GAS6 receptor antagonism impairs adipocyte differentiation and adipose tissue development in mice

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GAS6: growth arrest specific protein 6
SC: subcutaneous
GON: gonadal
EB: embryoid body

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ABSTRACT

A low Mr receptor tyrosine kinase inhibitor (R428) with high affinity and selectivity for the GAS6 (growth arrest specific protein 6) receptor Axl was used to study a potential role of GAS6 signaling in adiposity. In vitro, R428 caused a concentration-dependent inhibition of preadipocyte differentiation into mature adipocytes, as evidenced by reduced lipid uptake. Inhibition of Axl mediated signaling was confirmed by reduced levels of phospho-Akt activity. In vivo, oral administration of R428 during 5 weeks to mice kept on high fat diet resulted in significantly reduced weight gain and subcutaneous (SC) and gonadal (GON) fat mass. This was associated with marked adipocyte hypotrophy, enhanced macrophage infiltration and apoptosis. Thus, affecting GAS6 signaling through receptor antagonism using a low Mr Axl antagonist impairs adipocyte differentiation and reduces adipose tissue development in a murine model of nutritionally induced obesity.

INTRODUCTION

The TAM receptor protein tyrosine kinases, Tyro 3 (also called Rse, Sky, Brt, Tif, Dtk, Etk-2), Axl (also called Ark, Ufo, Tyro7) and Mer (also called Eyk, Nyk, Tyro12) play pivotal roles in innate immunity (Lemke and Rothlin, 2008). These receptors contain 2 immunoglobin-like domains and dual fibronectin type III repeats in the extracellular region, as well as a cytoplasmic kinase domain. The ligands for TAM receptors are GAS6 (growth arrest specific protein 6) and protein S (Hafizi and Dahlbäck, 2006). The high affinity interaction of GAS6 with Axl triggers anti-apoptotic signals, resulting in improved cell survival. In addition, Axl-mediated signaling has been implicated in tumor invasion, migration, angiogenesis, proliferation and adhesion (Li et al., 2009). GAS6 signaling stimulates cellular responses including activation of PI3' kinase – Akt, Erk and p38 MAP kinase cascades, the NF-kB pathway and STAT signaling. GAS6 is expressed during the clonal expansion of postconfluent 3T3-L1 preadipocytes (Shugart et al., 1995), and during differentiation of 3T3-F442A preadipocytes into mature adipocytes (Maquoi et al., 2005). We have shown that adipose tissue development is impaired in GAS6 deficient mice (Maquoi et al., 2005). Whereas GAS6, Tyro 3 and Mer are highly expressed in mature murine adipocytes, Axl is primarily expressed in the stromal-vascular cell fraction, including preadipocytes (Maquoi et al., 2005). Furthermore, Axl expression was found to be downregulated during in vitro porcine adipocyte differentiation (Suzuki et al., 2008). Protein array revealed higher Axl levels in subcutaneous adipose tissue of obese human subjects as compared to lean controls (Skopkova et al., 2007).

In the present study, we have evaluated a potential role of the GAS6 receptors in in vitro preadipocyte differentiation and in vivo adipose tissue formation, by using a low M_r inhibitor (R428), which exhibits high activity against Axl signaling (EC50/IC50 of 14nM) whereas its activity is limited to the tyrosine kinase subfamily, with 50 and > 100-fold higher selectivity for Axl than for Mer and Tyro3 (Holland et al., 2010).

METHODS

Culture and differentiation of embryonic stem (ES) cells

ES cells were derived from the C57Bl/6 mouse strain. They are feeder-dependent and were maintained in a pluripotent undifferentiated state *in vitro* by using TX-WES medium (Thrombogenics, Leuven, Belgium) (Schoonjans et al., 2003). Differentiation into adipocytes was induced essentially as described (Dani et al., 1997). Therefore, ES cells were harvested and resuspended at a cell density of 5×10^5 cells/ml in Glasgow/BHK 21 medium (Invitrogen, Paisley, UK) containing 0.23% sodium bicarbonate, 1 x MEM essential amino acids, 2 mM glutamine, 100 μM 2-mercaptoethanol and 10% fetal calf serum (Hyclone, Logan, UT) (differentiation medium). Microdrops of 20 μl were positioned for the hanging drop culture (day 0) and allowed to grow for 2 days and differentiate into embryoid bodies (EBs) (Desbaillets et al., 2000). The EBs were then transferred into bacteriological plates (day 2) and maintained for 3 days in suspension in differentiation medium only or supplemented with 10 nM all-*trans* retinoic acid, which activates early stage adipogenesis (Bost et al., 2002). After another 2 days in suspension in differentiation medium only, EBs were plated in gelatin-coated plates in differentiation medium only or supplemented with 85 nM insulin, 2 nM triiodothyronin and 1 μM rosiglitazone-maleate (day 7). Medium was changed every 2 days. At regular time points during the differentiation process, cell lysates were taken for RNA extraction. After 30 days, the extent of adipocyte differentiation was estimated by staining with Oil Red O. Briefly, cells were washed with phosphate buffered saline (PBS) and fixed for 5 min with a 1.5% glutaraldehyde solution in PBS. Cells were washed with PBS, stained for 2 hours with a 0.2% Oil Red O solution, washed and kept in tissue culture water. The Oil Red O stained lipid droplets were first characterized by light microscopy, then extracted in isopropanol and quantified from the absorbance at 490 nm.

Culture and differentiation of preadipocytes

3T3-F442A murine preadipocytes (Green and Kekinde, 1976) were cultured and differentiated as described elsewhere (Scroyen et al., 2010). On day 12, the cultures were stained for triglycerides with Oil Red O as described above. Experiments were performed with addition of R428 (50 nM-1 µM) or DMSO as control.

Axl cell-based assay

3T3-F442A preadipocytes were seeded in DMEM medium with 0.5% bovine calf serum iron-supplemented in 96-wells plates. After 24 hours starvation, 1 µM R428 (diluted in DMSO) or DMSO control was added to the cells for 1 hour. Cells were stimulated for 5 minutes at 37°C with preclustered, prewarmed antibody mixture (37.5 µg/ml mouse anti-Axl (R&D Systems, Minneapolis, MN) plus 100 µg/ml goat anti-mouse IgG (Jackson Immunoresearch, West Grove PA) in starvation medium, mixed at 4°C for 2 hours). Cells were fixed in 4% formaldehyde, quenched in 3% H_2O_2/0.1% sodium azide in wash buffer (0.1% TritonX-100 in PBS), blocked (in 5% BSA) and incubated overnight at 4°C with diluted anti-phospho-Akt Ser^{473} in blocking buffer under gentle shaking. After removal of the primary antibody, cells were incubated with diluted goat anti-rabbit-HRP (Jackson Immunoresearch), and developed with TMB substrate (optical density at 450 nm) (R&D Systems), after stopping the reaction with 2 M H_2SO_4.

mRNA expression analysis

Total DNA-free RNA was extracted from cells using the RNA Easy Qiagen kit (Qiagen, Valencia, CA) and the concentration was determined using the RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR).

The expression of GAS6 and its receptors was monitored by quantitative real-time PCR, using specific primers and probes (Gene expression assays, Mm00490389_m1 for GAS6, Mm00627285_m1 for Axl, Mm00444547_m1 for Tyro3 and Mm00434922_m1 for Mer;

Applied Biosystems, Foster City, CA). Reverse transcription reactions were performed from 50 ng of total RNA at 48°C during 60 min with the Taqman Reverse Transcription Kit supplemented with 5μM random hexamers (Applied Biosystems). Quantitative real time PCR was performed in the ABI 7500 fast sequence detector using the Taqman Fast Universal PCR Master Mix (Applied Biosystems). The data were normalized to the expression of β-actin, as housekeeping gene. Analysis of the Ct values was performed with the delta-delta Ct method using the 7500 Fast System SDS software (Applied Biosystems).

Animal model

Male wild-type C57Bl/6 mice were generated in the KULeuven animal facility. Mice were kept in individual microisolation cages on a 12h day/night cycle at 20-22°C and fed with a high fat diet (HFD, Harlan Teklad TD88137, Zeist, The Netherlands; 42% kcal as fat, caloric value 20.1kJ/g). Water was always available at libitum. At the age of 5 weeks, mice were kept on HFD for 3 weeks, and at the age of 8 weeks, one group (n = 12) was started on R428 at a dose of 75 mg/kg/day by oral gavage, and a second group (n = 12) was given 25 mg/kg, twice daily. Concentrations were adjusted to allow administration in the same volume for different doses. R428 (1-(6,7-dihydro-5*H*-benzo(6,7)cyclohepta(1,2-*c*)pyridazin-3-yl)-*N*3-((7-pyrrolidin-1-yl)-6,7,8,9-tetrahydro-5H-benzo(7)annulene-2-yl)-1*H*-1,2,4-triazole-3,5-diamine) (Holland et al., 2010) was a kind gift from Rigel Inc. (South San Francisco, CA). Food intake was monitored daily, and control groups were started receiving the same volume of vehicle (0.5% hydroxypropylmethylcellulose, 0.1% Tween 80) as the R428 treated groups, given as a single administration (n = 10) or twice daily (n = 11). The control groups were pair-fed as compared to the treated groups. The study was continued for 5 weeks and monitored as described below.

Body weight was measured daily, and body temperature was measured at weekly intervals using a rectal probe (TR-100, Fine Science Tools, Foster City, CA). Physical activity (voluntary wheel running) at night was monitored in cages equipped with a turning wheel linked to a computer to register full turns/12h (19:00-07:00). At the end of the experiments,

after overnight fasting, mice were euthanized by intraperitoneal injection of 60 mg/kg Nembutal (Abbott Laboratories, North Chicago, IL). Blood was collected via the retroorbital sinus on trisodium citrate (final concentration 0.01M); plasma was prepared by centrifugation at 4°C at 4000g for 5 min and stored at -80°C. Intra-abdominal (gonadal, GON) and inguinal subcutaneous (SC) fat pads were removed and weighed; portions were snap-frozen in liquid nitrogen for RNA extraction and paraffin sections (10µm) were prepared for histology and immunohistochemistry. Other organs including kidneys, lungs, spleen, pancreas, liver, heart and brain were also removed and weighed.

All animal experiments were approved by the local ethical committee (KULeuven P03112) and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

Analysis of adipose tissues

The size and density of adipocytes or blood vessels in the adipose tissues were determined by staining with haematoxylin/eosin under standard conditions or with the Bandeiraea Simplicifolia lectin, followed by signal amplification with the Tyramide Signal Amplification Cyanine System (Perkin Elmer, Boston, MA), as described (Laitinen, 1987; Van Hul and Lijnen, 2008). Blood vessel density was normalized to the adipocyte number. Analysis was performed using a Zeiss Axioplan 2 Imaging microscope with the AxioVision rel. 4.6 software (Carl Zeiss, Oberkochen, Germany).

Macrophage infiltration in adipose tissues was quantitated following staining with anti-Mac-3 antibody (Pharmingen, San Diego, CA) and expressed in percentage of the total section area. Apoptosis was monitored by staining with rabbit anti-active mouse caspase 3 (R&D Systems, Minneapolis, MN, USA), and expressed in percentage of cells.

Blood glucose concentrations were measured using Glucocard strips (Menarini Diagnostics, Firenze, Italy). Other metabolic parameters and liver enzymes, including triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, alkaline phosphatase, AST and ALT were determined using standard laboratory assays. Insulin (Mercodia, Uppsala, Sweden) and leptin

(R&D Systems Europe, Lille, France) levels were measured using commercial ELISA's, and PAI-1 antigen with a specific home-made ELISA (Declerck et al., 1995). Blood cell analysis, including white blood cells, neutrophils, lymphocytes, monocytes, eosinophils, basophils, red blood cells, platelet count, haemoglobin and haematocrit levels, was performed on a Cell-Dyn 3500R (Abbott Diagnostics, Abbott Park, IL).

To monitor the expression of GAS6 and its receptors, adipose tissues were homogenized using lysing matrix tubes (Qbiogene, Carlsbad, CA) in a Hybaid ribolyser (Thermo, Walham, MA). Total DNA-free RNA was extracted and quantitative RT-PCR was performed as described above, using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene.

Statistical analysis

For statistical analysis data are first averaged per mouse and are given as means ± SEM for the number of animals studied. Statistical significance between groups is evaluated by non-parametric Mann-Whitney U-test, or by 2-way ANOVA (only where specifically indicated). Correlations are examined using the nonparametric Spearman's rank correlation coefficient. Values of $p < 0.05$ are considered statistically significant.

RESULTS

Expression of GAS6 and its receptors at different stages of adipogenesis

To monitor the expression of GAS6 and its receptors at different stages of adipogenesis, we measured mRNA levels during differentiation of ES cells and of 3T3-F442A preadipocytes into mature adipocytes.

During differentiation of ES cells, expression of GAS6, Axl, Mer and Tyro3 increased with time. At day 30 of differentiation, significantly higher expression of GAS6, Axl, Mer and Tyro3 was observed in adipose EBs as compared to control EBs (Figure 1, left panel). Oil Red O staining revealed the presence of lipid droplets in about 80% of adipose EB outgrowths, as compared to 15% for control EBs (not shown).

As 3T3-F442A preadipocytes reached confluency, GAS6 mRNA level increased progressively. On treatment with the differentiation medium (day 4), GAS6 mRNA declined sharply to reach the level of preconfluent cultures. A similar expression pattern was observed for Mer, whereas mRNA levels for Axl and Tyro3 declined during differentiation (Figure 1, right panel). Oil Red O staining (OD 490nm of 0.80 – 1.20 as compared to 0.10 – 0.20 at the start) confirmed effective differentiation (not shown).

Effect of the Axl antagonist R428 on in vitro differentiation of 3T3-F442A preadipocytes

Addition of R428 (50 nM-1µM) resulted in a dose-dependent inhibition of differentiation of 3T3-F442A preadipocytes into mature adipocytes, as indicated by less Oil Red O positive lipid droplets on day 12 in the R428 treated cells then in the control cells treated with DMSO. Oil Red O staining ranged between 84 and 35 % of that of DMSO control at R428 concentrations between 50 nM and 1µM (mean of duplicate determinations in one experiment; data not shown). Inhibition of Axl signaling by R428 in differentiating preadipocytes was confirmed by the Axl cell-based assay, yielding lower values (A^{450nm}) for phospho-Akt activity upon treatment with 1µM R428 (0.26 ± 0.02; $n = 12$) as compared to medium control (0.51 ± 0.03; $n = 8$) or to DMSO control (0.37 ± 0.02; $n = 8$).

Effect of the Axl antagonist R428 on in vivo adipose tissue formation

Effect on body and fat pad weight and composition

To evaluate the effect of R428 treatment on ongoing development of obesity, 8 weeks old male mice – previously kept on HFD for 3 weeks – were given R428 as a single dose of 75 mg/kg/day or as two doses of 25 mg/kg/day. Control mice were kept on the same administration scheme with vehicle and were pair-fed. In the group receiving 2 x 25 mg/kg/day R428 one mouse was killed after 2 weeks, and in the corresponding control group two mice after 2 and 3 weeks (stopped eating).

At day 35, the last day of HFD feeding, the body weight in both groups treated with R428 was significantly lower than in the corresponding vehicle treated groups (Table 1). As compared to the start of the experiment, body weights at the end were significantly increased in both vehicle-treated groups ($p = 0.018$ and $p = 0.003$ for once and twice administration, respectively), but not in R428-treated groups ($p = 0.42$ and $p = 0.43$ for once and twice administration). Thus, the weight gain over 5 weeks (Fig. 2) was significantly lower in the R428-treated as compared to vehicle-treated groups: 0.79 ± 0.36 g versus 3.1 ± 0.59 g ($p = 0.015$) for the 75 mg/kg dose and 0.34 ± 0.45 g versus 4.1 ± 0.61 g ($p = 0.0004$) for the 2 x 25 mg/kg dose. Food intake in the different groups before start of the administrations ranged between 3.0 ± 0.11 g/day and 3.1 ± 0.06 g/day. The food intake during pair-feeding was not significantly different between groups (Table 1), suggesting a reduced feeding efficiency (weight gain normalized to caloric intake) in the R428-treated mice. Body temperature remained constant at 37-38°C over the 5 week experimental period and was not different between groups (not shown).

Voluntary wheel running at night was comparable for mice treated with 2 x 25 mg/kg of R428 or with vehicle (4280 ± 790 versus 6190 ± 1680 turns/night; $p = 0.32$), whereas for mice treated with one dose of 75 mg/kg this appeared somewhat reduced as compared to vehicle

(5410 ± 770 versus 9320 ± 1910 turns/night; p = 0.07). The isolated SC and GON fat mass was significantly lower in both R428-treated groups as compared to the corresponding vehicle-treated groups (Table 1). Liver weights were significantly reduced in both R428-treated groups, whereas spleen weight was somewhat reduced and pancreas weight somewhat enhanced in the 75 mg/kg/day R428 group (Table 1). Statistical analysis by 2-way ANOVA confirmed a significant lowering effect of treatment (R428 versus placebo) on body weight at the end (with and without fasting) and on SC and GON fat mass (all $p < 0.0001$). Treatment also significantly reduced the weight of liver ($p = 0.0005$), left kidney ($p = 0.02$) and brain ($p = 0.03$). The administration scheme (once 75 mg/kg versus twice 25 mg/kg) only affected liver weight ($p = 0.01$).

Adipocyte size was significantly reduced in SC ($p<0.005$) and GON ($p<0.01$) fat pads of mice treated with 2 x 25 mg/kg of R428 and in SC fat pads ($p<0.05$) of mice treated with 1 x 75 mg/kg as compared to the corresponding vehicle-treated groups (Figure 3 and Table 2). Blood vessel size was comparable for all groups. The blood vessel density in SC fat pads was significantly higher in both R428-treated groups ($p<0.01$ and $p<0.05$) as compared to the corresponding vehicle-treated groups. In contrast, we observed a lower blood vessel density in GON fat pads ($p<0.05$) of mice treated with 1 x 75 mg/kg. Normalized blood vessel density was lower in both SC and GON fat pads of both R428-treated groups.

Further analysis revealed that macrophage infiltration was higher in SC and GON fat pads of mice treated with 2 x 25 mg/kg of R428, whereas in fat pads of mice treated with 1 x 75 mg/kg of R428 it was comparable to the corresponding vehicle-treated groups (Table 2). Apoptosis was somewhat enhanced in the SC and GON adipose tissues of mice treated with R428 as compared to vehicle (Table 2). 2-Way ANOVA confirmed a significant effect of treatment on adipocyte size ($p < 0.0001$ for SC, $p = 0.03$ for GON), adipocyte density ($p < 0.0001$ for SC, $p = 0.001$ for GON), blood vessel size ($p = 0.02$ for GON), blood vessel density ($p = 0.0009$ for SC), normalized blood vessel density ($p = 0.03$ for SC, $p < 0.0001$ for

GON), and macrophage content ($p = 0.04$ for GON). The administration scheme had no effect on any of thes parameters.

Expression of GAS6 and its receptors Axl, Tyro3 and Mer could be demonstrated in SC and GON adipose tissues of mice in all 4 groups. A statistically significant effect of R428 was only observed for expression of GAS6 (1.4-fold upregulated; $p = 0.03$) and Mer (1.5-fold upregulated; $p = 0.04$) in the GON fat of mice treated with the 2x25 mg/kg dose. Expression of Axl or Tyro3 in adipose tissues was not affected by R428 treatment (Table 3). 2-Way ANOVA confirmed a significant enhancing effect of treatment on expression of GAS6 ($p = 0.005$) and Mer ($p = 0.004$) in GON fat, but no effects of the administration scheme.

Analysis of blood cell composition at the end of the experiment for both R428-treated groups as compared to vehicle revealed somewhat lower total white blood cell counts (1.9 ± 0.43 x 10^3/µl versus 2.4 ± 0.23 x 10^3/µl for the 2 x 25 mg/kg dose, $p = 0.06$; 1.7 ± 0.29 x 10^3/µl versus 3.1 ± 0.52 x 10^3/µl for the 75 mg/kg dose; $p = 0.04$). This was associated with significantly enhanced content of neutrophils ($40 \pm 2.4\%$ versus $16 \pm 2.3\%$ for the 2 x 25mg/kg and $35 \pm 2.7\%$ versus $16 \pm 2.3\%$ for the 75 mg/kg dose; $p < 0.0005$ for both doses) and monocytes ($5.7 \pm 1.7\%$ versus $1.6 \pm 0.34\%$ for the 2 x 25 mg/kg and $4.3 \pm 0.81\%$ versus $2.2 \pm 0.44\%$ for the 75 mg/kg dose; $p < 0.05$ for both doses), and reduced content of lymphocytes ($49 \pm 3.3\%$ versus $79 \pm 2.3\%$ for the 2 x 25 mg/kg and $56 \pm 3.2\%$ versus $78 \pm 3.2\%$ for the 75 mg/kg dose; $p < 0.001$ for both doses). Other blood cell fractions were not significantly affected by R428 treatment (data not shown).

Effect on plasma metabolic parameters

At the dose of 2 x 25 mg/kg R428, levels of glucose, total cholesterol, HDL cholesterol and triglycerides were reduced at the end of the experiment, whereas liver enzymes were not affected (Table 4). In contrast, at the dose of 1 x 75 mg/kg R428, metabolic parameters were not affected, whereas alkaline phosphatase and AST levels were enhanced. Insulin levels were not affected by R428 treatment, whereas PAI-1 levels were enhanced with both doses (p

< 0.05). Plasma leptin levels were lower in R428 treated animals, and correlated positively (all $r > 0.87$ and $p < 0.0001$) with SC and GON fat mass.

2-Way ANOVA showed a significant effect of treatment on levels of glucose ($p = 0.03$), PAI-1 ($p = 0.0009$), leptin ($p < 0.0001$), total cholesterol ($p = 0.007$), HDL cholesterol ($p = 0.008$), alkaline phosphatase ($p = 0.02$) and AST ($p = 0.03$). The administration scheme only affected alkaline phosphatase levels ($p = 0.007$).

DISCUSSION

Development of obesity arises from increased size of individual adipose cells caused by lipid accumulation, and/or from enhanced number of adipocytes upon differentiation of precursor cells into mature adipocytes under the appropriate nutritional and hormonal stimuli (Liu et al., 2003). Because GAS6 and its receptors Tyro 3, Axl and Mer are expressed by adipocytes and GAS6 deficient mice develop less adipose tissue, we hypothesized that the GAS6 signaling pathway may play a role in adipogenesis and adipose tissue formation. First, we have confirmed expression and modulation of GAS6 and its receptors during differentiation of ES cells and of preadipocytes into adipocytes, indicating their involvement already in early stages of adipogenesis. To study their functional role we used a low M_r inhibitor (R428) of tyrosine kinase activity that was identified as an orally bioavailable inhibitor with high selectivity for Axl over the other receptors (Holland et al., 2010). The doses used in the present study are based on available pharmacokinetic data indicating a long plasma half-life in mice (4 hours at 25 mg/kg and 13 hours at 75 mg/kg given by oral gavage), effective tissue distribution and high steady-state plasma drug concentrations. Thus, prolonged dosing at only 25 mg/kg b.i.d. generated a steady-state R428 concentration sufficient to block Axl signaling in the circulation (Holland et al., 2010).

We have confirmed that R428 inhibits Axl signaling in (pre)adipocytes, as monitored by decreased levels of Phospho-Akt. Furthermore, R428 had the potential to impair differentiation of preadipocytes into mature adipocytes, as evidenced by decreased lipid uptake. This is compatible with the concept that enhanced GAS6 levels are associated with the transient phase of growth arrest that precedes the phase of clonal expansion (Maquoi et al., 2005).

In the present study we have also shown the potential of R428 to impair adipose tissue development in mice pair-fed with HFD. This is associated with marked adipocyte hypotrophy in SC and GON adipose tissues. The weight of other organs was overall comparable with the exception of a reduced liver weight at both doses of R428. In the study of Holland et al. (Holland et al., 2010) prolonged dosing with R428 at 25 mg/kg b.i.d. for up

to 80 days in mice kept on normal chow, was not associated with significant weight loss. On HFD feeding in our study, however, the feeding efficiency of the mice (weight gain normalized to caloric intake) was significantly lower upon R428 treatment. A limitation of the present study is that no comprehensive energy balance has been made; we only monitored food intake, physical activity (voluntary wheel running) and body temperature.

At the dose of 2 x 25mg/kg R428 reduced levels of total cholesterol, HDL cholesterol and triglycerides were observed as compared to vehicle. Glucose levels were comparable to the 1 x 75mg/kg group, but were lower than in the corresponding vehicle group in which some animals had somewhat enhanced glucose levels. It is conceivable that these reduced levels are due to enhanced clearance, but the mechanism remains unclear, as Axl signaling has been implicated in many biological phenomena (Li et al., 2009).

We observed enhanced macrophage content in SC and GON adipose tissues of mice treated with the 2 x 25mg/kg dose, but not with the 75 mg/kg dose. It was shown previously that the Tyro3 family receptors limit macrophage activation (Lemke and Lu, 2003). The observation that higher degrees of cellular apoptosis were observed in the R428 treated adipose tissues is compatible with the notion that the GAS6 signaling pathway promotes cell survival. Because of marked variability between samples, these differences are, however, not statistically significant. Increased macrophage influx in adipose tissues may serve to help clear debris of apoptotic cells.

Previous studies have revealed that R428 has anti-angiogenic potential in breast cancer models (Holland et al., 2010), which is in line with the reported pro-angiogenic function of Axl (Li et al., 2009). It was also reported (Gallicchio et al., 2005) that Axl stimulation by GAS6 results in inhibition of the ligand-dependent activation of vascular endothelial growth factor (VEGF) receptor 2 and the consequent activation of an angiogenic program in vascular endothelial cells. In our study, GAS6 receptor antagonism had no effect on blood vessel size in the adipose tissues, whereas blood vessel density was somewhat enhanced in SC fat. This may be related to the fact that VEGFR2 expression in adipose tissues is not markedly affected by high fat feeding (Voros et al., 2005). However, when the blood vessel density is

normalized to the adipocyte number (because the number and/or size of adipocytes may affect blood vessel density (Cao et al., 2001)), a lower normalized density is observed in the fat tissues treated with R428.

The two different administration schemes used in our study (2 x 25 mg/kg/day or 75 mg/kg/day) may be associated with different peak levels and/or steady-state concentrations of R428, resulting in differential effects on some parameters. Overall, however, statistical analysis by 2-way ANOVA confirmed a beneficial effect on adiposity and metabolic parameters of the treatment (R428 versus vehicle), whereas the administration scheme had no effect on body and adipose tissue weight or composition, and only affected liver weight and alkaline phosphatase levels.

Thus, the GAS6 receptor antagonist R428 has the potential to impair preadipocyte differentiation and to reduce adipose tissue development in vivo, associated with marked adipocyte hypotrophy. Although R428 is a highly specific Axl antagonist, it cannot be excluded that at the doses achieved in vivo it also blocks Tyro 3 and/or Mer and may affect other pathways. Overall, however, affecting GAS6 signaling through the TAM receptor family appears to have the potential to impair adiposity.

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AUTHORSHIP CONTRIBUTIONS

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Wrote or contributed to the writing of the manuscript: Lijnen, Christiaens and Scroyen

Other: Lijnen acquired funding for the research

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Footnotes

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Legends for Figures

Fig. 1 Time course of the expression of GAS6 and its receptors Axl, Mer and Tyro3 during differentiation of embryonic stem (ES) cells or 3T3-F442A preadipocytes. For ES cells, open bars represent control embryoid bodies, and black bars correspond to adipose embryoid bodies. Data are means ± SEM of 3 experiments; * and ** $p < 0.05$ and $p < 0.01$ versus control

Fig. 2 Weight gain of mice kept on HFD during administration of vehicle (open symbols) or of the Axl antagonist R428 (closed symbols) at a dose of 2 x 25 mg/kg/day (A) or 75 mg/kg/day (B). Data are means ± SEM of 9-12 experiments.

Fig. 3 Haematoxylin/eosin staining of SC (panels A) and GON (panels B) adipose tissues of mice treated with 2 x 25mg/kg of the Axl antagonist R428 (panels 1) or with vehicle (panel 2).

Table 1 Effect of the Axl antagonistR428 treatment on adipose tissue and organ weights of C57Bl/6 wild-type mice kept on high fat diet

	2x25 mg/kg		1 x 75 mg/kg	
	Vehicle (n = 9)	R428 (n = 11)	Vehicle (n = 10)	R428 (n = 12)
Body weight start (g)	26.2 ± 0.91	24.9 ± 0.77	26.1 ± 0.90	25.2 ± 0.69
Body weight end (g)	30.3 ± 0.65	25.3 ± 0.74***	29.3 ± 0.64	26.0 ± 0.67**
Body weight end [(a)] (g)	28.3 ± 0.61	23.6 ± 0.62***	27.7 ± 0.58	24.3 ± 0.76**
Food intake (g/day)	3.17 ± 0.04	3.07 ± 0.06	3.11 ± 0.04	3.06 ± 0.06
SC fat (mg)	831 ± 58	421 ± 69***	690 ± 55	518 ± 51*
GON fat (mg)	1232 ± 63	685 ± 92***	1013 ± 81	794 ± 74*
Liver (mg)	1434 ± 54	1193 ± 61*	1258 ± 44	1097 ± 48*
Spleen (mg)	71 ± 2.4	83 ± 9.1	75 ± 3.4	67 ± 8.1**
Kidney left (mg)	168 ± 3.4	153 ± 3.6*	172 ± 3.8	162 ± 7.5
Kidney right (mg)	171 ± 5.3	163 ± 4.1	180 ± 5.7	169 ± 4.5
Pancreas (mg)	299 ± 4.7	297 ± 10	296 ± 5.0	322 ± 13*
Lung (mg)	159 ± 12	161 ± 6.5	182 ± 11	175 ± 8.4
Heart (mg)	136 ± 1.8	134 ± 3.0	135 ± 3.7	135 ± 3.5
Brain (mg)	430 ± 19	402 ± 7.8	427 ± 7.9	409 ± 2.7

[(a)] *after overnight fasting*

Data are means ± SEM of n experiments

*, ** and *** $p < 0.05$, $p < 0.01$ and $p < 0.005$ versus vehicle (non-parametric Mann-Whitney U)

Table 2 Effect of the Axl antagonist R428 treatment on adipose tissue composition of C57Bl/6 wild-type mice kept on high fat diet

	2 x 25 mg/kg		1 x 75 mg/kg	
	vehicle (n = 9)	R428 (n = 11)	vehicle (n = 10)	R428 (n = 12)
Adipocyte size (μm^2)				
SC fat	3295 ± 331	1807 ± 229***	2715 ± 224	1995 ± 152*
GON fat	3928 ± 218	2884 ± 230**	3474 ± 215	3513 ± 238
Adipocyte density (x $10^{-6}/\mu m^2$)				
SC fat	355 ± 47	646 ± 58***	409 ± 45	556 ± 43*
GON fat	263 ± 13	373 ± 30**	300 ± 17	303 ± 20
Blood vessel size (μm^2)				
SC fat	41± 2.6	40 ± 1.7	41 ± 1.8	40 ± 2.2
GON fat	55 ± 5.3	47 ± 2.8	62 ± 4.8	51 ± 3.1
Blood vessel density (x $10^{-6}/\mu m^2$)				
SC fat	333 ± 45	566 ± 45**	406 ± 47	465 ± 25*
GON fat	270 ± 9.0	323 ± 22	330 ± 14	275 ± 15*
Normalized blood vessel density				
SC fat	0.95 ± 0.044	0.89 ± 0.035	1.00 ± 0.036	0.89 ± 0.033*
GON fat	1.06 ± 0.037	0.88 ± 0.040*	1.11 ± 0.034	0.92 ± 0.035**
Macrophage infiltration (%)				
SC fat	9.7 ± 2.0	23 ± 6.0**	19 ± 3.2	18 ± 5.1
GON fat	13 ± 3.1	30 ± 5.0**	19 ± 2.3	21 ± 5.4
Apoptosis (%)				
SC fat	13 ± 3.9	22 ± 6.0	19 ± 4.3	24 ± 7.4
GON fat	16 ± 5.2	23 ± 6.1	14 ± 3.8	16 ± 5.6

Data are means ± SEM of n experiments

*, ** and *** $p < 0.05$, $p < 0.01$ and $p < 0.005$ versus vehicle (non-parametric Mann-Whitney U)

Table 3 Effect of the Axl antagonist R428 treatment on adipose tissue expression of GAS6 and its receptors

	2x25 mg/kg		1x75 mg/kg	
	Vehicle	R428	Vehicle	R428
GAS-6				
SC fat	4.28 ± 0.24	4.07 ± 0.26	4.39 ± 0.21	4.15 ± 0.13
GON fat	3.11 ± 0.10	2.58 ± 0.14*	2.97 ± 0.13	2.68 ± 0.15
Axl				
SC fat	4.45 ± 0.23	4.49 ± 0.24	4.55 ± 0.23	4.32 ± 0.12
GON fat	3.91 ± 0.14	3.63 ± 0.10	3.67 ± 0.12	3.78 ± 0.15
Mer				
SC fat	7.49 ± 0.33	7.16 ± 0.24	7.58 ± 0.23	7.15 ± 0.21
GON fat	6.74 ± 0.19	6.17 ± 0.16*	6.59 ± 0.07	6.20 ± 0.17
Tyro3				
SC fat	10.68 ± 0.27	10.44 ± 0.27	10.55 ± 0.22	10.53 ± 0.17
GON fat	9.97 ± 0.16	10.12 ± 0.09	9.82 ± 0.15	9.83 ± 0.13

Data are normalized ΔCt values and are expressed as means ± SEM of 9 to 12 determinations.

*, $p < 0.05$ versus vehicle (non-parametric Mann-Whitney U)

Table 4 Effect of the Axl antagonist R428 treatment on plasma metabolic parameters and liver enzymes of C57Bl/6 wild-type mice kept on HFD

	2x25 mg/kg		1x75 mg/kg	
	Vehicle	R428	Vehicle	R428
Glucose (mg/dl)	210 ± 8.9	162 ± 9.3**	169 ± 11	169 ± 11
PAI-1 (ng/ml)	1.5 ± 0.17	2.8 ± 0.38*	1.5 ± 0.15	2.3 ± 0.32*
Insulin (ng/ml)	2.1 ± 0.22	2.4 ± 0.30	1.9 ± 0.22	1.8 ± 0.20
Leptin (ng/ml)	7.3 ± 0.76	2.3 ± 0.64***	4.2 ± 0.98	2.7 ± 0.54
Cholesterol (mg/dl)	135 ± 3.6	108 ± 6.9*	120 ± 5.6	113 ± 6.2
HDL cholesterol (mg/dl)	119 ± 3.4	93 ± 6.8*	105 ± 4.4	100± 5.7
LDL cholesterol (mg/dl)	8.6 ± 1.3	12 ± 1.1	12 ± 1.9	11 ± 1.1
Triglycerides (mg/dl)	29 ± 3.0	21 ± 4.9*	22 ± 3.2	19 ± 1.9
Alkaline phosphatase (U/l)	170 ± 6.4	187 ± 20	121 ± 8.1	165 ± 8.0**
AST (U/l)	94 ± 10	101 ± 6.1	78 ± 3.7	105 ± 8.9*
ALT (U/l)	50 ± 7.6	39 ± 4.0	37 ± 2.0	48 ± 9.3

Data are means ± SEM of 8 to 12 determinations

*, ** and ***, $p < 0.05$, $p < 0.005$ and $p < 0.0005$ versus vehicle (non-parametric Mann-Whitney U)

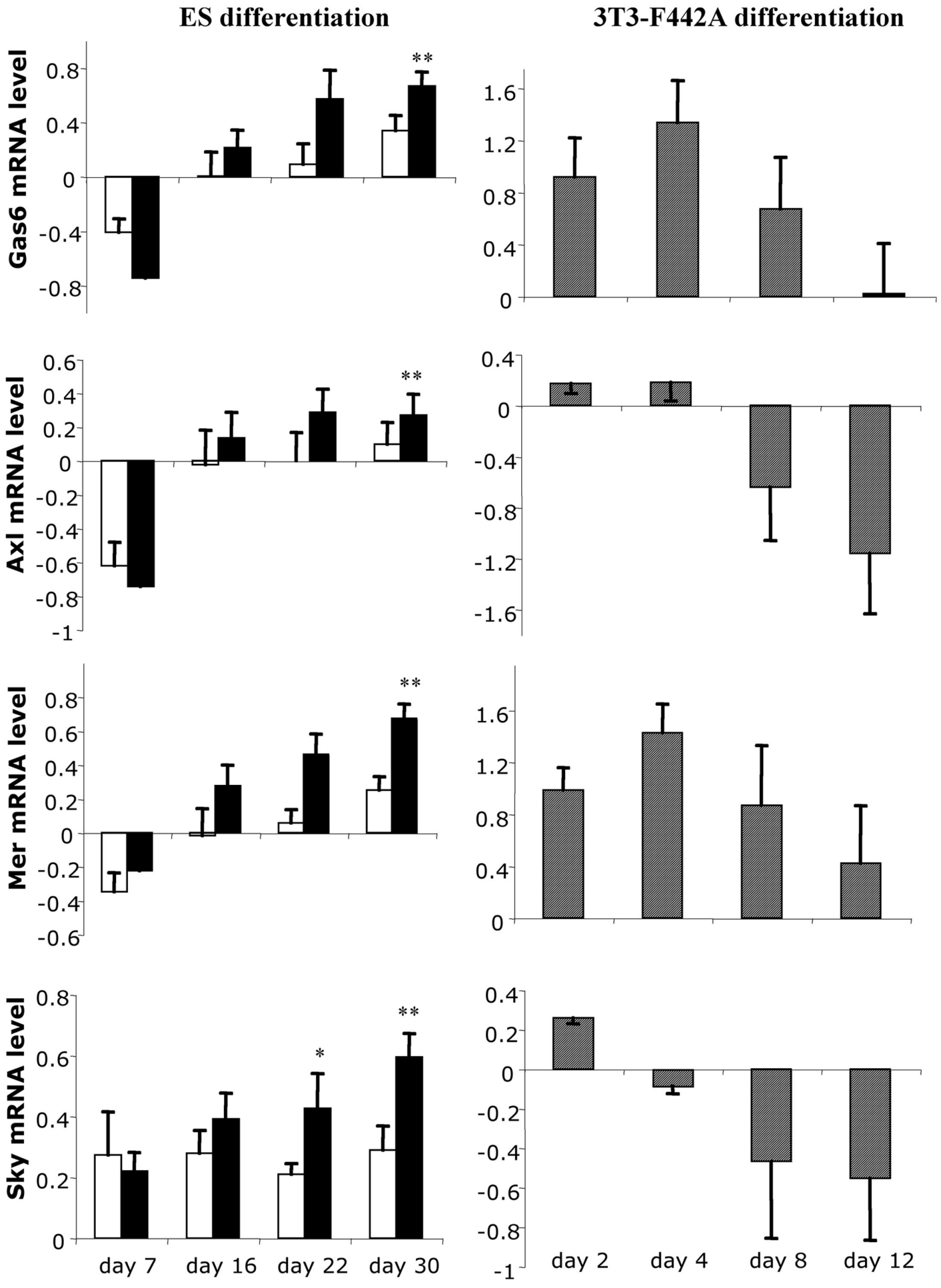
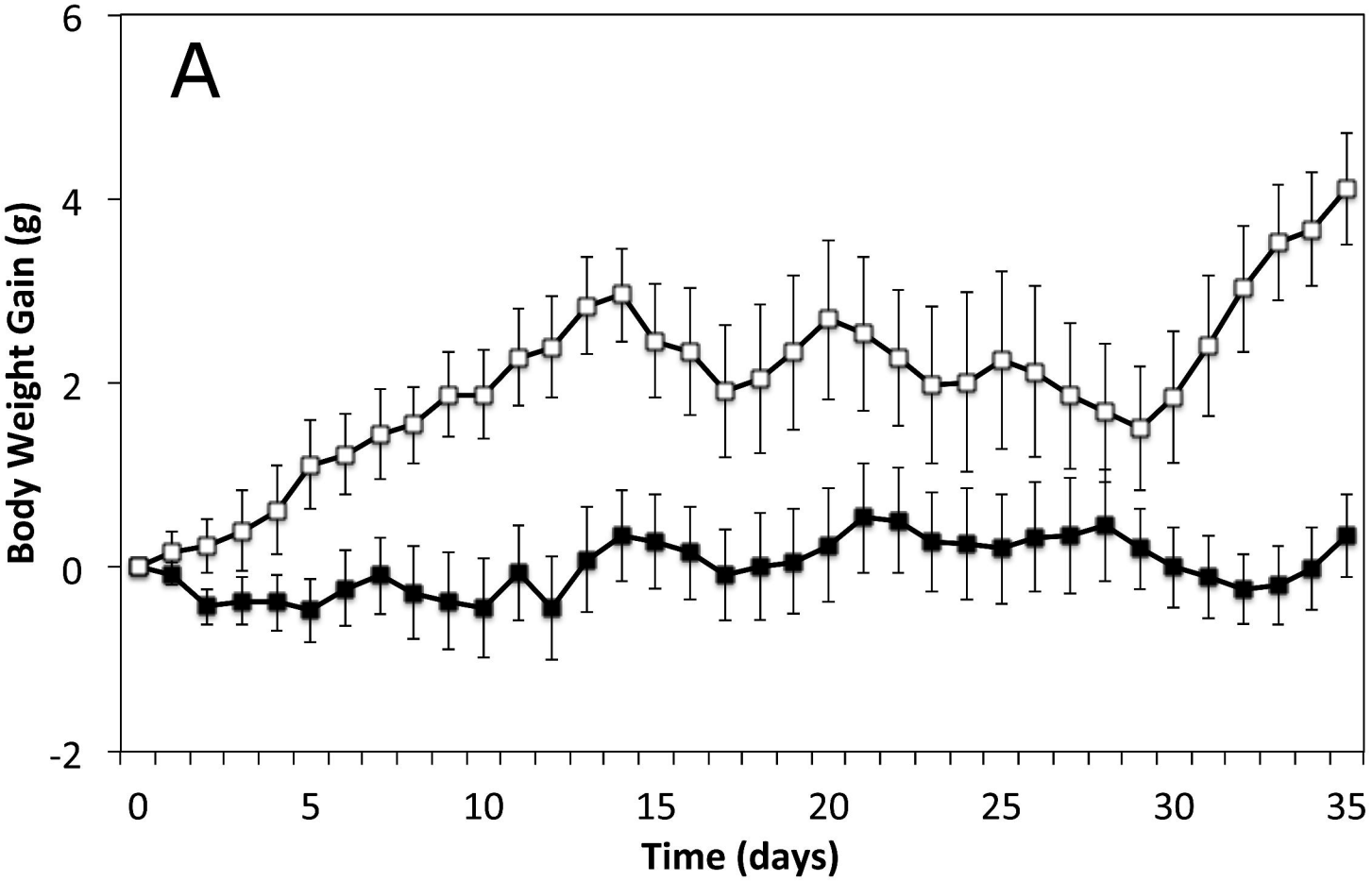

ES differentiation
3T3-F442A differentiation
Gas6 mRNA level
Axl mRNA level
Mer mRNA level
Sky mRNA level
**
*
day 7
day 16
day 22
day 30
day 2
day 4
day 8
day 12

Figure 1


A
Body Weight Gain (g)
6
4
2
0
-2
0
5
10
15
20
25
30
35
Time (days)

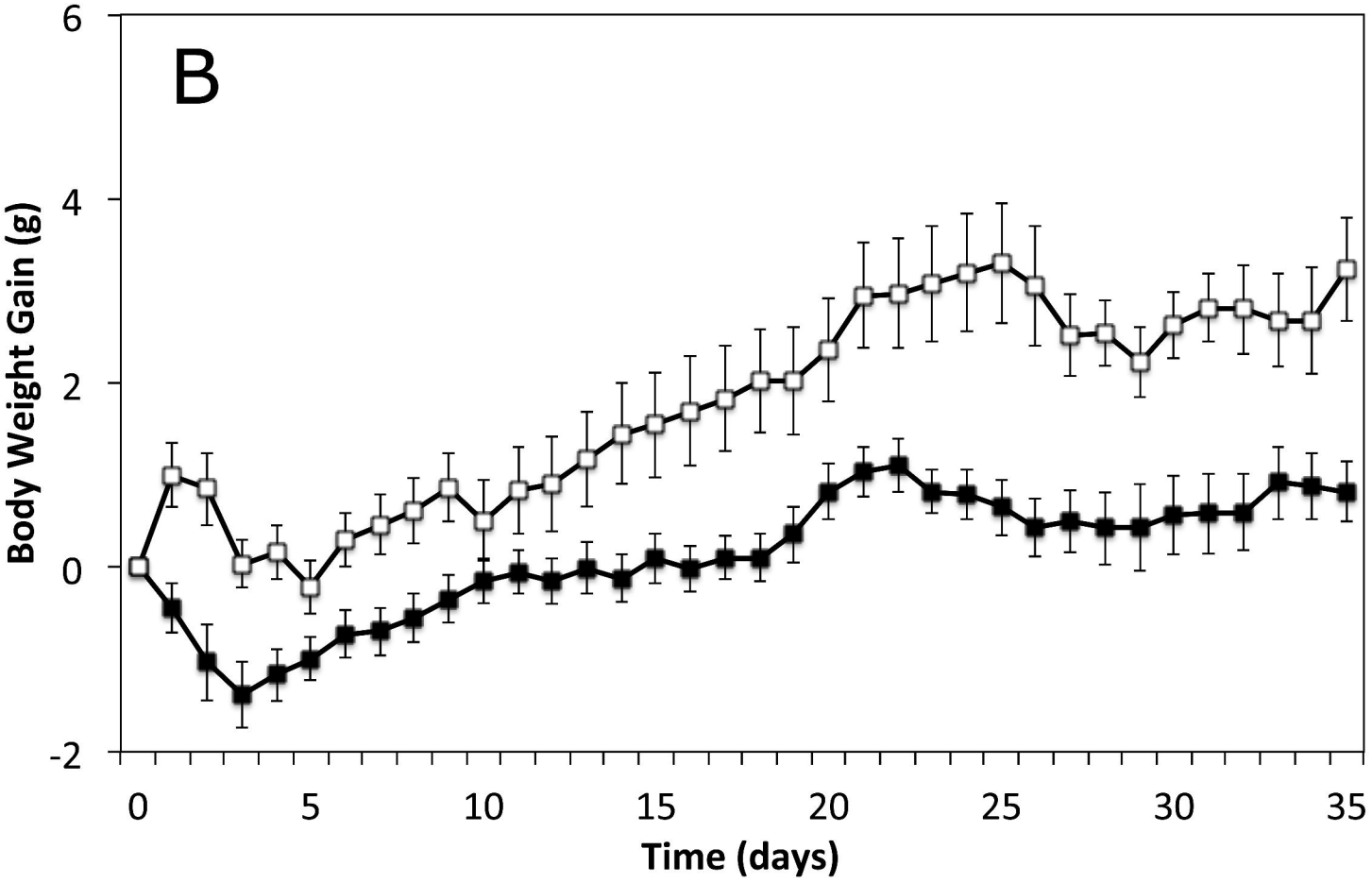

B
Body Weight Gain (g)
6
4
2
0
-2
0
5
10
15
20
25
30
35
Time (days)

Figure 2

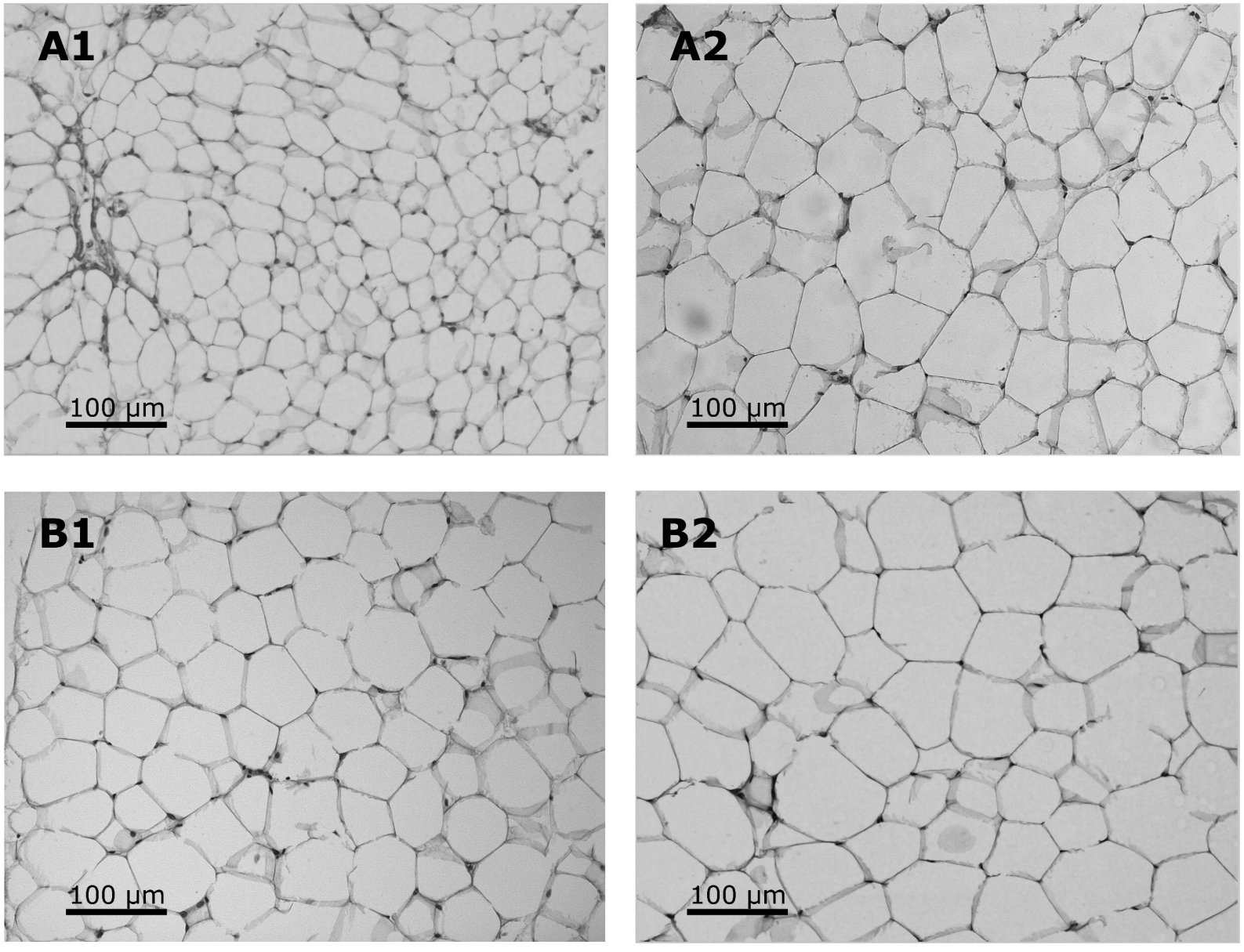


Figure 3