., 2014). The proposed mechanism leading to resistance to DIO of these mice is an augmentation of β-adrenergic signaling through adipocyte-specific absence of Alk7 (Guo et al., 2014). Both proposed mechanisms of Alk7 signaling in white adipocytes are depicted in Figure 2.

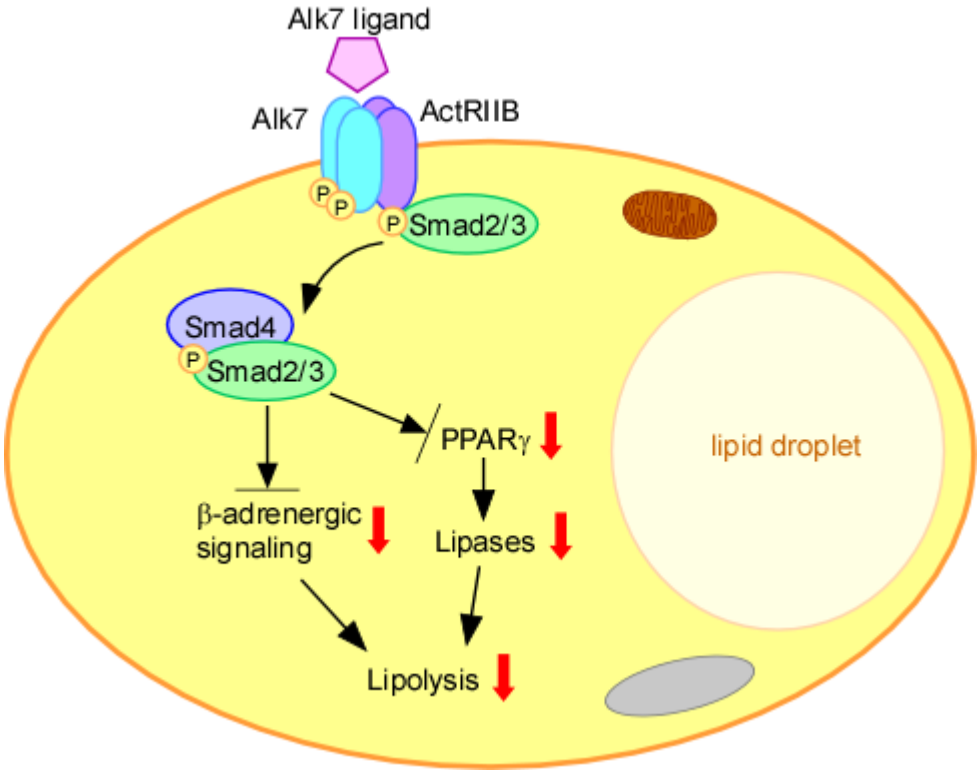

Figure 2: Proposed mechanisms of Alk7 signaling in white adipocytes. For details see main text.

The key aspect of recent Alk7 studies has been its role in WAT. The starting point of this work was the discovery that the *Acvr1c* gene encoding for Alk7 is the most upregulated gene by cGMP treatment in brown adipocytes. As cGMP mediates positive effects on brown adipocyte function, I was hypothesizing that Alk7 abundancy would also have beneficial functions in brown adipocytes. The first part of this work therefore concentrates on the crosstalk between the cGMP- and Alk7-signaling pathway as well as overexpression/activation models of Alk7 to decipher its role in brown adipocyte differentiation and function.
Indeed, I found a so far unknown crosstalk between the cGMP and Alk7 signaling pathway along with differential effects of Alk7 on development of the adipogenic and thermogenic program in brown adipocytes, i.e. decreasing the expression of adipogenic markers but increasing UCP1 expression, which is presented in Publication 1.

1.4 Lentiviral gene transfer for studying brown and beige fat *in vivo*

The second part of this work (Publication 2) focuses on the development and validation of an easy-to-handle and fast-to-accomplish method, which allows studying the role of various genes in differentiation and function of subcutaneous adipose tissues (i.e. brown and inguinal white) *in vivo*. In the first part of this work (Publication 1) it is demonstrated that Alk7 has differential effects on brown adipocyte differentiation and function *in vitro* with a possible benefit on thermogenic activity, as UCP1 is significantly upregulated by Alk7 activation. The next step was therefore to investigate effects of activated Alk7 in murine BAT *in vivo*. This can generally be performed in two ways. The first approach is pharmacological activation of Alk7 with specific ligands. In the case of Alk7 there are certain drawbacks to this approach. On the one hand, no established small molecule exists, which specifically acts through Alk7. On the other hand, administration of any Alk7 agonist not only targets Alk7 in BAT but also in other metabolically relevant tissues, i.e. pancreas and WAT, which would influence investigation of BAT-specific Alk7 in whole-body metabolism. The second approach - apart from pharmacological stimulation - is overexpression of a constitutively active form of Alk7 (caAlk7) in BAT. Following this approach, no further ligand administration is necessary. However, so far it has not been possible to induce overexpression of proteins specifically in BAT or WAT as no specific promoters for these tissues have been verified, i.e. the UCP1-promoter also drives protein expression in beige adipocytes (Kang et al., 2014). Therefore, the second part of this work (Publication 2) concentrates on the development and validation of a technique which circumvents the above mentioned problems: direct injection of lentiviral vectors into subcutaneous adipose tissues as a fast and efficient method to achieve specific expression (or knockdown) of a transgene in adipose tissue.

1.4.1 Characterisation of viral vectors

There are two ways of delivering genes into cells. One is the use of non-viral vectors, which can enter cells through chemical or physical methods to overcome the cell membrane. These non-viral vectors are characterised by transient gene expression and poor efficiency to deliver transgenes. Their use to deliver transgenes *in vivo* is low (~30%) in comparison to other gene delivery approaches (Journal of Gene Medicine Database, 2016; Yin et al., 2014). Viral vectors, on the other hand, possess advantages over non-viral vectors, including efficient transduction of cells. Depending on the needs of the experiment, several viral vectors with unique characteristics based on different viruses are available. Importantly, these viral vectors are modified in a way that they are replication deficient. Nevertheless, they are able to induce immune responses in the host individual (Kay, 2011). Some of the most important viral vectors used to transfer genes into a host genome are derived from retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAV), amongst others (Nayerossadat et al., 2012).

Viral vectors can be distinguished according to their genome. A large group of viruses are the RNA-containing *Retroviridae*, which can be divided into simple and complex retroviruses (Pfeifer and Verma, 2001). Simple retroviruses are single-stranded RNA viruses, whose genome is integrated into the host genome of dividing cells only (Miller et al., 1990). Hence, *in vivo* application of simple retroviruses is restricted as most cells in a tissue are terminally differentiated. Genes can be transferred into tissues with simple retroviruses after all, by transducing cells *in vitro* and explanting these into respective tissues, termed "*ex vivo* gene therapy" (Verma and Somia, 1997).

Lentiviruses are a subtype of retroviruses that feature the exceptional ability to transduce dividing as well as terminally differentiated, non-dividing cells (Lewis et al., 1992). The best-studied lentivirus is the human immunodeficiency virus (HIV). Integration into the host genome leads to stable transgene expression. However, integration of retroviral or lentiviral vector DNA into the host genome is mostly random and can lead to disruption of important endogenous genes or activate proto-oncogenes (Hacein-Bey-Abina et al., 2003; Li et al., 2002; Themis et al., 2005). On the other hand, lentiviral vectors (LVs) exhibit low immunogenicity in comparison to other viral systems due to the removal of most

genes encoding viral proteins (Breckpot et al., 2007). Maximal packaging size of LVs is up to 10 kb and tissue-/cell-specific expression can be achieved with different approaches, e.g. specific promoters, pseudotyping or connecting special fusogens on the surface of the virus capsule (Bouard et al., 2009; Breckpot et al., 2007; Yang et al., 2006). Production of LV yields virus particle concentrations of about $1x10^9$–$7x10^9$ inefectious particles (IP)/ml, depending on the method used for enrichment (Zimmermann et al., 2011). Over time, different LV systems were designed with self-inactivating vectors (SIN vectors) and split-genome packaging plasmids representing the most advanced LV systems, which result in high-titer LV preparations, improved efficacy as well as increased biosafety due to replication deficiency (Pauwels et al., 2009; Pfeifer and Hofmann, 2009). Importantly, LVs can be used for different *in vivo* applications to produce transgenic animals, e.g. lentiviral transgenesis (details see 1.4.2) (Pfeifer, 2004).
Adenoviruses are double-stranded, nonintegrating DNA viruses, which infect dividing and non-dividing cells. Production of adenoviral vectors yields high vector concentrations with a high transduction efficiency and a packaging capacity of up to 38 kb (Luo et al., 2007). However, transgene expression is lost quickly, especially in tissues with a high cell turnover (e.g. hematopoietic cells) due to episomal expression of the vector which is not passed down to daughter cells. Immune responses against adenoviruses are common and represent a major hurdle to the efficient and safe use of adenoviral vectors (Hendrickx et al., 2014).
AAV is a non-enveloped single-stranded DNA virus infecting dividing and non-dividing cells (Podsakoff et al., 1994) and inducing only mild immune responses in the host (Ferreira et al., 2014). The packaging genome of AAV is only up to 4 kb and predominantly persists as episome in host cells (Daya and Berns, 2008; Pfeifer and Verma, 2001). Productive replication in the host cell only takes place in the presence of a helper virus, e.g. adenovirus (Atchison et al., 1965) or herpes simplex virus (Buller et al., 1981). Nevertheless, wild-type AAV can integrate site-specific in the human chromosome 19 in the absence of helper virus (Linden et al., 1996). To achieve long-term gene expression, recombinant AAV can be applied, which either persist in the host cell in form of an episome or integrate into the host genome (McCarty et al., 2004).

1.4.2 Techniques to express transgenes in mice

Different methods to overexpress or knock down genes for *in vivo* experiments have been developed. The most commonly known and practised method is the use of plain transgenic mouse strains. Knockdown or overexpression of genes can further be induced by the application of inducible systems like the tetracycline-controlled transcriptional activation (Tet-On/Tet-Off) system or tissue specific expression with the cre-loxp recombination system. A faster technique to produce transgenic mice is the transfer of genes into embryos at a very early developmental stage, before any differentiation has taken place. The first experiments to implement this method have been performed with retroviral vector injections into fertilized oocytes (Jaenisch et al., 1975). It has been shown that retroviral vectors are not suitable for this method as their integrated DNA undergoes methylation and thereby causes silencing of the gene (Chan et al., 1998; Jahner and Jaenisch, 1985). Alternatively, plain DNA can be inserted into the pronucleus *via* microinjection (DNA-MI) (Hammer et al., 1985). DNA-MIs offer a very low efficiency (only ~2% of treated embryos develop into transgenic animals) because many embryos die during the procedure, gene transfer rates are low and the method is applicable only in certain mouse strains (Wall, 1996). The use of lentiviral transgenesis to generate transgenic animals has proven to be an efficient alternative to retroviruses and DNA-MIs (Pfeifer, 2004). Embryos in the zygote or morula stage are surrounded by a physical barrier called zona pellucida. To overcome this barrier, the LVs have to be either injected into the perivitelline space lying between the zona pellucida and the cytoplasmic membrane of the zygote (subzonal injections) or the zona pellucida is simply removed (denudation) (Pfeifer, 2004; Pfeifer et al., 2002). Application of lentiviral transgenesis yields more than 8-fold higher number of transgenics per treated embryo than does DNA-MI (Pfeifer, 2004). Utilization of LVs with specific promoters allows for targeted expression of the gene, e.g. overexpression of a constitutively active variant of the G-protein-coupled receptor Gq in BAT, WATi and gonadal WAT (WATg) under control of a UCP1-promoter (Klepac et al., 2016). However, this example also illustrates one drawback of lentiviral transgenesis, as so far no promoter is described, which specifically targets only one of these adipose depots. Furthermore, generating transgenic animals with

this method involves breeding of the animals and genotyping of the resulting offspring.

In search of a faster and more efficient method to generate tissue-specific transgenic animals, direct virus injections into different tissues have been developed. Injections of adenovirus as well as AAV into BAT and WAT have been performed before (Liu et al., 2014; Nagamatsu et al., 2001). However, both virus families have certain disadvantages in comparison to lentiviruses as mentioned above, i.e. stronger immunogenicity, episomal expression and small packaging capacity. Lentiviruses, on the other hand, efficiently transfer genes into white and brown adipocytes *in vitro* (Balkow et al., 2015; Haas et al., 2009; Mitschke et al., 2013) and have been shown to transduce WATi (Fujiwara et al., 2012; Gnad et al., 2014). Furthermore, they have been applied in the *in vitro* study in Publication 1 to analyse Alk7 signaling in brown adipocytes. Hence, in Publication 2 LVs were chosen for developing direct virus injections into adipose tissues *in vivo.* Moreover, one-and-the-same vector can be used to generate transgenic animals by subzonal injections, providing a second set of mice as experimental controls. Nevertheless, the described set-up/method could also be used for delivery of other viral vectors like adenoviral or AAV-derived vectors, with the above described advantages and drawbacks.

1.4.3 Gene therapy in obesity

The use of viral vectors for gene therapy is a constantly growing field for the treatment of many health issues. Figure 3A shows a maximum of 163 approved gene therapy clinical trials worldwide in 2015. Adenovirus, retrovirus, AAV and lentivirus made up more than 50% of vectors used for gene therapy clinical trials until February 2016 (Figure 3B). Furthermore, almost 2/3 of all indications addressed cancer diseases (Figure 3C). Thus, gene therapy resembles an important branch of therapeutical actions.

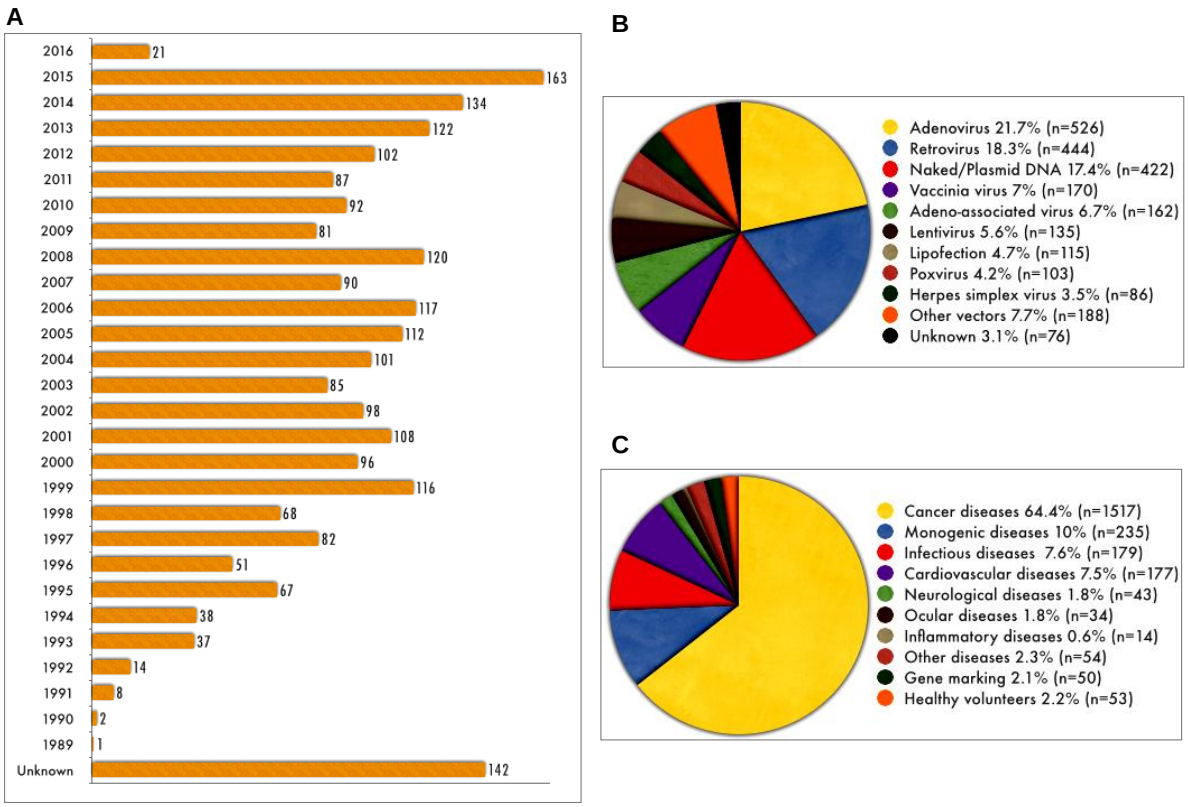

Figure 3: Gene therapy clinical trials worldwide. (A) Number of gene therapy clinical trials approved worldwide in the years 1989 – February 2016. (B) Proportion of vectors used in approved gene therapy clinical trials from 1989 – February 2016. (C) Proportion of indications addressed by approved gene therapy clinical trials from 1989 – February 2016. All graphs are modified from (Journal of Gene Medicine Database, 2016).

Many genes are involved in maintaining metabolic homeostasis, some of whose genetic variants have been associated with obesity in humans, e.g. leptin and its receptor, pro-opiomelanocortin (POMC), pro-protein convertase subtilisin/kexin 1 (PCSK1), and melanocortin 4 receptor (MC4R) (Farooqi and O'Rahilly, 2005). However, to date no gene therapies for the treatment of obesity in humans exist. On the other hand, research is focusing on the development of new gene therapeutic options in mice and rats, hoping to find a way to transfer promising results to humans. A plethora of gene therapy based approaches for the prevention and treatment of obesity in mice and rats have already been developed, a big portion of which are focused on adenovirus or AAV as method to transfer genes of interest into the animals (Gao and Liu, 2014). For example, it has been shown in rats that administration of an AAV carrying the gene for leptin into the brain is effective in prevention and treatment of DIO (Kalra and Kalra, 2002). Furthermore, a novel engineered hybrid capsid serotype of AAV (Rec2) has been designed to specifically transduce BAT of mice (Liu et al., 2014) and

can even be administered orally (Huang et al., 2016). However, so far there exists no method to target seperate adipose depots with lentiviral vectors specifically (e.g. only BAT or only WATi). As mentioned above, transgenesis with lentiviral vectors has certain advantages over other viral vectors, e.g. larger packaging capacity in comparison to AAV (see paragraph 1.4.1). Therefore, the second part of this work (Publication 2) focuses on the development and validation of a technique to fast and efficiently transduce subcutaneous adipose tissues (i.e. BAT and WATi). With this method, a plethora of genes can be studied in a timely manner, potentially leading to new gene therapeutic approaches for the treatment of obesity.

1.5 Thesis Outline

Obesity is a constantly growing health threat to civilisations in the whole world. One suggested solution in fighting obesity is the activation of BAT to increase energy expenditure. Various pathways are involved in the regulation of brown fat development and activity. This thesis sheds light on one of these pathways as well as introduces a fast and efficient method to study the function of different genes *in vivo*:

(1) Publication 1 introduces the role of Alk7 in brown adipocytes *in vitro*, focusing on a novel interplay of the Alk7 and cGMP signaling pathways and revealing differential effects of Alk7 on adipogenic and thermogenic differentiation.

Balkow A, Jagow J, Haas B, Siegel F, Kilic A, Pfeifer A. A novel crosstalk between Alk7 and cGMP signaling differentially regulates brown adipocyte function. *Mol Metab*. 2015; 4(8): 576-583.

(2) Publication 2 depicts the development and validation of a method for fast and efficient gene transfer into subcutaneous adipose tissues using direct injections of lentiviral vectors.

Balkow A, Hoffmann LS, Klepac K, Glöde A, Gnad T, Zimmermann K, Pfeifer A. Direct lentivirus injection for fast and efficient gene transfer into brown and beige adipose tissue. *J Biol Methods*. 2016; 3(3): e48.

2 Publication 1

2.1 Preamble

cGMP, together with PKGI, is an important factor in brown and white adipocyte differentiation and function (Haas et al., 2009; Mitschke et al., 2013). cGMP boosts the insulin signaling in brown adipocytes through inhibition of Rho/ROCK activity, thereby inducing mitochondrial biogenesis and adipogenic differentiation, characterized by UCP1 and PPARγ upregulation along with other factors (Haas et al., 2009). cGMP is involved in the regulation of a plethora of genes, amongst those also the ligand $TGF\beta_3$ in murine cardiac fibroblasts (Abdelaziz et al., 2001; Pilz and Casteel, 2003). The type I TGFβ receptor Alk7 has been shown to be highly expressed in WAT of mice and men and to a comparable extent in BAT of mice (Carlsson et al., 2009; Murakami et al., 2012). Importantly, Alk7 expression in the adipose tissue of obese patients is significantly reduced in comparison to lean controls (BMI difference ≥10 kg/m^2) and negatively correlates to several clinical parameters of metabolic disease (Carlsson et al., 2009), indicating that Alk7 is relevant for the maintainance of a healthy lean state. This notion is reinforced by the finding that global Alk7 knockout mice are, on the one hand, less prone to DIO in comparison to wildtype littermates, but on the other hand, develop insulin resistance and liver steatosis (Andersson et al., 2008). The molecular function of Alk7 has so far only been delineated in white adipocytes (Guo et al., 2014; Yogosawa and Izumi, 2013; Yogosawa et al., 2012), in which Alk7 abundancy is increased during differentiation (Kogame et al., 2006; Yogosawa et al., 2012). In the following publication, research is consequently focusing on the role of Alk7 in brown adipocytes, deciphering a novel crosstalk between the Alk7 and the cGMP signaling pathways.

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

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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

HIB1B 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.

MOLECULAR METABOLISM

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-

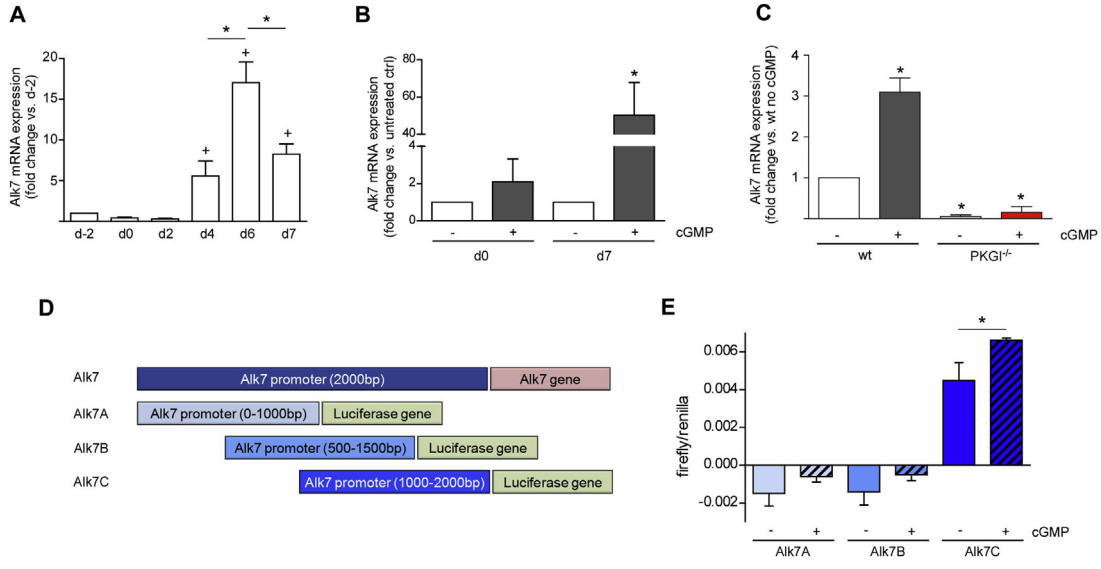

Figure 1: Alk7 expression is upregulated in mature adipocytes and modulated by the cGMP/PKGI pathway. **(A)** Alk7 mRNA expression in brown adipocytes at different time points during differentiation. **(B)** Alk7 mRNA expression after chronic 8-pCPT-cGMP (200 μM) treatment at day 0 and day 7, normalized 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.

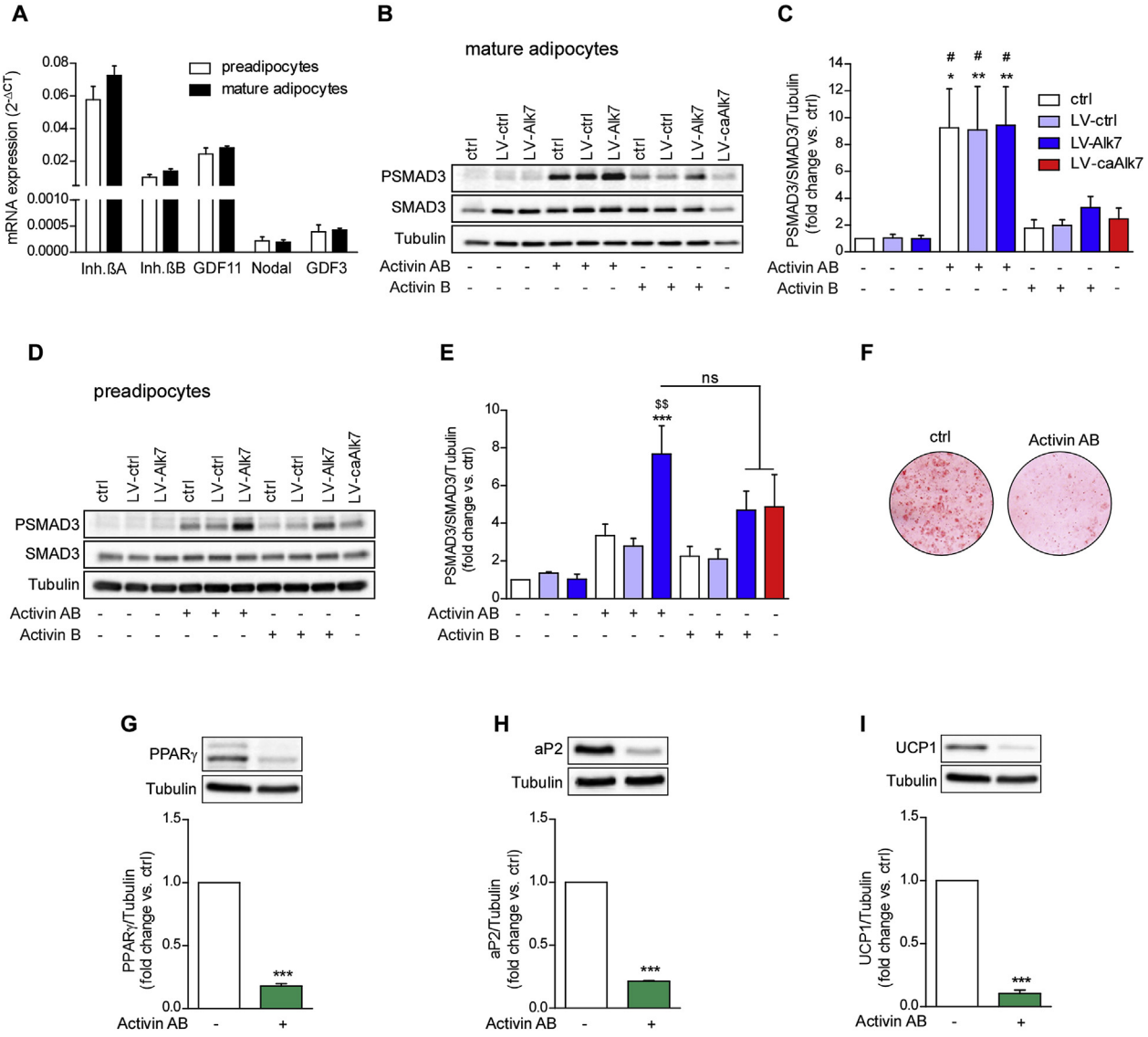

Figure 2: Alk7 signaling is activated by Activins and early activation of Alk7 diminishes adipogenic differentiation. **(A)** mRNA expression of Inhibin βA (Inh.βA), Inhibin βB (Inh.βB), GDF11, Nodal and GDF3 in preadipocytes and mature adipocytes. **(B–E)** Western blot analysis of SMAD3 phosphorylation (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).

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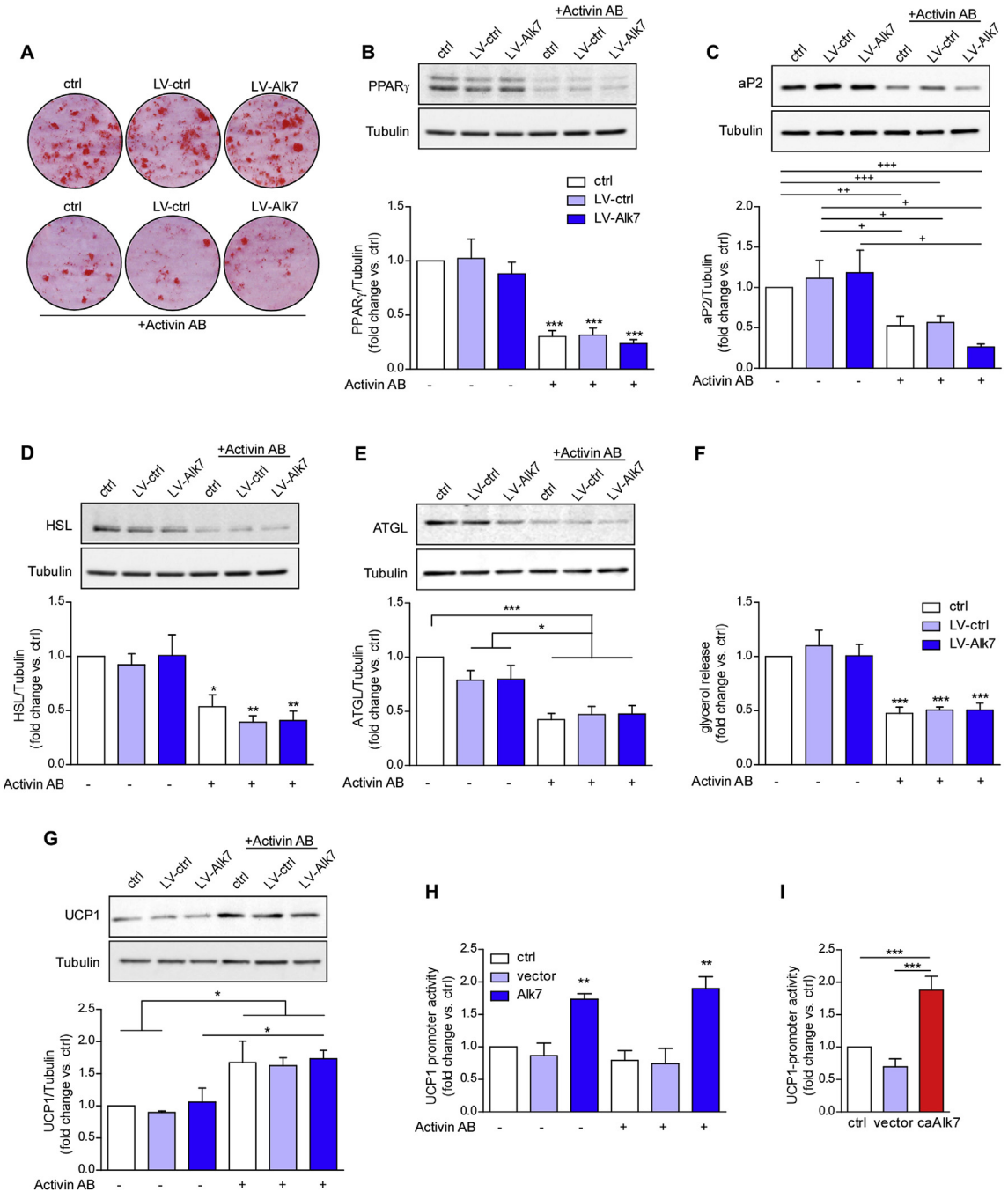

Figure 3: Activation of Alk7 in mature brown adipocytes reduces adipogenic differentiation but enhances UCP1 expression. **(A—G)** Analysis of adipocytes transduced with LV-ctrl or LV-Alk7 virus or untransduced cells treated with and without Activin AB (day 4 to day 7). **(A)** Representative Oil Red 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

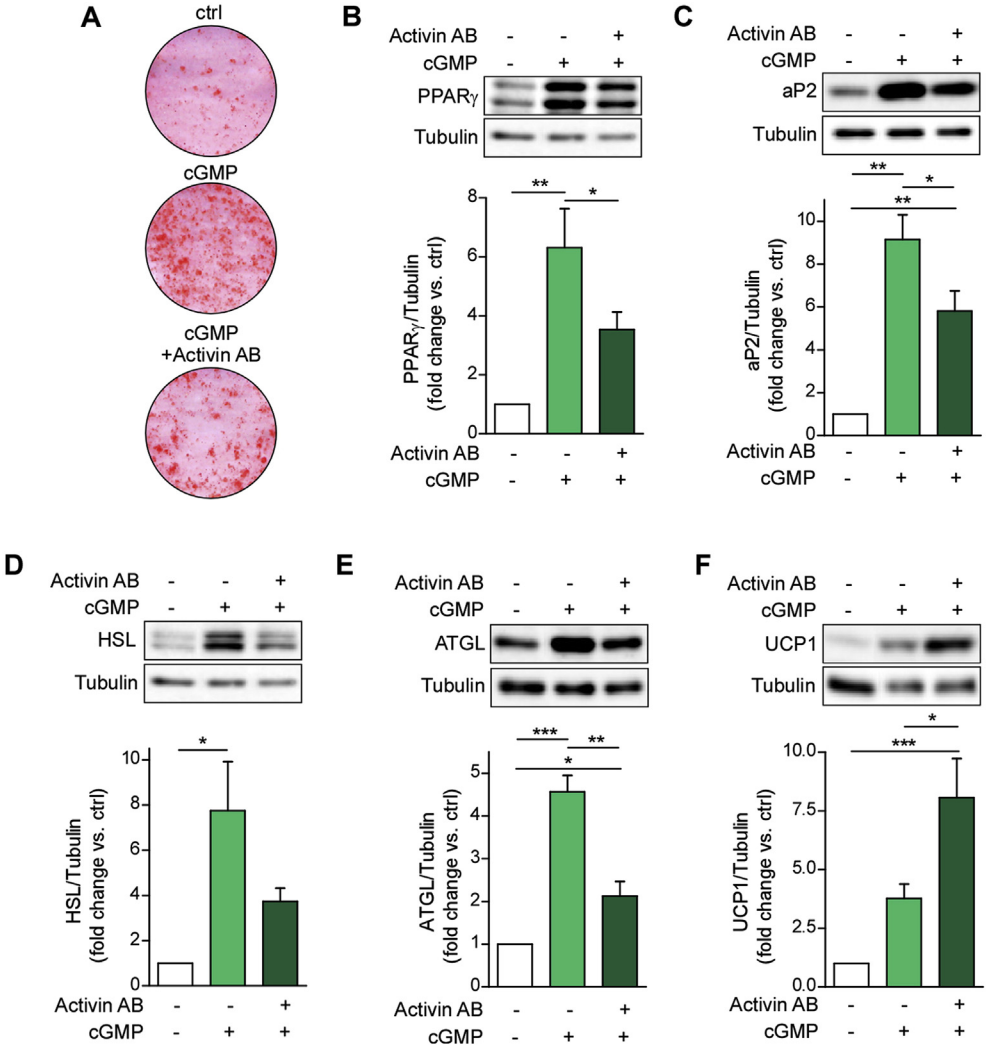

Figure 4: Differential regulation of cGMP effects by Alk7 activation. Analysis of BA chronically treated with 200 μM 8-Br-cGMP (day −2 to day 7) and additionally with Activin AB (day 4 to day 7). **(A)** Representative Oil Red O stainings. **(B–F)** Western blot analysis of PPARγ, aP2, HSL, ATGL and 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.

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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.

ACKNOWLEDGMENTS

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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

REFERENCES

[1] Cannon, B., Nedergaard, J., 2004. Brown adipose tissue: function and physiological significance. Physiological Reviews 84(1):277—359.
[2] Rosen, E.D., Spiegelman, B.M., 2014. What we talk about when we talk about fat. Cell 156(1—2):20—44.
[3] Kajimura, S., Seale, P., Spiegelman, B.M., 2010. Transcriptional control of brown fat development. Cell Metabolism 11(4):257—262.
[4] Pfeifer, A., Hoffmann, L.S., 2014. Brown, beige, and white: the new color code of fat and its pharmacological implications. Annual Review of Pharmacology and Toxicology 55:207—227.
[5] Bordicchia, M., Liu, D., Amri, E.Z., Ailhaud, G., Dessi-Fulgheri, P., Zhang, C., et al., 2012. Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. Journal of Clinical Investigation 122(3):1022—1036.
[6] Nisoli, E., Clementi, E., Paolucci, C., Cozzi, V., Tonello, C., Sciorati, C., et al., 2003. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science 299(5608):896—899.
[7] Haas, B., Mayer, P., Jennissen, K., Scholz, D., Berriel Diaz, M., Bloch, W., et al., 2009. Protein kinase G controls brown fat cell differentiation and mitochondrial biogenesis. Science Signaling 2(99):ra78.
[8] Jennissen, K., Siegel, F., Liebig-Gonglach, M., Hermann, M.R., Kipschull, S., van Dooren, S., et al., 2012. A VASP-Rac-soluble guanylyl cyclase pathway controls cGMP production in adipocytes. Science Signaling 5(239):ra62.
[9] Massague, J., 2008. TGFbeta in Cancer. Cell 134(2):215—230.
[10] Pereira, S., Teixeira, L., Aguilar, E., Oliveira, M., Savassi-Rocha, A., Pelaez, J.N., et al., 2014. Modulation of adipose tissue inflammation by FOXP3+ Treg cells, IL-10, and TGF-beta in metabolically healthy class III obese individuals. Nutrition 30(7—8):784—790.
[11] Carlsson, L.M., Jacobson, P., Walley, A., Froguel, P., Sjostrom, L., Svensson, P.A., et al., 2009. ALK7 expression is specific for adipose tissue, reduced in obesity and correlates to factors implicated in metabolic

disease. Biochemical and Biophysical Research Communications 382(2): 309–314.

[12] Murakami, M., Shirai, M., Ooishi, R., Tsuburaya, A., Asai, K., Hashimoto, O., et al., 2013. Expression of activin receptor-like kinase 7 in adipose tissues. Biochemical Genetics 51(3–4):202–210.

[13] Andersson, O., Korach-Andre, M., Reissmann, E., Ibanez, C.F., Bertolino, P., 2008. Growth/differentiation factor 3 signals through ALK7 and regulates accumulation of adipose tissue and diet-induced obesity. Proceedings of the National Academy of Sciences of the United States of America 105(20):7252–7256.

[14] Bertolino, P., Holmberg, R., Reissmann, E., Andersson, O., Berggren, P.O., Ibanez, C.F., 2008. Activin B receptor ALK7 is a negative regulator of pancreatic beta-cell function. Proceedings of the National Academy of Sciences of the United States of America 105(20):7246–7251.

[15] Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., et al., 2001. The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. Genes & Development 15(15):2010–2022.

[16] Andersson, O., Reissmann, E., Ibanez, C.F., 2006. Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. EMBO Reports 7(8):831–837.

[17] Tsuchida, K., Nakatani, M., Yamakawa, N., Hashimoto, O., Hasegawa, Y., Sugino, H., 2004. Activin isoforms signal through type I receptor serine/threonine kinase ALK7. Molecular and Cellular Endocrinology 220(1–2):59–65.

[18] Jornvall, H., Blokzijl, A., ten Dijke, P., Ibanez, C.F., 2001. The orphan receptor serine/threonine kinase ALK7 signals arrest of proliferation and morphological differentiation in a neuronal cell line. Journal of Biological Chemistry 276(7): 5140–5146.

[19] Bondestam, J., Huotari, M.A., Moren, A., Ustinov, J., Kaivo-Oja, N., Kallio, J., et al., 2001. cDNA cloning, expression studies and chromosome mapping of human type I serine/threonine kinase receptor ALK7 (ACVR1C). Cytogenetics and Cell Genetics 95(3–4):157–162.

[20] Yogosawa, S., Mizutani, S., Ogawa, Y., Izumi, T., 2012. Activin receptor-like kinase 7 suppresses lipolysis to accumulate fat in obesity through downregulation of peroxisome proliferator-activated receptor gamma and C/EBPalpha. Diabetes 62(1):115–123.

[21] Tsuchida, K., Nakatani, M., Hitachi, K., Uezumi, A., Sunada, Y., Ageta, H., et al., 2009. Activin signaling as an emerging target for therapeutic interventions. Cell Communication and Signaling 7:15.

[22] Derynck, R., Zhang, Y.E., 2003. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 425(6958):577–584.

[23] Chen, Y., Siegel, F., Kipschull, S., Haas, B., Frohlich, H., Meister, G., et al., 2013. miR-155 regulates differentiation of brown and beige adipocytes via a bistable circuit. Nature Communications 4:1769.

[24] Gnad, T., Scheibler, S., von Kugelgen, I., Scheele, C., Kilic, A., Glode, A., et al., 2014. Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature 516(7531):395–399.

[25] Enerback, S., 2010. Human brown adipose tissue. Cell Metabolism 11(4): 248–252.

[26] van Marken Lichtenbelt, W.D., Schrauwen, P., 2011. Implications of non-shivering thermogenesis for energy balance regulation in humans. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology 301(2):R285–R296.

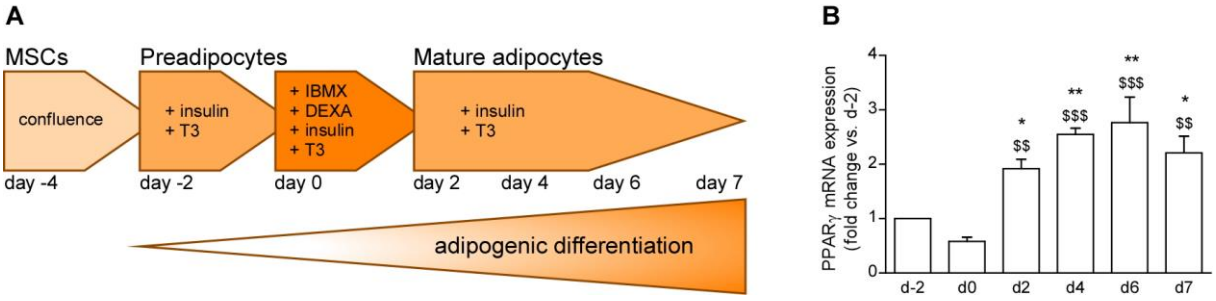
[27] Virtanen, K.A., Lidell, M.E., Orava, J., Heglind, M., Westergren, R., Niemi, T., et al., 2009. Functional brown adipose tissue in healthy adults. New England Journal of Medicine 360(15):1518–1525.

[28] Schwappacher, R., Kilic, A., Kojonazarov, B., Lang, M., Diep, T., Zhuang, S., et al., 2013. A molecular mechanism for therapeutic effects of cGMP-elevating agents in pulmonary arterial hypertension. Journal of Biological Chemistry 288(23):16557–16566.

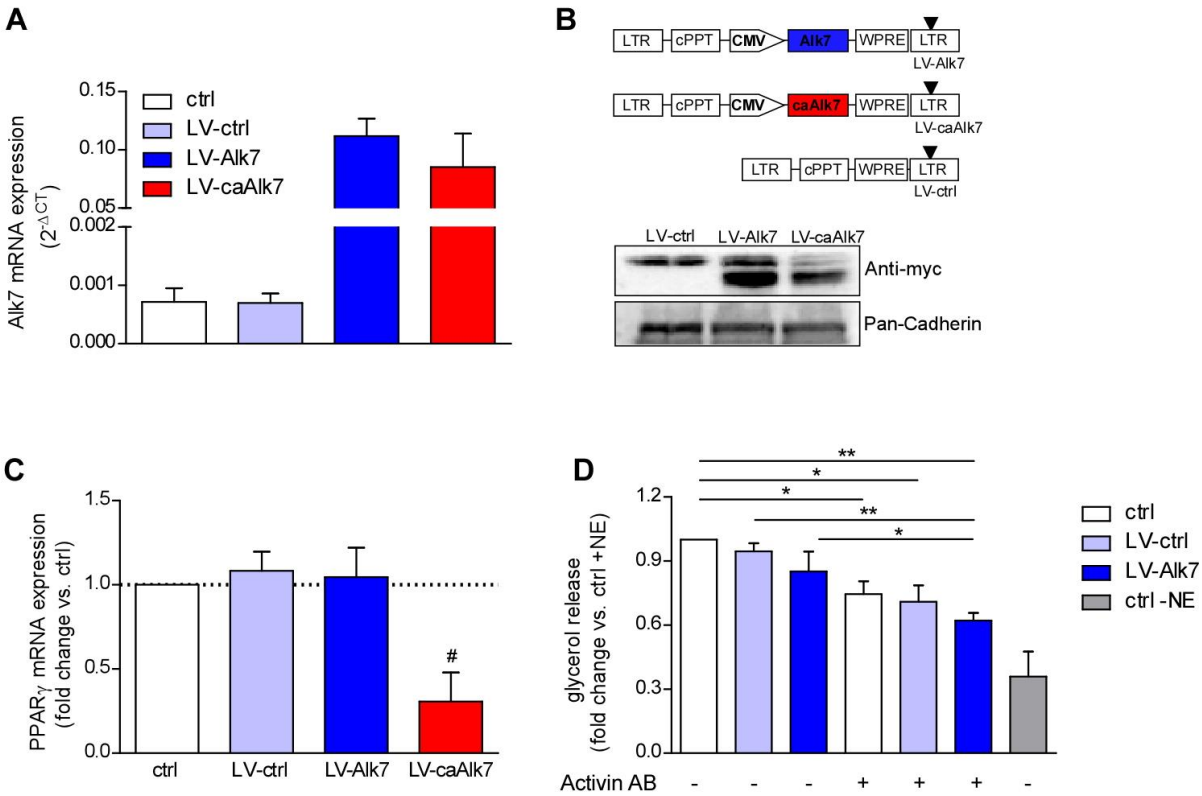
[29] Choy, L., Derynck, R., 2003. Transforming growth factor-beta inhibits adipocyte differentiation by Smad3 interacting with CCAAT/enhancer-binding protein (C/EBP) and repressing C/EBP transactivation function. Journal of Biological Chemistry 278(11):9609–9619.

[30] Guo, T., Marmol, P., Moliner, A., Bjornholm, M., Zhang, C., Shokat, K.M., et al., 2014. Adipocyte ALK7 links nutrient overload to catecholamine resistance in obesity. Elife 3:e03245.

Supplement 1: Balkow et al.



Supplement 2: Balkow et al.



Suppl. 1: Differentiation of brown adipocytes (A) Scheme of brown adipocyte differentiation protocol **(B)** PPARγ mRNA expression in brown adipocytes at different time points during differentiation. Data are presented as mean ± SEM from 3 independent experiments. *($p<0.05$; ANOVA), **($p<0.01$; ANOVA) significant difference vs. d-2; $^{\$\$}$($p<0.01$; ANOVA), $^{\$\$\$}$($p<0.001$; ANOVA) significant difference vs. d0.

Suppl. 2: Lentiviral overexpression of Alk7, PPARγ expression analysis and glycerol release. (A) mRNA analysis of Alk7 in ctrl, LV-ctrl, LV-Alk7 and LV-caAlk7 BA using primers concomitantly directed against human and murine Alk7. **(B)** Schematic representation of lentiviral constructs and representative Blot of BA overexpressing myc-tagged LV-Alk7 or LV-caAlk7 using antibody directed against the myc-tag. Pan-Cadherin serves as endogenous control. (**C**) mRNA analysis of PPARγ in ctrl, LV-ctrl, LV-Alk7 and LV-caAlk7 BA on day 3 of differentiation. (**D**) NE-induced lipolysis in brown adipocytes treated with and without Activin AB (day 4 to day 7) and stimulated with norepinephrine (1µM) for 2h. Data are presented as mean ± SEM from 3-5 independent experiments. $^{\#}$($p<0,05$; ANOVA) significant difference vs. all other conditions; *($p<0,05$; ANOVA), **($p<0,01$; ANOVA) significant difference vs d-2 or as indicated if control –NE is not taken into consideration.

Suppl. Table 1: qRT-PCR primers.

Primer	**Forward**	**reverse**	**Reference**
Murine Alk7	GTCAGAGTATCACGAGCAG GGCTCCTT	TTCACACACAGCTGGGAGATGG TCTTC	
MurineAlk7 (ABI)	GTACATGGCTCCCGAAATGC	GGCAACTGGTACTCCTCAACAAC	
Human+ Murine Alk7	CAGAATCCTAAAGTGGGAA CCAA	TTCATTGTATCATCAAGCATTTC	
HPRT	ACATTGTGGCCCTCTGTGTG CTCA	CTGGCAACATCAACAGGACTCCT CGT	Haas et al 2009
HPRT(ABI)	GTCCCAGCGTCGTGATTAGC	TCATGACATCTCGAGCAAGTCTT T	
Inhibin ßA	AGGCGGCGCTTCTCAAC	CCTCTATCTCCACATACCCGTTC T	
Inhibin ßB	CCTGAGTGAATGCACACCAC	CGAGTCCAGTTTCGCCTAGT	
GDF11	CAGCCCTCTCTGCTGTCATTT	TCCCCAGTTAGGGGTTTCAGT	
Nodal	AGCCAAGAAGAGGATCTGG TATGG	GACCTGAGAAGGAATGACGGTG AA	
GDF3	ATGCAGCCTTATCAACGGCT T	AGGCGCTTTCTCTAATCCCAG	
PPARγ	TCCGTAGAAGCCGTGCAAGA GATCA	CAGCAGGTTGTCTTGGATGTCCT CG	

2.3 Epilogue

The presented publication highlights several new findings regarding Alk7 and brown adipocytes:

1. Alk7 is vastly expressed in mature brown adipocytes, resembling findings that have been made in the white adipocyte cell line 3T3-L1 as well as in primary white adipocytes (Kogame et al., 2006; Yogosawa et al., 2012).
2. Alk7 expression is highly dependent on the cGMP/PKGI signaling pathway, as its expression is upregulated in cGMP-treated cells and diminished in $PKGI^{-/-}$ cells. This effect seems to appear at the transcriptional level through direct interaction of the cGMP/PKGI signaling pathway with the Alk7 promoter.
3. Brown adipocytes express several ligands for Alk7 and mediate downstream actions *via* the canonical SMAD3 signaling pathway, with Activin AB serving as the more potent ligand to activate Alk7 in comparison to Activin B.
4. A tight regulation of Alk7 signaling is crucial for normal adipocyte differentiaton, i.e. activation of Alk7 early in adipocyte development inhibits cell differentiation.
5. Alk7 activation in terminally maturing adipocytes differentially regulates expression of adipogenic and thermogenic proteins and, most importantly, enhances UCP1 expression on a transcriptional level.
6. cGMP effects in brown adipocytes are attenuated by Alk7 activation, with the major except for UCP1 abundance, which is further boosted by Activin AB treatment, resulting in a synergistic effect.

Alk7 is known to be a marker for mature white adipocytes since 2006 (Kogame et al., 2006; Yogosawa et al., 2012), but expression levels of Alk7 during differentiation of brown adipocytes as well as of Alk7 ligands have not been analyzed up to now. Furthermore, SMAD3 phosphorylation by Alk7 activation has been presented in several cell types, e.g. white adipocytes (Yogosawa et al., 2012), PC12 cells (Jornvall et al., 2001) and human trophoblast cells (Munir et al., 2004), but never in brown adipocytes. Additionally, one proposed mechanism of Alk7 signaling in white adipocytes is through downregulation of PPARγ expression, thereby negatively regulating lipolysis (Yogosawa and Izumi, 2013; Yogosawa et al., 2012). The same mechanism seems to apply in brown adipocytes as presented here. Importantly, the presented publication features a

novel impact of Alk7 on UCP1 expression. In contrast, it has been demonstrated before, that other members of the TGFβ/SMAD3 signaling pathway rather suppress UCP1 expression in brown and beige adipocytes (Fournier et al., 2012; Singh et al., 2014). The here demonstrated data are therefore highlighting Alk7 as an exceptional receptor among the TGFβ-receptor family. Specific activation of Alk7 in BAT could serve beneficial for UCP1-dependent energy expenditure. Moreover, a link between the cGMP and Alk7 signaling pathways has so far not been investigated. This newly unraveled link could be responsible for Alk7 reduction in adipose tissue of obese patients, as it has also been found that components of the cGMP-signaling pathway (i.e. PKGI, phosphorylated eNOS and phosphorylated VASP) are downregulated in WATg of obese mice (Handa et al., 2011). We propose that Alk7 in brown adipocytes functions as an endogenous brake of the cGMP-signaling pathway, avoiding overstimulation of the adipogenic program by cGMP and at the same time enhancing UCP1 expression to facilitate energy expenditure. Promoting cGMP signaling to enhance Alk7 signaling in BAT could therefore be of clinical benefit for obese patients.

3 Publication 2

3.1 Preamble

The study presented in Publication 1 is based on an *in vitro* approach, which unravels Alk7 as a novel target for the upregulation of UCP1, thereby possibly mediating a beneficial effect on BAT activity and energy expenditure *in vivo*. However, the available techniques so far made it almost impossible to overexpress and activate Alk7 specifically in BAT. For example, the UCP1 promoter can be activated in brown and in beige adipocytes, resulting in transgene expression in BAT and WATi if global transgenic mice are produced with subzonal injections (Kang et al., 2014; Klepac et al., 2016). Furthermore, so far no small molecule exists, which binds to Alk7 specifically (i.e. Activin AB and B also activate Alk4 and Alk5). Additionally, administration of such a small molecule would target and activate Alk7 in all Alk7 expressing tissues. To overcome this problem, a method for direct lentivirus injection into subcutaneous adipose depots was developed and validated in the following publication. LVs were chosen to be employed in this technique, as they have several advantages over other viral vectors, e.g. genome integration into dividing as well as terminally differentiated cells (Lewis et al., 1992), leading to stable transgene expression, large packaging capacity (Pfeifer, 2004; Pfeifer and Hofmann, 2009) and lower immunogenicity in comparison to other viral systems like adenovirus (Breckpot et al., 2007).

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PROTOCOL

Direct lentivirus injection for fast and efficient gene transfer into brown and beige adipose tissue

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Competing interests: The authors have declared no competing interests exist.

Abbreviations used: BAT, brown adipose tissue; UCP1, uncoupling protein 1; WAT, white adipose tissue; WATi, inguinal white adipose tissue; PE, polyethylene; GFP, green fluorescent protein; CMV, cytomegalovirus; aP2, fatty acid-binding protein 4; AAV, adeno-associated virus

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ABSTRACT

Brown adipose tissue is a special type of fat contributing to energy expenditure in human newborns and adults. Moreover, subcutaneous white adipose tissue has a high capacity to adapt an energy-consuming, brown-like/beige phenotype. Here, we developed an easy to handle and fast to accomplish method to efficiently transfer genes into brown and beige fat pads *in vivo*. Lentiviral vectors are directly injected into the target fat pad of anesthetized mice through a small incision using a modified, small needle connected to a microsyringe, which is well suited for infiltration of adipose tissues. Expression of the target gene can be detected in brown/beige fat one week after injection. The method can be applied within minutes to efficiently deliver transgenes into subcutaneous adipose tissues. Thus, this protocol allows for studying genes of interest in a timely manner in murine brown/beige fat and could potentially lead to new gene therapies for obesity.

Keywords: beige adipose tissue, brown adipose tissue, gene transfer, lentivirus

BACKGROUND

There is a high medical need to develop new strategies to treat overweight and obesity as these conditions have reached pandemic dimensions. Obesity and its comorbidities, such as type 2 diabetes, cardiovascular disease and certain kinds of cancer, are a major threat to global health [1,2]. Brown adipocytes are promising targets as they consume energy and could be used to increase energy expenditure and facilitate weight loss and thereby counteract obesity.

Brown adipocytes are rich in mitochondria and express specifically the uncoupling protein 1 (UCP1). UCP1 is activated by free fatty acids and uncouples the proton gradient within the mitochondria leading to generation of heat instead of ATP. Cold exposure leads to sympathetic activation of lipolysis and consequent activation of UCP1 by the released free fatty acids [3]. Newborn humans and small mammals possess brown adipose tissue (BAT) that produces heat to maintain body temperature [4]. Importantly, metabolically active BAT can also be found in human adults [5,6,7] and activation and/or recruitment of human BAT can increase whole body metabolism [8,9].

Apart from interscapular BAT, inducible brown adipocytes, so called beige or brite (brown-in-white) cells, have been found disseminated in white adipose tissue (WAT). Beige cells also contain a high number of UCP1-expressing mitochondria and consume energy similar to "classical" brown adipocytes [10]. The number of beige adipocytes can be increased by different stimuli like cold exposure or by certain drugs [11]. Among WAT depots the subcutaneous, inguinal WAT (WATi) has a high susceptibility to browning in mice and men [12,13,14]. Even though the knowledge about brown and beige adipocytes has increased significantly during the last years there are still many open questions.

Lentiviruses are enveloped, single stranded RNA viruses that belong to the family of *Retroviridae*. An important difference between lentiviruses and simple retroviruses is that lentiviruses have the unique ability to infect non-dividing, terminally differentiated eukaryotic cells. Therefore, they are widely used as an efficient method for gene transfer [15].

Here we describe a protocol of direct lentivirus injection into subcutaneous brown and beige adipose depots for efficient gene transfer into brown/beige adipocytes. The role of target genes can thereby be studied in brown and beige fat *in vivo* as well as in whole body metabolism without the need to establish transgenic mouse lines. Furthermore, this method could also serve as a potential gene therapy approach which specifically targets subcutaneous beige or brown fat depots.

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Development of the protocol

Lentiviruses efficiently transfer genes into white and brown adipocytes *in vitro* [16,17] and have been shown to transduce WAT [18,19]. We have developed and optimized the protocol for lentivirus injection into BAT and to induce beige cells in WATi. Importantly, the custom adapted injection device allows for the injection of small volumes through a very small needle to avoid significant tissue damage. The set-up provides clear visual control during injection without contamination of the glass syringe by virus.

Experimental design

The use of lentivirus allows employing a large variety of constructs. Lentivirus can either be used to overexpress genes or to knockdown genes using shRNA or miRNA [20]. For every experiment it is important to use respective controls. The buffers used for dissolving the viral particles, *e.g.,* phosphate buffered saline (PBS) or Hank's balanced salt solution (HBSS), can be used for mock injections. Moreover, either "empty" viruses without transgene cassette or viral vectors carrying a reporter gene like green fluorescent protein (GFP) can be injected as controls. Especially for shRNAs or miRNAs a scrambled control vector should be used. It is possible to have intra-individual, internal controls within one mouse by injecting the gene of interest into only one fat lobe and the respective control into the other. This is useful if overexpression or knockdown efficiency within one mouse is investigated but should not be applied if whole body metabolism is studied.

Mice that have been injected with lentivirus can be included into long term studies, because the lentivector integrates into the host genome. Expression of the transgene is stable for at least 6 weeks [19].

Comparison with other methods

Until now, fat tissue specific overexpression or knockdown of target genes was mostly accomplished by generating specific transgenic or knockout mouse models by subzonal injections of lentivirus into fertilized oocytes or using the Cre-lox recombination system, respectively [21]. Even though these methods are proficient in transferring or deleting the gene of interest into specific tissues by tissue-specific promoters, they are also very time consuming and expensive. Furthermore, a large number of mice are needed for breeding to establish a specific mouse line.

Direct lentiviral injection into brown/beige fat, does not involve breeding or genotyping of mouse lines and hence dramatically reduces the time required to express a gene of interest in fat pads as well as number of animals and costs. Another advantage of the method described here is the gene transfer into specific fat pads (*e.g.,* only WATi, but not BAT). So far this cannot be accomplished with transgenesis as there are no promoters available which specifically target only one of these fat pads, *e.g.,* the UCP1 promoter is active in both brown and beige cells [22].

The described set-up/method can also be used to deliver genes of interest into existing transgenic animals as well as for delivery of other viral vectors [23], like vectors derived from adenovirus or adeno-associated virus (AAV). AAV-derived particles are considered biohazard level 1; however, a major disadvantage is the small packaging capacity of classical AAV vectors [24,25]. An important feature of lentiviral vectors is their integration into the host genome ensuring long-term expression. Moreover, one-and-the-same vector can be used to generate transgenic animals by subzonal injection (lentiviral transgenesis) [20].

Using the method described here, it is possible to generate genetically modified mice within minutes.

Limitations

We recommend using mice of at least 4 weeks of age for injections into BAT and WATi. The presented method is primarily designed for subcutaneous fat tissues, which are easily accessible via a small skin incision. In principal, injections into visceral fat are possible, but injection into the epididymal adipose tissue would require opening the abdominal cavity. In the adipose tissue, not only adipocytes will be transduced with this method but all present cells (*e.g.,* preadipocytes, fibroblasts, immune cells, endothelial cells). Adipocyte-specific expression can be achieved with specific promoters to control expression of the target gene, *e.g.,* the fatty acid-binding protein 4 (aP2) promoter for all fat cells or the UCP1 promoter for only brown and beige fat cells [22]. To achieve cell-specific transduction, one could design viruses that have an adipocyte-specific fusogen that delivers the virus to designated cell types only [26]. ASC-1, PAT2, and P2RX5 could serve as specific surface markers for white, beige and brown adipocytes [27].

MATERIALS

General considerations on safety issues

All experiments have to be performed in accordance to relevant guidelines and protocols and have to be approved by local authorities including ethics/animal health committees. The study described herein has been approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, NRW, Germany. Working with lentiviruses is restricted to biosafety level 2 (BSL-2) facilities. Appropriate personal protection equipment such as gloves and lab coat are required when working with these vectors. The paper "State-of-the-Art Lentiviral Vectors for Research Use: Risk Assessment and Biosafety Recommendations" by Katia Pauwels *et al*. (2009) [28] provides a good overview about risk assessment and biosafety recommendations when working with lentiviral vectors. Mice injected with replication-deficient virus are usually classified as BSL-1 organisms and can therefore be handled as any other BSL-1 laboratory mouse. However, as legislation might differ between countries, it is essential to review the local guidelines and precautions for handling viruses in BSL-2 facilities as well as lentiviral-injected mice before starting with the procedure.

Animals

We have successfully overexpressed transgenes with direct lentivirus injection into brown (interscapular BAT) and beige (WATi) adipose tissue in C57Bl/6 mice at 4 weeks of age or older. Other mouse strains might be as suitable for the method as C57Bl/6 mice, but adjustment of the protocol might be necessary. For example when using ob/ob mice, it is necessary to adjust the number of viral particles depending on the increase in adipose tissue mass compared to wild-type controls. We recommend testing the procedure with Trypan blue solution or with a reporter construct (*e.g.,* GFP) before starting with the actual experiments.

Reagents

- ✓ Ethanol 70%
- ✓ Isoflurane (Abbott), 1–2.5% (w/v) in oxygen. **CAUTION:** Isoflurane is harmful if inhaled, swallowed or upon skin contact. Wear appropriate personal protection equipment and use devices with carbon filters to minimize exposure to isoflurane.
- ✓ Trypan blue solution, 0.4%, **CAUTION:** Trypan blue might be

hazardous to your health, wear appropriate personal protection equipment such as gloves when working with trypan blue.

- ✓ Packaging plasmids pMDLg/pRRE, RSV-rev and pMD.G for lentivirus production
- ✓ High titer lentivirus encoding the desired gene or reporter. The virus is produced according to established protocols [21]. The lentiviral vector was obtained by cloning GFP into the Bam HI and Sal I sites of the vector p156rrlsinPPT, which carries a ubiquitous CMV promoter. **CAUTION:** Adhere to biosafety regulations and only use the virus within a BSL-2 facilities. The virus can only be handled within a safety cabinet. Wear appropriate personal protection equipment such as gloves and lab coat.
- ✓ HBSS (Hank's Balanced Salt Solution, Life technologies)
- ✓ Carprofen (Rimadyl, Pfizer)

Reagent setup

Virus solution. High titer lentivirus should be prepared according to established protocols [21,29]. In short, HEK 293T cells (ATCC) are seeded on poly-L-lysine-coated 150-mm2 dishes and co-transfected with the lentiviral vector plasmid as well as the packaging plasmids pMDLg/pRRE, RSV-rev and pMD.G. After transfection, cells are incubated at 37°C and 3% CO_2 overnight. The transfection medium is then exchanged and cells are further incubated at 37°C and 10% CO_2. The secreted virus is harvested after another 24 h and 48 h by collecting the supernatant of the cells. This supernatant is centrifuged by an ultracentrifuge (Beckman Coulter) with SW32Ti rotor at 61,700 g at 17°C for 2 h to pellet the secreted virus. The virus pellet is resuspended in HBSS. Combined virus suspensions after 24 h and 48 h are concentrated by centrifugation over a 20% (w/v) sucrose cushion in a SW55Ti rotor (Beckman Coulter) at 53,500 g at 17°C for 1.5 h and again solved in HBSS. The physical titer of the lentivirus can be assessed by colorimetric reverse transcriptase (RT) assay (Roche). Dilute lentivirus in HBSS, to achieve desired volume for injection (*i.e.* 20–30 µl for each fat lobe). In this protocol 1000 ng/25 µl (BAT) and 1000 ng/30 µl (WATi) of lentivirus were injected into each fat lobe.

Equipment

- ✓ Surgical swabs (Paul Hartmann)
- ✓ Animal hair clipper (AESCULAP GT420)
- ✓ Isoflurane anesthesia system (Vapor, Dräger)
- ✓ Nanopass33 (33G needle for pen injectors, Terumo Corp.)
- ✓ Fine-Bore Polyethylene (Polythene) Tubing (ID 0.28 mm, OD 0.61 mm, Smiths Medical)
- ✓ Microsyringe (Hamilton)
- ✓ Tungsten carbide scissors (Fine Science Tools)
- ✓ Surgical scissors (Fine Science Tools)
- ✓ Curved forceps (Fine Science Tools)
- ✓ Straight forceps (Fine Science Tools)
- ✓ Michel suture clips (Fine Science Tools)
- ✓ Michel clip applicator (Fine Science Tools)
- ✓ Heating pad

Equipment setup

Injection device. Cut off outer plastic rim around the pen-needle (**Fig. 1A**) and connect it to the polyethylene (PE) tubing (20–30 cm in length). Be careful not to puncture the tubing with the pen needle which is sharp at both sides. Connect a Hamilton microsyringe to the other side of the tubing (**Fig. 1B**). To test whether all connections are tight and correctly sealed, flush the injection device with PBS. The virus solution can now be drawn up with the microsyringe into the plastic tubing and will be visible within the tubing. The injection device is now ready to use.

CAUTION: Adhere to biosafety regulations in terms of handling and disposal of virus and virus containing material.

PROCEDURE

In vitro testing of lentiviral vectors

We recommend testing your lentiviral construct *in vitro* before injecting it into mice. Such a test could be performed as follows:

1. Seed preadipocytes and let attach.
2. Transduce with different amounts of virus (*e.g.,* 50 ng and 200 ng per 6-well).
3. Differentiate the cells according to standard differentiation protocol [19,30].
4. Expression of the transgene can be quantified using qRT-PCR for mRNA expression, Western blotting for protein expression (**Fig. S1A-S1B**) or microscopic analysis at different time points during differentiation if using a reporter gene (**Fig. S1C-S1D**).

In vivo injection of lentiviral vectors

5. Inject mice with analgesic (Carprofen 5 mg/kg body weight) 30 min before starting the procedure.
6. Under the safety cabinet draw up the desired amount of virus. We recommend using a volume of 20–30 µl in each fat lobe of BAT and WATi.
7. Anaesthetize mice with isoflurane: Anesthesia is induced with 2.5% (w/v) isoflurane and maintained with 1% (w/v) isoflurane in O_2. **CAUTION:** Test if anesthesia is deep enough by pinching the skin between the toes. No withdrawal should be observed.

8. Shave the small area in the interscapular region for injections into BAT (**Fig. 1C**) and at the flanks, proximal of hip joints for injections into WATi/beige fat with hair clipper (**Fig. 1D**). Clean the skin with ethanol.
9. Make a 0.5–0.8 cm incision in the skin using surgical scissors (see red lines in **Fig. 1C** and **1D**).
10. Hold skin open with tweezers to expose fat pad. Visualize BAT with surrounding/adjacent white fat pads (**Fig. 1E**) or the dorsal tip of the WATi pad (**Fig. 1F**). For injections into WATi use tweezers to shift the fat pad upwards through the incision and expose the pad without completely removing it from its native position.
11. Take the injection device and carefully insert the pen needle into the fat pad. Only when the needle is inserted deep enough start to inject lentiviral vectors into multiple (5–10) distinct spots in the fat pad using the fine needle (total 20–30 µl per lobe).
12. After the virus solution is injected completely, carefully take out the needle and dispose the needle together with the tubing according to local regulations.
13. Close the incision with Michel suture clips.
14. Post surgery, monitor health status of the mice daily and treat the mice with analgesic (Carprofen 5 mg/kg body weight) for two days or longer if you have hints that the mouse is still in pain (not normal behavior, *e.g.,* less exploring and rearing; outer appearance, *e.g.,* shaggy fur, blurry eyes, inflammation of the wound). Recovery from the small surgery is usually very quick and the suture clips can be removed or are lost generally after 7–14 days. **CAUTION:** always refer to approval by local ethics/animal health committees.
15. Analysis: The injected mice can be retained for any desired time period from days up to several weeks and can even be used for further *in vivo* experiments after the appropriate recovery time (*e.g.*, metabolic measurements, special dietaries, glucose tolerance tests).

Timing

Steps 5-6, preparation and pre-treatment of mice: 30 min
Steps 7-13: 10-15 min
Step 14: 2 days or longer
Step 15: Any desired time point

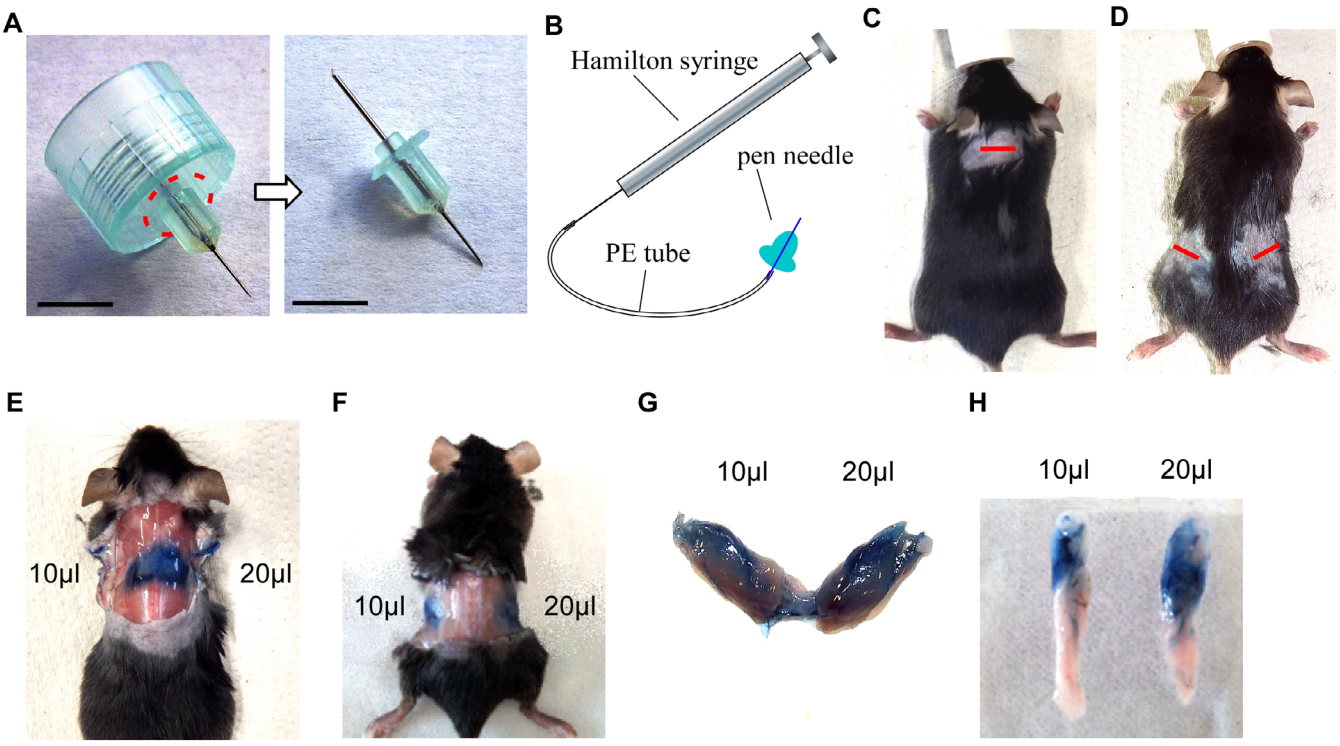

Figure 1. Set up of injection device and establishment of protocol. A. Original pen needle (dashed line indicates the cutting area) (left) and needle after cutting off plastic rim (right). Scale bar = 0.5 cm. **B.** Illustration showing setup of injection device. **C** and **D.** Pictures of anesthetized and shaved mouse, incision sites are marked by a red line in the interscapular region (C) or at the flanks, proximal of hips (D). **E.** *Situs* after injection of 10 µl and 20 µl Trypan blue solution in BAT. **F.** *Situs* after injection of 10 µl and 20 µl Trypan blue solution in the upper region of WATi. **G.** Dissected BAT from (E). **H.** Dissected WATi from (F).

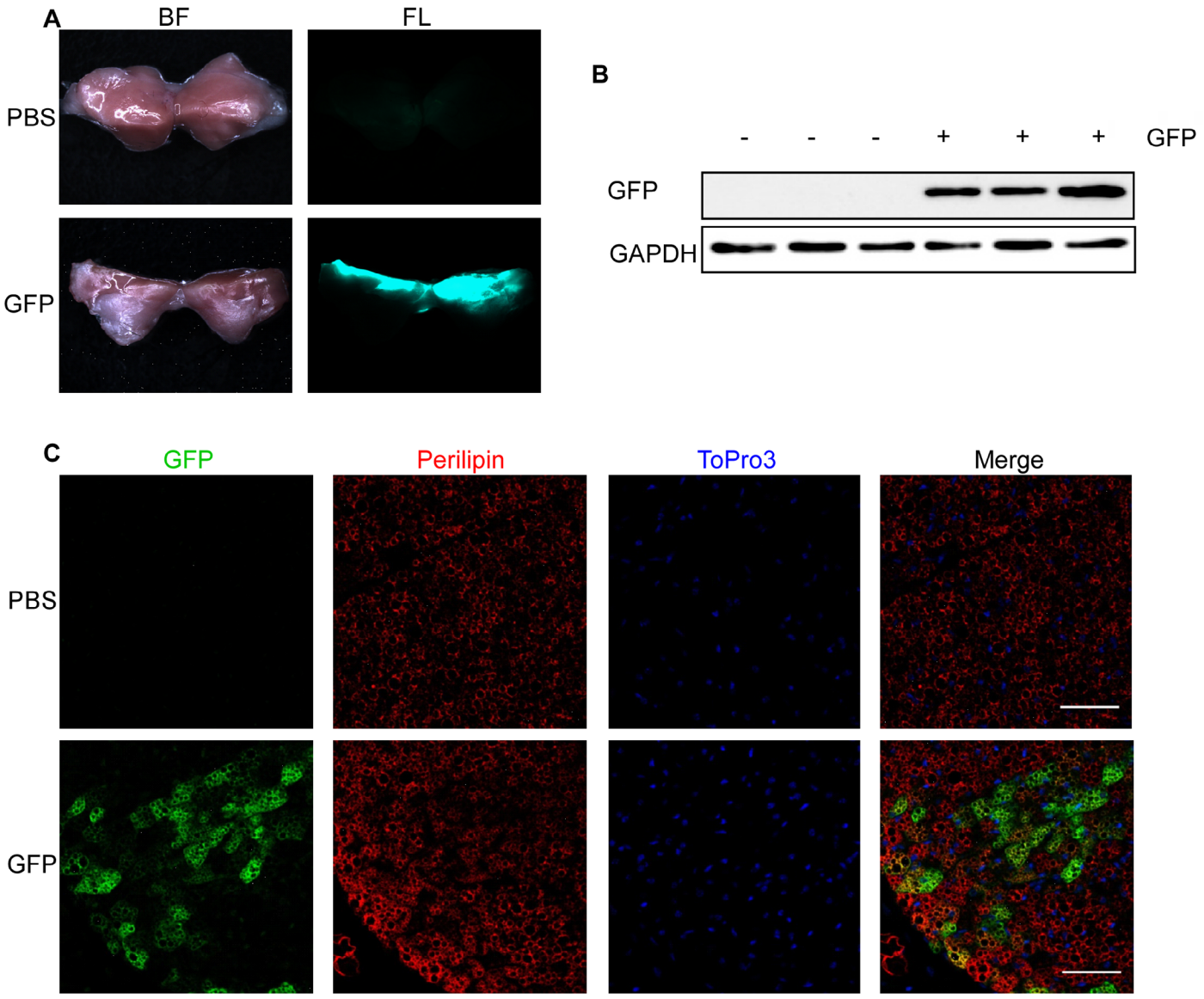

Figure 2. GFP fluorescence in interscapular BAT after lentivirus injection. A-C. 1000 ng RT of lentivirus carrying GFP under control of a CMV promoter (GFP) in 25 µl HBSS or 25 µl PBS were injected into each interscapular BAT lobe of 4-week-old male mice and analyzed after 1 week. **A.** Bright field (BF, left) and fluorescent images (FL, right) of PBS (upper panel) and GFP (lower panel) injected BAT. **B.** GFP expression assessed by Western blotting. Respective blots of GFP and the loading control GAPDH are shown. **C.** GFP expression assessed by immunohistological staining. PFA-fixed BAT sections were triple stained with antibodies directed against GFP (green) and Perilipin (red) as well as with ToPro3 (blue) to stain the nuclei. Scale bar = 50 µm.

ANTICIPATED RESULTS

In vitro testing of lentiviral vectors

To test that your lentiviral vector is working in principle, it is recommended to test it *in vitro* before injecting it into your mice. We transduced brown (BA) and inguinal white adipocytes (WAi) with two different amounts of lentivirus carrying GFP under the control of a cytomegalovirus (CMV) promoter (*i.e.,* 50 ng and 200 ng per 150,000 seeded cells) and differentiated the cells to mature adipocytes according to standard protocol. Proteins from terminally differentiated cells were isolated and Western blotting was performed to analyze GFP expression, which was only present in transduced BA and WAi but not in untransduced control (ctrl) cells (**Fig. S1A-S1B**). During differentiation GFP expression was confirmed by means of fluorescent microscopy, showing expression of GFP in preadipocytes (d-2) as well as in mature BA and WAi (d7) (**Fig. S1C-S1D**).

Establishment of protocol using Trypan blue solution

Before injecting precious lentivirus solutions, we recommend to get used to the protocol with the use of Trypan blue solution. After following the protocol as outlined in the PROCEDURE section the adipose tissue pads should appear in a blue color as shown in **Figure 1E-1H**.

GFP expression in BAT and WATi

One week after injection of 1000 ng lentivirus carrying GFP under the control of a cytomegalovirus (CMV) promoter per BAT lobe, fluorescence was clearly visible in the dissected fat pads (**Fig. 2A**). The results were validated using Western blotting (**Fig. 2B**), which showed expression of GFP in lentivirus-injected mice but not in mice injected with PBS. Furthermore, we performed immunofluorescent staining of GFP, Perilipin (adipocyte marker) and ToPro3 (nucleus staining) in PFA-fixed sections of lentivirus-injected BAT pads to analyze brown adipocyte specific GFP expression (**Fig. 2C**). Similar results were obtained after injection of WATi (**Fig. 3A-3C**). Even though the same amount of lentivirus (*i.e.,* 1000 ng/fat lobe) was injected into BAT and WATi, expression levels of GFP were significantly lower in WATi in comparison to BAT (**Fig. 4A**). This is probably due to the larger overall size of WATi, which reduces the number of viral particles per gram of tissue. We recommend using a higher amount of lentivirus for injections into WATi than into BAT. Sirius Red staining of lentivirus-injected BAT pads detected no damage in the injected tissue due to the small injection needle used (**Fig. 4B**).

In conclusion, we present a fast and easy applicable method to efficiently transfer genes into murine brown and beige adipocytes using lentiviral vectors.

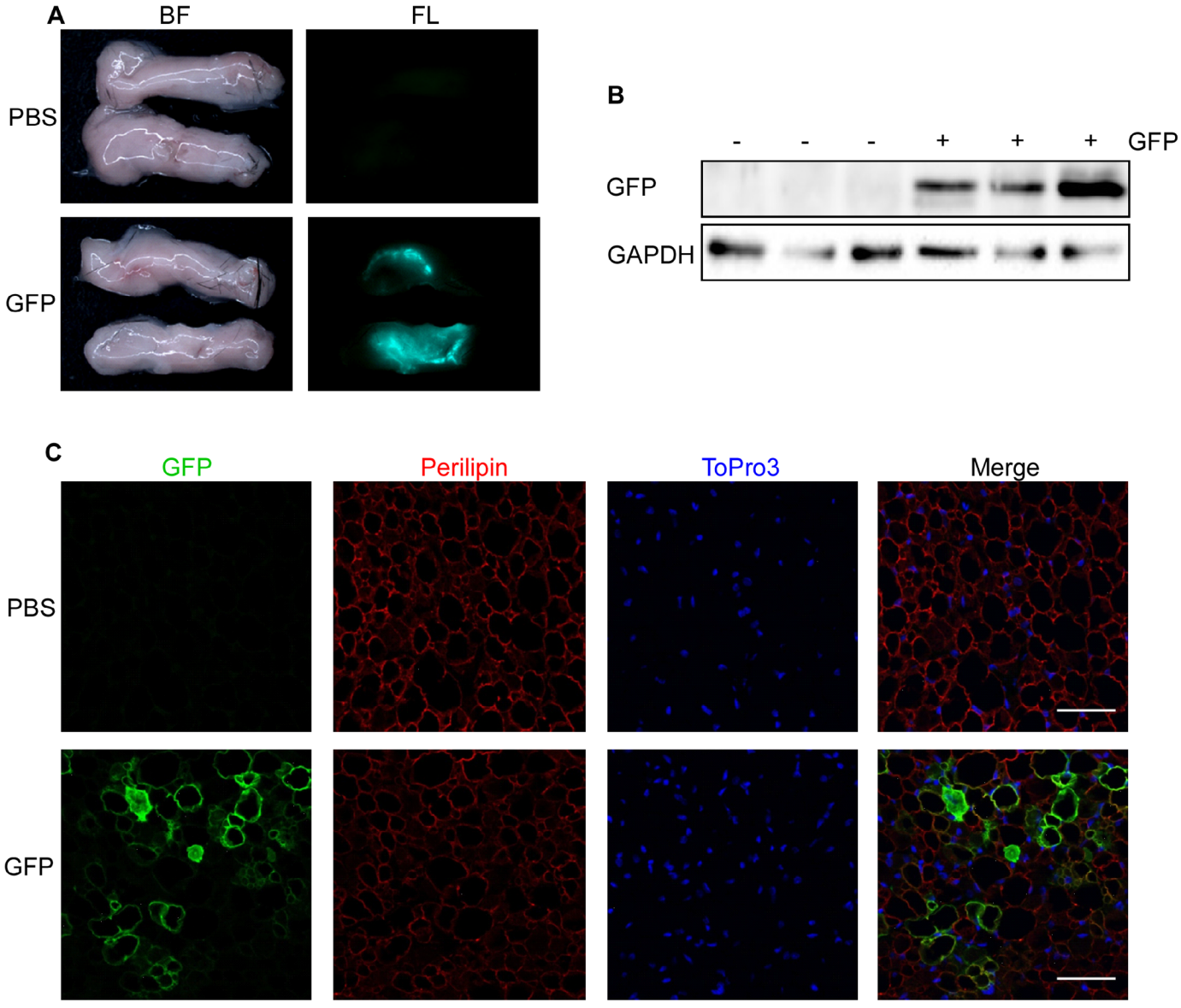

Figure 3. Expression of GFP in inguinal WAT (WATi) after lentivirus injection. A-C. 1000 ng RT of lentivirus carrying GFP under control of a CMV promoter (GFP) in 30 µl HBSS or 30 µl PBS were injected into each WATi pad of 10-week-old male mice and analyzed after 1 week. **A.** Bright field (BF, left) and fluorescent images (FL, right) of PBS (upper panel) and GFP (lower panel) injected WATi. **B.** GFP expression assessed by Western blotting. Respective blots of GFP and the loading control GAPDH are shown. **C.** GFP expression assessed by immunohistological staining. PFA-fixed WATi sections were triple stained with antibodies directed against GFP (green) and Perilipin (red) as well as with ToPro3 (blue) to stain the nuclei. Scale bar = 50 µm.

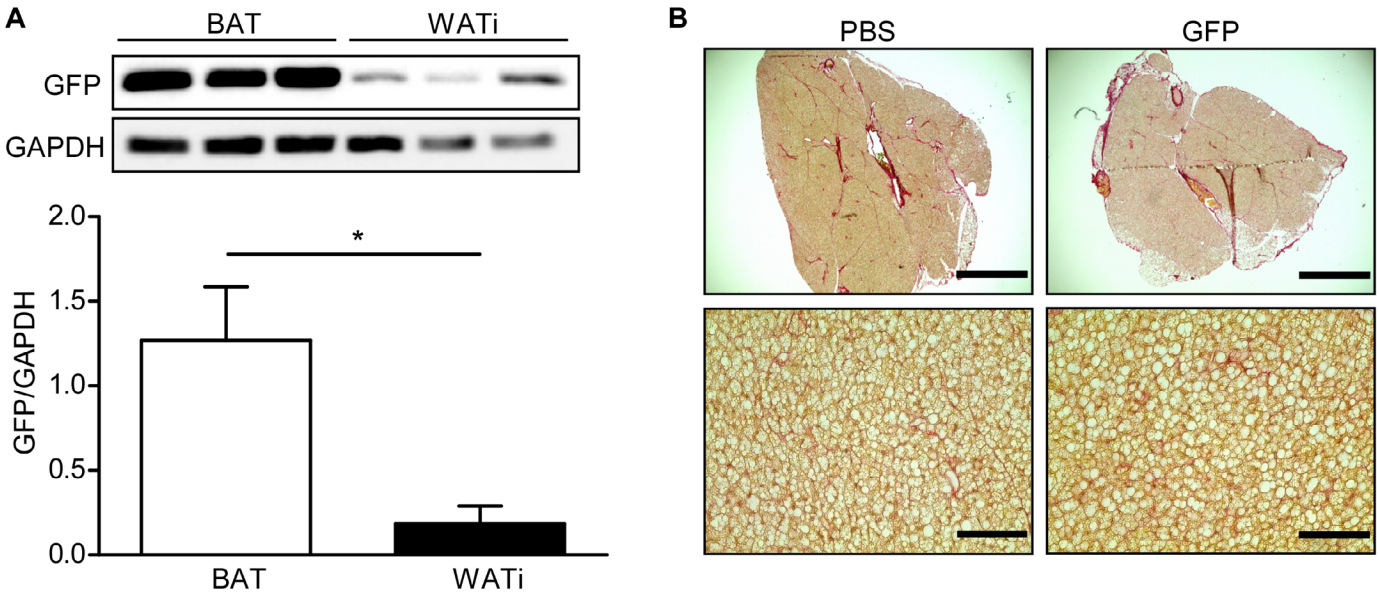

Figure 4. Comparison of GFP expression in BAT and WATi and Sirius Red staining of BAT. A. GFP expression in BAT and WATi after injection of 1000 ng RT of lentivirus carrying GFP into each fat lobe assessed by Western blotting (top) and quantitative analysis (bottom). *$P < 0.05$ (n = 3). **B.** Representative BAT sections of PBS- and GFP-injected mice, stained with Sirius Red for collagen (I and III) fibres. Scale bar top = 1 mm; scale bar bottom = 100 µm.

TROUBLESHOOTING

Potential problems and their causes and solutions are listed in **Table 1**.

Table 1. Troubleshooting.

Step	Problems	Causes	Suggestions
6	Impossible to draw up virus	• Virus solution is too viscous	• Dilute in larger volume of virus solvent
		• PE tubing is damaged	• Use a new piece of tubing and be very careful when connecting it to the pen needle
		• Pen needle is damaged or clogged	• Use a new pen needle
7	Anaesthesia does not work	• Isoflurane dose is too low	• Carefully increase dose of isoflurane until mouse loses consciousness and does not react to pinch test anymore. Check mouse constantly for signs of awakening.
		• Isoflurane anesthesia system is not set up properly	• Check all connections
14	Incision becomes inflamed	• Surgical instruments were not properly cleaned	• Sterilize and autoclave all used surgical instruments properly
		• Hair was not properly removed and entered the wound	• Remove fur in a larger area around the incision. Use ethanol to clean sticking hair
		• Transferred gene induces inflammation	
15	No or too low expression of transferred gene	• Fat pads were not injected properly	• Practice the method using Trypan blue solution (see **Fig. 1E-1H**) to make sure the correct spots are injected. Be careful not to put the needle too deep or too shallow. Try to inject in as many different spots as possible to ensure expression in the whole tissue.
		• Low expression efficiency of lentiviral vector	• Test efficiency of lentiviral vector *in vitro,* if possible try to increase amount of injected lentivirus
		• Analysis is done too early	• We recommend waiting at least 48–72 h post injection before starting with analysis to ensure proper transduction of the tissues and expression of the transferred gene.

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Authors' contributions

AB wrote the manuscript, performed experiments and analyzed data, LH wrote the manuscript, designed protocols and performed experiments, KK and AG performed experiments, TG helped writing the manuscript and performed experiments, KZ designed protocols and reviewed the manuscript, AP designed protocols and wrote the manuscript.

References

1. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, et al. (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet 384: 766-781. doi: 10.1016/S0140-6736(14)60460-8. PMID: 24880830
2. Pfeifer A, Hoffmann LS (2014) Brown, beige, and white: the new color code of fat and its pharmacological implications. Annu Rev Pharmacol Toxicol 55: 207-227. doi: 10.1146/annurev-pharmtox-010814-124346. PMID: 25149919
3. Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. Physiol Rev 84: 277-359. doi: 10.1152/physrev.00015.2003. PMID: 14715917
4. Klingenspor M, Herzig S, Pfeifer A (2012) Brown fat develops a brite future. Obes Facts 5: 890-896. doi: 10.1159/000346337. PMID: 23296106
5. van Marken Lichtenbelt, D. (Wouter) , Vanhommerig JW, Smulders NM, Drossaerts JMAFL, Kemerink GJ, et al. (2009) Cold-activated brown adipose tissue in healthy men. N Engl J Med 360: 1500-1508. doi: 10.1056/NEJMoa0808718. PMID: 19357405
6. Cypess AM, Lehman S, Williams G, Tal I, Rodman D, et al. (2009) Identification and importance of brown adipose tissue in adult humans. N Engl J Med 360: 1509-1517. doi: 10.1056/NEJMoa0810780. PMID: 19357406
7. Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, et al. (2009) Functional brown adipose tissue in healthy adults. N Engl J Med 360: 1518-1525. doi: 10.1056/NEJMoa0808949. PMID: 19357407
8. Yoneshiro T, Aita S, Matsushita M, Kayahara T, Kameya T, et al. (2013) Recruited brown adipose tissue as an antiobesity agent in humans. J Clin Invest 123: 3404-3408. doi: 10.1172/JCI67803. PMID: 23867622
9. van der Lans AA, Hoeks J, Brans B, Vijgen GH, Visser MG, et al. (2013) Cold acclimation recruits human brown fat and increases nonshivering thermogenesis. J Clin Invest 123: 3395-3403. doi: 10.1172/JCI68993. PMID: 23867626
10. Harms M, Seale P (2013) Brown and beige fat: development, function and therapeutic potential. Nat Med 19: 1252-1263. doi: 10.1038/nm.3361. PMID: 24100998
11. Rosen ED, Spiegelman BM (2014) What we talk about when we talk about fat. Cell 156: 20-44. doi: 10.1016/j.cell.2013.12.012. PMID: 24439368
12. Seale P, Conroe HM, Estall J, Kajimura S, Frontini A, et al. (2010) Prdm16 determines the thermogenic program of subcutaneous white adipose tissue in mice. J Clin Invest 121: 96-105. doi: 10.1172/JCI44271. PMID: 21123942
13. Schulz TJ, Huang TL, Tran TT, Zhang H, Townsend KL, et al. (2010) Identification of inducible brown adipocyte progenitors residing in skeletal muscle and white fat. Proc Natl Acad Sci U S A 108: 143-148. doi: 10.1073/pnas.1010929108. PMID: 21173238
14. Hoffmann LS, Larson CJ, Pfeifer A (2016) cGMP and Brown Adipose Tissue. Handb Exp Pharmacol 233: 283-299. doi: 10.1007/164_2015_3. PMID: 25903412
15. Pfeifer A, Verma IM (2001) Gene therapy: promises and problems. Annu Rev Genomics Hum Genet 2: 177-211. doi: 10.1146/annurev.genom.2.1.177. PMID: 11701648
16. Haas B, Mayer P, Jennissen K, Scholz D, Berriel Diaz M, et al. (2009) Protein kinase G controls brown fat cell differentiation and mitochondrial biogenesis. Sci Signal 2: doi: 10.1126/scisignal.2000511. PMID: 19952371
17. Mitschke MM, Hoffmann LS, Gnad T, Scholz D, Kruithoff K, et al. (2013) Increased cGMP promotes healthy expansion and browning of white adipose tissue. FASEB J 27: 1621-1630. doi: 10.1096/fj.12-221580. PMID: 23303211
18. Fujiwara K, Hasegawa K, Ohkumo T, Miyoshi H, Tseng Y, et al. (2012) Necdin controls proliferation of white adipocyte progenitor cells. PLoS One 7: doi: 10.1371/journal.pone.0030948. PMID: 22292082
19. Gnad T, Scheibler S, von Kügelgen I, Scheele C, Kilić A, et al. (2014) Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature 516: 395-399. doi: 10.1038/nature13816. PMID: 25317558
20. Pfeifer A, Lehmann H (2010) Pharmacological potential of RNAi--focus on miRNA. Pharmacol Ther 126: 217-227. doi: 10.1016/j.pharmthera.2010.03.006. PMID: 20388525
21. Pfeifer A, Hofmann A (2009) Lentiviral transgenesis. Methods Mol Biol 530: 391-405. doi: 10.1007/978-1-59745-471-1_21. PMID: 19266335
22. Kang S, Kong X, Rosen ED (2014) Adipocyte-specific transgenic and knockout models. Methods Enzymol 537: 1-16. doi: 10.1016/B978-0-12-411619-1.00001-X. PMID: 24480338
23. Liu X, Magee D, Wang C, McMurphy T, Slater A, et al. (2014) Adipose tissue insulin receptor knockdown via a new primate-derived hybrid recombinant AAV serotype. Mol Ther Methods Clin Dev 1: doi: 10.1038/mtm.2013.8. PMID: 25383359
24. Verma IM, Somia N (1997) Gene therapy -- promises, problems and prospects. Nature 389: 239-242. doi: 10.1038/38410. PMID: 9305836
25. Daya S, Berns KI (2008) Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev 21: 583-593. doi: 10.1128/CMR.00008-08. PMID: 18854481
26. Yang L, Bailey L, Baltimore D, Wang P (2006) Targeting lentiviral vectors to specific cell types in vivo. Proc Natl Acad Sci U S A 103: 11479-11484. doi: 10.1073/pnas.0604993103. PMID: 16864770
27. Ussar S, Lee KY, Dankel SN, Boucher J, Haering M, et al. (2014) ASC-1, PAT2, and P2RX5 are cell surface markers for white, beige, and brown adipocytes. Sci Transl Med 6: 247-103. doi: 10.1126/scitranslmed.3008490. PMID: 25080478
28. Pauwels K, Gijsbers R, Toelen J, Schambach A, Willard-Gallo K, et al. (2009) State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. Curr Gene Ther 9: 459-474. PMID: 20021330
29. Trueck C, Zimmermann K, Mykhaylyk O, Anton M, Vosen S, et al. (2012) Optimization of magnetic nanoparticle-assisted lentiviral gene transfer. Pharm Res 29: 1255-1269. doi: 10.1007/s11095-011-0660-x. PMID: 22274554
30. Jennissen K, Haas B, Mitschke MM, Siegel F, Pfeifer A (2013) Analysis of cGMP signaling in adipocytes. Methods Mol Biol 1020: 175-192. doi: 10.1007/978-1-62703-459-3_11. PMID: 23709033

Supplementary information

Figure S1. Example for testing of lentiviral constructs *in vitro* in brown and white adipocytes.

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/123.

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Direct lentivirus injection for fast and efficient gene transfer into brown and beige adipose tissue

Balkow et al.

Supplementary Information

Figure S1. Example for testing of lentiviral constructs *in vitro* in brown and white adipocytes.

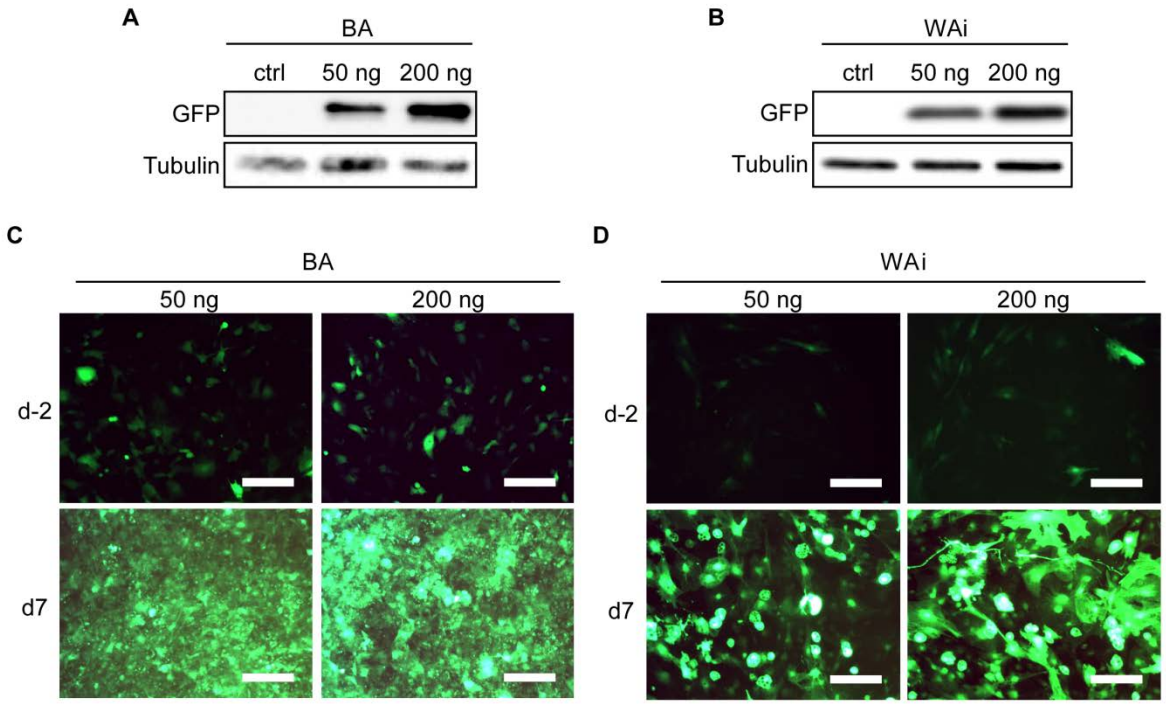

Figure S1. Example for testing of lentiviral constructs *in vitro* in brown and white adipocytes. Brown adipocytes (BA) differentiated from preadipocytes isolated from BAT of newborn mice and white adipocytes (WAi) differentiated from preadipocytes isolated from inguinal white adipose tissue of 12-weeks old mice were transduced with CMV-GFP lentivirus (50 ng or 200 ng RT); untransduced cells served as control (ctrl). **A** and **B.** Western blotting of GFP expression in BA and WAi; tubulin serves as loading control. **C** and **D.** Microscopic pictures of GFP expression in BA and WAi in preadipocytes (d-2) and mature adipocytes (d7). Scale bars = 20 µm.

Supplementary information of this article can be found online at http://www.jbmethods.org/jbm/rt/suppFiles/123.

3.3 Epilogue

The presented method for direct lentivirus injections into subcutaneous adipose tissues has several advantages over production of transgenic mouse lines:

1. It is time saving in many aspects:
    - Production of a new transgenic mouse line usually takes several months, whereas direct lentiviral injections can be applied within minutes to overexpress or knock down a gene of interest.
    - Even if a transgenic mouse line already exists, regular breeding and genotyping to receive the right amount of mice with the desired genotype is very time consuming. By means of direct lentiviral injections, it is possible to generate as many transgenic mice, including appropriate controls, as needed. No genotyping or breeding is necessary.
    - The use of a transgenic mouse line requires waiting until mice of the right genotype reach a desired age before experiments can be performed. Direct lentiviral injections, on the other hand, can be performed in any mice older than 4 weeks.
2. The above mentioned points also reduce costs for housing, breeding and genotyping of mice.
3. Only mice with the correct genotype will be produced, thereby reducing animal numbers dramatically.
4. Transgenes can be expressed in only one adipose depot specifically.
5. Already existing transgenic mouse lines can further be modified with this technique.
6. One-and-the-same vector can be used to produce global transgenic mice with the help of subzonal injections, thereby serving as an additional experimental control to direct lentivirus injections.

Overall, brown and beige adipocytes are in the focus of research as possible targets to treat obesity. The method presented here could be of substantial interest for any researcher investigating signaling pathways in adipose tissues. It makes the study of transgenes for their influence on brown/beige fat activity and energy expenditure *in vivo* easily accessible. By this, a plethora of genes can be studied in a timely manner, potentially leading to new gene therapies for the treatment of obesity or at least identifying novel targets for pharmaceutical interventions.

4 Conclusion

In recent years, activation of brown and beige fat has emerged as potential target to increase energy expenditure and thereby fight obesity in humans. Factors involved in regulation of adipose tissue function can either derive from adipose tissue itself or from other organs like the pancreas, liver, muscle, heart and brain. Examples for such factors are adiopnectin (Hu et al., 1996; Scherer et al., 1995), insulin (Renold et al., 1950), FGF21 (Nishimura et al., 2000; Xu et al., 2009), irisin (Bostrom et al., 2012), natriuretic peptides (Crandall et al., 1989) and endocannabinoids (Cota et al., 2003), respectively.

In this thesis, the impact of the type I TGFβ receptor Alk7 on brown adipocyte function is unraveled. Activation of Alk7 by Activin AB differentially regulates adipogenic and thermogenic pathways, leading to an upregulation of UCP1 expression (Balkow et al., 2015). Activin AB/B could therefore be a novel adipose derived factor which increases UCP1 expression in brown adipocytes. Importantly, the Alk7 signaling pathway is closely connected to the cGMP-signaling pathway in brown adipocytes; probably representing a potential safety mechanism to ensure that overstimulation of the adipogenic program by cGMP is avoided (Balkow et al., 2015). As obese patients exhibit decreased Alk7 expression (Carlsson et al., 2009), it could be of clinical benefit to enhance cGMP signaling in BAT to endogenously increase Alk7 expression and by this boost UCP1 activity as well as energy expenditure. However, testing this hypothesis requires further *in vivo* studies.

The second part of the thesis is dealing with the development and validation of an easy-to-handle and fast applicable method to transduce brown and beige adipose tissue *via* direct lentiviral injections *in vivo* (Balkow et al., 2016). As the procedure is undertaken within less than an hour to efficiently produce transgenic mice, it could be of substantial interest for any researcher investigating various signaling pathways in adipose tissues, without the need of creating whole new transgenic mouse lines. By this, a multitude of genes can be

studied in a timely manner, potentially identifying novel targets for pharmaceutical interventions or even new gene therapies for the treatment of obesity.

Furthermore, the presented method can be applied to investigate Alk7-effects in BAT *in vivo*. This could be conducted by overexpressing either caAlk7 or known ligands for Alk7 (i.e. Activin AB/B) and subsequently analyzing transgenic mice for aspects of whole-body metabolism, e.g. by submission to a HFD or a cold stimulus. The Alk7-induced upregulation of UCP1 expression should increase energy expenditure, subsequently resulting in resistance to DIO as well as to increased cold tolerance.

In conclusion, the presented work in this thesis is uncovering two novel approaches for the investigation of brown adipocyte differentiation and function, thereby possibly revealing new targets to fight the obesity pandemic.

5 References

Abdelaziz N., Colombo F., Mercier I., and Calderone A. (2001). Nitric oxide attenuates the expression of transforming growth factor-beta(3) mRNA in rat cardiac fibroblasts via destabilization. Hypertension *38*, 261-266.

Adeva-Andany M.M., Gonzalez-Lucan M., Donapetry-Garcia C., Fernandez-Fernandez C., and Ameneiros-Rodriguez E. (2016). Glycogen metabolism in humans. BBA Clin *5*, 85-100.

Andersson O., Korach-Andre M., Reissmann E., Ibanez C.F., and Bertolino P. (2008). Growth/differentiation factor 3 signals through ALK7 and regulates accumulation of adipose tissue and diet-induced obesity. Proc Natl Acad Sci U S A *105*, 7252-7256.

Andersson O., Reissmann E., and Ibanez C.F. (2006). Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. EMBO Rep *7*, 831-837.

Atchison R.W., Casto B.C., and Hammon W.M. (1965). Adenovirus-Associated Defective Virus Particles. Science *149*, 754-756.

Ayala J.E., Bracy D.P., Julien B.M., Rottman J.N., Fueger P.T., and Wasserman D.H. (2007). Chronic treatment with sildenafil improves energy balance and insulin action in high fat-fed conscious mice. Diabetes *56*, 1025-1033.

Balkow A., Hoffmann L.S., Klepac K., Glöde A., Gnad T., Zimmermann K., and Pfeifer A. (2016). Direct lentivirus injection for fast and efficient gene transfer into brown and beige adipose tissue. Journal of Biological Methods *3*, e48.

Balkow A., Jagow J., Haas B., Siegel F., Kilic A., and Pfeifer A. (2015). A novel crosstalk between Alk7 and cGMP signaling differentially regulates brown adipocyte function. Mol Metab *4*, 576-583.

Berg J.M., Tymoczko J.L., and Stryer L. (2002). Triacylglycerols Are Highly Concentrated Energy Stores. In Biochemistry (New York, W H Freeman).

Bernard D.J., Lee K.B., and Santos M.M. (2006). Activin B can signal through both ALK4 and ALK7 in gonadotrope cells. Reprod Biol Endocrinol *4*, 52.

Bertolino P., Holmberg R., Reissmann E., Andersson O., Berggren P.O., and Ibanez C.F. (2008). Activin B receptor ALK7 is a negative regulator of pancreatic beta-cell function. Proc Natl Acad Sci U S A *105*, 7246-7251.

Bondestam J., Huotari M.A., Moren A., Ustinov J., Kaivo-Oja N., Kallio J., Horelli-Kuitunen N., Aaltonen J., Fujii M., Moustakas A.*, et al.* (2001). cDNA cloning, expression studies and chromosome mapping of human type I serine/threonine kinase receptor ALK7 (ACVR1C). Cytogenet Cell Genet *95*, 157-162.

Bordicchia M., Liu D., Amri E.Z., Ailhaud G., Dessi-Fulgheri P., Zhang C., Takahashi N., Sarzani R., and Collins S. (2012). Cardiac natriuretic peptides act via p38 MAPK to induce the brown fat thermogenic program in mouse and human adipocytes. J Clin Invest *122*, 1022-1036.

Bostrom P., Wu J., Jedrychowski M.P., Korde A., Ye L., Lo J.C., Rasbach K.A., Bostrom E.A., Choi J.H., Long J.Z.*, et al.* (2012). A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature *481*, 463-468.

Bouard D., Alazard-Dany D., and Cosset F.L. (2009). Viral vectors: from virology to transgene expression. Br J Pharmacol *157*, 153-165.

Bray G.A., Fruhbeck G., Ryan D.H., and Wilding J.P. (2016). Management of obesity. Lancet.

Breckpot K., Aerts J.L., and Thielemans K. (2007). Lentiviral vectors for cancer immunotherapy: transforming infectious particles into therapeutics. Gene Ther *14*, 847-862.

Buller R.M., Janik J.E., Sebring E.D., and Rose J.A. (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. J Virol *40*, 241-247.

Cannon B., and Nedergaard J. (2004). Brown adipose tissue: function and physiological significance. Physiol Rev *84*, 277-359.

Carlsson L.M., Jacobson P., Walley A., Froguel P., Sjostrom L., Svensson P.A., and Sjoholm K. (2009). ALK7 expression is specific for adipose tissue, reduced in obesity and correlates to factors implicated in metabolic disease. Biochem Biophys Res Commun *382*, 309-314.

Chan A.W., Homan E.J., Ballou L.U., Burns J.C., and Bremel R.D. (1998). Transgenic cattle produced by reverse-transcribed gene transfer in oocytes. Proc Natl Acad Sci U S A *95*, 14028-14033.

Cinti S., Cigolini M., Gazzanelli G., and Bosello O. (1985). An ultrastructural study of adipocyte precursors from epididymal fat pads of adult rats in culture. J Submicrosc Cytol *17*, 631-636.

Cinti S., Zancanaro C., Sbarbati A., Cicolini M., Vogel P., Ricquier D., and Fakan S. (1989). Immunoelectron microscopical identification of the uncoupling protein in brown adipose tissue mitochondria. Biol Cell *67*, 359-362.

Cota D., Marsicano G., Tschop M., Grubler Y., Flachskamm C., Schubert M., Auer D., Yassouridis A., Thone-Reineke C., Ortmann S.*, et al.* (2003). The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. J Clin Invest *112*, 423-431.

Crandall D.L., Ferraro G.D., and Cervoni P. (1989). Effect of experimental obesity and subsequent weight reduction upon circulating atrial natriuretic peptide. Proc Soc Exp Biol Med *191*, 352-356.

Cypess A.M., Lehman S., Williams G., Tal I., Rodman D., Goldfine A.B., Kuo F.C., Palmer E.L., Tseng Y.H., Doria A.*, et al.* (2009). Identification and importance of brown adipose tissue in adult humans. N Engl J Med *360*, 1509-1517.

Daya S., and Berns K.I. (2008). Gene therapy using adeno-associated virus vectors. Clin Microbiol Rev *21*, 583-593.

Derynck R., and Zhang Y.E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature *425*, 577-584.

Deutsche Adipositas-Gesellschaft (DAG), Deutsche Diabetes Gesellschaft (DDG), Deutsche Gesellschaft für Ernährung (DGE), and Deutsche Gesellschaft für Ernährungsmedizin (DGEM) (2014). Interdisziplinäre Leitlinie der Qualität S3 zur „Prävention und Therapie der Adipositas". *2. Auflage*.

Dickson L.M., Gandhi S., Layden B.T., Cohen R.N., and Wicksteed B. (2016). Protein kinase A induces UCP1 expression in specific adipose depots to increase energy expenditure and improve metabolic health. Am J Physiol Regul Integr Comp Physiol *311*, R79-88.

Enerbäck S. (2010). Human brown adipose tissue. Cell Metab *11*, 248-252.

Farooqi I.S., and O'Rahilly S. (2005). Monogenic obesity in humans. Annu Rev Med *56*, 443-458.

Fedorenko A., Lishko P.V., and Kirichok Y. (2012). Mechanism of fatty-acid-dependent UCP1 uncoupling in brown fat mitochondria. Cell *151*, 400-413.

Feng X.H., and Derynck R. (2005). Specificity and versatility in tgf-beta signaling through Smads. Annu Rev Cell Dev Biol *21*, 659-693.

Ferreira V., Petry H., and Salmon F. (2014). Immune Responses to AAV-Vectors, the Glybera Example from Bench to Bedside. Front Immunol *5*, 82.

Flegal K.M., Kit B.K., Orpana H., and Graubard B.I. (2013). Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. JAMA *309*, 71-82.

Forest C., Joffin N., Jaubert A.M., and Noirez P. (2016). What induces watts in WAT? Adipocyte *5*, 136-152.

Foster D.O., and Frydman M.L. (1978). Nonshivering thermogenesis in the rat. II. Measurements of blood flow with microspheres point to brown adipose tissue as the dominant site of the calorigenesis induced by noradrenaline. Can J Physiol Pharmacol *56*, 110-122.

Fournier B., Murray B., Gutzwiller S., Marcaletti S., Marcellin D., Bergling S., Brachat S., Persohn E., Pierrel E., Bombard F.*, et al.* (2012). Blockade of the

activin receptor IIb activates functional brown adipogenesis and thermogenesis by inducing mitochondrial oxidative metabolism. Mol Cell Biol *32*, 2871-2879.

Francis S.H., Busch J.L., Corbin J.D., and Sibley D. (2010). cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol Rev *62*, 525-563.

Friebe A., and Koesling D. (2009). The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models. Nitric Oxide *21*, 149-156.

Frontini A., and Cinti S. (2010). Distribution and development of brown adipocytes in the murine and human adipose organ. Cell Metab *11*, 253-256.

Frontini A., Vitali A., Perugini J., Murano I., Romiti C., Ricquier D., Guerrieri M., and Cinti S. (2013). White-to-brown transdifferentiation of omental adipocytes in patients affected by pheochromocytoma. Biochim Biophys Acta *1831*, 950-959.

Fujiwara K., Hasegawa K., Ohkumo T., Miyoshi H., Tseng Y.H., and Yoshikawa K. (2012). Necdin controls proliferation of white adipocyte progenitor cells. PLoS One *7*, e30948.

Galic S., Oakhill J.S., and Steinberg G.R. (2009). Adipose tissue as an endocrine organ. Mol Cell Endocrinol *316*, 129-139.

Gao M., and Liu D. (2014). Gene therapy for obesity: progress and prospects. Discov Med *17*, 319-328.

Garton A.J., Campbell D.G., Carling D., Hardie D.G., Colbran R.J., and Yeaman S.J. (1989). Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. Eur J Biochem *179*, 249-254.

Gesta S., Tseng Y.H., and Kahn C.R. (2007). Developmental origin of fat: tracking obesity to its source. Cell *131*, 242-256.

Gnad T., Scheibler S., von Kugelgen I., Scheele C., Kilic A., Glode A., Hoffmann L.S., Reverte-Salisa L., Horn P., Mutlu S.*, et al.* (2014). Adenosine activates brown adipose tissue and recruits beige adipocytes via A2A receptors. Nature *516*, 395-399.

Grundy S.M., Brewer H.B., Jr., Cleeman J.I., Smith S.C., Jr., and Lenfant C. (2004). Definition of metabolic syndrome: Report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation *109*, 433-438.

Guo T., Marmol P., Moliner A., Bjornholm M., Zhang C., Shokat K.M., and Ibanez C.F. (2014). Adipocyte ALK7 links nutrient overload to catecholamine resistance in obesity. Elife *3*, e03245.

Haas B., Mayer P., Jennissen K., Scholz D., Berriel Diaz M., Bloch W., Herzig S., Fassler R., and Pfeifer A. (2009). Protein kinase G controls brown fat cell differentiation and mitochondrial biogenesis. Sci Signal *2*, ra78.

Hacein-Bey-Abina S., Von Kalle C., Schmidt M., McCormack M.P., Wulffraat N., Leboulch P., Lim A., Osborne C.S., Pawliuk R., Morillon E.*, et al.* (2003). LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science *302*, 415-419.

Hamad A.M., Clayton A., Islam B., and Knox A.J. (2003). Guanylyl cyclases, nitric oxide, natriuretic peptides, and airway smooth muscle function. Am J Physiol Lung Cell Mol Physiol *285*, L973-983.

Hammer R.E., Pursel V.G., Rexroad C.E., Jr., Wall R.J., Bolt D.J., Ebert K.M., Palmiter R.D., and Brinster R.L. (1985). Production of transgenic rabbits, sheep and pigs by microinjection. Nature *315*, 680-683.

Hammond R.A., and Levine R. (2010). The economic impact of obesity in the United States. Diabetes Metab Syndr Obes *3*, 285-295.

Handa P., Tateya S., Rizzo N.O., Cheng A.M., Morgan-Stevenson V., Han C.Y., Clowes A.W., Daum G., O'Brien K.D., Schwartz M.W.*, et al.* (2011). Reduced vascular nitric oxide-cGMP signaling contributes to adipose tissue inflammation during high-fat feeding. Arterioscler Thromb Vasc Biol *31*, 2827-2835.

Harms M., and Seale P. (2013). Brown and beige fat: development, function and therapeutic potential. Nat Med *19*, 1252-1263.

Heaton G.M., Wagenvoord R.J., Kemp A., Jr., and Nicholls D.G. (1978). Brown-adipose-tissue mitochondria: photoaffinity labelling of the regulatory site of energy dissipation. Eur J Biochem *82*, 515-521.

Hendrickx R., Stichling N., Koelen J., Kuryk L., Lipiec A., and Greber U.F. (2014). Innate immunity to adenovirus. Hum Gene Ther *25*, 265-284.

Hoffmann L.S., Etzrodt J., Willkomm L., Sanyal A., Scheja L., Fischer A.W., Stasch J.P., Bloch W., Friebe A., Heeren J.*, et al.* (2015). Stimulation of soluble guanylyl cyclase protects against obesity by recruiting brown adipose tissue. Nat Commun *6*, 7235.

Hu E., Liang P., and Spiegelman B.M. (1996). AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem *271*, 10697-10703.

Huang W., McMurphy T., Liu X., Wang C., and Cao L. (2016). Genetic Manipulation of Brown Fat Via Oral Administration of an Engineered Recombinant Adeno-associated Viral Serotype Vector. Mol Ther *24*, 1062-1069.

Hull D., and Segall M.M. (1965). The contribution of brown adipose tissue to heat production in the new-born rabbit. J Physiol *181*, 449-457.

Inuzuka M., Tamura N., Yamada N., Katsuura G., Oyamada N., Taura D., Sonoyama T., Fukunaga Y., Ohinata K., Sone M.*, et al.* (2010). C-type natriuretic peptide as a new regulator of food intake and energy expenditure. Endocrinology *151*, 3633-3642.

Jaenisch R., Fan H., and Croker B. (1975). Infection of preimplantation mouse embryos and of newborn mice with leukemia virus: tissue distribution of viral

DNA and RNA and leukemogenesis in the adult animal. Proc Natl Acad Sci U S A *72*, 4008-4012.

Jahner D., and Jaenisch R. (1985). Retrovirus-induced de novo methylation of flanking host sequences correlates with gene inactivity. Nature *315*, 594-597.

Jornvall H., Blokzijl A., ten Dijke P., and Ibanez C.F. (2001). The orphan receptor serine/threonine kinase ALK7 signals arrest of proliferation and morphological differentiation in a neuronal cell line. J Biol Chem *276*, 5140-5146.

Journal of Gene Medicine Database (2016). Gene Therapy Clinical Trials Worldwide. Retrieved from http://www.wiley.com/legacy/wileychi/genmed/clinical/

Kalra P.S., and Kalra S.P. (2002). Obesity and metabolic syndrome: long-term benefits of central leptin gene therapy. Drugs Today (Barc) *38*, 745-757.

Kang S., Kong X., and Rosen E.D. (2014). Adipocyte-specific transgenic and knockout models. Methods Enzymol *537*, 1-16.

Kang Y., and Reddi A.H. (1996). Identification and cloning of a novel type I serine/threonine kinase receptor of the TGF-beta/BMP superfamily in rat prostate. Biochem Mol Biol Int *40*, 993-1001.

Kay M.A. (2011). State-of-the-art gene-based therapies: the road ahead. Nat Rev Genet *12*, 316-328.

Khalil A.M., Dotimas H., Kahn J., Lamerdin J.E., Hayes D.B., Gupta P., and Franti M. (2016). Differential Binding Activity of TGF-beta Family Proteins to Select TGF-beta Receptors. J Pharmacol Exp Ther.

Kim S.H., and Plutzky J. (2016). Brown Fat and Browning for the Treatment of Obesity and Related Metabolic Disorders. Diabetes Metab J *40*, 12-21.

Kim S.J., Tang T., Abbott M., Viscarra J.A., Wang Y., and Sul H.S. (2016). AMPK Phosphorylates Desnutrin/ATGL and Hormone-Sensitive Lipase To Regulate Lipolysis and Fatty Acid Oxidation within Adipose Tissue. Mol Cell Biol *36*, 1961-1976.

Klepac K., Kilic A., Gnad T., Brown L.M., Herrmann B., Wilderman A., Balkow A., Glode A., Simon K., Lidell M.E.*, et al.* (2016). The Gq signalling pathway inhibits brown and beige adipose tissue. Nat Commun *7*, 10895.

Kogame M., Matsuo S., Nakatani M., Kurisaki A., Nishitani H., Tsuchida K., and Sugino H. (2006). ALK7 is a novel marker for adipocyte differentiation. J Med Invest *53*, 238-245.

Krotkiewski M., Bjorntorp P., Sjostrom L., and Smith U. (1983). Impact of obesity on metabolism in men and women. Importance of regional adipose tissue distribution. J Clin Invest *72*, 1150-1162.

Lee Y.H., Petkova A.P., Mottillo E.P., and Granneman J.G. (2012). In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. Cell Metab *15*, 480-491.

Lewis P., Hensel M., and Emerman M. (1992). Human immunodeficiency virus infection of cells arrested in the cell cycle. EMBO J *11*, 3053-3058.

Li Z., Dullmann J., Schiedlmeier B., Schmidt M., von Kalle C., Meyer J., Forster M., Stocking C., Wahlers A., Frank O.*, et al.* (2002). Murine leukemia induced by retroviral gene marking. Science *296*, 497.

Linden R.M., Ward P., Giraud C., Winocour E., and Berns K.I. (1996). Site-specific integration by adeno-associated virus. Proc Natl Acad Sci U S A *93*, 11288-11294.

Liu X., Magee D., Wang C., McMurphy T., Slater A., During M., and Cao L. (2014). Adipose tissue insulin receptor knockdown via a new primate-derived hybrid recombinant AAV serotype. Mol Ther Methods Clin Dev *1*.

Luo J., Deng Z.L., Luo X., Tang N., Song W.X., Chen J., Sharff K.A., Luu H.H., Haydon R.C., Kinzler K.W.*, et al.* (2007). A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. Nat Protoc *2*, 1236-1247.

McCarty D.M., Young S.M., Jr., and Samulski R.J. (2004). Integration of adeno-associated virus (AAV) and recombinant AAV vectors. Annu Rev Genet *38*, 819-845.

Miles D.C., Wakeling S.I., Stringer J.M., van den Bergen J.A., Wilhelm D., Sinclair A.H., and Western P.S. (2013). Signaling through the TGF beta-activin receptors ALK4/5/7 regulates testis formation and male germ cell development. PLoS One *8*, e54606.

Miller D.G., Adam M.A., and Miller A.D. (1990). Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol *10*, 4239-4242.

Mitschke M.M., Hoffmann L.S., Gnad T., Scholz D., Kruithoff K., Mayer P., Haas B., Sassmann A., Pfeifer A., and Kilic A. (2013). Increased cGMP promotes healthy expansion and browning of white adipose tissue. FASEB J *27*, 1621-1630.

Miyashita K., Itoh H., Tsujimoto H., Tamura N., Fukunaga Y., Sone M., Yamahara K., Taura D., Inuzuka M., Sonoyama T.*, et al.* (2009). Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. Diabetes *58*, 2880-2892.

Munir S., Xu G., Wu Y., Yang B., Lala P.K., and Peng C. (2004). Nodal and ALK7 inhibit proliferation and induce apoptosis in human trophoblast cells. J Biol Chem *279*, 31277-31286.

Murakami M., Shirai M., Ooishi R., Tsuburaya A., Asai K., Hashimoto O., Ogawa K., Nishino Y., and Funaba M. (2012). Expression of activin receptor-like kinase 7 in adipose tissues. Biochem Genet *51*, 202-210.

Nagamatsu S., Nakamichi Y., Ohara-Imaizumi M., Ozawa S., Katahira H., Watanabe T., and Ishida H. (2001). Adenovirus-mediated preproinsulin gene transfer into adipose tissues ameliorates hyperglycemia in obese diabetic KKA(y) mice. FEBS Lett *509*, 106-110.

Nayerossadat N., Maedeh T., and Ali P.A. (2012). Viral and nonviral delivery systems for gene delivery. Adv Biomed Res *1*, 27.

Ng M., Fleming T., Robinson M., Thomson B., Graetz N., Margono C., Mullany E.C., Biryukov S., Abbafati C., Abera S.F.*, et al.* (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. Lancet *384*, 766-781.

Nicholls D.G., Bernson V.S., and Heaton G.M. (1978). The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation. Experientia Suppl *32*, 89-93.

Nishimura T., Nakatake Y., Konishi M., and Itoh N. (2000). Identification of a novel FGF, FGF-21, preferentially expressed in the liver. Biochim Biophys Acta *1492*, 203-206.

Nisoli E., Clementi E., Paolucci C., Cozzi V., Tonello C., Sciorati C., Bracale R., Valerio A., Francolini M., Moncada S.*, et al.* (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science *299*, 896-899.

Pauwels K., Gijsbers R., Toelen J., Schambach A., Willard-Gallo K., Verheust C., Debyser Z., and Herman P. (2009). State-of-the-art lentiviral vectors for research use: risk assessment and biosafety recommendations. Curr Gene Ther *9*, 459-474.

Pfeifer A. (2004). Lentiviral transgenesis. Transgenic Res *13*, 513-522.

Pfeifer A., and Hoffmann L.S. (2014). Brown, beige, and white: the new color code of fat and its pharmacological implications. Annu Rev Pharmacol Toxicol *55*, 207-227.

Pfeifer A., and Hofmann A. (2009). Lentiviral transgenesis. Methods Mol Biol *530*, 391-405.

Pfeifer A., Ikawa M., Dayn Y., and Verma I.M. (2002). Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc Natl Acad Sci U S A *99*, 2140-2145.

Pfeifer A., Klatt P., Massberg S., Ny L., Sausbier M., Hirneiss C., Wang G.X., Korth M., Aszodi A., Andersson K.E.*, et al.* (1998). Defective smooth muscle regulation in cGMP kinase I-deficient mice. EMBO J *17*, 3045-3051.

Pfeifer A., and Verma I.M. (2001). Gene therapy: promises and problems. Annu Rev Genomics Hum Genet *2*, 177-211.

Pilz R.B., and Casteel D.E. (2003). Regulation of gene expression by cyclic GMP. Circ Res *93*, 1034-1046.

Podsakoff G., Wong K.K., Jr., and Chatterjee S. (1994). Efficient gene transfer into nondividing cells by adeno-associated virus-based vectors. J Virol *68*, 5656-5666.

Potter L.R. (2011). Guanylyl cyclase structure, function and regulation. Cell Signal *23*, 1921-1926.

Reissmann E., Jornvall H., Blokzijl A., Andersson O., Chang C., Minchiotti G., Persico M.G., Ibanez C.F., and Brivanlou A.H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. Genes Dev *15*, 2010-2022.

Renold A.E., Marble A., and Fawcett D.W. (1950). Action of insulin on deposition of glycogen and storage of fat in adipose tissue. Endocrinology *46*, 55-66.

Ricquier D., and Kader J.C. (1976). Mitochondrial protein alteration in active brown fat: a soidum dodecyl sulfate-polyacrylamide gel electrophoretic study. Biochem Biophys Res Commun *73*, 577-583.

Roberts H.J., Hu S., Qiu Q., Leung P.C., Caniggia I., Gruslin A., Tsang B., and Peng C. (2003). Identification of novel isoforms of activin receptor-like kinase 7 (ALK7) generated by alternative splicing and expression of ALK7 and its ligand, Nodal, in human placenta. Biol Reprod *68*, 1719-1726.

Rubio A., Raasmaja A., and Silva J.E. (1995). Thyroid hormone and norepinephrine signaling in brown adipose tissue. II: Differential effects of thyroid hormone on beta 3-adrenergic receptors in brown and white adipose tissue. Endocrinology *136*, 3277-3284.

Ryden M., Imamura T., Jornvall H., Belluardo N., Neveu I., Trupp M., Okadome T., ten Dijke P., and Ibanez C.F. (1996). A novel type I receptor serine-threonine kinase predominantly expressed in the adult central nervous system. J Biol Chem *271*, 30603-30609.

Saito M., Okamatsu-Ogura Y., Matsushita M., Watanabe K., Yoneshiro T., Nio-Kobayashi J., Iwanaga T., Miyagawa M., Kameya T., Nakada K.*, et al.* (2009). High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. Diabetes *58*, 1526-1531.

Sandoval-Guzman T., Gongrich C., Moliner A., Guo T., Wu H., Broberger C., and Ibanez C.F. (2012). Neuroendocrine control of female reproductive function by the activin receptor ALK7. FASEB J *26*, 4966-4976.

Sauzeau V., Le Jeune H., Cario-Toumaniantz C., Smolenski A., Lohmann S.M., Bertoglio J., Chardin P., Pacaud P., and Loirand G. (2000). Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced Ca2+ sensitization of contraction in vascular smooth muscle. J Biol Chem *275*, 21722-21729.

Scherer P.E., Williams S., Fogliano M., Baldini G., and Lodish H.F. (1995). A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem *270*, 26746-26749.

Seale P., Bjork B., Yang W., Kajimura S., Chin S., Kuang S., Scime A., Devarakonda S., Conroe H.M., Erdjument-Bromage H.*, et al.* (2008). PRDM16 controls a brown fat/skeletal muscle switch. Nature *454*, 961-967.

Shi Y., and Massague J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Singh R., Braga M., and Pervin S. (2014). Regulation of brown adipocyte metabolism by myostatin/follistatin signaling. Front Cell Dev Biol *2*, 60.

Sjoholm K., Palming J., Lystig T.C., Jennische E., Woodruff T.K., Carlsson B., and Carlsson L.M. (2006). The expression of inhibin beta B is high in human adipocytes, reduced by weight loss, and correlates to factors implicated in metabolic disease. Biochem Biophys Res Commun *344*, 1308-1314.

Smith R.E. (1961). Thermogenic activity of the hibernating gland in the cold-acclimated rat. Physiologist, 113.

Stern J.H., Rutkowski J.M., and Scherer P.E. (2016). Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. Cell Metab *23*, 770-784.

Tamura N., Doolittle L.K., Hammer R.E., Shelton J.M., Richardson J.A., and Garbers D.L. (2004). Critical roles of the guanylyl cyclase B receptor in endochondral ossification and development of female reproductive organs. Proc Natl Acad Sci U S A *101*, 17300-17305.

Tang K.M., Wang G.R., Lu P., Karas R.H., Aronovitz M., Heximer S.P., Kaltenbronn K.M., Blumer K.J., Siderovski D.P., Zhu Y.*, et al.* (2003). Regulator of G-protein signaling-2 mediates vascular smooth muscle relaxation and blood pressure. Nat Med *9*, 1506-1512.

ten Dijke P., and Hill C.S. (2004). New insights into TGF-beta-Smad signalling. Trends Biochem Sci *29*, 265-273.

Themis M., Waddington S.N., Schmidt M., von Kalle C., Wang Y., Al-Allaf F., Gregory L.G., Nivsarkar M., Holder M.V., Buckley S.M.*, et al.* (2005). Oncogenesis following delivery of a nonprimate lentiviral gene therapy vector to fetal and neonatal mice. Mol Ther *12*, 763-771.

Tsuchida K. (2004). Activins, myostatin and related TGF-beta family members as novel therapeutic targets for endocrine, metabolic and immune disorders. Curr Drug Targets Immune Endocr Metabol Disord *4*, 157-166.

Tsuchida K., Sawchenko P.E., Nishikawa S., and Vale W.W. (1996). Molecular cloning of a novel type I receptor serine/threonine kinase for the TGF beta superfamily from rat brain. Mol Cell Neurosci *7*, 467-478.

van Marken Lichtenbelt W.D., and Schrauwen P. (2011). Implications of nonshivering thermogenesis for energy balance regulation in humans. Am J Physiol Regul Integr Comp Physiol *301*, R285-296.

van Marken Lichtenbelt W.D., Vanhommerig J.W., Smulders N.M., Drossaerts J.M., Kemerink G.J., Bouvy N.D., Schrauwen P., and Teule G.J. (2009). Cold-activated brown adipose tissue in healthy men. N Engl J Med *360*, 1500-1508.

Vazquez-Vela M.E., Torres N., and Tovar A.R. (2008). White adipose tissue as endocrine organ and its role in obesity. Arch Med Res *39*, 715-728.

Vegiopoulos A., Muller-Decker K., Strzoda D., Schmitt I., Chichelnitskiy E., Ostertag A., Berriel Diaz M., Rozman J., Hrabe de Angelis M., Nusing R.M.*, et al.* (2010). Cyclooxygenase-2 controls energy homeostasis in mice by de novo recruitment of brown adipocytes. Science *328*, 1158-1161.

Verma I.M., and Somia N. (1997). Gene therapy -- promises, problems and prospects. Nature *389*, 239-242.

Virtanen K.A., Lidell M.E., Orava J., Heglind M., Westergren R., Niemi T., Taittonen M., Laine J., Savisto N.J., Enerback S.*, et al.* (2009). Functional brown adipose tissue in healthy adults. N Engl J Med *360*, 1518-1525.

Vitali A., Murano I., Zingaretti M.C., Frontini A., Ricquier D., and Cinti S. (2012). The adipose organ of obesity-prone C57BL/6J mice is composed of mixed white and brown adipocytes. J Lipid Res *53*, 619-629.

Wall R. (1996). Transgenic livestock: progress and prospects for the future. Theriogenology *45*, 57-68.

Wang H., Jiang J.Y., Zhu C., Peng C., and Tsang B.K. (2006). Role and regulation of nodal/activin receptor-like kinase 7 signaling pathway in the control of ovarian follicular atresia. Mol Endocrinol *20*, 2469-2482.

Watanabe R., Yamada Y., Ihara Y., Someya Y., Kubota A., Kagimoto S., Kuroe A., Iwakura T., Shen Z.P., Inada A.*, et al.* (1999). The MH1 domains of smad2 and smad3 are involved in the regulation of the ALK7 signals. Biochem Biophys Res Commun *254*, 707-712.

World Health Organization (2016). Obesity and Overweight, Fact Sheet No. 311. Retrieved from http://www.who.int/mediacentre/factsheets/fs311/en/

Xu J., Lloyd D.J., Hale C., Stanislaus S., Chen M., Sivits G., Vonderfecht S., Hecht R., Li Y.S., Lindberg R.A.*, et al.* (2009). Fibroblast growth factor 21 reverses hepatic steatosis, increases energy expenditure, and improves insulin sensitivity in diet-induced obese mice. Diabetes *58*, 250-259.

Yang L., Bailey L., Baltimore D., and Wang P. (2006). Targeting lentiviral vectors to specific cell types in vivo. Proc Natl Acad Sci U S A *103*, 11479-11484.

Yin H., Kanasty R.L., Eltoukhy A.A., Vegas A.J., Dorkin J.R., and Anderson D.G. (2014). Non-viral vectors for gene-based therapy. Nat Rev Genet *15*, 541-555.

Ying S., Cao H., Hu H., Wang X., Tang Y., and Huang C. (2016). Alk7 Depleted Mice Exhibit Prolonged Cardiac Repolarization and Are Predisposed to Ventricular Arrhythmia. PLoS One *11*, e0149205.

Yogosawa S., and Izumi T. (2013). Roles of activin receptor-like kinase 7 signaling and its target, peroxisome proliferator-activated receptor gamma, in lean and obese adipocytes. Adipocyte *2*, 246-250.

Yogosawa S., Mizutani S., Ogawa Y., and Izumi T. (2012). Activin receptor-like kinase 7 suppresses lipolysis to accumulate fat in obesity through downregulation of peroxisome proliferator-activated receptor gamma and C/EBPalpha. Diabetes *62*, 115-123.

Zhi J., Melia A.T., Guerciolini R., Chung J., Kinberg J., Hauptman J.B., and Patel I.H. (1994). Retrospective population-based analysis of the dose-response (fecal fat excretion) relationship of orlistat in normal and obese volunteers. Clin Pharmacol Ther *56*, 82-85.

Zimmermann K., Scheibe O., Kocourek A., Muelich J., Jurkiewicz E., and Pfeifer A. (2011). Highly efficient concentration of lenti- and retroviral vector preparations by membrane adsorbers and ultrafiltration. BMC Biotechnol *11*, 55.

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Summary

The obesity pandemic is increasing worldwide and is a major threat to human health. Comorbidities associated with obesity are type 2 diabetes, cardiovascular disorders, non-alcoholic fatty liver disease and even some types of cancer. Presently, we lack reliable and easy applicable medication without major side-effects to treat obesity. Recents studies have unvailed the existance of energy-consuming brown adipose tissue (BAT) in human adults. This tissue has therefore become the focus of research to develop novel anti-obesity therapies.

The presented thesis depicts two novel approaches to investigate the development and function of brown adipocytes. The first approach identifies the type I TGFβ receptor Activin receptor-like kinase 7 (Alk7) as a novel cyclic guanosin monophosphate (cGMP)-regulated target in brown adipocytes. cGMP is an important second messenger in adipocytes that activates PKGI to induce adipogenic and thermogenic differentiation. Interestingly, exogenous cGMP treatment of brown adipocytes increases Alk7 expression. Activin AB is a potent ligand activating Alk7 downstream-signaling in brown adipocytes, which is mediated by phosphorylation of SMAD3. Activation of Alk7 during terminal differentiation of brown adipocytes differentially regulates adipogenic and thermogenic protein expression. It induces downregulation of several adipogenic markers and upregulation of the major thermogenic marker UCP1. Importantly, this effect is augmented in cGMP-treated brown adipocytes. Alk7 could therefore serve as potential endogenous brake of the cGMP signaling pathway, avoiding overstimulation of the adipogenic program by cGMP and at the same time enhancing UCP1 expression to facilitate energy expenditure.

To be able to analyze effects of the Alk7/cGMP signaling pathway as well as of other signaling pathways in BAT *in vivo*, the second part of this work describes the development and validation of an easy-to-handle and fast-to-accomplish method for direct lentiviral injections into subcutaneous adipose tissues. Lentiviral vectors are directly injected into the target fat pad of anesthetized mice through a small incision using a microsyringe connected to a modified, small needle, which is well suited for infiltration of adipose tissues. Expression of the target gene can be detected in the respective adipose tissue as early as one week after injection and is stable over months due to the use of lentiviral vectors, which integrate stably into the host genome. Delivery of transgenes into

the fat pads of one mouse is carried out within minutes. The method therefore allows for studying genes of interest in murine brown/beige fat in a timely manner. Consequently, it could be of substantial interest for any researcher investigating signaling pathways in adipose tissues, potentially leading to new gene therapies for the treatment of obesity.

In conclusion, two approaches targeting brown/beige adipocytes are unraveled in this thesis. These could be useful for the development of novel treatments for obesity. The first approach reveals Alk7 as a potential target to increase thermogenic capacity in brown adipocytes. The second approach describes an efficient method to produce adipose-specific transgenic mice, which allows the study of several genes/signaling pathways with respect to their metabolic consequences *in vivo*.