GH action influences adipogenesis of mouse adipose tissue-derived mesenchymal stem cells

Nicoleta C Olarescu^{1,2}, Darlene E Berryman^{3,4}, Lara A Householder^{3,4}, Ellen R Lubbers³, Edward O List³, Fabian Benencia⁴, John J Kopchick^{3,4} and Jens Bollerslev^{1,2}

¹Section of Specialized Endocrinology, Department of Endocrinology, Oslo University Hospital, Rikshospitalet, PO Box 4950, N-0424 Oslo, Norway

²Faculty of Medicine, University of Oslo, Oslo, Norway

³Edison Biotechnology Institute, Ohio University, Athens, Ohio, USA

⁴Heritage College of Osteopathic Medicine, Ohio University, Athens, Ohio, USA

Correspondence
should be addressed
to N C Olarescu
Email
nicola@ous-hf.no

Abstract

GH influences adipocyte differentiation, but both stimulatory and inhibitory effects have been described. Adipose tissue-derived mesenchymal stem cells (AT-MSCs) are multipotent and are able to differentiate into adipocytes, among other cells. Canonical Wnt/ β -catenin signaling activation impairs adipogenesis. The aim of the present study was to elucidate the role of GH on AT-MSC adipogenesis using cells isolated from male GH receptor knockout (GHRKO), bovine GH transgenic (bGH) mice, and wild-type littermate control (WT) mice. AT-MSCs from subcutaneous (sc), epididymal (epi), and mesenteric (mes) AT depots were identified and isolated by flow cytometry ($Pdgfra^{+}Sca1^{+}Cd45^{-}Ter119^{-}$ cells). Their *in vitro* adipogenic differentiation capacity was determined by cell morphology and real-time RT-PCR. Using identical *in vitro* conditions, adipogenic differentiation of AT-MSCs was only achieved in the sc depot, and not in epi and mes depots. Notably, we observed an increased differentiation in cells isolated from sc-GHRKO and an impaired differentiation of sc-bGH cells as compared to sc-WT cells. *Axin2*, a marker of Wnt/ β -catenin activation, was increased in mature sc-bGH adipocytes, which suggests that activation of this pathway may be responsible for the decreased adipogenesis. Thus, the present study demonstrates that i) adipose tissue in mice has a well-defined population of $Pdgfra^{+}Sca1^{+}$ MSCs; ii) the differentiation capacity of AT-MSCs varies from depot to depot regardless of GH genotype; iii) the lack of GH action increases adipogenesis in the sc depot; and iv) activation of the Wnt/ β -catenin pathway might mediate the GH effect on AT-MSCs. Taken together, the present results suggest that GH diminishes fat mass in part by altering adipogenesis of MSCs.

Key Words

- growth hormone
- adipose tissue-derived mesenchymal stem cells
- adipogenesis
- Wnt/ β -catenin signaling

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Introduction

Recent studies have indicated that healthy adipose tissue (AT) expansion, especially of the subcutaneous (sc) fat depot, is essential for protecting against ectopic fat

deposition in muscles and the liver and against resulting insulin resistance (Hocking *et al.* 2013, Shulman 2014). AT expansion is achieved by increasing the size of individual

cells (hypertrophy) or by recruiting new adipocytes from the resident pool of progenitors (hyperplasia) (Rosen & Spiegelman 2014). Growth hormone (GH) decreases fat mass, and this effect is thought to be mediated by the GH stimulation of lipolysis and inhibition of lipogenesis (Moller & Jorgensen 2009). Indeed, both in mice and in humans, GH deficiency has been shown to lead to increased fat mass, whereas GH excess, such as in acromegaly, is associated with a decrease in fat mass (Berryman *et al.* 2004, Jorgensen *et al.* 2011, Olarescu *et al.* 2012). In contrast to the well-known lipolytic action, GH's effect on adipocyte differentiation is more controversial and difficult to reconcile, particularly because the effects seen in cell lines differ from observations in primary cells (Morikawa *et al.* 1982, Green *et al.* 1985, Doglio *et al.* 1986, Hausman & Martin 1989, Corin *et al.* 1990, Clarkson *et al.* 1995, Wabitsch *et al.* 1995, 1996a,b, Flint *et al.* 2006, Zhao *et al.* 2014). For example, activation of one of the main GH dependent intracellular signaling proteins, STAT5, has been shown to decrease differentiation in primary rat preadipocytes (Hansen *et al.* 1998, Richter *et al.* 2003), but it has also been shown to increase differentiation in mouse preadipocyte cell lines (3T3-L1, 3T3-F442A) (Yarwood *et al.* 1999, Shang & Waters 2003, Stewart *et al.* 2004). When interpreting the discrepant results of the effect of GH on adipogenesis, one should take into account that: i) genuine variance exists between the response of primary cells and the response of cell lines to GH stimulation; ii) pools of cells at different stages of differentiation were studied; and iii) the experiments were performed under serum-containing conditions that may have obscured GH's effects by making cells refractory to GH stimulation.

Mesenchymal stem cells (MSCs) are multipotent cells that are present in adult marrow. They can replicate as undifferentiated cells and have the potential to differentiate to lineages of mesenchymal tissues, including fat, bone, and cartilage. The self-renewal and differentiation of MSCs are regulated in part by the Wnt/ β -catenin signaling pathway. That is, activation of Wnt/ β -catenin signaling decreases the adipogenic potential of MSCs (Ross *et al.* 2000, Lowe *et al.* 2011). Although MSCs have primarily been described in bone marrow, they are present in and can be isolated from several organs, including AT (Pittenger *et al.* 1999, Zuk *et al.* 2002). Platelet-derived growth factor receptor, α polypeptide (*Pdgfra*) and stem cell antigen 1 (*Sca1*) have recently been identified as being selective markers of mouse MSCs. *Pdgfra*⁺*Sca1*⁺ MSCs have augmented growth potential and robust tri-lineage differentiation as compared to standard culture-selected MSCs (Houlihan *et al.* 2012). In addition, the selective isolation of

MSCs by flow cytometry avoids cellular contamination that can complicate other culture methods.

In the present study, we hypothesized that GH action influences the fate of AT-derived MSCs (AT-MSCs) by decreasing their differentiation potential toward adipocytes. To avoid the unspecific effects of the growth factors that are present in fetal bovine serum (FBS) and to study how these cells behave *ex vivo*, we used two genetically engineered mouse lines with decreased or increased GH action. More specifically, we used a dwarf mouse line that is completely insensitive to GH (GH receptor knockout (GHRKO) mice) and a giant mouse line with extra copies of the bovine GH gene (bovine GH transgenic (bGH) mice). Thus, our primary aim was to elucidate the role of GH on AT-MSC adipogenesis using cells isolated from these mice and control littermates.

Materials and methods

Animals

Four-month-old male GHRKO, bGH, and WT mice were used. The production of these mice has been previously described (Zhou *et al.* 1997, Berryman *et al.* 2004). Mice were either produced on a pure C57BL/6J background or backcrossed more than ten generations into C57BL/6J mice, and they were bred in the animal facility at the Edison Biotechnology Institute, Ohio University, Athens, OH, USA. All mice were kept under specific pathogen-free conditions on a 14 h light:10 h darkness cycle; they were allowed to consume normal chow (Prolab RMH 3000 LabDiet; PMI Richmond, Richmond, IN, USA) and water *ad libitum*. Procedures were approved by the Ohio University Animal Care and Use Committee.

Body composition

Fat, lean, and fluid mass were analyzed with a quantitative NMR apparatus (Minispec, Bruker Optics, Billerica, MA, USA) according to a previously described protocol (List *et al.* 2009).

White adipose tissue depot samples

All of the mice were killed by cervical dislocation. Subcutaneous/inguinal (sc) and all intra-abdominal depots – epididymal (epi), mesenteric (mes), and retro-peritoneal (retro) – were collected using sterile dissection techniques.

Isolation of adipose tissue stromal vascular fractions

In order to obtain a sufficient number of cells for further *in vitro* culture, different white adipose tissue (WAT) depots from three mice were collected together and washed three times with fresh PBS containing 1% antibiotic–antimycotic (cat. no. 15240-062, Gibco). The tissue was minced and digested with collagenase solution – HBSS with Ca and Mg (cat. no. 14025092, Gibco) supplemented with 2 mg/ml collagenase, type 1 (cat. no. LS004196, Worthington Biochemical Corp., Lakewood, NJ, USA), 3 mM CaCl₂, and 2% BSA – for 60 min at 37 °C and 150–250 r.p.m. on a horizontal shaker. The mature adipocyte fraction was removed, and the stromal vascular fraction (SVF) suspension was filtered through a 70 µm sterile filter and spun down by centrifugation at 280 *g* for 5 min at 4 °C. The pellet was resuspended by tapping, and the red blood cells were lysed in the suspension by adding 1 ml of ice-cold sterile H₂O for 6 s as previously described (Houlihan *et al.* 2012). The SVF was spun down again by centrifugation, and the pellets were resuspended in fresh PBS.

Fluorescence-activated cell sorting

After being incubated with specific antibodies, SVF samples were analyzed and sorted on a FACS Aria flow cytometer with FACSDiva software (Becton Dickinson, San Jose, CA, USA). Two surface markers for mouse AT-MSC (*Pdgfra* and *Sca1*) and two negative surface markers (*Cd45* and *Ter119*) were used to identify and sort a pure population of mouse AT-MSCs, following a previously validated protocol (Houlihan *et al.* 2012). The specific fluorochrome-conjugated MABs (1:100 dilution) used were from eBioscience (San Jose, CA, USA) (Table 1). Output data was recorded by the FACS Diva software.

Cell culture

Sufficient cells were obtained for *in vitro* experiments from the sc, epi, and mes depots but not from the retro depot. The fluorescence-activated cell sorted (FACS) populations

of AT-MSCs were seeded at a density of 5000 cells/cm² in growth medium – DMEM-F12 (cat. no. 21885-108, Gibco), 10% FBS (cat. no. 12662-029, Gibco), 2 mM glutamine (cat. no. 35050-038, Invitrogen), and 1% antibiotic–antimycotic – and cultured for two passages. The cells were then seeded at a density of 40 000 cells/well in 12-well plates (Costar, Cambridge, MA, USA) and maintained in a humidified incubator at 37 °C with 5% CO₂. Confluent cultures were induced to differentiate using differentiation media – DMEM-F12, 0.5 mM IBMX (cat. no. I5879, Sigma), 1 µM dexamethasone (cat. no. D4902, Sigma), 0.2 mM indomethacin (cat. no. I7378-56, Sigma), and 10 µg/ml insulin (cat. no. I9278, Sigma) – for 7 days.

Pictures were taken for visualization of the lipid droplet formation, and the cells were lysed with QIAzol lysis reagent (Qiagen).

Real-time RT-PCR

RNA was isolated using an ethanol precipitation method. cDNA was synthesized with Maxima First Strand cDNA Synthesis Kits (cat. no. K1642, Thermo Scientific, Waltham, MA, USA), and quantitative real-time RT-PCR was performed with Maxima SYBR Green/Fluorescein qPCR Master Mix (cat. no. K0242, Thermo Scientific). Data were normalized to ribosomal protein S3 (*Rps3*), which is one of the most stable housekeeping genes for WAT (Lubbers *et al.* 2013). Primer sequences are listed in Table 2.

Statistical analysis

Data are expressed as means ± S.E.M. First, one-way ANOVA was performed to assess the total variability within the mice or cells data. Then, two-tailed unpaired Student's sample *t*-tests were used to evaluate differences between genetically engineered mouse lines and WT mice and between cells isolated from these mouse lines and control littermates.

Table 1 Antibodies used for primary isolation and immunophenotyping

Protein and fluorochrom	eBioscience cat. no.	Clone	Isotype
SCA1: Alexa Fluor 700	56-5981-80	D7	Rat IgG2a κ
PDGFRα: PE	12-1401-81	APA5	Rat IgG2a κ
CD45: APC-eFluor 780	47-0451-80	30-F11	Rat IgG2a κ
TER119: PerCP-Cyanine5.5	45-5921-80	TER119	Rat IgG2b κ
Isotype Alexa Fluor 700	56-4301-80	eBRG1	Rat IgG1 κ
Isotype PE	12-4301-81	eBRG1	Rat IgG1 κ
Isotype APC-eFluor 780	47-4714-80	P3.6.2.8.1	Mouse IgG1 κ
Isotype PerCP-Cyanine5.5	45-4301-80	eBRG1	Rat IgG1 κ
7-AAD viability staining solution	00-6993-50	–	–

Table 2 Real-time RT-PCR primer pairs. All of the primers are listed 3' to 5'

Gene	Primers	Product length
<i>Rps3</i>	F: ATCAGAGAGTTGACCGCAGTT R: AATGAACCGAAGCACACCATA	183
<i>Pparγ</i>	F: GGGATATTTTGGCATACTCTG R: AAAGACAACGGACAAATCAC	195
<i>Adiponectin</i>	F: CTCTCCTGTTCTCTTAATCCT R: ACCAAGAAGACCTGCATCTC	218
<i>Fabp4</i>	F: ACACCGAGATTTCTTCAAACCTG R: CCATCTAGGGTTATGATGCTCTTC	88
<i>Axin2</i>	F: CGCCAACGACAGCGAGTT R: CGGTAAGGAGGGACTCCATCTA	90

F, forward; R, reverse.

Results

Body weight and body composition

There was a significant association between genotype and body weight, fat, lean, and fluid mass. bGH mice had significantly increased total body weight vs WT and GHRKO mice (44.5 ± 4.0 g vs 28.66 ± 2.1 g and 13.5 ± 1.7 respectively, $P < 0.001$). As expected, GHRKO mice had the lowest total body weight but the highest percentage of body fat (Fig. 1).

bGH mice had both increased lean mass (81.2 ± 1.5 vs $65.4 \pm 2.5\%$, $P < 0.001$) and fluid mass (16.8 ± 0.7 vs $15.0 \pm 0.4\%$, $P < 0.01$) as compared to GHRKO mice, whereas no significant differences were observed for lean and fluid mass when the mice were compared to WT (data not shown).

Selective isolation of *Pdgfr α* ⁺*Sca1*⁺ cells from different AT depots using flow cytometry

Using a recently described protocol (Houlihan *et al.* 2012), we identified and sorted a population of mouse AT-MSCs on the basis of their positive surface markers of *Pdgfr α* and *Sca1* and their lack of *Cd45* and *Ter119*, two markers of hematopoietic stem cells. The algorithm used is presented in Fig. 2. The percentage of *Pdgfr α* ⁺*Sca1*⁺ cells showed a wide variability within each mouse genotype (data not shown). However, a well-delimited population of cells that complies with the AT-MSC criteria was identified and sorted (Fig. 2D).

Adipogenic differentiation of WT *Pdgfr α* ⁺*Sca1*⁺ AT-MSCs

Adipogenesis was achieved only in the cells derived from the sc depot in all of the genotypes. Cells from the epi and

mes depots showed impaired differentiation ability, even when we used *in vitro* conditions that were identical to those for the sc depot (Fig. 3A). When stimulated to differentiate, none of the other depots showed increased *Ppar γ* gene expression (Fig. 3A), and very few epi cells presented lipid accumulation (data not shown), although a slight increase in *adiponectin* and *Fabp4* gene expression was observed (Fig. 3B and C). Consequently, the assessment of the difference in the adipogenetic ability among different genotypes was performed just for the sc depot.

In vitro culture and adipogenic potential of GHRKO, bGH, and WT AT-MSCs from sc depot

As shown in Fig. 4A, the overnight cell culture of sorted AT-MSCs led to the cells attaching to the plastic surface, and no cell death was observed in the media. The cells gradually acquired a fibroblastic appearance and divided.

Figure 1

Total body weight and fat mass in 4-month-old male GHRKO, bGH, and WT mice. GHRKO mice had decreased total body weight but a higher percentage of body fat vs both WT and bGH mice. bGH mice had increased total body weight but a lower percentage of body fat vs both WT and GHRKO mice. No differences were observed between the different genotypes of WTs. Data are presented as means $\pm$ S.E.M. WT, wild-type littermate control mice; GHRKO, growth hormone receptor knockout mice; bGH, bovine growth hormone transgenic mice. * $P < 0.05$, *** $P \leq 0.001$.

Figure 2

Fluorescence-activated cell sorting algorithm of AT-MSCs. First, a scatter gate was defined to account for cell debris and aggregates (A). Second, a population of cells negative for markers of hematopoietic lineage

(Q3, $Cd45^{-}Ter119^{-}$) was separated (B). Finally, a population of cells positive for markers of mouse MSCs ($Pdgfr\alpha^{+}Sca1^{+}$) that were not present with isotype control antibodies was identified (C) and sorted (D).

No phenotypic difference was observed among the GHRKO, bGH, and WT cells. The cells were stimulated to undergo adipogenic differentiation when 100% confluence was achieved. Upon differentiation, sc AT-MSCs from all of the genotypes showed increases in *Ppar γ* , *Fabp4*, and *adiponectin*, which are markers for adipogenesis (Fig. 4B). Moreover, the GHRKO cells revealed the highest amount of lipid accumulation (Fig. 4B) and the highest mRNA levels for the adipogenic markers. Conversely, bGH cells showed less lipid accumulation and lower levels of *Ppar γ* , *adiponectin*, and *Fabp4* mRNA as compared to WT and GHRKO cells (Fig. 4B). We also observed that lipid droplets in GHRKO cells were smaller and had a multilocular appearance as compared to those in WT and bGH cells (Fig. 4B).

Activation of Wnt/ β -catenin is associated with decreased adipogenesis of bGH cells

Activation of the Wnt/ β -catenin pathway has been shown to lead to decreased adipogenesis (Ross *et al.* 2000, Christodoulides *et al.* 2009) (Fig. 5A). Thus, we determined

Axin2 mRNA levels as a marker for the activation of Wnt/ β -catenin. *Axin2* increased during adipogenesis of WT and bGH cells but not in GHRKO cells (Fig. 5B). Moreover, the bGH cells showed a significantly higher expression of *Axin2* vs WT cells, which suggests that the impairment of adipogenesis may be mediated by increased Wnt/ β -catenin activity.

Discussion

In the present study, we isolated a population of AT-MSCs ($Pdgfr\alpha^{+}Sca1^{+}Cd45^{-}Ter119^{-}$ cells) from different depots of dwarf GHRKO, giant bGH, and WT mice by flow cytometry. Furthermore, we cultured and assessed the adipogenic potential of the isolated cells based on the hypothesis that GH influences adipogenesis of mouse AT-MSCs. We found that the expression of key adipogenic transcriptional markers is higher in sc adipocytes differentiated from GHRKO and lower in sc cells differentiated from bGH as compared to WT cells. Thus, GH appears to play an important role by inhibiting the adipogenesis of

Figure 3

Different adipogenic potential of FACS sorted WT AT-MSCs. Adipogenesis was achieved in the sc depot but not in the epi and mes depots. Data are presented as means $\pm$ s.e.m. Undiff, undifferentiated; sc, subcutaneous; epi, epididymal; mes, mesenteric. ** $P \leq 0.01$, *** $P \leq 0.001$ vs undifferentiated control.

sc AT-MSCs. We also demonstrated that AT-MSCs isolated from various depots have different adipogenic potential. More specifically, the present data indicate that only the cells isolated from the sc depot achieve a mature adipocyte phenotype *in vitro*. Finally, we suggested that the decreased adipogenesis of bGH cells might be attributed to an increase in Wnt/ β -catenin signaling.

In a recent study, SVFs isolated from the sc depots of 20-month-old GHRKO mice were found to have greater differentiation capacity as compared to controls (Stout *et al.* 2014). However, SVFs are a mixture of cells that, in addition to AT-MSCs, contain preadipocytes at different stages of commitment, fibroblasts, leukocytes, epithelial cells, endothelial cells, and moreover, other cells that

comprise the vasculature and nerve tissue (Cawthorn *et al.* 2012). Thus, contamination of the *in vitro* culture with non-adipogenic cells may have altered the responses of the adipogenic cells. To remove the undesirable effects of cellular contamination, we decided to selectively isolate AT-MSCs by flow cytometry. AT-MSCs are a functionally distinct subpopulation of SVFs (Cawthorn *et al.* 2012). The most widespread technique used to distinguish between SVFs and AT-MSCs is assaying for cell surface specific markers. Therefore, we isolated mouse AT-MSCs on the basis of their positive expression of *Pdgfra* and *Sca1*, two known markers of AT-MSCs (Houlihan *et al.* 2012). Importantly, we sorted a pure population of AT-MSCs, because *Pdgfra* is expressed only by AT-MSCs and is not present on committed preadipocytes (Cawthorn *et al.* 2012). Our results indicate evidence for a large population of *Pdgfra*⁺*Sca1*⁺ cells in adipose tissue in mice, even though prior reports have found these cells in bone (Houlihan *et al.* 2012). Obtaining a pure population of cells was important, because contamination with other SVFs (which is possible in different proportions among different genotypes/ depots) could influence the differentiation ability of the isolated cells, regardless of their GH signaling status.

A key aspect of MSC culture environment is the necessity of serum, which constitutes a basic source of nutrients, hormones, growth factors, and micronutrients that are essential for maintaining proper properties and growth conditions. The choice of serum has significant importance in culturing MSCs (Shahdadfar *et al.* 2005). A culture media with a concentration of 10% FBS contains ~6 ng/ml GH and 7 ng/ml insulin-like growth factor 1 (IGF1), which is sufficient to impair the effects of exogenous added GH and IGF1 (Kurtz *et al.* 1985, Cool *et al.* 2005). To specifically address the GH effect on adipogenesis and at the same time to avoid any unspecific effects of the GH present in serum, we used cells that lack GH action. We report an increased level of adipogenesis of cells that lack GH action, as measured by the gene expression of adipocyte-related markers (*Ppar γ* , *Fabp4*, and *adiponectin*) and lipid accumulation. Moreover, we observed decreased adipogenesis of the cells that have an extra copy of bovine GH, which suggests a negative paracrine effect of GH on the adipogenesis of AT-MSCs. *Ppar γ* , a member of the nuclear receptor superfamily, is both necessary and sufficient for adipogenesis (Rosen & MacDougald 2006). Its expression rapidly increases after the induction of adipogenesis, and maximal levels of expression are attained in mature adipocytes (Gregoire *et al.* 1998). During the terminal phase of

Figure 4

Phenotypic appearance of sc WT AT-MSCs after isolation (A) and comparison of the adipogenic properties between GHRKO, bGH, and WT cells (B). (A) *In vitro* appearance of sc WT AT-MSCs at 1, 3, and 7 days after FACS isolation. The cells adhered to the plastic surface and presented a round form at 1 day after isolation. At 3 and 7 days, the cells started to elongate, to acquire a characteristic fusiform appearance, and to divide. (B) The adipogenesis of sc AT-MSCs from GHRKO, bGH, and WT mice at

7 days of differentiation. GHRKO cells achieved a higher differentiation, whereas bGH cells showed an impaired differentiation, as quantified by lipid accumulation and the gene expression of the adipogenic markers. Data are presented as means $\pm$ s.e.m. Undiff, undifferentiated; 7d Diff, 7 days differentiated; WT, wild-type littermate control mice; GHRKO, growth hormone receptor knockout mice; bGH, bovine growth hormone transgenic mice. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. 10 $\times$ magnification.

differentiation, adipocytes synthesize FABP4, a protein directly related to lipid metabolism, and adiponectin, a classical adipokine (Lowe *et al.* 2011).

Another typical sign of adipogenesis is the accumulation of intracellular lipid droplets. *In vivo*, white mature adipocytes contain a large, single lipid droplet, which displaces the nucleus to the periphery. Lipid accumulation *in vitro* may not necessarily replicate lipid accumulation seen *in vivo*, but a tendency toward larger and fewer lipid droplets is evident in old cultures of mature adipocytes. Morphologically, brown adipocytes differ from white adipocytes in terms of their multilocular appearance, which is caused by their numerous small lipid droplets (Lee *et al.* 2013). Of note, in the present study, the lipid droplet morphology in GHRKO cells was closer to brown than to white adipocyte appearance, which raises the question of whether the function is similar. In fact, an

argument for a potential increase in the metabolic activity of sc fat in GHRKO mice comes from the observation that GHRKO mice, even when they are obese, are extremely insulin-sensitive (List *et al.* 2011, Sackmann-Sala *et al.* 2014). This is also supported by human studies that showed that individuals who carry mutations in the *GHR* gene and lack GH signaling, although they are often obese, are protected from metabolic dysfunction (Guevara-Aguirre *et al.* 2011). However, this model is not necessarily valid in subjects with adult-onset GH deficiency who are not protected against type 2 diabetes or metabolic syndrome. Thus, it seems that the time point during the lifespan when GH action is disrupted is of major importance with regard to its metabolic consequences.

GH excess in mice and humans has been shown to decrease fat and to increase lean mass but also to induce insulin resistance (Vijayakumar *et al.* 2011, Olareanu *et al.*

Figure 5

Activation of Wnt/ β -catenin signaling decreases adipogenesis of MSCs (A). Axin2, a marker of Wnt/ β -catenin activation, is increased in mature sc-bGH adipocytes, which suggests that the decreased adipogenic potential of these cells might be attributed to increased Wnt/ β -catenin activity (B). Data are presented as means $\pm$ s.e.m. Undiff, undifferentiated; 7d Diff, 7 days differentiated; WT, wild-type littermate control mice; GHRKO, growth hormone receptor knockout mice; bGH, bovine growth hormone transgenic mice. * $P < 0.05$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-15-0012>.

2012). As has been suggested by previous investigations (Berryman *et al.* 2004, 2010, Jara *et al.* 2014), we confirm that the percentage of fat mass is markedly elevated in GHRKO and significantly decreased in bGH relative to WT male mice at 4 months of age. Furthermore, there is clear evidence in the literature that adipose tissue is impacted in a depot-specific manner in mice with altered GH signaling (Berryman *et al.* 2004, 2011). In this respect, the accumulation of fat in GHRKO is not uniform and has been shown to localize mainly to the sc depot, whereas intra-abdominal depots are proportional in size to those of WT (Berryman *et al.* 2010). The present study provides a possible mechanism for explaining the increased sc fat depot in GHRKO mice, which could be a result of increased adipogenesis. In accordance with this, recent studies have demonstrated that GH action is AT

depot-specific (Berryman *et al.* 2011, Lubbers *et al.* 2013, Sackmann-Sala *et al.* 2014, Stout *et al.* 2014). This may explain the differences in immune cell content, adipokine production, cellular composition, cellular senescence, plasticity, and extracellular matrix content among different AT depots that finally lead to various metabolic outcomes.

In addition to the known lipolytic activity of GH, the inhibition of adipogenesis, as shown in the present study, may contribute to the systemic insulin resistance that is associated with GH excess. Both processes lead to increased circulating free fatty acids (FFAs) either via the release of FFAs from AT through lipolysis or via the incapacity to store excess FFAs in newly recruited adipocytes because of the diminished capacity of differentiation. Recent evidence has unequivocally demonstrated a role of FFA and ectopic lipid accumulation in the pathogenesis of insulin resistance in muscles and the liver (Hocking *et al.* 2013, Shulman 2014).

Importantly, we observed depot-specific differences in the ability of sorted AT-MSCs to differentiate in a medium containing IBMX, dexamethasone, indomethacin, and insulin, with the sc depot being the only depot to fully achieve adipogenesis. Regional differences in MSC replication, differentiation, subtype abundance, susceptibility to apoptosis or senescence, and capacity for adipogenesis, even when they originate from the same individual, have been previously described (Tchkonja *et al.* 2013). The regional differences are heritable over many cell generations, which suggests that the multipotentiality of MSCs is epigenetically restricted (Reinisch *et al.* 2014).

In terms of adipogenesis, we also found that GH-induced intracellular signaling may activate the canonical WNT/ β -catenin pathway in AT-MSCs, a process that could explain the negative influence of GH on adipogenesis. Indeed, several reports have implicated WNT/ β -catenin as a molecular switch that, when activated, represses adipogenesis (Christodoulides *et al.* 2009). Furthermore, the activation of Wnt/ β -catenin leads to impaired insulin stimulation of glucose uptake in mature adipocytes (Gustafson & Smith 2010), which could represent yet another mechanism for explaining GH-induced insulin resistance (i.e., GH's diabetogenic effect).

The present study has some limitations. First, the experiments were performed only in 4-month-old male mice, which did not allow us to evaluate age- and sex-related influences. Although differences in body composition between male and female mice have been demonstrated (Berryman *et al.* 2010), no effect of estrogens

on the *in vitro* differentiation potential of AT-MSCs has been reported (Veronesi *et al.* 2014). On the other hand, we decided to perform the present study in relatively young animals to account for previously reported age-related variations in MSC properties (Kim *et al.* 2012). Second, we did not evaluate the possible effects IGF1, which is present in FBS or is produced upon GH stimulation. However, the differences in the adipogenesis observed in the present study were not a result of IGF1 action because GHRKO cells, which lack GH signaling, may eventually produce less IGF1 that would act in a paracrine manner and favor adipogenesis. Moreover, bGH cells showed impaired adipogenesis in the present study, an opposite effect of the pro-adipogenic action of IGF1 that has been described in the literature (Scavo *et al.* 2004). Third, the results regarding the activation of the canonical WNT/ β -catenin pathway by GH should be considered with caution, because they were based on the measurement of a single WNT/ β -catenin signaling marker, namely, *Axin2*. Further studies to demonstrate that the inhibition of WNT/ β -catenin activation in bGH cells leads to an increased adipogenesis are needed before a definite conclusion can be made. Finally, although we isolated AT-MSCs using two specific surface antibodies in an attempt to select cells that were at the same stage of differentiation, we cannot rule out that prior *in vivo* exposure did not influence the intrinsic properties of the isolated cells.

In conclusion, the present results suggest that GH diminishes the amount of AT by altering the adipogenesis of AT-MSCs. Consequently, a reduced AT expandability potential might represent a novel mechanism for further explaining GH-induced insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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