

Accepted: 16 March 2020

This article is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND). Usage and distribution for commercial purposes as well as any distribution of a cXJUYX a UHfjU fYei jFYgk fjtTb dYfa jggcb"

---

**Conclusion:** Obesity leads to ovarian leptin resistance and major time-dependent changes in gene expression in CCs, which in early obesity may be caused by increased leptin signalling in place in the obese mother.

Obesity is considered one of the major public health challenges of modern times and has been linked to various comorbidities, such as metabolic syndrome, type 2 diabetes, cancer, stroke [1] and infertility [2]. Obese women have increased risk of menstrual dysfunctions and anovulation, pregnancy complications, and poor reproductive outcome [3]. In mouse models, obesity is characterised by lipid accumulation in the ovary and ensuing lipotoxicity [4] and

*Animal protocol*

Breeding pairs from mouse strains C57BL/6J (B6) and B6.Cg-Lep



Cell Physiol Biochem 2020;54:417-437



pSTAT5 (1:200, sc-81524, Santa Cruz Biotechnology), GP against PTP1B (1:200, sc-1718, Santa Cruz Biotechnology), MM against SOCS3 (1:500, sc-51699, Santa Cruz Biotechnology) in cell lysates. The results are summarized in Table 2. Proteins were detected after incubation of the membranes with secondary GP anti-rabbit alkaline phosphatase-conjugated antibody (1:30000, A3687, Sigma Aldrich), GP anti-mouse alkaline phosphatase-conjugated antibody (1:30000, A4187, Sigma Aldrich), or RP anti-goat horseradish peroxidase-conjugated antibody (1:75000, A50-100P, Bethyl, Montgomery, Alabama, USA) for 2 h at RT. Immune complexes were visualized using the alkaline phosphatase visualization procedure or ECL substrate visualization. Blots were scanned using GeneSnap™ (SynGene, Brno, Czech Republic) and GeneTools™ (SynGene, Brno, Czech Republic). The results are summarized in Table 2.

*Immunohistochemistry and immunofluorescent staining*

Sections were fixed in 4% paraformaldehyde (PFA) in phosphate buffered formalin (NBF, 432173427, Poch, Gliwice, Poland) at 4°C for 24 h, and subsequently dehydrated in a series of ethanol solutions (70%, 80%, 90%, 100%) for 1 h each. Tissue was incubated in blocking solution (ab64261, Abcam, Cambridge, UK) for 1 h at RT and primary RP anti-SOCS3 antibody (1:1000, ab16030, Abcam) or primary

RP anti-PTP1B antibody (1:500, ab189179, Abcam) added overnight at 4°C. The negative control sections were incubated with RP anti-immunoglobulin G (IgG, ab37415, Abcam) or without primary antibody. The primary antibody complexes were detected after incubating the tissue with biotinylated goat anti-rabbit IgG (H+L) (ab64261, Abcam) for 60 min, and streptavidin peroxidase for 40 min. Staining was evident after



*Statement of Ethics*



The establishment of leptin resistance in the ovary of diet induced obese mice. (A) Experimental design: animals were maintained on chow diet (CD) or high fat diet (HFD) for 4 weeks (wk) or 16 wk. Protein abundance of components of the leptin signalling pathway in ovarian extracts analysed by Western blot or real-time PCR (RT-PCR). Abundance of (B) SOCS3 protein, (C) phosphorylation of STAT3, (D) leptin receptor (ObR), (E) phosphorylation of tyrosine 985 of leptin receptor, (F) phosphorylation of Janus kinase 2, (G) phosphorylation of STAT5. (H) Heatmap showing fold change in expression of mRNA of leptin signalling components measured in whole ovary or theca and stroma enriched (TC) fraction determined by RT-PCR. Immunohistochemical localisation of SOCS3 protein during follicle development in ovaries of mice subjected to diet-induced obesity (4 wk and 16 wk). Positive staining in brown, counterstaining with heamatoxylin. Negative control stained with polyclonal rabbit IgG (I) 4 wk CD and (J) 4 wk HFD, localisation of SOCS3 in primary follicle (K) 4 wk CD and (L) 4 wk HFD, antral follicles (M) 16 wk CD and (N) 16 wk HFD. Staining is present in oocyte, granulosa and theca cells. Oval-headed arrow indicates oocyte; large-headed arrow indicates granulosa cells and small-headed arrow indicates theca cells. Scale bars represent 100  $\mu$ m. (O-P) nuclear counterstaining with DAPI in blue. (O-P) negative control 16 wk CD performed with polyclonal rabbit IgG, SOCS3 localised in (Q-R) primordial follicles 16 wk CD, (S-T) primary follicles 16 wk CD. Images

Ys<sup>3</sup> j ± j Y<sup>oe</sup> a cō s<sup>3</sup> Y<sup>oe</sup> - μ<sup>o</sup> μ<sup>o</sup> c<sup>o</sup> ± a « ± «<sup>o</sup> - o<sup>a</sup> o<sup>p</sup> o<sup>p</sup> Y<sup>i</sup> o<sup>i</sup> o<sup>o</sup> a « c' # ' « a - j o<sup>o</sup> a - o<sup>o</sup> c<sup>o</sup> H<sup>o</sup> s<sup>o</sup> Y<sup>o</sup> - o<sup>o</sup> Y<sup>i</sup> Y<sup>o</sup> a<sup>o</sup> ob/ob «<sup>2</sup> s<sup>o</sup> o<sup>o</sup> a<sup>o</sup> o<sup>o</sup> o<sup>o</sup> s<sup>o</sup> o<sup>o</sup> a<sup>o</sup> « c' # ' / μ<sup>o</sup> 1Q-T) and observing a weaker intensity of SOCS3 in both oocyte and GC in ovaries from *ob/ob* ( ± j j<sup>o</sup> c<sup>o</sup> j a<sup>o</sup> s<sup>o</sup> μ<sup>o</sup> / μ<sup>o</sup> ( · \* o<sup>o</sup> c<sup>o</sup> - s<sup>o</sup> μ<sup>o</sup> Y<sup>o</sup> «<sup>3</sup> Y<sup>o</sup> μ<sup>o</sup> j ± j j<sup>o</sup> c<sup>o</sup> j a<sup>o</sup> s<sup>o</sup> μ<sup>o</sup> / μ<sup>o</sup> ) ( o<sup>o</sup> j j<sup>o</sup> L<sup>o</sup> μ<sup>o</sup> ± o<sup>o</sup> o<sup>o</sup> a<sup>o</sup> o<sup>o</sup> o<sup>o</sup> j Y<sup>o</sup> o<sup>o</sup> j - j o<sup>o</sup> o<sup>o</sup> μ<sup>o</sup> « c<sup>o</sup> o<sup>o</sup> j - o<sup>o</sup> s<sup>o</sup> μ<sup>o</sup> μ<sup>o</sup> o<sup>o</sup> j - μ<sup>o</sup> o<sup>o</sup> # ' Y<sup>o</sup> j - j o<sup>o</sup> Y<sup>o</sup> «<sup>o</sup> j j<sup>o</sup> ) abundant in tissues from the *ob/ob* mouse [34]. Furthermore, we also inferred that impaired leptin signalling in the ovary is likely to have direct implications for the oocyte, since the gamete was shown to express SOCS3.

*Cumulus cell transcriptome analysis: global transcriptome of CCs reflects body weight*

Next, we repeated the protocol and subjected the animals to superovulation in order to collect CCs and analyse the transcriptome from 4 wk and 16 wk DIO protocols (Fig. 2A). A total of 50-80 CCs per animal were collected, from which RNA-seq libraries were generated using a Smart-seq2 oligo-dT method [31, 32], with separate RNA-seq libraries made from the CC from each female (see Supplementary Table 1). We then used Principal Component Analysis (PCA) to study the distribution of our samples according to global gene expression (Fig. 2B). We decided to include the HFDLG from the 16 wk HFD group as a control, to test whether the transcriptional response could be linked to the BW of the animals; indeed, the HFDLG samples clustered together with 16 wk CD of a similar weight (Fig. 2B). The correlation between PC1 and BW was  $r=0.777$  ( $p=3.026e-06$ ) (Fig. 2C; Supplementary Table 2), which substantiates the physiological effect driven by BW, rather than the nature of the diet itself, (Fig. 2D).

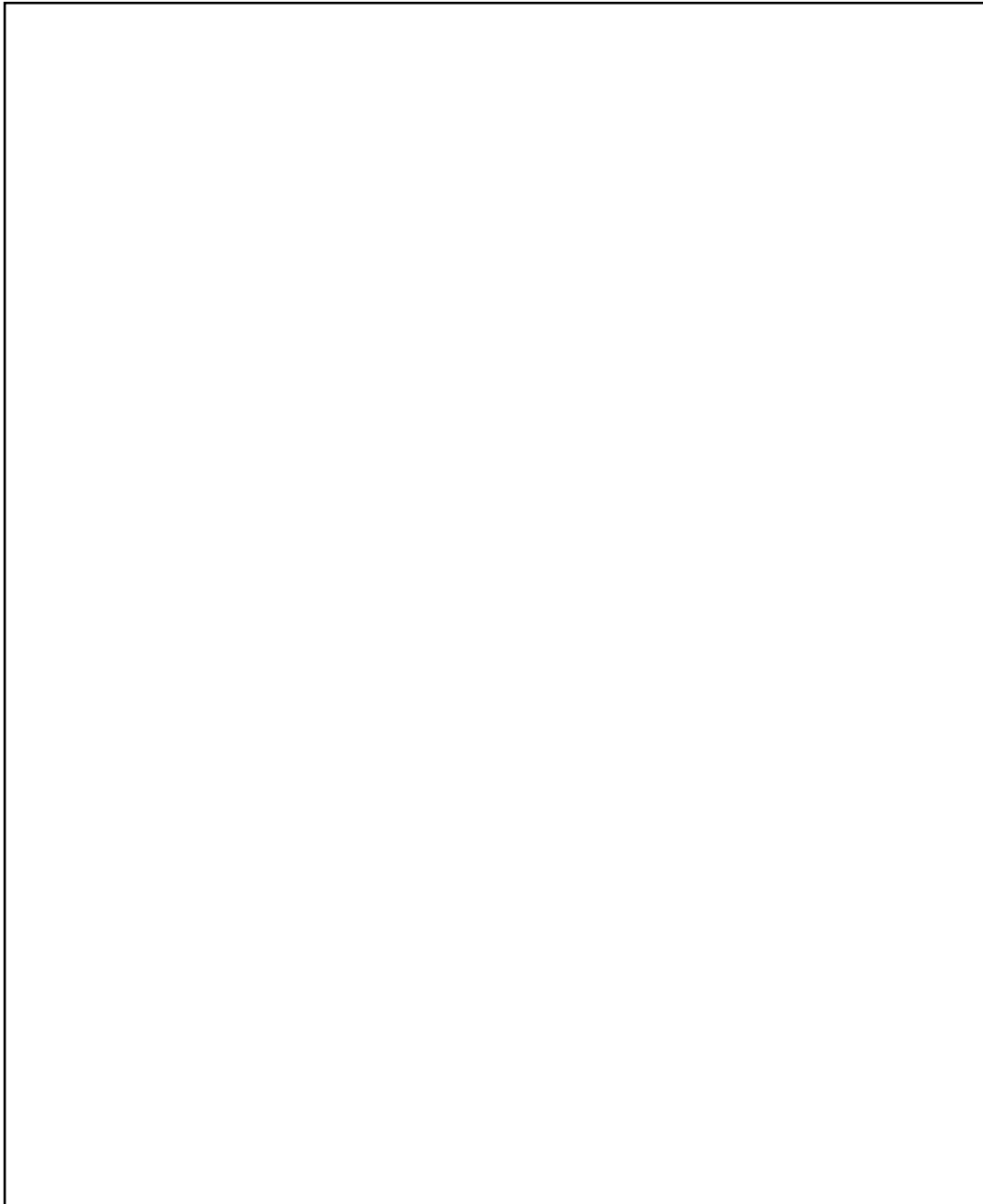
Next, we aimed to identify DEGs in CCs: for this analysis, we excluded the 3 HFDLG outliers from the 16 wk HFD, so as to ensure a minimum of 13 g of BW difference between CD 16 wk and HFD 16 wk and a BW difference of 5 g between CD 4 wk and HFD 4 wk (Fig. 2E). We identified 1408 DEGs in CCs (373 upregulated and 624 downregulated; Fig. 2D; Supplementary Table 3) and 846 DEGs in 16 wk HFD (203 upregulated and 643 downregulated; Fig. 2E; Supplementary Table 3) (Fig. 2F).

Cumulus cell transcriptome analysis in diet induced-obese mice reveals strong correlation with body

the 16 wk DEGs and discovered that *Nfib* was upregulated and *Ptgs2* and *Trim28* transcripts were downregulated in CCs (Supplementary Fig. 6A-C). The altered expression of these markers in CCs during late obesity might indicate direct consequences for oocyte and embryo quality, as previously proposed [24, 37–39].



«C f i a j - - > j f f Y « 3 a @ f t s o j Y o d @ ± f « ± o » j - Y μ > ± o s - « f i a j - - 3 Y d « - - « - Y j - - @ Y j . which suggests an adaptive response of CCs to changes in the physiology of the mother. In a parallel approach, we intersected the DEGs datasets from 4 wk HFD and 16 wk HFD and from 0 d j . - - i f i - Y o e © © « a » j o 3 j j a o d j o 3 « o Y © j - - « Y o - - / Y e . / Y j a o Y Y Y . © s Y o e - o j @ . « C i f i - ( d j : Y o : o e - o j @ o e © - @ Y j Y . f i a j - - Y « 3 a @ f t s o j Y f » « o d : 3 S s a Y . 3 S f l / - - i - - i - - i © j a o s q u ( s > j . - 3 Y d o d j © « - o - - Y a Y Y s a o j μ Y j @ f t s o j Y f i a j s o . 3 S f l / - . *MICAL Like (Micall) 1* (FDR = 0.0002), but other genes like



Pharmacologically hyperleptinemic mouse model shows leptin effects in the transcriptome of cumulus cells during early obesity. (A) Experimental design: mice were fed chow diet (CD) or high fat diet (HFD) for 4 weeks (wk) (4 wk DIO) or injected with saline (CONT) or 100  $\mu$ g of leptin (LEPT) for 16 days, followed by superovulation and collection of cumulus cells from cumulus-oophorus-complexes. RNA-seq analysis of cumulus cells from 4 wk DIO mice revealed significant changes in gene expression. Principal component analysis of global transcriptome shows LEPT effect is the main source of variance in the transcriptome. Heatmaps presenting fold of change in expression of genes associated with the following pathways: (E) epigenetic regulation; (F) actin cytoskeleton organisation; (G) glucose metabolism; (H) long chain fatty acid oxidation in CC. Gene ontology analysis performed with Gene Ontology (GO) terms. Genes in yellow upregulated by both treatments.

and *DNA methyltransferase (Dnmt) 3a* (Fig. 4E), which suggested epigenetic dysregulation. Another important effect that could be attributed to leptin in early stages of obesity was the repression of genes mediating actin-cytoskeleton reorganisation (Fig. 4F; Supplementary Table 7). Furthermore, we assessed the potential impact of leptin on genes involved in CC [43]. Here, we questioned how lack of leptin signalling could be detrimental metabolically. For instance, the oocyte is unable to metabolise glucose due to low phosphofructokinase activity [43], highlighting the importance of glycolytic activity of CCs in the generation of pyruvate [44]. This function appeared to be decreased in 16 wk HFD, which could be the result of the establishment of leptin resistance in the ovary. As a consequence, the transport of pyruvate into the oocyte would be decreased, which could directly impact the tricarboxylic acid cycle (TCA) and adenosine triphosphate (ATP) generation (Supplementary Fig. 9B) [45]. Leptin is also known to be key for free fatty acid (FFA) metabolism, promoting their oxidation and regulating the homeostasis of triglycerides in a cell [46, 47]. Thus, disruption of leptin signalling in 16 wk HFD CCs (Supplementary Fig. 9A) could be relevant for lipotoxicity and stress previously described in obese ovaries [48] (Supplementary Fig. 9E, 9F). In general, hyperactivation of leptin signalling in CCs seemed to be linked primarily to impaired cell membrane transport and endocytosis, but also cell metabolism and gene expression regulation.

The present study characterises the molecular mechanisms underlying the establishment of leptin resistance in the ovary of DIO mice. Furthermore, making use of sensitive methods for reduced-cell number RNA-seq, we studied the transcriptome of the somatic cells surrounding the oocyte from mice subjected to DIO for 4 wk and 16 wk, as well as validated model for pharmacological hyperleptinemia – a system presenting exclusively increased circulating levels of leptin amongst all features of obesity, which allowed us to pinpoint the exclusive effects of leptin-SOCS3 ovarian hyperactivation during early-onset of obesity.

Leptin is a major adipokine, which was initially linked to satiety [6]. The establishment of leptin resistance at different levels in the body has been documented in recent years as one of the outcomes of obesity. Accordingly, leptin signalling is deregulated in the hypothalamus [49] and liver of obese human and mice [50]. However, the same was not

pSTAT5 signalling *per se* could compromise oocyte maturation and fertility during obesity. Importantly, we observed that SOCS3 staining in the oocyte occurred mainly in response to ObRb activation, since the *ob/ob* mouse presented weaker staining. This suggests a direct impact of disrupted ovarian leptin signalling on oocyte quality through SOCS3 activation. Indeed, at 16 wk DIO, we observed different levels of *Socs3* transcribed in various ovarian components. Our RNA-seq data revealed that *Socs3* was increased at 4 wk HFD, but decreased at 16 wk HFD in CCs, whereas in the TC fraction it was upregulated at both time points. This may suggest blunted ObRb signalling in CCs at 16 wk HFD, once the transcription of the major components of the pathway was inhibited (Supplementary Fig. 9A). Therefore, leptin signalling in CCs seems to be highly sensitive to obesity and maternal metabolic performance.

Having an understanding of the impact of obesity on leptin signalling in the ovary, we then analysed the transcriptome of CCs from DIO mice. A major observation of this study is that, on the other hand, other studies showed functional changes in the ovary, including depletion of CCs in diet composition, as well as variable length of exposure to diet, might account for the differences between studies. Also the aforementioned study did not present a global gene expression profile. HFDLG CCs was similar to that in 16 wk CD, clearly demonstrating the impact of maternal diet on the ovary. of gene signatures altered in early vs late stages of obesity. After 4 wk HFD, mainly genes related to nutrient mobilisation to the oocyte, as well as the uptake of signalling molecules from the oocyte, is fundamental for COCs expansion and oocyte maturation [23]. Indeed, the genes *Micall1* and *Unc-51 Like Kinase (Ulk) 4* are important mediators of endocytosis [57, 58], and were shown to be regulated by *Stat3*. Furthermore, amongst the genes upregulated in both 4 wk HFD and LEPT we found *Lcn2*, associated with lipid and hormone transport [59], *Claudin 22*, a component of tight junctions [60], and *Anxa11*, known to be involved in transmembrane secretion [61]. This is suggestive of the effects of leptin in altering transmembrane transport in the early-onset of obesity.

Other genes upregulated in CCs include *Arachidonate 15-Lipoxygenase (Alox15)*, and the transcription factor *Hes Related Family BHLH Transcription Factor With YRPW Motif (Hey) 1*. LEPT (Supplementary Table 8). The transcriptional repressor HEY1 is directly activated by Notch Receptor (NOTCH) 2 during follicular development, and both HEY1 and NOTCH2 were shown to be increased in proliferating granulosa cells and can contribute to ovarian overstimulation and premature follicular failure [62]. These effects further demonstrate the impact of leptin on the ovary.



somatic cells, an instrumental system for oocyte maturation [64]. Indeed, the oocyte is in extreme need of the metabolites generated in CCs, but also signalling factors such as growth differentiation factor (GDF) 9 secreted by the oocyte and required to orchestrate CCs function. Leptin seemed to support the TCA cycle at 4 wk HFD (Supplementary Fig. 9B), which suggested to us that at this early stage the boost in leptin signalling in CCs could be responsible for the observed increase in leptin signalling in CCs (Supplementary Fig. 9D). However, at 16 wk HFD the inferred drop in CC leptin signalling (Supplementary Fig. 9D), which invariably suggests compromised oocyte quality. The aforementioned events are an important part of COC expansion, a complex mechanism triggered by luteinizing hormone (LH), in which bidirectional exchange of metabolites and signalling factors between the oocyte and CCs leads to maturation of the gamete and resumption of meiosis [22]. This process is tightly regulated by immune mediators, particularly interleukin (IL) 6 [65]. Indeed, as well as IL6 response, in particular mediating innate immunity through IL6, has been described before [55]. Consequently, the detrimental effect of obesity could be related to increased leptin signalling at 4 wk HFD, but most likely through its failure at 16 wk HFD (Supplementary Fig. 9A). Generally, in the early stages of obesity, leptin downregulated potentially important epigenetic mediators and genes involved in cytoskeletal organisation in CCs.

For example, *Micall1* or *Dync1h*, involved in protein transport, positioning of cell compartments, and movement of structures within the cell [66] to be decreased in 4 wk and 16 wk HFD. Furthermore, the most increased gene in 16 wk HFD was the *Guanylate-binding protein (Gbp) 8* (Supplementary Table 5), a component of cellular response to interferon-gamma [67]. Another gene upregulated at 16 wk HFD was *Rhou*, a gene that encodes for a transcription factor. As a result of increased leptin signalling and its mediators at this stage, the activated pathways may well be an outcome of lipotoxicity previously described in the obese ovary [48]. Thus, during obesity ovarian cells are trying to accommodate the surplus of lipid compounds, which is likely to activate mechanisms of cellular reorganisation. Overall, early changes in CC transport, gene expression and epigenetic modifications to accommodate the lipid surplus. Functional studies in CCs with variable levels of leptin and

Graphical representation of the main temporal changes in the ovary of obese mice. During early obesity (4 weeks of diet-induced obesity, DIO) increased leptin signalling affects the transcriptome of cumulus cells (CCs). RNA-seq analysis revealed mainly alterations in genes involved in cytoskeleton organisation and glucose metabolism. During late obesity (16 wk DIO) leptin resistance is established, which causes accumulation of SOCS3 in the ovary. Transcriptome analysis of CCs at this timepoint indicated alterations in genes involved in glucose metabolism and transport.



In conclusion, we found that the ovaries of obese mice develop leptin resistance and that global gene expression in CCs was strikingly correlated with BW. Mechanistically, failure in ovarian leptin signalling was mediated by SOCS3 overexpression, and inhibition of pTyr985 and pJAK2. Initially, during the onset of obesity the hyperactivation of leptin signalling was followed by inhibition of genes associated with epigenetic regulations in CCs. Conversely, in late obesity, the hyperactivation of leptin signalling was followed by inhibition of genes associated with epigenetic regulations in CCs.

- 1 Schelbert KB: Comorbidities of Obesity. *Prim Care Clin Off Pract* 2009;36:271–285.
- 2 Pasquali R, Patton L, Gambineri A: Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* 2007;14:482–487.

27 Couse JF, Yates MM, Rodriguez KF, Johnson JA, Poirier D, Korach KS: The Intraovarian Actions of Estrogen & the Female Gonad. *Endocrinology* 2006;147:3666–3678.

29 Galvão A, Wolodko K, Rebordão MR, Skarzynski D, Ferreira-Dias G: TGFB1 modulates *in vitro* secretory activity and viability of equine luteal cells. *Cytokine* 2018;110:316–327.

30 Zhao S, Fernald RD: Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. *J Comput Biol* 2005;12:1047–1064.

31 Picelli S, Björklund ÅK, Faridani OR, Sagasser S, Winberg G, Sandberg R: Smart-seq2 for sensitive full-length RNA-seq from single cells. *Nat Protoc* 2014;9:171–181.

32 Picelli S, Faridani OR, Björklund ÅK, Winberg G, Sagasser S, Sandberg R: Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* 2014;9:171–181.

33 Love MI, Huber W, Anders S: Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.

34 Duan J, Choi Y-H, Hartzell D, Della-Fera MA, Hamrick M, Baile CA: Effects of Subcutaneous Leptin Injections on the Female Gonad. *Endocrinology* 2006;147:3666–3678.

35 Eden E, Lipson D, Yogev S, Yakhini Z: Discovering Motifs in Ranked Lists of DNA Sequences. *PLoS Comput Biol* 2007;3:e39.

36 Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z: GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* 2009;10:48.

38 Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL: Human cumulus cell gene expression as a

