Growth Hormone Action Influences Adipogenesis of Mouse Adipose Tissue-Derived Mesenchymal Stem Cells

Nicoleta C Olarescu1,2, Darlene E Berryman3,4, Lara A Householder3,4, Ellen R Lubbers3, Edward O List3, Fabian Benencia4, John J Kopchick3,4, and Jens Bollerslev1,2

1Section of Specialized Endocrinology, Department of Endocrinology, Oslo University Hospital, Rikshospitalet, Oslo, Norway
2Faculty of Medicine, University of Oslo, Norway
3Edison Biotechnology Institute, Ohio University, Athens, OH
4Heritage College of Osteopathic Medicine, Ohio University, Athens, OH

Abstract

Growth hormone (GH) influences adipocyte differentiation, but both stimulatory and inhibitory effects have been described. Adipose tissue-derived mesenchymal stem cells (AT-MSC) are multipotent, able to differentiate into adipocytes, among other cells. Canonical Wnt/β-catenin signaling activation impairs adipogenesis.

The aim of this study was to elucidate the role of GH on AT-MSC adipogenesis using cells isolated from male GH receptor gene knockout (GHRKO), bovine GH transgenic (bGH) and wild-type littermate control (WT) mice.

AT-MSC from subcutaneous (sc), epididymal (epi), and mesenteric (mes) AT depots were identified and isolated by flow cytometry (PDGFRα+Sca-1+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-MSC was only achieved in the sc depot, but 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 compared with sc-WT cells. Axin-2, a marker of Wnt/β-catenin activation, was increased in mature sc-bGH adipocytes suggesting that activation of this pathway may be responsible for the decreased adipogenesis.

Thus, we demonstrate that 1) adipose tissue in mice has a well-defined population of Sca-1+PDGFRα+ MSC cells; 2) the differentiation capacity of AT-MSC varies from depot to depot regardless of GH genotype; 3) the lack of GH action increases adipogenesis in sc depot; and 4) activation of Wnt/β-catenin pathway might mediate the GH effect on AT-MSC.

Taken together, our results suggest that GH diminishes fat mass, in part, by altering adipogenesis of MSC.
Keywords
growth hormone; adipose tissue derived mesenchymal stem cells; adipogenesis; Wnt/β-catenin signaling

Introduction

Recent studies indicate that healthy adipose tissue (AT) expansion, especially of the subcutaneous fat depot, is essential for protecting against ectopic fat deposition in muscle and liver and the 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 and Spiegelman 2014). Growth hormone (GH) decreases fat mass, an effect thought to be mediated by its stimulation of lipolysis and inhibition of lipogenesis (Moller and Jorgensen 2009). Indeed, both in mice and humans, GH deficiency leads to increased fat mass, while 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 (Clarkson, et al. 1995; Corin, et al. 1990; Doglio, et al. 1986; Flint, et al. 2006; Green, et al. 1985; Hausman and Martin 1989; Morikawa, et al. 1982; Wabitsch, et al. 1996a; Wabitsch, et al. 1995; Wabitsch, et al. 1996b; Zhao, et al. 2014). For example, activation of one of the main GH dependent intracellular signaling proteins, STAT5, decreases differentiation in primary rat preadipocytes (Hansen, et al. 1998; Richter, et al. 2003), whereas it increases differentiation in mouse preadipocyte cell lines (3T3-L1, 3T3-F442A) (Shang and Waters 2003; Stewart, et al. 2004; Yarwood, et al. 1999). When interpreting the GH discrepant results on adipogenesis one should take into account that: 1) genuine variance exist between the response of primary cells and cell lines to GH stimulation; 2) a pool of cells at different stages of differentiation have been studied; and 3) the experiments are performed under serum-containing conditions that may obscure GH’s effects by making cells refractory to GH stimulation.

Mesenchymal stem cells (MSC) are multipotent cells present in adult marrow that 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 MSC are regulated, in part, by the Wnt/β-catenin signaling pathway. That is, activation of Wnt/β-catenin signaling decreases the adipogenic potential of MSC (Lowe, et al. 2011; Ross, et al. 2000). Although MSCs are primarily described in bone marrow, they are present and can be isolated from several organs including adipose tissue (AT) (Pittenger, et al. 1999; Zuk, et al. 2002). Platelet-derived growth factor receptor α (PDGFR-α) and stem cell antigen 1 (Sca-1) have recently been identified as selective markers of mouse MSC. PDGFR-α+Sca-1+ MSC have augmented growth potential and robust tri-lineage differentiation, compared with standard culture-selected MSC (Houlihan, et al. 2012). In addition, the selective isolation of MSC 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 mesenchymal stem cells (AT-MSC) by decreasing their differentiation potential towards adipocytes. To avoid the unspecific effects of growth factors present in FBS and to study how these cells behave \textit{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 (growth hormone receptor knock-out or GHRKO mice) and a giant mouse line with extra copies of the bovine growth hormone gene (bovine growth hormone transgenic or 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.

\section*{Materials and methods}

\subsection*{Animals}

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

\subsection*{Body Composition}

Fat, lean and fluid mass was analyzed with a quantitative NMR apparatus (Minispec, Bruker Optics, Billerica, MA) using a previously described protocol (List, et al. 2009).

\subsection*{White adipose tissue (WAT) depot samples}

All mice were sacrificed by cervical dislocation. Subcutaneous/inguinal (sc) and all intraabdominal depots – epididymal (epi), mesenteric (mes), and retroperitoneal (retro) – were collected using sterile dissection techniques.

\subsection*{Isolation of adipose tissue stromal vascular fraction (SVFs)}

To obtain a sufficient number of cells for further \textit{in vitro} culture, different WAT depots from three mice were collected together and washed three times with fresh PBS containing 1% antibiotic-antimycotic (Gibco, Grand Island, NY, cat. no. 15240-062). The tissue was minced and digested with collagenase solution – HBSS with Ca and Mg (Gibco, Grand Island, NY, cat. no. 14025092) supplemented with 2 mg/ml Collagenase, Type 1 (Worthington Biochemical Corp., Lakewood, NJ, cat. no. LS004196), 3 mM CaCl2 and 2% BSA – for 60 min, at 37°C, and 150 – 250 rpm. The mature adipocytes fraction was removed, and the SVFs suspension was filtered through a 70-µm sterile filter and spun down by centrifugation at 280 g at 4 °C for 5 min. 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 SVFs was spun down again by centrifugation, and the pellets were resuspended in fresh PBS.
Fluorescence-activated cell sorting (FACS)

After incubation with specific antibodies, SVFs samples were analyzed and sorted on a FACSARia flow cytometer using FACSDiva software (Becton Dickinson, San Jose, CA). Two surface markers for mouse AT-MSC (Sca-1 and PDGFR-α) and two negative surface markers (CD45 and Ter119) were used to identify and sort a pure population of mouse AT-MSC, following a previously validated protocol (19). The specific fluorochrome-conjugated monoclonal antibodies (1/100 dilution), used were from eBioscience (San Jose, CA) (Table 1). Output data was recorded by using the FACS Diva software.

Cell culture

Sufficient cells were obtained for in vitro experiments from sc, epi and mes depots, but not from the retro depot. The FACS sorted populations of AT-MSC were seeded at a density of 5,000 cells per cm$^2$ in growth medium – DMEM-F12 (GIBCO, Grand Island, NY, cat. no. 21885-108), 10% FBS (GIBCO, Grand Island, NY, cat. no 12662-029), 2 mM glutamine (Invitrogen, cat no. 35050-038) and 1% antibiotic-antimycotic – and cultured for two passages. The cells were then seeded at a density of 40,000 cells per well in 12-well plates (Costar, Cambridge, MA) 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 (SIGMA, Saint Louis, MO, cat no. I5879), 1 µM dexamethasone (SIGMA, cat. no. D4902), 0.2 mM indomethacin (SIGMA, cat. no. I7378-56), 10 µg/ml insulin (SIGMA cat no. I9278) – for 7 days.

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

Real-time PCR

RNA was isolated using ethanol precipitation method. cDNA was synthesized using Maxima First Strand cDNA Synthesis Kits (Thermo Scientific, cat. no. K1642), and quantitative real-time RT-PCR was performed using Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, cat. no. K0242). Data were normalized to ribosomal protein S3 (RPS3) as one of the most stable housekeeping gene for WAT (Lubbers, et al. 2013). Primer sequences are listed in Table 2.

Statistical analysis

Data are expressed as mean ± SEM. First, one-way ANOVA was performed to assess the total variability within the mice or cells data. Further, unpaired Student’s sample t tests (two-tailed) were used to evaluate differences between genetically engineered mouse lines and WT mice and between cells isolated from these mouse lines and control littermates.

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 control and GHRKO
mice (44.5 ± 4.0 g vs 28.6 ± 2.1 g, and 13.5 ± 1.7, P<0.001). As expected, GHRKO mice had the lowest total body weight but the highest percentage of body fat (Figure 1).

bGH mice had both increased lean mass (81.2 ± 1.5 vs 65.4 ± 2.5 %, P<0.001) and fluid mass (16.8 ± 0.7 vs 15.0 ± 0.4 %, P<0.01) compared with GHRKO mice whereas no significant difference was observed for lean and fluid mass, when the mice were compared with their WT littermates controls (data not shown).

Selective isolation of PDGFR-α+ Sca-1+ cells from different AT depots using flow cytometry

Using a recently described protocol (19), we identified and sorted a population of mouse AT-MSC on the basis of their positive surface markers of PDGFR-α and Sca-1 and the lack of CD45 and Ter119, two markers of hematopoietic stem cells. The algorithm used is presented in Figure 2. The percentage of PDGFR-α+ Sca-1+ 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 (Figure 2D).

Adipogenic differentiation of WT PDGFR-α+ Sca-1+ positive AT-MSC

Adipogenesis was achieved only in the cells derived from the sc depot in all genotypes. Cells from the epi and mes depots showed impaired differentiation ability, even if we used identical in vitro conditions as for the sc depot (Figure 3A). When stimulated to differentiate, none of the latter depots increased PPARγ gene expression (Figure 3A), very few epi cells presented lipid accumulation (data not shown) although a slight increase of adiponectin and FABP4 gene expression was observed (Figure 3B,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-MSC from sc depot

As shown in Figure 4A the overnight cell culture of sorted AT MSC led to the cells attaching to the plastic surface with no cell death observed in the media. The cells gradually acquired a fibroblastic appearance and divided. 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-MSC cells from all the genotypes showed increases in PPARγ, FABP4 and adiponectin, markers for adipogenesis (Figure 4B). Moreover, the GHRKO cells revealed the highest amount of lipid accumulation (Figure 4B) and the highest mRNA level for the adipogenic markers. Conversely, bGH cells showed less lipid accumulation and lower levels of PPARγ, adiponectin and FABP4 mRNA compared with WT and GHRKO cells (Figure 4B). We also observed that lipid droplets in GHRKO cells were smaller and had a multilocular appearance when compared to WT and bGH cells (Figure 4B).

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

Activation of Wnt/β-catenin pathway leads to decreased adipogenesis (Christodoulides, et al. 2009; Ross et al. 2000) (Figure 5A). Thus, we determined Axin-2 mRNA levels as a marker for activation of Wnt/β-catenin. Axin-2 increased during the adipogenesis in WT and bGH
cells but not in GHRKO cells (Figure 5B). Moreover, the bGH cells showed a significantly higher expression of Axin-2 vs WT cells, suggesting that the impairment of adipogenesis may be mediated by increased Wnt/β-catenin activity.

Discussion

In this study, we isolated a population of AT-MSC (PDGFRα+ Sca-1+ CD45− Ter119− cells) from different depots of dwarf GHRKO, giant bGH, and control 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-MSC. We found that expression of key adipogenic transcriptional markers is higher in sc adipocytes differentiated from GHRKO and lower in sc cells differentiated from bGH than WT cells. Thus, GH appears to play an important role by inhibiting the adipogenesis of sc AT-MSC. We also demonstrate that AT-MSC isolated from various depots have different adipogenic potential. More specifically, our data indicate that only the cells isolated from the sc depot achieve a mature adipocyte phenotype in vitro. Finally, we show that the decrease adipogenesis of bGH cells might be attributed to an increase of Wnt/β-catenin signaling.

In a recent study, SVFs isolated from sc depot of 20 months old GHRKO mice were found to have greater differentiation capacity compared to controls (Stout, et al. 2014). However, SVFs are a mixture of cells containing in addition to AT-MSC, preadipocytes at different stages of commitment, fibroblasts, leukocytes, epithelial cells, endothelial cells, and moreover other cells comprising the vasculature and nerve tissue (Cawthorn, et al. 2012). Thus, contamination of the in vitro culture with non-adipogenic cells might have altered the responses of the adipogenic cells. To remove the undesirable effects of cellular contamination, we decided to selective isolate AT-MSC by flow cytometry. As mentioned, AT-MSC is a functionally distinct SVF subpopulation (Cawthorn et al. 2012). The most widespread technique used to distinguish between SVFs and AT-MSC is assaying for cell surface specific markers. Therefore, we isolated mouse AT-MSCs on the basis of their positive expression of PDGFR-α and Sca-1, two known markers of AT-MSC (Houlihan et al. 2012). Importantly, we sorted a pure population of AT-MSC, since PDGFR-α is expressed only by AT-MSC and is not present on committed preadipocytes (Cawthorn et al. 2012). Our results indicate evidence for a large population of PDGFR-α+ Sca-1+ cells present in adipose tissue in mice, since reports prior to this have found these cells in bone. Obtaining a pure population of cells was important, because the contamination with other SVFs (possible in different proportions among different genotypes/depots) may have influenced 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 essential for maintaining proper properties and growth conditions. The choice of serum has significant importance in culturing MSC (Shahdadfar, et al. 2005). A culture media with a concentration of 10% bovine fetal serum (FBS) contains approximately 6 ng/m GH and 7 ng/ml IGF-I, sufficient to impair the effects of exogenous added GH and IGF-I (Cool, et al. 2005; Kurtz, et al. 1985). To specifically address the GH effect on adipogenesis and at the

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same time to avoid unspecific effects of the GH present in serum, we used cells lacking GH action. We report an increased level of adipogenesis in cells lacking GH action, as measured by gene expression of adipocytes-related markers (PPARγ, FABP4 and adiponectin) and lipid accumulation. Moreover, we observed decreased adipogenesis of the cells that have an extra copy of bovine GH, suggesting a negative paracrine effect of GH on the adipogenesis of AT-MSC. PPARγ, a member of the nuclear-receptor superfamily, is both necessary and sufficient for adipogenesis (Rosen and MacDougald 2006). Its expression rapidly increases after induction of adipogenesis, and maximal levels of expression are attained in mature adipocytes (Gregoire, et al. 1998). During the terminal phase of differentiation, adipocytes synthetize 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, displacing the nucleus to the periphery. Lipid accumulation in vitro may not necessarily replicate lipid accumulation seen in vivo, but a tendency towards larger and fewer lipid droplets is evident in old cultures of mature adipocytes. Morphologically, brown adipocytes differ from white adipocytes by their multilocular appearance caused by their numerous small lipid droplets (Lee, et al. 2013). Of notice, in our study the lipid droplet morphology in GHRKO cells was closer to brown than to white adipocytes appearance, raising the question of whether the function is similar. In fact, an argument for a potential increased metabolic activity of sc fat in GHRKO mice comes from the observation that GHRKO mice, even if obese, are extremely insulin sensitive (List, et al. 2011; Sackmann-Sala, et al. 2014). This is also supported by human studies showing that individuals, who carry mutations in the GHR gene and lack GH signaling, although obese, are protected from metabolic dysfunction (Guevara-Aguirre, et al. 2011). However, this model is not necessarily valid in subjects with adult onset growth hormone 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 regards to the metabolic consequences.

GH excess in mice and humans decreases fat and increases lean mass, but also induces insulin resistance (Olarescu et al. 2012; Vijayakumar, et al. 2011). As suggested by previous investigations, we confirm that the percent fat mass is markedly elevated in GHRKO, and significantly decreased in bGH, relative to WT male mice at 4 months of age (Berryman et al. 2004; Berryman, et al. 2010; Jara, et al. 2014). 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; Berryman, et al. 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 intraabdominal depots are proportional in size to those of WT (Berryman et al. 2010). The current study provides a possible mechanism to explain the increased sc fat depot in GHRKO mice, which could be due to increased adipogenesis. In accordance, recent studies demonstrate 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 will lead to various metabolic outcomes.

In addition to the known lipolytic activity of GH, inhibition of adipogenesis, as shown in this study, may contribute to the systemic insulin resistance associated with GH excess. Both processes lead to increased circulating free fatty acids (FFA), by releasing them from AT through lipolysis or by the incapacity to store the excess FFA in newly recruited adipocytes due to the diminished capacity of differentiation. Recent evidence demonstrates unequivocally a role of FFA and ectopic lipid accumulation in the pathogenesis of insulin resistance in muscle and the liver (Hocking et al. 2013; Shulman 2014).

Noteworthy, we observed depot specific differences in the ability of sorted AT-MSC cells to differentiate in a medium containing IBMX, dexamethasone, indomethacin and insulin, with sc depot being the only depot fully achieving adipogenesis. Although interesting, regional differences in MSC replication, differentiation, subtype abundance, susceptibility to apoptosis or senescence, and capacities for adipogenesis, despite originating from the same individual were previously described (Tchkonia, et al. 2013). The regional differences are heritable over many cell generations, suggesting that multipotentiality of MSCs is epigenetically restricted (Reinisch, et al. 2014).

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

Our study has some limitations. First, the experiments were performed only in 4 months old, male mice, that did not allow us to evaluate the age and sex related influences. Although differences in body composition between male and females mice were demonstrated (Berryman et al. 2010), no effect of estrogens on the in vitro differentiation potential of AT-MSC has been reported (Veronesi, et al. 2014). On the other hand, we decided to perform the study in relative young animals to account for age-related alterations in MSC properties (Kim, et al. 2012). Second, we did not evaluate the possible effects IGF-I, present in FBS or produced upon GH stimulation. However, the differences in the adipogenesis observed in this study were not due to IGF-I action, because GHRKO cells, by lacking GH signaling, may eventually produce less IGF-I that will act in a paracrine manner and favor adipogenesis. Moreover, bGH cells showed impaired adipogenesis in our study, an opposite effect compared with the pro-adipogenic action of IGF-I described in the literature (Scavo, et al. 2004). Thirdly, the results regarding activation of canonical WNT/β-catenin pathway by GH should be considered with caution since they are based on measuring a single WNT/β-catenin signaling marker, namely Axin-2. 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 statement is made. Finally, although we isolated AT-MSC cells using two
specific surface antibodies in an attempt to select cells that are at the same stage of
differentiation we cannot rule out that the prior in vivo exposure did not influence the
intrinsic properties of the isolated cells.

In conclusion, our results suggest that GH diminishes the amount of AT by altering the
adipogenesis of AT-MSC. Consequently, a reduced AT expandability potential might
represent a novel mechanism to further explain GH induced insulin resistance.

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Figure 1. Total body weight and fat mass in 4 months 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 control and GHRKO mice. No difference was observed between different genotypes of WTs. Data are presented as mean ± SEM; WT, wild-type littermate control mice; GHRKO, growth hormone receptor knockout mice; bGH, bovine growth hormone transgenic mice; *, p<0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
Figure 2. Fluorescence-activated cell sorting algorithm of AT-MSC
First, a scatter gate was defined to account for cells’ 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 MSC (PDGRFα+Sca-1+), that was not present with isotype control antibodies (C), was identified, and sorted (D).
Figure 3. Different adipogenic potential of FACS sorted WT AT-MSC
Adipogenesis was achieved in the sc depot, but not in epi and mes depots. Data are presented as mean ± SEM; Undiff, undifferentiated. Sc, subcutaneous, Epi, epididymal, Mes, mesenteric; **, p ≤ 0.01; ***, p ≤ 0.001 vs undifferentiated control.
Figure 4. Phenotypic appearance of sc WT AT-MSC after isolation (A) and comparison of the adipogenic properties between GHRKO, bGH and WT cells (B)

Figure A presents in vitro appearance of sc WT AT-MSC at 1, 3 and 7 days after FACS isolation. The cells adhere to plastic surface and present round form at 1 day after isolation. Thereafter at 3 and 7 days the cells start to elongate, and to acquire a characteristic fusiform appearance, and to divide. Figure B presents the adipogenesis of sc AT-MSC from GHRKO, bGH and WT mice at 7 days of differentiation. GHRKO cells achieved a higher differentiation, while bGH showed an impaired differentiation as quantified by the lipid accumulation and gene expression for the adipogenic markers; Data are presented as mean ± SEM; 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≤ 0.01; ***, p≤ 0.001; 10× magnification.
Figure 5. Activation of Wnt/β catenin decreases adipogenesis

Activation of Wnt/β catenin signaling decreases adipogenesis of MSC (A). Axin-2, a marker of Wnt/β catenin activation, is increased in mature sc-bGH adipocytes suggesting that the decreased adipogenic potential of these cells might be attributed to increased Wnt/β catenin activity. Data are presented as mean ± SEM; 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.
## Table 1

Antibodies used for primary isolation and immunophenotyping

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</tr>
</tbody>
</table>
Table 2

Real-time RT-PCR primer pairs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rps3</td>
<td>F: ATCAGAGAGTTGACCAGTT</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>R: AATGAAACCGAACACCATA</td>
<td></td>
</tr>
<tr>
<td>PPARγ</td>
<td>F: GGGATATTTTTGGCATACTCTG</td>
<td>195</td>
</tr>
<tr>
<td></td>
<td>R: AAAGACAAACGACAAATCAC</td>
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</tr>
<tr>
<td>Adiponectin</td>
<td>F: CTCTCTGTCTCTTAATCCT</td>
<td>218</td>
</tr>
<tr>
<td></td>
<td>R: ACCAAGAAGACCTGATCTC</td>
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</tr>
<tr>
<td>FABP4</td>
<td>F: AACCAGAGATTTCAAAACTG</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>R: CCATCTAGGGATATGCTCTTC</td>
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</tr>
<tr>
<td>Axin-2</td>
<td>F: CGCCAACGACAGCGAGTT</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>R: CGGTAAGGGAGGACTCCATCTA</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse. All the primers are listed 3’ to 5’