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The impact of maternal obesity on leptin signalling in the ovary and effects on oocyte and cumulus cells regulation in mice

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Articles presentation

Original article:

Wołodko K, Walewska E, Adamowski M, Castillo-Fernandez J, Kelsey G, Galvão A. **Leptin Resistance in the Ovary of Obese Mice is Associated with Profound Changes in Cumulus Cells Transcriptome.** *Cellular Physiology and Biochemistry* 2020;54:417-437. (MNiSW=140)

Review article:

Wołodko K, Castillo-Fernandez J, Kelsey G, Galvão A. **Revisiting the Impact of Local Leptin Signaling in Folliculogenesis and Oocyte Maturation in Obese Mothers.** *International Journal of Molecular Sciences* 2021;22:4270. (MNiSW=100)

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Streszczenie

Otyłość jest obecnie powszechną chorobą cywilizacyjną, często związana z niepłodnością. Rozrost tkanki tłuszczowej prowadzi do zaburzenia równowagi hormonalnej, szczególnie podwyższonego poziomu leptyny we krwi, co potencjalnie wpływa na prawidłowe funkcjonowanie jajników. Pomimo iż leptynooporność została wykazana wcześniej w wielu narządach takich jak podwzgórze, wątroba, czy mięśnie, nie zbadano domniemanych zmian w szlaku sygnałowym leptyny w jajniku podczas rozwoju otyłości. Co ważne, hormon ten reguluje wiele procesów zachodzących w jajniku, z udowodnionym działaniem zależnym od dawki. Leptyna reguluje steroidogenezę, kontroluje rezerwę pęcherzykową, jak również dojrzewanie oocytu, czy owulację. W badaniach wykorzystano mysie modele otyłości indukowanej dietą (ang. diet- induced obesity, DIO) oraz farmakologicznej hiperleptynemii (ang. pharmacological hyperleptinemia, LEPT) celem scharakteryzowania szlaku sygnałowego leptyny w jajniku podczas rozwoju otyłości oraz zbadania wpływu leptyny na funkcjonowanie komórek ziarnistych oraz komórek otoczki.

Myszy poddano wysokotłuszczowej (ang. high fat diet, HFD) lub standardowej diecie (ang. chow diet, CD) przez okres 4 i 16 tygodni (tyg) oraz nastrzykiwano leptyną lub solą fizjologiczną przez okres 16 dni. W początkowym okresie po 4 tyg stosowania HFD odnotowano hiperaktywację szlaku sygnałowego leptyny w jajniku, co objawiało się zwiększoną fosforylacją tyrozyny 985 receptora leptyny (Tyr985ObRb) oraz zakonserwowanego inhibitora szlaku, ang. suppressor of cytokine signalling 3 (SOCS3). Z drugiej strony, po 16 tyg stosowania HFD szlak sygnałowy leptyny został zahamowany, co objawiało się spadkiem fosforylacji cząsteczki Janus kinase 2 (pJAK2) z jednoczesnym wzrostem ekspresji białka SOCS3, co potwierdza rozwój leptynooporności. Analizy transkryptomu komórek ziarnistych (ang. cumulus cells, CCs), które towarzyszą oocytowi, wykazały znaczne zmiany skorelowane z masą ciała samicy. Co więcej, podczas wczesnej otyłości (4 tyg HFD) hiperaktywacja szlaku sygnałowego leptyny została powiązana ze wzrostem ekspresji genów związanych z metabolizmem glukozy, lecz spadkiem ekspresji genów związanych z regulacjami epigenetycznymi oraz organizacją cytoszkieletu komórkowego. Przeciwnie, podczas późnej otyłości (16 tyg HFD) odnotowano zmiany w ekspresji genów związanych ze ścieżkami zapalnymi i przebudową morfologiczną.

W przedstawionych badaniach wykazano po raz pierwszy rozwój leptynooporności w jajniku otyłych myszy oraz scharakteryzowano zmiany w czasie w transkryptomie CCs podczas rozwoju otyłości, podkreślając szczególną rolę wzmożonej aktywacji szlaku sygnałowego leptyny. Opisywane rezultaty mogą przyczynić się do wynalezienia nowych narzędzi, które znajdą zastosowanie w technikach wspomaganego rozrodu i pomogą monitorować postęp choroby i pogorszenie funkcjonowania jajników podczas przebiegu otyłości.

Co więcej, wpływ matczynej otyłości i lokalnych zmian w szlaku sygnałowym leptyny na folikulogenezę i dojrzewanie gamet zostały opisane w pracy przeglądowej. Scharakteryzowane zostały domniemane zmiany w jakości oocytu oraz rozwoju wczesnozarodkowym. Ponadto, wykorzystując transkryptom oocytów i komórek ziarnistych z różnych stadiów rozwojowych podczas folikulogenezy u kobiet, opublikowany przez zespół Zhang'a, scharakteryzowaliśmy ekspresję komponentów szlaku sygnałowego leptyny. Przypuszcza się, iż obniżona aktywność szlaku sygnałowego leptyny podczas otyłości może przyczyniać się do przyspieszonej utraty pęcherzyków jajnikowych, zaburzonego formowania pęcherzyków antralnych, czy nieprawidłowego dojrzewania oocytu.

Abstract

Obesity is a prevalent disease worldwide, recurrently associated with infertility. Expansion of adipose tissue leads to endocrine imbalance, particularly increased circulating leptin levels, which potentially affects ovarian function. Although systemic leptin resistance was demonstrated in various organs like hypothalamus, liver or muscles, no previous study characterized putative changes in leptin signalling in the ovary in the course of obesity. Importantly, leptin is a key regulator of ovarian function, with known dose- dependent effects on ovarian cells. Indeed, leptin was shown to regulate steroidogenesis, control follicular reserve, as well as oocyte maturation and ovulation. Thus, we used diet- induced obese (DIO) and pharmacologically hyperleptinemic (LEPT) mice protocols to characterize leptin signaling in the ovary during obesity progression and study the effects of leptin on cumulus and theca cells function.

Mice were subjected to high- fat diet (HFD) or chow diet (CD) for 4 or 16 weeks (wk), and treated with leptin or saline for 16 days. We initially observed after 4 wk HFD the hyperactivation of leptin signalling in the ovary, measured by increased phosphorylation of tyrosine 985 of leptin receptor (Tyr985ObRb) and its conserved inhibitor the suppressor of cytokine signalling 3 (SOCS3). On the other hand, after 16 wk HFD treatment leptin signalling was repressed, evidenced by decreased phosphorylation of Janus kinase 2 (pJAK2) with parallel upregulation of SOCS3 protein levels, supporting the establishment of leptin resistance. Transcriptome analysis of cumulus cells (CCs), the somatic companions of the oocyte, showed dramatic changes which correlated with maternal body weight. Furthermore, in early obesity (4 wk HFD) the hyperactivation of leptin signalling was linked to increased glucose metabolism but decreased epigenetic regulation and cytoskeleton organization. Conversely, during late obesity (16 wk HFD) changes in gene expression pointed to maintenance of inflammatory pathways and morphological rearrangement.

The present study revealed for the first time the establishment of leptin resistance in the ovary of obese mice, and characterized temporally changes in CCs gene expression during obesity progression, highlighting the specific role of increased leptin signalling. These results represent a potential tool in assisted reproductive clinics, helping characterizing levels of disease progression and ovarian failure throughout obesity.

Finally, the effects of maternal obesity and changes in local leptin signalling on folliculogenesis and oocyte maturation were described in the review. Potential outcomes for oocyte quality and early embryo development were characterized. Interestingly, we used transcriptome of oocyte and GC isolated from different stages of folliculogenesis in women by Zhang's, to characterize leptin signalling component expression. It is suggested that decreased leptin signalling during obesity might contribute to accelerated follicle loss, disturbed antral follicle formation or improper oocyte maturation.

Introduction

Worldwide epidemic of obesity has reached unprecedented level. Obesity is known for its detrimental effects for health with multiple associated comorbidities, as type 2 diabetes, cardiovascular disease, metabolic syndrome [1] or infertility [2]. Obese women present anovulation or menstrual dysfunction, poor reproductive outcomes or pregnancy complications [3]. In general, obesity in woman is associated with endocrine imbalance, increased levels of androgens and ovarian failure [4]. Robker et al. showed, that obesity in women changes levels of ovarian metabolites, hormones and gene expression [5]. Moreover, studies in mice confirmed the detrimental effects of obesity on fertility, and enabled scientists to study the underlying molecular mechanisms. The accumulation of lipids in oocytes and accompanying granulosa cells (GCs) and cumulus cells (CCs) leads to lipotoxicity, cellular stress, inflammation and apoptosis [6] with oocyte mitochondrial dysfunction [7]. Furthermore, obesity was linked to ovarian dysfunction, particularly impaired follicle growth, gamete maturation and disturbed steroidogenesis [8, 9]. Even though literature describes main pathological readouts in ovaries of obese mothers, the underlying mechanisms leading to pathogenesis remain understudied. Therefore, understanding the temporal course of events in the ovaries of obese mothers is fundamental to delineate treatment strategies adequate to levels of disease progression.

Leptin is the main adipokine secreted by adipose tissue and mainly regulates appetite at central level [10]. Moreover, this pleiotropic hormone regulates also other processes, amongst them angiogenesis [11], immune responses [12], or neurons maturation [13]. Its effects on reproduction were studied at central level where leptin affects the release of gonadotropins [14], but also periferically in the ovary, where it regulates folliculogenesis [15], ovulation [16], steroidogenesis [9] or follicular pool activation [17]. Interestingly, it was shown that leptin affects the expression of enzymes involved in progesterone synthesis in a dose- dependent manner [9]. Moreover, leptin controls the activation of follicular pool, as lower leptin serum levels facilitated the transition from primary to secondary follicles [17]. Furthermore, leptin was also shown to control ovulation, supporting CCs expansion through cyclooxygenase 2 and hyaluronic acid synthase 2 (HAS2) activity [16]. Therefore, leptin plays a major role in ovarian physiology.

Leptin signals through its membrane receptor, ObR. After binding to the dimerized receptor, Janus kinase 2 (JAK2) is phosphorylated and transfers phosphate groups to tyrosine 985, 1077 and 1138 of the receptor within BOX2 (Fig. 1, [18]). This mediates the recruitment and activation of: (i) SH2- domain containing protein tyrosine phosphatase (SHP-2), which, in turn, binds to its adapter molecule Grb-2 and activates downstream signaling, resulting in extracellular signal regulated kinase (ERK) 1/2 activation, (ii) signal transducer and activator of transcription (STAT) 5 activation, and (iii) STAT3 activation. Importantly, insulin signalling pathway can cross talk with leptin signalling, as JAK2 recruits SH2B adaptor protein 1 (SH2B1), which binds insulin receptor substrate (IRS) and initiates phosphatidylinositol 3 kinase (PI3K) pathway (Fig. 1, [18]). Regulation of activation of long isoform of ObR (ObRb) and canonical leptin signalling pathway is mainly done by two inhibitors, the signal transduction- protein tyrosine phosphatase 1B (PTP1B), which dephosphorylates JAK2, and suppressor of cytokine signalling 3 (SOCS3), which blocks phosphorylation of tyrosine 985 od leptin receptor (Tyr985ObRb) and Tyr1077ObRb and further signal propagation [19]. Moreover, leptin and its receptor are ubiquitously expressed in ovarian cells- GCs, oocytes in women [20] and rodents [21].

Despite increasing circulating levels of leptin during obesity progression [22], its signalling eventually fails with the establishment of leptin resistance, previously shown in multiple organs such as hypothalamus [23], liver [24] or muscle [25]. However, organs like the heart [26] or kidneys [27] of obese humans do not seem to develop leptin resistance. In the present work we studied leptin signalling in the ovaries of obese mice. Furthermore, the effects of increased leptin level during obesity on ovarian physiology and particularly the impact on cumulus- oocyte complexes were also uncovered. Therefore, we hypothesised that leptin signalling is disturbed in the ovaries of obese mice, with a major impact for the regulation of gene expression in cumulus cells. Indeed, we have shown that during early obesity hyperactivation of leptin signalling is linked to the decrease in the expression of genes associated with glucose metabolism, but increase in the expression of genes linked to epigenetic dysregulation and cytoskeletal organization in CCs. On the other hand, during late obesity there is an increase in expression of genes associated with inflammation and morphogenesis. Cumulus cells, the oocyte somatic companions, closely cooperate with the gamete regulating its growth, metabolism [28] and meiosis resumption [29]. Moreover, the CCs transcriptome analysis have been

commonly used in assisted reproduction clinics, as a predictor of oocyte competence. Hence, a better knowledge of the processes taking place in the ovary and at follicular level during obesity progression will help us understanding the molecular mechanisms underpinning ovarian failure and infertility.

Aims

The main aims of the present study were to characterise the expression of leptin signalling components in the ovary of mice during obesity progression, and also to identify alterations in gene signatures in CCs during early and late obesity, particularly measuring the contribution of leptin in the process.

The following specific aims were pursued:

1. Diet induced obesity model (DIO) characterisation and the description of leptin signalling components expression in mouse ovary during DIO through:

a. Characterisation of mRNA level of leptin signalling components in the ovary of obese mice during estrous cycle followed by mRNA and protein expression of leptin signalling components in ovarian extracts of obese mice in estrus stage and after superovulation (SO);

b. Leptin signalling inhibitor SOCS3 immunolocalisation in the ovary of diet- induced obese mice and characterisation of its protein expression in germinal vesicle (GV) stage oocyte during obesity;

c. Cumulus cells transcriptome analysis during obesity progression- identification of early and late obesity markers.

2. Pharmacological hyperleptinemic (LEPT) mouse model validation and description of leptin signalling components expression in the ovary through:

a. Leptin dose and length of treatment validation;

b. Characterisation of mRNA and protein expression of leptin signalling components in ovarian extracts from hyperleptinemic mice (estrus stage) and leptin signalling inhibitor SOCS3 immunolocalisation in the ovary of hyperleptinemic mice and genetic obese mice with deficiency of leptin (ob/ob);

c. Cumulus cells transcriptome analysis in LEPT protocol.

3. Characterisation of the impact of increased leptin signalling pathway activation on cumulus cells transcriptome changes during early obesity.

Materials & methods

All experiments were approved by Local Committee for the Ethical Treatment of Experimental Animals of Warmia- Mazury University (Agreement No. 80/2015, 38/2018), Olsztyn, Poland.

Studies were conducted on C57BL/6J (B6) and B6.Cg-Lepob/J (ob/ob) mice. Whole ovaries, plasma and ovarian cells- CCs, theca- enriched fraction and GV oocytes were collected for further analysis. Two experimental models were used in studies. In the first model DIO B6 mice received high fat diet (HFD) or chow diet (CD) for 4 or 16 weeks (wk) [30]. These two timepoints are corresponding to early and late obesity. Mice phenotype was characterised and estrous cycle was monitored. DIO mice significantly gained body weight (BW) and fat mass (FM) already at 4 wk, with an average gain in BW of 13 grams after 16 wk of HFD [30]. Monitoring estrous cycle revealed higher prevalence of estrus counts in 4 wk HFD group compared with controls fed CD, while in 16 wk HFD group there was a reduction in proestrus counts [30]. High levels of insulin and leptin were confirmed after 4 and 16 wk of HFD [30], with the establishment of impaired glucose tolerance and insulin resistance at 16 wk HFD [30]. In the second experimental model of LEPT, B6 mice were intraperitoneally injected with leptin or saline for 16 days [30]. 16 days of leptin treatment resulted in consistent drop in BW and FM and increased incidence of estrus [30].

In the first experiment animals were synchronised in order to collect ovaries and study leptin signalling expression on mRNA level. Samples were collected from animals in estrus stage, 18-20 h after pregnant's mare serum gonadotropin (PMSG) administration, whereas after 16-18 h of human chorionic gonadotropin (hCG) administration, samples were collected in diestrus stage. Subsequently in the second study, LEPT and DIO experiments were performed, and this time ovaries were collected in estrus stage, after monitorisation of estrous cycle for 12 consecutive days for further mRNA and protein analysis. GV oocytes and theca- enriched fraction were additionally collected from DIO protocol in this experiment. After puncturing the ovaries, GV oocytes were isolated and further mechanically denuded from surrounding GCs. The ovarian reminiscents were then centrifuged, washed and collected as theca- enriched fraction [30]. Specific markers for theca cells (TCs) and GC confirmed the purity of the samples (data not shown). Finally, another experiment was conducted in order to collect ovaries and cumulus- oocyte complexes (COCs), after SO protocol, followed by the collection of CCs

after removing metaphase II (MII) oocytes, in LEPT and DIO protocols. Mice were injected with PMSG followed after 48 h by hCG. Subsequently, 18 h after hCG administration, animals were sacrificed and material was collected [30]. Briefly, ovaries were collected for further mRNA and protein analysis while COCs were digested with hyaluronidase for 3 minutes, followed by collection of MII oocytes and centrifugation of remaining CCs. Cells were collected in lysis buffer and immediately frozen in -80ºC until being shipped to United Kingdom (UK) for further analysis.

Ovaries were freed from adipose tissue and further collected, followed by mRNA and protein extraction according to the protocol, or processed for immunostaining analysis [30]. Either mRNA and protein expression of leptin signalling components-leptin receptor ObRb, pTyr985ObRb, pTyr1077ObRb, pTyr1138ObRb, phosphorylated JAK2 (pJAK2), phosphorylated STAT3 (pSTAT3), pSTAT5, PTP1B, SOCS3 were studied by Real-time PCR (RT-PCR) and Western blotting (WB), accordingly. Leptin signalling inhibitor SOCS3 was localised in the ovary of DIO, LEPT and ob/ob mice by immunohistochemistry (IHC) stainings. The specificity of our results was confirmed by immunofluorescence assay [30]. Subsequently, CCs were collected from SO animals from COCs and RNA sequencing (RNA-seq) libraries were prepared following described protocol [30]. Samples were sequenced on Illumina Nextseq 500 instrument in Babraham Institute, UK. This sensitive method enables to analyse gene expression in very little amounts of material, in this case approximately 50 CCs from individual animal. Furthermore, theca- enriched fraction was collected after puncturing the ovary and removing GCs and GV oocytes [30]. The mRNA level of leptin signalling components was analysed in theca- enriched fraction, and SOCS3 protein level was characterised in GV oocytes. Briefly, approximately 100 oocytes were collected into 20ul of sodium dodecyl sulfate (SDS) loading buffer and immediately frozen in -80ºC. Samples were denaturated for 5 minutes in 95ºC after thawing, placed in acrylamide gel and electrophoretically separated in WB technique. The results were normalised against β-actin levels in each individual sample.

Statistical analysis was performed using GraphPad Prism 7.0. Statistical differences were determined using test adequate for each experimental design, sample quantity and Gauss distribution. Significance was defined as values of $p<0.05$. Transcriptome data was quantified and analysed in SeqMonk version v1.45.4. Differential expression analysis was performed using DESeq2 implemented in SeqMonk with a false discovery rate (FDR) < 0.05.

Results & discussion

Characterisation of leptin signalling components expression in mouse ovaries during diet- induced obesity

Leptin and its receptor were shown to be highly abundant in murine ovarian cells [21], with the highest staining intensity of leptin detected in oocytes. Nevertheless no previous studies has characterised leptin signalling pathway in the ovary during obesity. Firstly, we collected ovaries from synchronised animals in estrus and diestrus stage of the cycle. *Obr, Jak2, Sh2b, Stat3, Stat5a, Stat5b, Ptp1b* and *Socs3* mRNA expression was analysed in ovarian extracts by RT-PCR (Fig. 2). Despite previous report showing increased ObR expression in the ovaries of rats in proestrus stage [31], no differences were noted regarding expression of *Obr,* or other components of the pathway *Jak2*, *Sh2b, Stat3, Stat5a*. Nevertheless, we observed increased abundance of *Stat5b* (Fig. 2F; $p<0.05$), *Ptp1b* (Fig. 2G; $p<0.01$) and *Socs3* (Fig. 2H; $p<0.05$) in diestrus comparing to estrus stage. Interestingly, STAT5b was previously shown to interact with SOCS3 promoter during prolactin synthesis regulation [32], and therefore the increase of *Socs3* transcription in our ovaries could have been mediated by *Stat5b*. Different expression of leptin signalling components in the ovary during estrous cycle might be associated with fluctuations in estradiol level, as leptin was shown to be positively correlated with estradiol in polycystic ovary syndrome (PCOS) patients [33]. Secondly, B6 mice were submitted to DIO and estrous cycle was monitored. We collected ovaries from mice in estrus phase of ovarian cycle, as leptin was previously shown to support ovulation [34]. Ovaries were either collected for the analysis of mRNA and protein level of leptin signalling components, or punctured with a needle for collection of theca- enriched fraction. Hyperactivation of leptin signalling was noted after 4 wk of HFD comparing to CD, as corroborated also by the increase in SOCS3 expression, and the tendency for increased phosphorylation of STAT3 [30]. On the other hand, after 16 wk of HFD, local leptin resistance was established, underscored by the decrease phosphorylation of Tyr985 of leptin receptor, decreased phosphorylation of JAK2 and upregulation of SOCS3 protein levels [30]. The phosphorylation of STAT5 was significantly decreased at both timepoints [30]. Functionally, STAT5 in mouse ovary was shown to be crucial in prolactin signalling and cell proliferation during follicular growth [35], being the reduction of its activity potentially significant for oocyte maturation and fertility during

obesity. Noteworthy, no changes were seen in the phosphorylation of other tyrosine domains of ObR, either in the expression of second leptin signalling inhibitor PTP1B. Subsequently, we studied the expression of mRNA of leptin signalling components and compared the results in whole ovarian tissue and in ovarian cells in order to understand weather different ovarian compartments responded similarly to the obesogenic diet. Despite no changes observed in early obesity, the mRNA of *Socs3* was increased in late obesity in both whole ovary and theca- enriched fraction in comparison to the expression in CD group [30]. Moreover, the same was observed for *Ptp1b* expression after 16 wk HFD [30]. Thus, we have shown molecular mechanisms underlying the establishment of local leptin resistance in the murine ovary during obesity progression. Finally, in the third experiment DIO animals were subjected to SO protocol in order to induce ovulation. Ovaries were collected and mRNA and protein levels of leptin signalling components were analysed. We noted decrease in mRNA level of *Obrb* (Fig. 3A; $p<0.01$) and *Jak2* (Fig. 3E; $p<0.05$) after 16 wk of HFD comparing to CD, with no changes in the expression of other leptin signalling components. Moreover, on protein level, a diminished leptin signalling was observed already after 4 wk of HFD, which was manifested by decrease in ObRb expression (Fig. 3A; $p<0.01$), with parallel increase in PTP1B (Fig. 3H; $p<0.05$) and SOCS3 (Fig. 3I; $p<0.05$) expression. Furthermore, after 16 wk of HFD an increase in phosphorylation of Tyr985ObRb (Fig. 3B; $p<0.05$), pSTAT3 (Fig. 3F; $p<0.05$) and PTP1B (Fig. 3H; $p<0.05$) was seen. Also, phosphorylation of STAT5 was decreased in this timepoint (Fig. 3G; $p<0.05$). Hormonal manipulation in SO protocol seems to affect the expression of leptin signalling components at ovarian level. Contrarily to the analysis in cycling animals without hormonal stimulation, SO protocol revealed increase in phosphorylation of Tyr985ObRb and no changes in phosphorylation of JAK2 either in the expression of SOCS3 in late obesity. Moreover, PTP1B expression was increased in both timepoints studied, which might imply different leptin pathway regulation. Surprisingly, although PTP1B is acting on dephosphorylation of JAK2 [36], pJAK2 remained unchanged. Similarities between the results in cycling and stimulated mice comprised the decreased in phosphorylation of STAT5 after 16 wk of HFD comparing to CD. In general, hormonal treatment for stimulation of ovulation in mice during early obesity determined the transcription of leptin signalling pathway inhibitors. Conversely, during late obesity leptin signaling through ObRb was retained, with an increase in Tyr985ObRb, pSTAT3 and no changes in the expression of SOCS3.

Our results suggested that SOCS3 might be an important mediator of leptin resistance establishment in the ovary in mice during obesity. In order to further instigate this hypothesis, we performed immunostainings in ovarian slides from DIO mice and assessed SOCS3 expression in GV oocytes collected from obese mice. Immunohistochemistry revealed the presence of SOCS3 protein in oocytes from all developmental stages from primordial to preovulatory stages [30]. Moreover, SOCS3 protein was localised in other ovarian cells such as TCs, GCs or ovarian stromal cells. Staining specificity was confirmed in the experiment with immunofluorescence detection of SOCS3 in sections from DIO and ob/ob ovaries. As SOCS3 is expected to be less abundant in tissues from animals with genetic leptin deficiency, we confirmed weaker stainings in oocytes and GCs in sections from ob/ob homozygous mice comparing to wild type [30]. This fact suggests that SOCS3 abundance in the oocytes occurred mainly in response to ObRb activation and implies a direct impact of disrupted leptin signalling on oocyte quality. Importantly, we have also compared the IHC staining of SOCS3 and PTP1B in 16 wk HFD [30], and as PTP1B presented almost no staining in the oocyte, we have confirmed SOCS3 relevance in leptin resistance establishment and its consequences for the gamete. Furthermore, as the gamete presented high SOCS3 expression level, we decided to confirm the protein abundance in pooled GV oocytes in WB. SOCS3 level in oocytes collected from mice after 16 wk of HFD was significantly increased comparing to the oocytes collected from CD (Fig. 4; $p<0.05$). Throughout oogenesis, oocytes accumulate transcripts and proteins required later during nuclear and cytoplasmic maturation, which largely determine the quality of female gamete. We have shown a significant increase in SOCS3 abundance in mice gametes during obesity, which might have direct consequences for processes during meiotic maturation and fertilization.

Finally, CCs were collected from SO animals in order to study the transcriptome of these cells during early and late obesity. A total of 50-80 CCs per animal were collected, followed by RNA-seq libraries generation using a Smart-seq2 oligo-dT method [30]. Primarily, after using Principal Component Analysis to study the distribution of our samples according to global gene expression profile, we observed that principal component 1 (PC1) was mainly driven by BW, which was confirmed by the correlation between female BW and CCs transcriptome [30]. Subsequently, differently expressed genes (DEGs) were identified in early and late obesity after DESeq2 analysis (FDR<0.05). A total of 997 DEGs in 4 wk HFD group and 846 genes after 16 wk of HFD were identified. Interestingly, only 52 genes were common between these two timepoints,

underscoring the differences in gene signature and pathophysiology during early and late obesity. Within these genes, we identified 5 main clusters of DEGs [30]. The first 2 clusters comprised 33 genes downregulated during early and late obesity. Gene with the most significantly changed expression was *MICAL Like 1 (Micall1)*, an important mediator of endocytosis [37], but also *Dynein cytoplasmic 1 heavy chain (Dync1h)* involved in protein transport and positioning of cell compartments [38] was identified in this group. In the next cluster we found genes like *Annexin 11 (Anxa11)* or *Exportin 5 (Xpo5),* involved in transmembrane transport, which were upregulated at 4wk HFD, but inhibited in 16 wk protocol. Contrarily, the next cluster comprised genes downregulated at early and upregulated at late obesity with the most significantly changed gene *Ras homology family member U (Rhou),* which is known for regulating cell morphology [39]. Finally, the last cluster with genes upregulated throughout obesity comprised only 3 genes [30]. Gene ontology (GO) analysis of DEGs in early obesity revealed that genes upregulated at this timepoint were associated with nitrogen and lipid metabolism and transport, cell stress and reactive oxygen species generation, while transcripts with downregulated expression were linked to macromolecule biosynthesis and gene expression, chromatin organisation/ histone modification and regulation of cell cycle. Furthermore, after 16 wk of HFD treatment, upregulated genes in CCs were associated with negative regulation of development and cellular component organisation, while pathways which presented downregulated expression were mapped to localisation, transport and positive regulation of metabolism [30]. In general, we showed strong correlation between BW and CCs transcriptome, what confirms that female physiology impacts ovarian cells condition. Moreover, we have identified the gene signatures during early and late stages of obesity. This discovery might contribute to improving assisted reproductive technologies (ART), as CCs are commonly sampled for the analysis of oocyte competence or embryo quality, as well as to finding new markers indicative of ovarian pathology progression in obese females.

Characterisation of leptin signalling in ovaries in pharmacologically hyperleptinemic mice

After identifying major changes in leptin signalling in the ovary throughout obesity, we validated a model of pharmacological hyperleptinemia, in which mice were exposed to high systemic levels of leptin, feature commonly observed in obesity, but lacking all the remaining traits of obesity. Thus, we validated the LEPT model and

injected intraperitoneally mice with different doses of leptin for 9 or 16 days [30]. Then, we studied the expression of 5 markers in ovaries of these mice- *steroidogenic acute regulatory protein (Star)* as a marker of steroidogenesis*, Obrb, Ptp1b,*2 markers of leptin signalling activation, *interleukin 6 (Il6), tumor necrosis factor alpha (Tnfa*), 2 markers of inflammation. Firstly, animals were injected with 25 μg of leptin or saline in the control group for 9 days. Treatment did not affect the ovarian expression of markers comparing to control group. The same was observed for 100 μg of leptin injected for 9 days. Nevertheless, prolonging the protocol for 16 days of 100 μg of leptin treatment affected the expression of studied markers, as manifested by an increase in markers level comparing to controls. We then used 100 μg of leptin for 16 days in our final experiment. Ovaries of LEPT mice were collected for mRNA and protein analysis of leptin signalling components. While leptin treatment for 9 days did not change the protein expression of leptin signalling components [30], after 16 days of leptin treatment there was a significant decrease in protein expression of ObRb, and phosphorylation of Tyr985ObRb, pSTAT5 with a parallel increase in SOCS3 protein expression [30]. Also, *Socs3* mRNA level was significantly upregulated after 16 days of leptin treatment. Finally, SOCS3 immunolocalisation in the ovary was performed. We showed that SOCS3 expression pattern was similar to the one observed in DIO model, with SOCS3 equally staining in oocytes of follicles from all developmental stages, TCs, GCs and stroma. The specificity of the results was confirmed by immunofluorescent staining in ovarian slides collected from ob/ob mice with genetic leptin deficiency, as previously described, with decreased intensity staining. Collectively, we have generated the model of LEPT with a hyperactivation of leptin signalling pathway in the ovary, manifested by increased SOCS3 expression, which allowed us to pinpoint the direct consequences of ObRb hyperactivation in the ovary during early obesity.

Next, we analysed the transcriptome of CCs collected from LEPT mice submitted to SO protocol. After DESeq2 analysis, 2026 DEGs were identified comparing to control group injected with saline [30]. Gene ontology analysis revealed that upregulated genes were associated primarily with cellular and cytoskeleton organisation, as well as with immune responses. Taking into consideration immune- mediating established role of leptin [40], these results suggest the contribution of leptin to mounting ovarian inflammation in early obesity. Conversely, downregulated genes in LEPT were linked to

cell metabolism, chromatin organisation and histone modifications. Thus, we have described the leptin- driven changes in CCs transcriptome in mice.

Characterisation of the impact of increased activation of leptin signalling pathway on cumulus cells transcriptome changes during early obesity

Based on the hypothesis, that early obesity is characterised by hyperactivation of leptin signalling, we overlapped 4 wk HFD and LEPT CCs transcriptome datasets. This analysis enabled us to pinpoint leptin- driven changes in CCs transcriptome during early obesity. Expression of 144 genes was upregulated in both 4 wk HFD and LEPT group, mainly associated with response to toxins, transport and glucose metabolism. More specifically, genes with the highest significance in changes in gene expression were *Lipocalin 2 (Lcn2)* associated with lipid transport [41], *Claudine 22 (Cldn22)* a component of tight junctions [42], *Anxa 11* involved in transmembrane secretion [43]. This suggested the importance of leptin- mediated changes in lipid metabolism, as well as transmembrane transport in the early onset obesity. Moreover, *Glucose-6-phosphate dehydrogenase x- linked (G6pdx*) associated with glucose metabolism was also identified in this group [30]. On the other hand, 177 downregulated genes in both protocols were associated mainly with metabolism and gene expression regulation. Amongst them, there was a subset of genes coding for epigenetic factors, like *Dna segment, chr 14, abbott 1 expressed o (Tasor), Lysine (k)-specific methyltransferase 2d (Kmt2d/Mll2), Methyl-cpg binding domain protein (Mbd) 2*, and *DNA methyltransferase (Dnmt) 3a,* which suggested potential involvement in epigenetic dysregulation. Moreover, another leptin- driven effect in early obesity was the repression of genes associated with cytoskeleton reorganisation. Cytoskeletal core builds transzonal projections (TZPs) forming routs between somatic cells and the oocyte. The impairment in cellular transport might have drastic consequences for ovarian regulation, as communication between oocyte and somatic cell is crucial for oocyte growth and maturation [44]. Indeed, glucose metabolites are delivered to the oocyte, as well as oocyte secretes factors like growth differentiation factor 9 (GDF9) for CCs to regulate their growth and development. This bidirectional communication is crucial for proper oocyte maturation, meiotic resumption and ovulation. We have also confirmed the role of leptin during early obesity on changes of expression of genes involved in CCs metabolism. Thus, the expression of genes associated with glucose metabolism and free fatty acid (FFA) oxidation was analysed. The gamete is unable to metabolise glucose due to low phosphofructokinase activity [45],

and relies on CCs glycolytic activity to produce pyruvate [46]. We distinguished clear similarities in the profiles of expression between early obesity and LEPT in terms of glucose and FFA metabolism, which tended to be upregulated in both groups. Interestingly this functions appeared to be decreased during late obesity [30], presumably as a result of established leptin resistance. Furthermore, this might have further consequences as the transport of pyruvate into the oocyte would be decreased, which could also affect tricarboxylic acid cycle (TCA) and adenosine triphosphate (ATP) generation. Also, as leptin role on FFA oxidation and triglyceride homeostasis regulation is well known, the disruption of leptin signalling in late obesity might account for lipotoxicity and cellular stress observed in ovaries in obese females. Leptin seems to have a supportive effect on CCs metabolism during early obesity, manifested by the positive response on oocyte competence and GDF9 signalling [30]. However, after 16 wk of HFD, the decreased metabolic capacity was followed by downregulation of main paracrine mediators of oocyte maturation and responsiveness to GDF9, such as *gremlin 1 (Grem1), epidermal growth factor 1 (Egf1), Has2*, suggestive of compromised oocyte quality [30].

In general, we presented that hyperactivation of leptin signalling in CCs during early obesity seemed to be linked to impaired cell membrane transport and endocytosis, but also cell metabolism and gene expression regulation. Thus, the detrimental effect of obesity could be related to increased SOCS3 in earlier stages of the disease, followed by leptin resistance establishment at 16 wk HFD. Furthermore, after 4 wk of HFD, increased leptin contributed to the downregulation of expression of major epigenetic factors and cytoskeleton organisation. Inversely, during late obesity when leptin resistance is established, inflammatory responses were shown to be activated, presumably due to lipotoxicity described in the ovary during obesity previously, followed by cellular reorganisation in order to accommodate surplus lipids.

The importance of leptin signalling during folliculogenesis and oocyte maturation

Folliculogenesis is a complex process, vulnerable to hormonal imbalance and environmental disruptors. Female lifetime reproductive potential depends on proper follicle awakening and depletion of the follicular pool. Moreover, the growth and maturation of ovarian follicles and oocytes, depends on a plethora of factors. In the recent review paper [18], we have presented the role of leptin signalling and its potential disturbances during obesity, on folliculogenesis and oocyte developmental competence

in mice and women. Furthermore, major molecular pathways preconizing developmental changes in gametes and somatic cells during primordial follicle activation, preovulatory follicle formation and oocyte maturation were discussed. Indeed, SOCS3 overexpression could contribute to putative ERK phosphorylation inhibition and PI3K signalling disturbances during primordial follicle pool activation. Furthermore, leptin effects on estradiol synthesis [47], the main player in preovulatory follicle formation, are well known. During preovulatory follicle formation, altered leptin signaling can potentially affect not only steroidogenesis, but also the bidirectional communication between oocyte and somatic cells, which is key for antrum formation and oocyte growth [48]. We have shown that leptin receptor b hyperactivation during early obesity might dysregulate the expression of genes associated with transzonal projections [30], with notorious consequences for preovulatory follicle development. Finally, leptin signalling disturbances during obesity establishment might also lead to potential dysregulations in oocyte maturation and metabolism, meiosis resumption and early embryo development due to leptin involvement in these processes [49].

The endocrine imbalance observed in obese mothers, as well as hyperinsulinemia and hyperleptinemia can affect directly the follicular pool, reducing the number of primordial follicles and compromising the fertility. We have used data on oocyte and granulosa cell transcriptome gathered by Zhang [50], throughout folliculogenesis in women. After checking the expression level of leptin signalling components in oocytes and GC in primordial, primary, secondary and antral follicle, we have shown that expression of *Stat3, Ptpn2, Ptp1b* were distinctively higher than other components in both oocytes and GC. Moreover, *Socs3* presented very low expression in both cell types under physiological conditions, particularly in later stages of folliculogenesis [18]. This evidences, alongside with our findings of hyperactivation of leptin signalling in ovaries in early obesity followed by leptin resistance establishment in obese females might contribute to ovarian failure. Furthermore, we have also checked the expression of 134 DEGs in CCs of mice on HFD for 4 wk and LEPT in GCs from different follicular stages in women. Interestingly, most of the genes were actively transcribed in GC from antral and preovulatory follicles [18], amongst them *Ptpn2* and *Ptp1b*. This suggests that leptin-driven changes in CCs during early obesity, might be important already in GC from preantral to preovulatory stages of follicles.

In conclusion, dysregulation of leptin signalling might equally affect ovarian follicles on different developmental stages. Leptin signalling inhibitors, through

hyperactivation of leptin receptor and activity of downstream effector genes, may affect primordial follicle pool awakening, preovulatory follicle formation or oocyte developmental competence acquirement.

Summary of the results

The analysis undertaken in the scope of the following dissertation revealed the following results:

1. After initial leptin signalling hyperactivation during early obesity in the ovary in mice, there is an establishment of leptin resistance during late obesity, which is manifested by the decrease in pJAK2 and increased expression of SOCS3. SOCS3 was localised in the mouse ovary during obesity, while its increased expression after 16 wk of high-fat diet was confirmed in GV oocytes.
2. Hormonal stimulation protocol in mice changes leptin signalling expression during obesity in comparison to cycling animals. After initial inhibitors production during early obesity, signal propagation is retained in late obesity, with increase in phosphorylation of Tyr985ObRb, STAT3 and no changes in the phosphorylation of JAK2 or expression of pathway inhibitors PTP1B or SOCS3.
3. Global gene expression in cumulus cells was shown to be correlated with female body weight.
4. The analysis revealed for the first time the temporal changes in gene expression in cumulus cells during obesity progression. Early obesity caused an increase in expression of genes associated with metabolism and cell stress, but decrease in the ones connected with biomolecule synthesis and chromatin organisation. On the other hand, during late obesity, there was an augmentation in the negative regulation of development and decrease in localisation and transport.
5. We have validated the model of pharmacological hyperleptinemia, with a hyperactivation of leptin signalling in the ovary but lacking all the remaining traits of obesity. Genes associated with cytoskeleton organisation and immune responses were upregulated in cumulus cells of these mice, while cell metabolism and chromatin organisation were diminished.
6. The overlay of the differential analysis of the CCs transcriptome between early obesity and hyperleptinemic mice, revealed leptin- mediated changes. Thus, expression of genes associated with responses to toxins, transport and glucose metabolism was upregulated, while epigenetic regulation and cytoskeleton reorganisation were repressed.

7. Current knowledge on potential effects of altered leptin signalling on folliculogenesis and oocyte maturation in mice and women had been presented in the review.

Conclusions

In conclusion, characterisation of leptin signalling in the ovary of mice during obesity progression was studied for the first time. It was observed in ovaries from mice in early obesity stage the hyperactivation of leptin signalling, which was followed by the establishment of local leptin resistance during late obesity. This was mainly manifested by decreased phosphorylation of JAK2 and parallel upregulation of SOCS3 protein expression. Furthermore, the hormonal treatment of diet induced obese mice, through a protocol for superovulation, affected the expression of leptin signalling pathway components in the ovary. Indeed, during late obesity leptin signalling through its receptor ObRb still mediated the phosphorylation of JAK2, without overexpression of SOCS3. The validation of a pharmacological hyperleptinemia mouse model, showing hyperactivation of leptin signalling in the ovary but lacking remaining traits of obesity, helped dissecting leptin-driven changes in follicle components, particularly the CCs. Moreover, sensitive methods for RNA sequencing enabled to characterise the CCs transcriptome and reveal temporal changes in gene expression during obesity progression. It was found that female body weight is highly correlated with CCs transcriptome. Furthermore, it was shown that during early obesity hyperactivation of leptin signalling in CCs contributes to diminished expression of genes associated with epigenetic regulation and cytoskeleton organisation, but increased metabolism. Subsequently, during late obesity when leptin resistance was established, altered gene signatures were mainly linked to inflammatory responses and morphological rearrangements. The present work shed light on our understanding of the pathophysiology of the ovaries in the course of obesity development. This certainly contributes to the characterisation of markers indicative of disease progression in the ovary.

Finally, the description of leptin- mediated effects during obesity on folliculogenesis and oocyte developmental competence in mice and women were presented in the review work. The analysis of gene expression of leptin signalling components in oocyte and GC transcriptome in women during folliculogenesis revealed that *Stat3*, *Ptpn2* and *Ptp1b* were highly abundant, while *Socs3* was expressed on low levels. Moreover, the analysis of expression of DEGs common in early obesity and LEPT protocol in CCs collected from mice, in GC during folliculogenesis in women, evidenced that *Ptpn2* and *Ptp1b* were highly expressed during antral and preovulatory stages, while *Socs3* was more abundant during earlier stages of folliculogenesis.

Hence, a better understanding of the pathophysiology of ovarian failure during obesity might help us find innovative tools to monitor the disease progression. Thus, we could prevent pregnancy failure and ensuring birth of a healthy offspring.

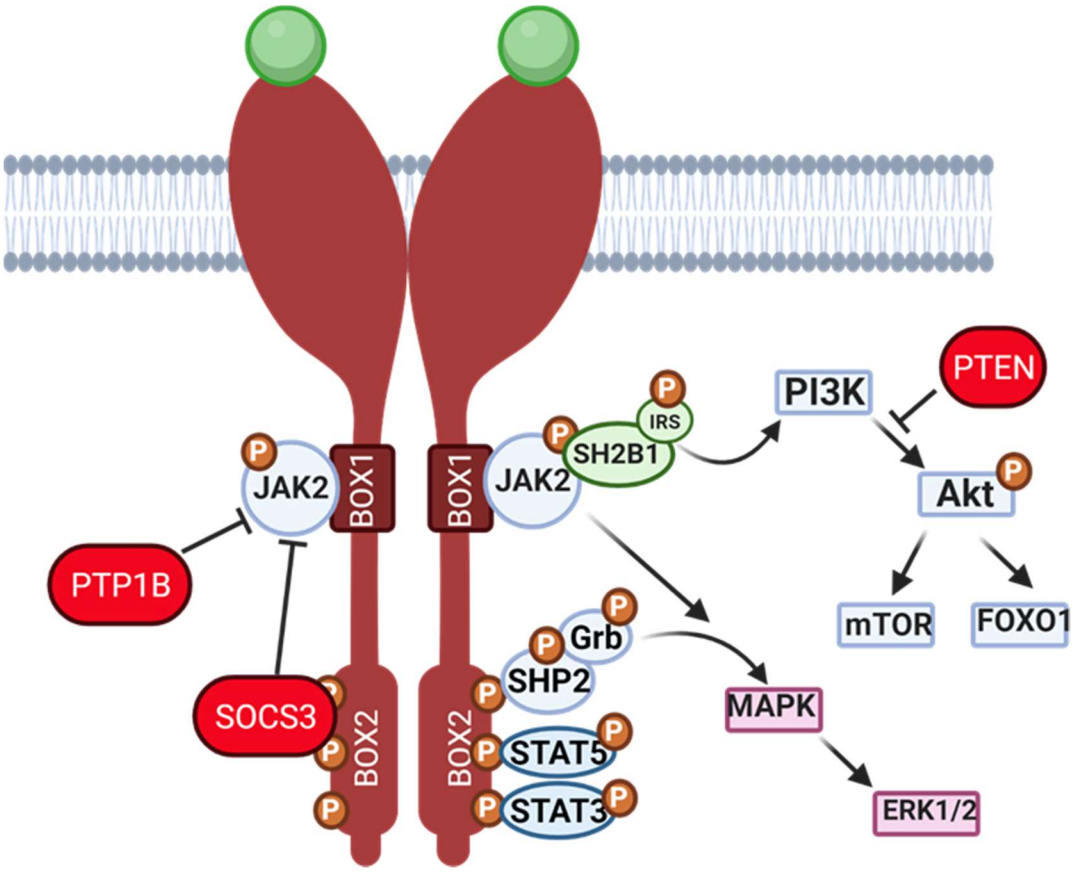

Figure 1. Schematic representation of leptin signalling pathway cascade. Leptin binds to its dimerised membrane receptor and signal propagation starts. Janus Kinase 2 (JAK2) phosphorylation causes transition of phosphate groups to three tyrosines within BOX2 of ObR and activation of i) SH2- Domain Containing Protein Tyrosine Phosphatase (SHP-2), which, in turns, binds its adapter molecule Grb-2 and activates downstream signalling resulting in Extracellular Signal Regulated Kinase (ERK) 1/2 activation, ii) Signal Transducer and Activator of Transcription (STAT) 5 activation and iii) STAT3 activation. The phosphorylation of JAK2 activates also MAPK signalling pathway and promotes SH2B Adaptor Protein 1 (SH2B1) and Insulin Receptor Substrate (IRS) binding, which initiates Phosphatidylinositol 3 Kinase (PI3K) pathway, that leads to phosphorylation of Protein Kinase B (Akt), Mammalian Target of Rapamycin (mTOR) and Forkhead Box O1 (FOXO1) activation. During hyperactivation of leptin signalling pathway two main inhibitors can be transcribed- Protein Tyrosine Phosphatase (PTP) 1B, which dephosphorylates JAK2 and Suppressor of Cytokine Signalling (SOCS) 3 which blocks tyrosine and JAK2 phosphorylation. Created with BioRender.com.

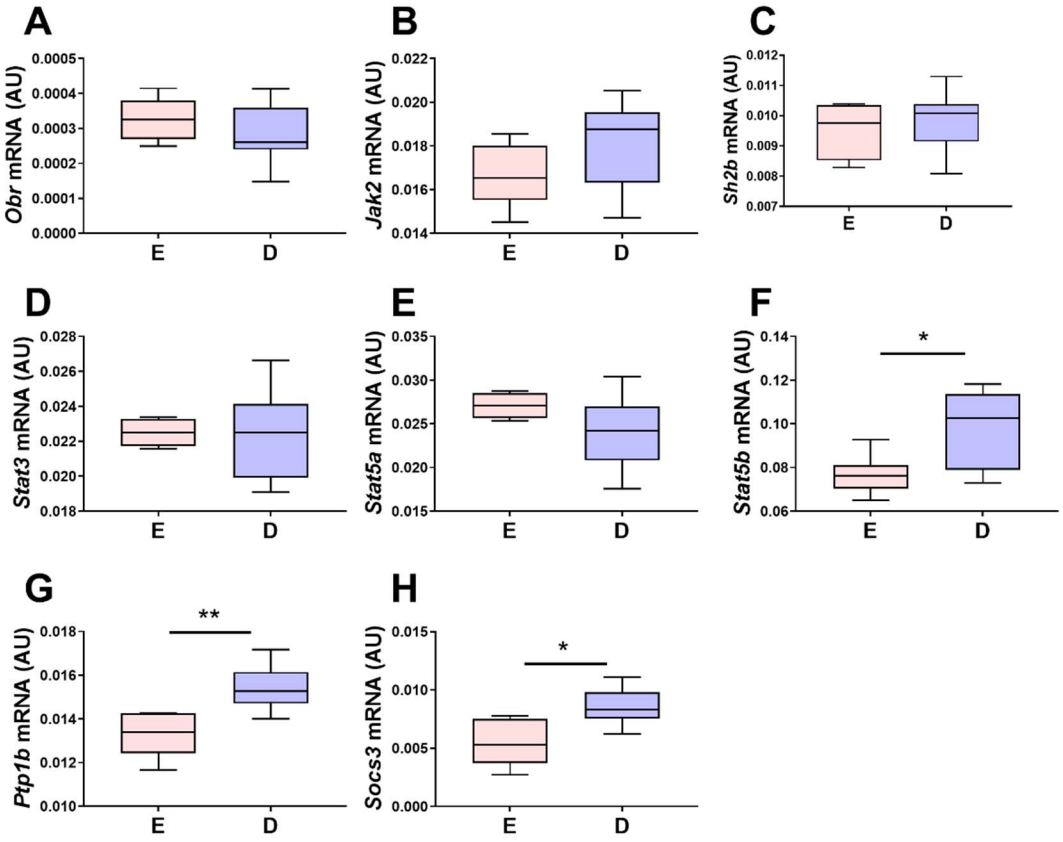

Figure 2. The expression of components of leptin signalling pathway in the ovary in estrus and diestrus stage of the cycle in mice. Abundance of (A) leptin receptor (Obr), (B) Janus kinase 2 (Jak2), (C) SH2B adapter protein (Sh2b), (D) signal transducer and activator of transcription 3 (Stat3), (E) Stat5a, (F) Stat5b, (G) protein tyrosine phosphatase 1b (Ptp1b), (H) suppressor of cytokine signaling 3 (Socs3). mRNA expression of ribosomal protein L37 (Rpl37) was used to normalise the expression data. Each bar represents the mean ± SD. Differences between groups analysed by Mann-Whitney test. N=8. * $p<0.05$; ** $p<0.01$.

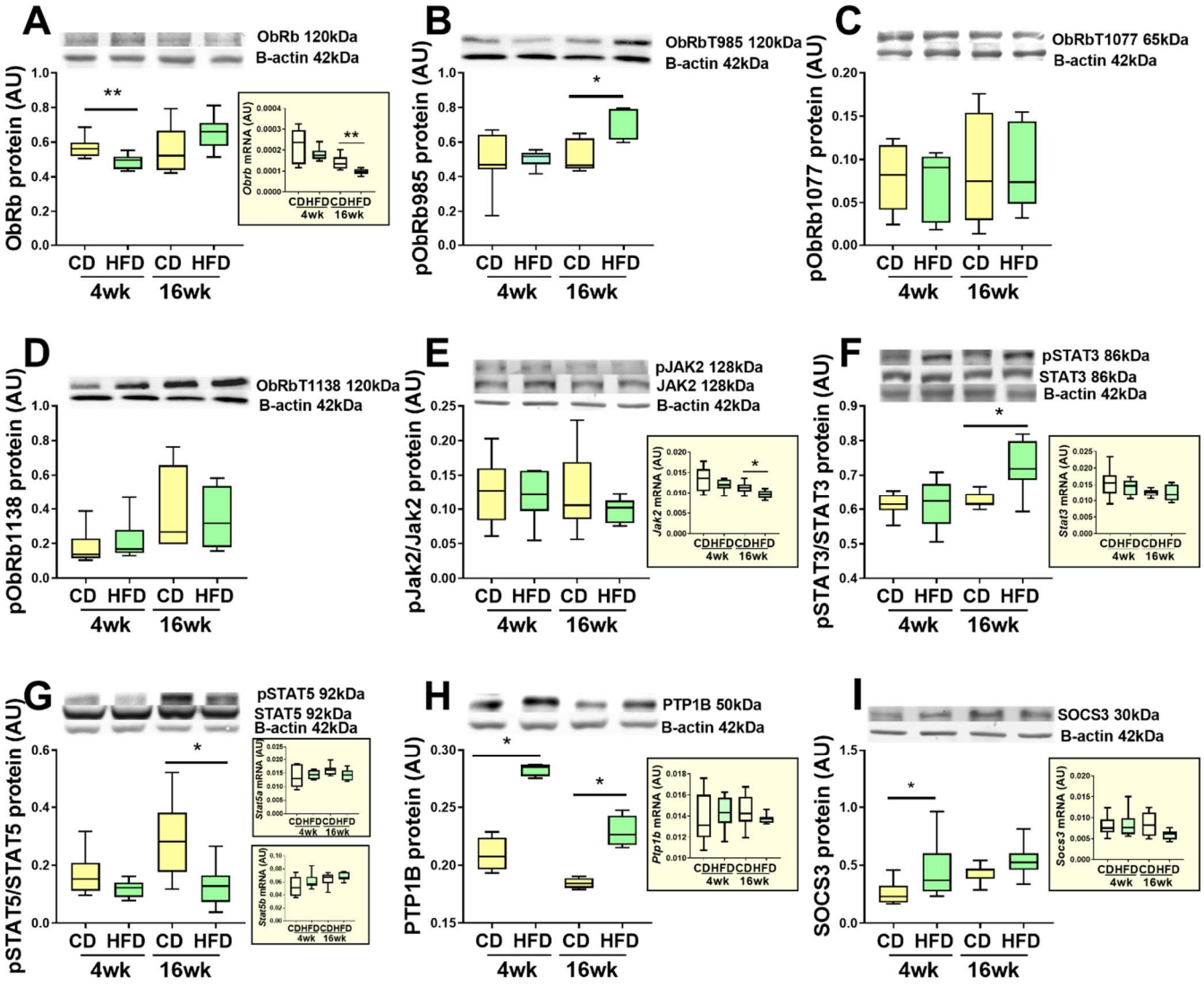

Figure 3. Abundance of mRNA (yellow box) and protein of leptin signalling pathway components in ovarian extracts collected from animals maintained on chow diet (CD) or high fat diet (HFD) for 4 weeks (wk) or 16 wk and sacrificed after superovulation protocol. Expression of (A) long isoform of leptin receptor (ObRb), phosphorylation of (B) tyrosine 985 of leptin receptor, (C) tyrosine 1077 of leptin receptor, (D) tyrosine 1138 of leptin receptor, (E) Janus kinase 2 (JAK2), (F) signal transducer and activator of transcription 3 (STAT3), (G) STAT5, expression of (H) protein tyrosine phosphatase 1B (PTP1B) and (I) suppressor of cytokine signalling 3 (SOCS3) determined by Real-time PCR and Western blotting. mRNA expression of Rpl37 and protein expression of B-actin were used to normalize the expression data. Each bar represents the mean ± SD. Differences between groups were analysed by Mann- Whitney test. N=4-8 for immunoblots and N=8 for RT PCR analysis. * $p<0.05$; ** $p<0.01$.

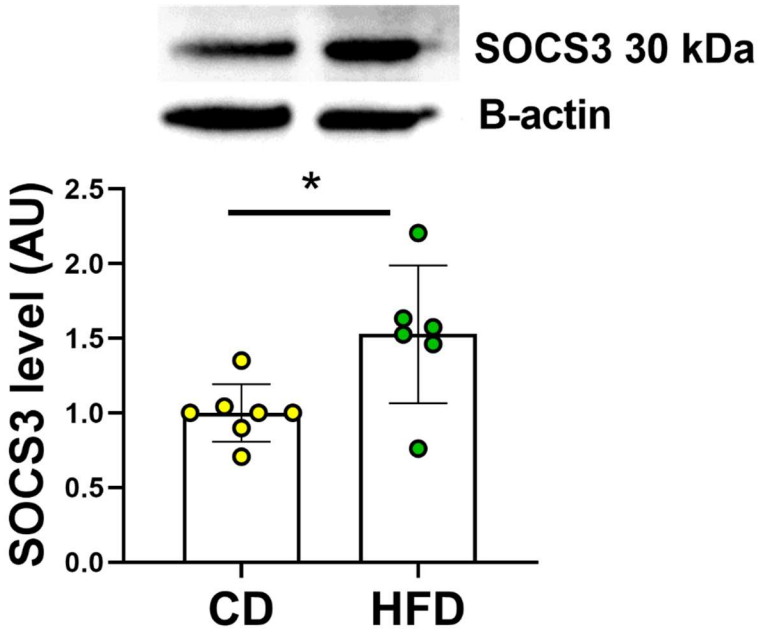

Figure 4. SOCS3 abundance in GV oocytes analysed by Western blot in mice fed chow diet (CD) or high fat diet (HFD) for 16 weeks. Protein expression of B-actin was used to normalise the expression data. Each bar represents the mean ± SD. Differences between groups analysed by Mann- Whitney test. N=6. * $p<0.05$. Representative picture in the upper panel.

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Information about nature of participation and authors' contribution

„Leptin Resistance in the Ovary of Obese Mice is Associated with Profound Changes in Cumulus Cells Transcriptome" authored by: Karolina Wołodko, Edyta Walewska, Marek Adamowski, Juan Castillo-Fernandez, Gavin Kelsey, Antonio Galvao, published in Cellular Physiology and Biochemistry, 2020; 2020;54:417-437

Authors' name and surname	Nature of participation	Contribution in %
PhD student- mgr inż. Karolina Wołodko	Data acquisition, analysis and interpretation of the data, manuscript preparation	45%
mgr Edyta Walewska	Data acquisition, analysis and interpretation of the data	5%
mgr Marek Adamowski	Data acquisition and analysis	5%
dr Juan Castillo-Fernandez	Bioinformatic analysis and interpretation of data, revising the manuscript	5%
prof. Gavin Kelsey	Supervision, funding, revising the manuscript	5%
dr hab. Antonio Galvao	Conception and design, funding acquisition, acquisition of data, bioinformatic analysis and interpretation of data, writing and revising of the manuscript	35%

„Revisiting the Impact of Local Leptin Signaling in Folliculogenesis and Oocyte Maturation in Obese Mothers" authored by: Karolina Wołodko, Juan Castillo-Fernandez, Gavin Kelsey, Antonio Galvao, published in International Journal of Molecular Sciences, 2021;22:4270

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PhD student- mgr inż. Karolina Wołodko	Conception and design, review preparation, schemes preparation	60%
dr Juan Castillo-Fernandez	Bioinformatic analysis, revising the review	5%
prof. Gavin Kelsey	Supervision, revising the review	5%
dr hab. Antonio Galvao	Conception and design, supervision, revising the review	30%

I hereby declare that all co-authors agreed to use these articles in the Karolina Wołodko's dissertation.

Podpis

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Original Paper

Leptin Resistance in the Ovary of Obese Mice is Associated with Profound Changes in the Transcriptome of Cumulus Cells

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Key Words
Obesity • Cumulus cell • Leptin • SOCS3 • Transcriptome

Abstract
Background/Aims: Obesity is associated with infertility, decreased ovarian performance and lipotoxicity. However, little is known about the aetiology of these reproductive impairments. Here, we hypothesise that the majority of changes in ovarian physiology in diet-induced obesity (DIO) are a consequence of transcriptional changes downstream of altered leptin signalling. Therefore, we investigated the extent to which leptin signalling is altered in the ovary upon obesity with particular emphasis on effects on cumulus cells (CCs), the intimate functional companions of the oocyte. Furthermore, we used the pharmacological hyperleptinemic (LEPT) mouse model to compare transcriptional profiles to DIO. ***Methods:*** Mice were subjected to DIO for 4 and 16 weeks (wk) and leptin treatment for 16 days, to study effects in the ovary in components of leptin signalling at the transcript and protein levels, using Western blot, Real-time PCR and immunostaining. Furthermore, we used low-cell RNA sequencing to characterise changes in the transcriptome of CCs in these models. ***Results:*** In the DIO model, obesity led to establishment of ovarian leptin resistance after 16 wk high fat diet (HFD), as evidenced by increases in the feedback regulator suppressor of cytokine signalling 3 (SOCS3) and decreases in the positive effectors phosphorylation of tyrosine 985 of leptin receptor (ObRb-pTyr985) and Janus kinase 2 (pJAK2). Transcriptome analysis of the CCs revealed a complex response to DIO, with large numbers and distinct sets of genes deregulated at early and late stages of obesity; in addition, there was a striking correlation between body weight and global transcriptome profile of CCs. Further analysis indicated that the transcriptome profile in 4 wk HFD CCs resembled that of LEPT CCs, in the upregulation of cellular trafficking and impairment in cytoskeleton organisation. Conversely, after 16 wk HFD CCs showed expression changes indicative of augmented inflammatory responses, cell morphogenesis, and decreased metabolism and transport, mainly as a consequence of the physiological changes of obesity.

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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 the ovary, whereas in late obesity are likely to be a consequence of metabolic changes taking place in the obese mother.

Introduction

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 oxidative stress [5]. Nonetheless, the exact mechanisms underlying ovarian pathogenesis in the course of obesity remain uncharacterised.

Leptin is a cytokine secreted by the adipose tissue (adipokine) [6]. Indeed, soon after the introduction to an obesogenic environment, large amounts of leptin can be found in the circulation [7], making this adipokine one of the early-onset obesity markers. Leptin controls food intake through its action at the central nervous system [8]; however, as a pleiotropic adipokine, leptin contributes to the regulation of numerous processes in the body, such as immune response [9] or angiogenesis [10]. Concerning the reproductive tract, leptin has been shown to control the neuroendocrine reproductive axis [11] and folliculogenesis [12]. Furthermore, leptin has been linked to ovulation [13] and embryo development [14]. Leptin is detected in most cell types in the murine ovary, with the highest staining intensity seen in the oocyte [15]. Nevertheless, no previous consideration has been made whether leptin signalling is dysregulated in the obese ovary. The leptin receptor b (ObRb) is a type I cytokine receptor, which signals through the association with the tyrosine kinase Janus kinase 2 (JAK2). Upon leptin binding and dimerization of the receptor, JAK2 is recruited [16], mediating the phosphorylation of three conserved tyrosine residues on the intracellular domain of the receptor: tyrosine (Tyr) 985, Tyr1077 and Tyr1138. Subsequently, signalling molecules are recruited to these activated tyrosines [17] and, as a result, the signal transducer and activator of transcription (STAT) 5 and/or STAT3 are also phosphorylated and translocated into the nucleus, where they regulate transcription [8, 18]. During sustained activation of ObRb, the expression of both suppressor of cytokine signalling 3 (SOCS3) and tyrosine-protein phosphatase 1B (PTP1B) is initiated as a negative feedback response [19, 20]. While PTP1B dephosphorylates JAK2, SOCS3 binds to receptor domains within JAK2 and Tyr985 and terminates signal transduction.

To date, little is known about the integrity of leptin signalling in the ovary during obesity progression, and its particular impact on the cumulus oophorous complex (COC). Cumulus cells (CCs) are vital regulators of oocyte growth and metabolism [21], controlling meiosis resumption [22], as well as the ovulation process itself [23]. Thus, a better knowledge of the pathophysiology of the events taking place in these cells in the course of obesity will allow us to understand the molecular mechanisms leading to impaired ovarian performance and infertility. Furthermore, the transcriptional signature of CCs has been used to predict oocyte competence or embryo quality [24, 25], demonstrating the importance of such references in assisted reproduction techniques. Here, we first characterise the establishment of leptin resistance in whole ovaries from diet induced obesity (DIO) mice fed for 4 and 16 weeks (wk). Subsequently, we analysed the transcriptome of CCs throughout obesity progression and identify temporally altered gene expression signatures. Finally, using a mouse model for pharmacological hyperleptinemia (LEPT), we pinpoint the transcriptional events mediated by increased leptin signalling in CCs in the early stages of obesity.

Materials and Methods

Animal protocol

Breeding pairs from mouse strains C57BL/6J (B6) and B6.Cg-Lep^{ob}/J (*ob/ob*) were obtained from the Jacksons Laboratory (The Jacksons Laboratory, Bar Harbour, Maine, USA). At 8 wk of age B6 animals were divided into 2 groups (n=12/ group). In DIO, the control group was fed *ad libitum* chow diet (CD, 11% energy in kcal from fat, 5053, rodent diet 20, LabDiet IPS, London, UK), while the experimental group received high fat diet (HFD, 58% energy in kcal from fat, AIN-76A 9G03, LabDiet IPS). Mice were maintained on the respective diet for 4 or 16 wk. Regarding the pharmacological hyperleptinemia protocol, 8 wk old B6 female mice fed CD were divided into two groups (n=15/group): i) saline 16 days (d) (CONT); ii) leptin 16 d (Recombinant Mouse Leptin, GFM26, Cell Guidance Systems, Cambridge, UK). The animals were injected intraperitoneally twice a day, at 09:00 h and 21:00 h and total dosage of 100 µg/day of leptin was administrated. Concerning the *ob/ob* model, mice were kept from weaning until twelve wk of age on CD. For all protocols, mice were housed with a 12 h light/12 h dark cycle at room temperature (23°C, RT).

For phenotype characterisation of DIO, changes in body composition were monitored every 4 wk by nuclear magnetic resonance (NMR, Bruker, Rheinstetten, Germany), whereas in the LEPT model animals were phenotyped every three days. Body weight (BW), fat mass (FM), lean mass (LM), adiposity index (AI, fat mass/lean mass), and food intake (FI) were measured. For DIO and LEPT models vaginal cytology was done at 9:00 h, for twelve consecutive days. After vaginal lavage with saline, the smears were placed on clean glass slides and stained with Diff Quick Staining Set (Diff - Quick Color Kit, Rapid Staining Set, Medion Grifols Diagnostics AG, Duedingen, Switzerland), for cell identification as previously described [26]. The results were analysed for percent of time spent in oestrous stage (further explained in Supplementary Fig. 1J, 1K; Supplementary Fig. 2G – for all supplemental material see www.cellphysiolbiochem.com). All samples for mRNA, protein analysis and staining were collected at the oestrus stage.

A superovulation protocol was used for COC collection and further isolation of a pure population of CCs after removing the metaphase II (MII) oocytes. This developmental state of granulosa cells (GC) was preferred to ensure a tightly controlled level of progression. The animals were injected with pregnant mare's serum gonadotropin (PMSG, G4877, 5IU, Sigma Aldrich, Saint Louis, Missouri, USA) followed after 48 h by human chorionic gonadotropin (hCG, Chorulon, 5IU, MSD Animal Health, Boxmeer, Holland). Subsequently, 18 h after hCG injection the animals were sacrificed.

Ovary collections

Mice were sacrificed by cervical dislocation and the reproductive tracts collected and rinsed with phosphate buffered saline (PBS, 0.1 M, pH=7.4). Ovaries were then removed from the genitalia and cleaned of adipose tissue. Ovaries were stored either in TRI Reagent (T9424, Sigma Aldrich) for mRNA, or radioimmunoprecipitation assay buffer (RIPA, 89901, Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with protease inhibitor cocktail (PIC, P8340, Sigma Aldrich), phenylmethylsulfonyl fluoride (PMSF, P7626, Sigma Aldrich) and phosphatase inhibitor (88667, Thermo Fisher Scientific), for protein analysis. Samples were stored at -80°C, except for the analysis of phosphorylated proteins, in which samples were isolated immediately after sacrificing the animals.

Ovarian cell isolation protocol

For theca and stroma enriched (TC) fraction collection, immediately after culling the animals, ovaries were transferred to Dulbecco's modified Eagle's medium (DMEM, D/F medium; 1:1 (v/v), D-8900, Sigma Aldrich) with 3% bovine serum albumin (BSA, 735078, Roche Diagnostics GmbH, Mannheim, Germany), 20 µg/ml gentamicin (G1397, Sigma Aldrich) and 250 µg/ml amphotericin (A2942, Sigma Aldrich). For the TC fraction, ovaries were punctured with a 16 gauge needle as described before [27]. Briefly, after removing GC and oocytes, the remaining tissue was washed twice with media and stored in TRI Reagent for mRNA analysis (n=8/group). For CCs, after superovulating the animals, COCs were retrieved from oviducts, and further digested with hyaluronidase (H3506, 400 µg/ml, Sigma Aldrich). After removing the oocytes, a total of approximately 50 pure CCs were collected from one individual animal from either 4 wk or 16 wk DIO (n=5/ condition), as well as the 16 d LEPT (n=at least 3/ condition) protocol.

RNA isolation and cDNA synthesis

For mRNA extraction, either whole ovaries or TC fraction were collected from mice in oestrus stage, placed in 1 ml of TRI Reagent in 1.5 ml eppendorf tubes (n=8/ group) and mechanically disrupted with a lancet. The suspension was pipetted up and down vigorously and incubated for 5 minutes (min) at RT. After centrifugation (9400 g, 4°C, 15 min), the supernatant was transferred to a fresh tube and thoroughly mixed with 100 µl of 1-Bromo-3-chloropropane (BCP, BP151, Molecular Research Centre, Cincinnati, Ohio, USA), followed by incubation at RT for 10 min. Subsequently, samples were centrifuged (13500 g, 4°C, 15 min) and the aqueous phase transferred to a new tube, before being mixed with an equal volume of isopropanol and incubated at -80°C for 60 min. Another centrifugation (20000 g, 4°C, 15 min) to pellet down the RNA, which was then washed three times with 75% ethanol and incubated overnight at -80°C. Next day, samples were centrifuged (20000 g, 4°C, 15 min) and the RNA pellet dried and resuspended in 20 µl of RNAse free water (W4502, Sigma Aldrich), supplemented with RNAse Inhibitor (RiboProtect, RT35, BLIRT, Gdańsk, Poland). Finally, RNA quality and concentration were assessed with NanoDrop. Absorbance ratio at 260 nm and 280 nm (A260/A280) was determined and the quality and concentration of isolated mRNA confirmed.

A total of 1 µg of RNA was reversely transcribed using Maxima First Strand cDNA Synthesis Kit for Real-time polymerase chain reaction (PCR) (K1642, Thermo Scientific) according to the manufacturer's instructions. The cDNA was stored at -20°C until the real-time PCR was carried out.

Real-time polymerase chain reaction

Real-time PCR was performed in a 7900 Real-Time PCR System (Applied Biosystems, Warrington, UK) using Maxima SYBR Green/ROX qPCR Master Mix (K0223, Thermo Scientific). Primers were designed using Primer 3.0 v.0.4.0. software [28], based on gene sequences from GeneBank (NCBI), as described before [29]. All primers were synthesised by Sigma Aldrich. Primer sequences, expected PCR products length, and GeneBank accession numbers are reported in Table 1. The total reaction volume was 12 µl, containing 4 µl cDNA (10µg), 1 µl each forward and reverse primers (80 nM or 160 nM), and 6 µl SYBR Green PCR master mix. Real-time PCR was carried out as follows: initial denaturation (10 min at 95°C), followed by 45 cycles of denaturation (15 s at 95°C) and annealing (1 min at 60°C). After each PCR, melting curves were obtained by stepwise increases in temperature from 60 to 95°C to ensure single product amplification. In each real-time assay, both the target gene and a housekeeping gene (HKG) - *Ribosomal Protein L37* (*Rpl37*, primers in Table 1) or *Eukaryotic Translation Initiation Factor 5A* (*Eif5a*, primers in Table 1) - were run simultaneously and reactions were carried out in duplicate wells in a 384-well optical reaction plate (4306737, Applied Biosystems). The HKG selection was performed with NormFinder, in each experimental group. Real-time PCR results were analysed with the Real-time PCR Miner algorithm [30].

Western blot

Protein expression was assessed by western blot (n=5/group). Ovaries from mice in oestrus stage were collected into RIPA supplemented with inhibitors and mechanically disrupted with a lancet. Then, lysates were incubated for one hour on ice, with mixing every 15 min. Subsequently, samples were centrifuged (20000 g, 4°C, 15min) and the supernatant was collected and stored at -80°C until the analysis. The protein concentration was assessed using bicinchoninic acid assay (BCA, BCA1-1KT, Sigma Aldrich). A total of 10-40 µg of protein was loaded on 8-14% acrylamide gel, and after electrophoresis proteins were transferred to polyvinylidene difluoride (PVDF) or nitrocellulose membrane depending on the antibodies to be used. Membranes were blocked in 5% BSA (A2153, Sigma Aldrich) and incubated with primary antibodies (AB) overnight at 4°C. Leptin receptor and its phosphorylated domains were evaluated using the following antibodies: mouse monoclonal (MM) against leptin receptor (ObR; 1:500, sc-8391, Santa Cruz Biotechnology, Dallas, Texas, USA), goat polyclonal (GP) against phosphorylated Tyr 985 ObRb (pTyr985ObRb; 1:500, sc-16419, Santa Cruz Biotechnology), rabbit polyclonal (RP) against phosphorylated Tyr 1077 ObRb (pTyr1077ObR; 1:500, 07-1317, Merck Millipore, Burlington, Vermont, USA), GP against phosphorylated Tyr 1138 ObRb (pTyr1138ObRb; 1:500, sc-16421, Santa Cruz Biotechnology). The expression of other leptin signalling pathway components was assessed using the following antibodies: RP against JAK2 (1:200, sc-294, Santa Cruz Biotechnology), RP against phosphorylated Tyr 1007/1008 JAK2 (pJAK2; 1:200, sc-16566-R, Santa Cruz Biotechnology), RP against STAT3 (1:200, sc-482, Santa Cruz Biotechnology), MM against phosphorylated Tyr 705 pSTAT3 (1:200, sc-8059, Santa Cruz Biotechnology), RP against STAT5 (1:200, sc-835, Santa Cruz Biotechnology), MM against phosphorylated Tyr 694/699

Table 1. Specific primer sequences used for quantitative real-time PCR

Functional pathway	Gene name	Gene symbol	GeneBank Accession no.	Sequences 5'-3'	Length (base pair)
Leptin signalling	Leptin receptor	Obrb	NM_146146.2	CCTCCAGGAGAGATGCTCACAC TGACTGTGCGTGGAACAGGT	111
	Janus kinase 2	Jak2	NM_001048177.2	GGTGTTTCACAAAATCAGGAATG TGTGCAGTTGACCATAATCTCC	119
	SH2B adaptor protein 1	Sh2b	NM_001289539.1	GGACCCAGCGAGAGTAACGA GCAGCAATGGAGGCAGAACT	101
	Signal transducer and activator of transcription 3	Stat3	NM_011486.5	CGATGCCTGTGGGAAGAGTC CTGTCACTACGGCGGCTGTT	97
	Signal transducer and activator of transcription 5A	Stat5a	NM_001164062.1	GGGACAATGCCTTTGCTGAG AGCCCCGGTTGCTCTGTACT	123
	Signal transducer and activator of transcription 5B	Stat5b	NM_001113563.2	CAGGACAACAATGCCACAGC TTTGGCCGATCAGGAAACAC	172
	Protein tyrosine phosphatase, non-receptor type 2	Ptpn2	NM_008977.3	CGCTCTGGCACCTTCTCTCT GGAAAGGCAGGATCTCTCGA	283
	Protein tyrosine phosphatase non receptor type 1	Ptp1b	NM_011201.3	TGGCCACAGCAAGAAGAAAA GGAAAGGCAGGATCTCTCGA	151
	Suppressor of cytokine signaling 3	Socs3	NM_007707.3	GCGAGAAGATTCCGCTGGTA TACTGATCCAGGAACTCCCGA	151
Reference genes	Ribosomal protein L37	Rpl37	NM_026069.3	CTGGTCGGATGAGGCACCTA AAGAACTGGATGCTGCGACA	108
	Eukaryotic translation initiation factor 5A	Eif5a	NM_001166594.1	CCTCAGCCACCTTCCCAAT AAATGTCAATGCCAACCAGATG	150

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 were normalized with β-actin (1:10000, MM, A2228, Sigma-Aldrich). All antibodies specifications are summarised 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 (1:20000, 31321, Thermo Scientific), RP anti-goat alkaline phosphatase-conjugated (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 in a Molecular Imager VersaDoc MP 4000 System (BioRad, Hercules, California, USA) and specific bands quantified using ImageLab Software (BioRad). Finally, band density for each protein was normalised against β-actin.

Immunohistochemistry and immunofluorescent staining

Ovaries collected from mice in oestrus stage (n=3/group) were fixed in 4% neutral phosphate-buffered formalin (NBF, 432173427, Poch, Gliwice, Poland) at 4°C for 24 h, and subsequently dehydrated in ethanol. Paraffin embedded ovarian tissues were sectioned into 5 μm slices. For antigen retrieval, sections were heated in citrate buffer (10 mM, pH=6.0). 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

Table 2. Specification of antibodies used for Western blot

Antibody name and specificity	Company, Cat no, RRID no	Antibody dilution
Mouse monoclonal against leptin receptor (ObR)	Santa Cruz Biotechnology Cat# sc-8391, RRID:AB_627882	1:500
Goat polyclonal against phosphorylated Tyr-985 of leptin receptor (p-ObR Tyr985)	Santa Cruz Biotechnology Cat# sc-16419, RRID:AB_2234640	1:500
Rabbit polyclonal against phosphorylated Tyr 1077 ObR (p-ObR Tyr1077)	Millipore Cat# 07-1317, RRID:AB_1977322	1:500
Goat polyclonal against phosphorylated Tyr 1138 ObR (p-ObR Tyr1138)	Santa Cruz Biotechnology Cat# sc-16421, RRID:AB_2288076	1:500
Rabbit polyclonal against Janus kinase 2 (Jak2)	Santa Cruz Biotechnology Cat# sc-294, RRID:AB_631854	1:200
Rabbit polyclonal against phosphorylated Tyr 1007/1008 Jak2 (pJak2)	Santa Cruz Biotechnology Cat# sc-16566-R, RRID:AB_653287	1:200
Rabbit polyclonal against Signal transducer and activator of transcription 3 (STAT3)	Santa Cruz Biotechnology Cat# sc-482, RRID:AB_632440	1:200
Mouse monoclonal against phosphorylated Tyr 705 Signal transducer and activator of transcription 3 (pSTAT3)	Santa Cruz Biotechnology Cat# sc-8059, RRID:AB_628292	1:200
Rabbit polyclonal against Signal transducer and activator of transcription 5 (STAT5)	Santa Cruz Biotechnology Cat# sc-835, RRID:AB_632446	1:200
Mouse monoclonal against phosphorylated Tyr 694/699 Signal transducer and activator of transcription 5 (pSTAT5)	Santa Cruz Biotechnology Cat# sc-81524, RRID:AB_1129712	1:200
Goat polyclonal against Protein tyrosine phosphatase 1B (PTP1B)	Santa Cruz Biotechnology Cat# sc-1718, RRID:AB_2174942	1:200
Mouse monoclonal against Suppressor of cytokine signalling 3 (SOCS3)	Santa Cruz Biotechnology Cat# sc-51699, RRID:AB_630243	1:500
Mouse monoclonal against β-actin	Sigma Aldrich Cat# A2228, RRID:AB_476697	1:10000

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 15 s incubation in 3,3-diaminobenzidine (DAB) peroxidase substrate solution (Rabbit-specific HRP/DAB (ABC) Detection IHC Kit, ab64261, Abcam). Subsequently, samples were counterstained with haematoxylin (MHS16, Sigma Aldrich) and mounted. Sections were examined using Axio Observer Systems Z1 microscope (Carl Zeiss Microscopy GmbH, Hannover, Germany) and Zeiss ZEN 2.5 lite Microscope Software (Carl Zeiss, Germany). For immunofluorescence (IF), 5 µm sections were deparaffinised and rehydrated in an ethanol series. Next, tissues were permeabilised in 0.3% Triton X-100 (T8787, Sigma Aldrich), followed by antigen retrieval in citrate buffer (10 mM, pH=6.0) for 40 min at 90°C and blocking in 2% BSA (A2153, Sigma Aldrich) with 0,3 M glycine (G8898, Sigma Aldrich) in PBS-0.1% Tween 20 (P7949, Sigma Aldrich) (PBST) solution. Sections were then incubated with 0.3% Sudan Black (199664, Sigma Aldrich) in 70% ethanol for 10 min at RT, followed by washes in PBST. Slides were subsequently incubated with RP anti-SOCS3 antibody (1:200, ab16030, Abcam) overnight at 4°C. The negative control sections were incubated with RP anti-IgG (1:200) as before, or without primary antibody. On the next day slides were washed in PBST, followed by incubation with cyanine 3 (Cy3)- donkey polyclonal anti-rabbit IgG (H+L) (711-165-152, Jackson ImmunoReserach, Cambridgeshire, UK), and a series of washes in PBST. Finally, slides were covered with a drop of Prolong Gold medium with diamidino-2-phenylindole (DAPI) and sealed with cover slips. Images were captured using 40x/1.2A or 63x/1.4A oil immersion objectives on a LSM800 confocal microscope (Carl Zeiss, Germany).

Enzyme-linked immunosorbent assay

Animals in oestrus were sacrificed (n=8/group) and blood samples collected after puncturing the heart. Blood samples were centrifuged (1800 g, 4°C, 10 min) and plasma stored at -80°C. Levels of circulating leptin and insulin were assessed with enzyme-linked immunosorbent assay (ELISA) kit, according to the manufacturer's instructions (Mouse Leptin ELISA Kit, 90030; Crystal Chem, Zaandam, Netherlands; Rat/Mouse Insulin ELISA Kit, EZRMI-13K; Merck Millipore). The intra- and interassay coefficients of variation (CVs) were as follows: for Leptin ELISA kit <10% both and for Insulin ELISA kit 8.35% and 17.9%, respectively. To determine SOCS3 in ovarian extracts, ELISA test was used (ELISA KIT for SOCS3; SEB684Mu, Cloud- Clone, Texas, USA). Briefly, the tissue was minced in lysis buffer (n=8/group), centrifuged (10000 g, 4°C, 5 min) and protein concentration in the lysate determined with BCA test. All tests and assessments were performed according to the manufacturer's instructions.

RNA-seq library generation

Once the 16 wk HFD group presented divergence in BW gain, we identified 3 animals with less than 33 g of body weight that we designated as HFD low gainers (HFDLG) and excluded them from the regular HFD group for the further description of differently expressed genes (DEGs) between CD and HFD. CCs were collected into RLT buffer (1053393, Qiagen, Hilden, Germany) and kept at -80°C until library generation. Subsequently, RNA sequencing (RNA-seq) libraries were prepared following a previously described protocol [31, 32], with minor changes. Briefly, mRNA was captured using Smart-seq2 oligo-dT pre-annealed to magnetic beads (MyOne C1, Invitrogen, Carlsbad, California, USA). The beads were resuspended in 10 µl of reverse transcriptase mix (100 U, SuperScript II, Invitrogen; 10 U, RNAsin, Promega, Madison, Wisconsin, USA), 1 × Superscript II First-Strand Buffer, 2.5 mM ditiotreitol (DTT, Invitrogen), 1 M betaine (Sigma Aldrich), 9 mM magnesium chloride ($MgCl_2$, Invitrogen), 1 µM Template-Switching Oligo (Exiqon, Vedbaek, Denmark), 1 mM deoxyribonucleotide triphosphate (dNTP) mix (Roche) and incubated for 60 min at 42°C followed by 30 min at 50°C and 10 min at 60°C [31, 32]. Amplification of the cDNA was then undertaken after adding 11 µl of 2 × KAPA HiFi HotStart ReadyMix and 1 µl of 2 µM ISPCR primer [31, 32], followed by the cycle: 98°C for 3 min, then 9 cycles of 98°C for 15 s, 67°C for 20 s, 72°C for 6 min and finally 72°C for 5 min. Finally, the cDNA was purified using a similar volume of AMPure beads (Beckman Coulter, Brea, California, USA) and eluted into 20 µl of nuclease- free water (P1195; Promega). All libraries were prepared from 100 to 200 pg of cDNA using the Nextera XT Kit (Illumina, San Diego, California, USA), according to the manufacturer's instructions. The final cDNA libraries were purified using a 0.7:1 volumetric ratio of AMPure beads before pooling and sequencing on an Illumina Nextseq500 instrument in 75-base pair (bp) single-read high output mode at the Babraham Institute Sequencing Facility. A total of 5-10 million mappable reads per sample were obtained, with an average of 30-50 million reads per condition.

Library mapping and trimming

Trim Galore v0.4.2 was used with default parameters on raw Fastq sequence files. Mapping of the RNA-seq data was done with Hisat v2.0.5 against the mouse GRCm38 genome, as guided by known splice sites taken from Ensemble v68.

RNA-seq differential expression analysis

Mapped RNA-seq reads were quantified and analysed using SeqMonk version v1.45.4 (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Differential expression analysis was performed using DESeq2 [33] implemented in SeqMonk setting a false discovery rate (FDR) < 0.05.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0. The D'Agostino-Pearson omnibus normality test was performed followed by nonparametric Mann-Whitney test or multiple unpaired t-test with statistical significance determined using the Bonferroni- Sidak method, depending on the experiment. The data are shown as the mean ± SD of three or more independent replicates. Significance was defined as values of $p < 0.05$.

Statement of Ethics

All experiments were approved by Local Committee for the Ethical Treatment of Experimental Animals of Warmia- Mazury University (Agreement No. 80/2015, 15/2018, 38/2018), Olsztyn, Poland and were performed accordingly to the Guide for Care and Use of Laboratory Animals, endorsed by European legislation.

Results

Leptin signalling is impaired in the ovary of diet-induced obese mice

Initially we sought to characterise changes in leptin signalling in the ovary throughout DIO. Thus, mice were subjected to HFD for 4 and 16 wk and whole ovaries and TC fraction were collected for mRNA or protein analysis (Fig. 1A). Oestrous stage was followed for twelve consecutive days, confirming that samples were collected in oestrus (Supplementary Fig. 1J, 1K) in cycling animals. Mice significantly gained BW and FM already at 4 wk, with an average absolute gain in BW of 13 grams (g) in the 16 wk HFD group (Supplementary Fig. 1A; $p<0.0001$). Three animals with comparable FI but BW gain less than 13 g were excluded from the statistical analysis and designated as HFD-low gainers (HFDLG). Also, after 4 and 16 wk HFD we confirmed high plasma levels of insulin (Supplementary Fig. 1F; $p<0.01$, $p<0.001$ respectively) and leptin (Supplementary Fig. 1G; $p<0.01$, $p<0.001$ respectively) and the establishment of impaired glucose tolerance and insulin resistance at 16 wk HFD (Supplementary Fig. 1H, 1I; $p<0.01$, $p<0.001$ respectively). Monitoring oestrous cycle revealed that the 4 wk HFD group had higher prevalence of oestrus counts compared with controls fed CD, whereas in the 16 wk HFD group there was a reduction in pro-oestrus (Supplementary Fig. 1J, 1K; $p<0.05$, $p<0.01$ respectively).

Next, we isolated protein from whole ovaries and studied the abundance of components of the leptin signalling pathway. Whilst we found initial hyperactivation of leptin signalling pathway as demonstrated by upregulation of SOCS3 protein (Fig. 1B; Supplementary Fig. 3A; $p<0.05$ both) and a tendency to increased phosphorylation of STAT3 (Fig. 1C; $p=0.06$), after 16 wk HFD local leptin resistance was clearly established. This was evidenced by the decrease in abundance of leptin receptor (Fig. 1D; $p<0.01$), and a trend towards decreased phosphorylation of pTyr985 ObRb (Fig. 1E; $p=0.09$) and decreased phosphorylation of JAK2 (Fig. 1F; $p<0.05$), along with upregulation of SOCS3 (Fig. 1B; $p<0.001$, Supplementary Fig. 3A; $p<0.05$). In contrast, no differences were found in phosphorylation of other Tyr residues of ObRb (Supplementary Fig. 3B and 3C) or in PTP1B expression (Supplementary Fig. 3D). Additionally, there was reduced phosphorylation of STAT5 after 4 and 16 wk HFD (Fig. 1G; both $p<0.01$).

Next, we sought to characterise the extent to which various ovarian components responded similarly to increased circulating leptin during obesity. We performed real-time PCR analysis of whole ovaries and TC fraction. Despite no significant changes after 4 wk HFD, the mRNA of *Socs3* was increased after 16 wk HFD in both whole ovary (Fig. 1H; $p<0.05$) and TC (Fig. 1H; $p<0.001$), in comparison to the CD group. Additionally, the mRNA level of *Ptp1b* was increased in both whole ovary and TC after 16 wk HFD (Fig. 1H; $p<0.01$, $p<0.001$ respectively).

The aforementioned results suggested that SOCS3 could be an important player in the establishment of leptin resistance in the ovary of obese mice. Therefore, we examined SOCS3 localisation in ovaries of DIO and the genetically obese model: mice with a mutation in the obese gene *(ob/ob)*. Immunohistochemistry (IHC) revealed the presence of SOCS3 protein in oocytes from follicles in all developmental stages (Fig. 1K-N; Supplementary Fig. 4E, 4F, 4I, 4J); in addition, theca cells and GC from the respective follicles were stained, as well as the ovarian stroma (Fig. 1K-N). Importantly, we compared the IHC staining of SOCS3 and PTP1B in 16 wk HFD ovaries, which suggested that SOCS3 is the major ObRb inhibitor being expressed in the oocyte and GC, as PTP1B protein presented almost no staining in the oocyte (Supplementary Fig. 4G, 4H, 4K, 4L). As a control for the specificity of the IHC

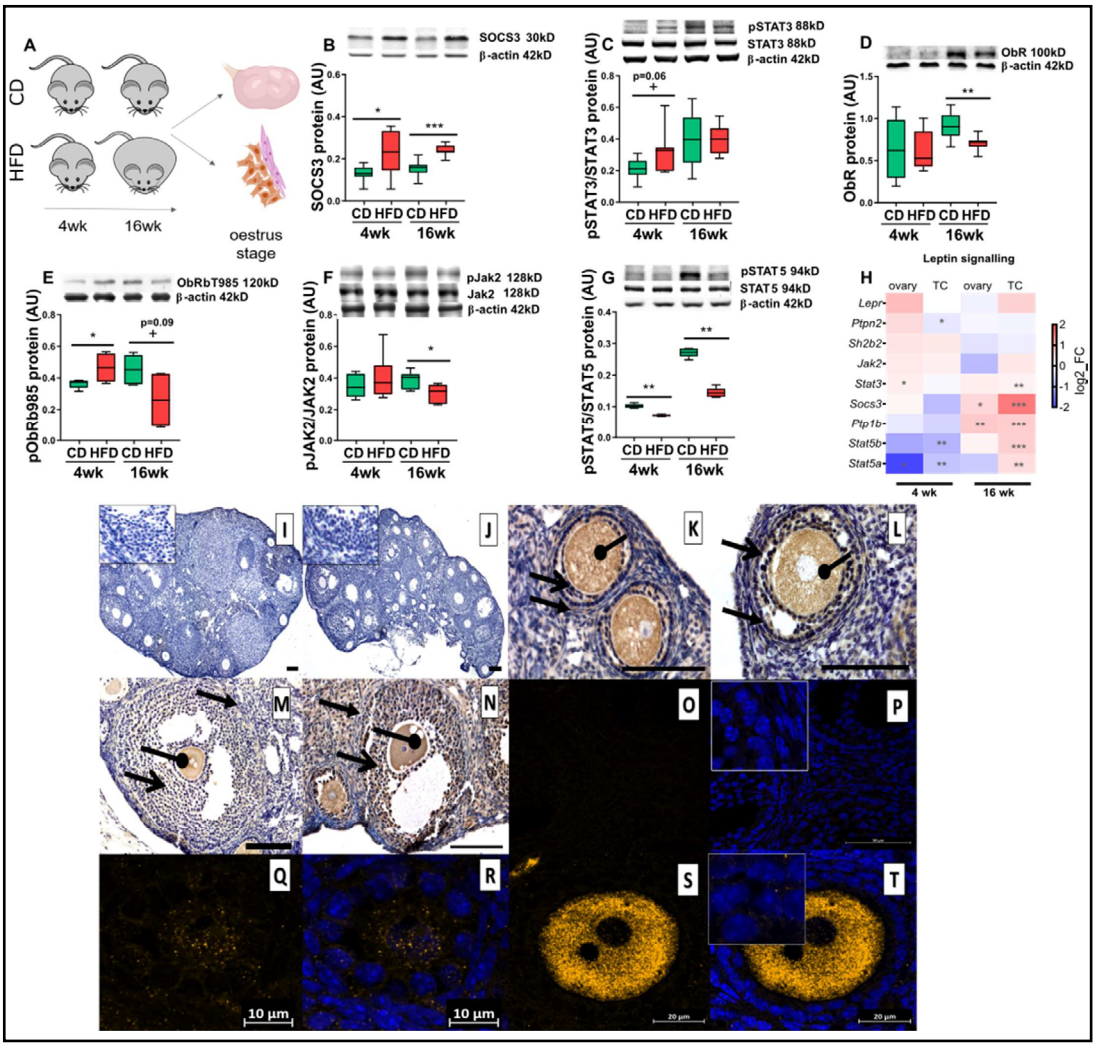

Fig. 1. The establishment of leptin resistance in the ovary of diet induced obese mice. (A) Experimental design: animals were maintained on chow diet (CD) or high fat diet (HFD) for 4 weeks (wk) or 16 wk. Protein abundance of components of the leptin signalling pathway in ovarian extracts analysed by 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 µm. The staining was confirmed by immunofluorescent localisation of SOCS3 (Q-T). Positive staining in orange, 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 are representatives of 3 biological replicates. Inserts in left top corners are magnifications of granulosa cells. mRNA expression of Rpl37 and protein expression of β-actin were used to normalize the expression data. Each bar represents the mean ± SD. Differences between groups were analysed by Mann-Whitney test. N=4-8 for immunoblots and N=8 for RT-PCR analysis. * $p<0.05$; ** $p<0.01$; ***$p<0.001$; + p=0.06 or p=0.09.

data, we used confocal microscopy for immunofluorescence detection of SOCS3 on sections from DIO and leptin-deficient *ob/ob* (-/-) ovaries, confirming the localisation of SOCS3 (Fig. 1Q-T) and observing a weaker intensity of SOCS3 in both oocyte and GC in ovaries from *ob/ob* (Supplementary Fig. 4T, 4V) compared to wild type (+/+) (Supplementary Fig. 4S, 4U). These IF results confirmed the specificity of the staining, since SOCS3 is expected to be less abundant in tissues form 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 profile, and found that principal component 1 (PC1) was mainly driven by BW (Fig. 2B). Here 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, on the global gene expression profile of CCs.

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 (Supplementary Fig. 1A). After DESeq2 analysis (FDR <0.05), a total of 997 DEGs in 4 wk HFD (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) were identified. Surprisingly, amongst the DEGs only 52 genes were common between the 4 wk and 16 wk comparisons (Fig. 2F), highlighting the differences in pathophysiology of early and late stages of obesity. Gene ontology (GO) [35, 36] analysis of the DEG lists showed that transcripts with increased abundance in 4 wk HFD were primarily linked to nitrogen and lipid metabolism and transport, but also cell stress and reactive oxygen species generation (Supplementary Table 4). Transcripts downregulated after 4 wk HFD were mapped to pathways involved in regulation of macromolecule biosynthesis and gene expression, as well as chromatin organisation/histone modification and regulation of cell cycle (Supplementary Fig. 5A; Supplementary Table 4). After 16 wk HFD treatment, upregulated genes were associated with negative regulation of development and cellular component organisation, while pathways highlighted for downregulated genes included localisation, transport and positive regulation of metabolism (Supplementary Fig. 5B; Supplementary Table 5). Therefore, in this analysis we identified the gene signatures in CCs altered at the onset and later development of DIO.

Finally, we wished to examine the impact of BW as a factor on gene expression in CCs. Therefore, we examined the expression of the 846 DEGs identified in 16 wk HFD group in the 16 wk HFDLG and CD samples. Strikingly, for this set of genes, the HFDLGs presented an expression pattern closer to 16 wk CD than to 16 wk HFD (Fig. 2G), revealing a very strong correlation between BW and global gene expression profile in CCs indicative of the impact of female physiology on CCs gene expression. As CCs represent an important accessible source of biomarkers for the assessment of reproductive potential of the mother, they are often sampled in assisted reproductive technologies (ART) to profile biomarkers of oocyte competence or embryo quality. Thus, we looked for known markers of embryo quality [25] in

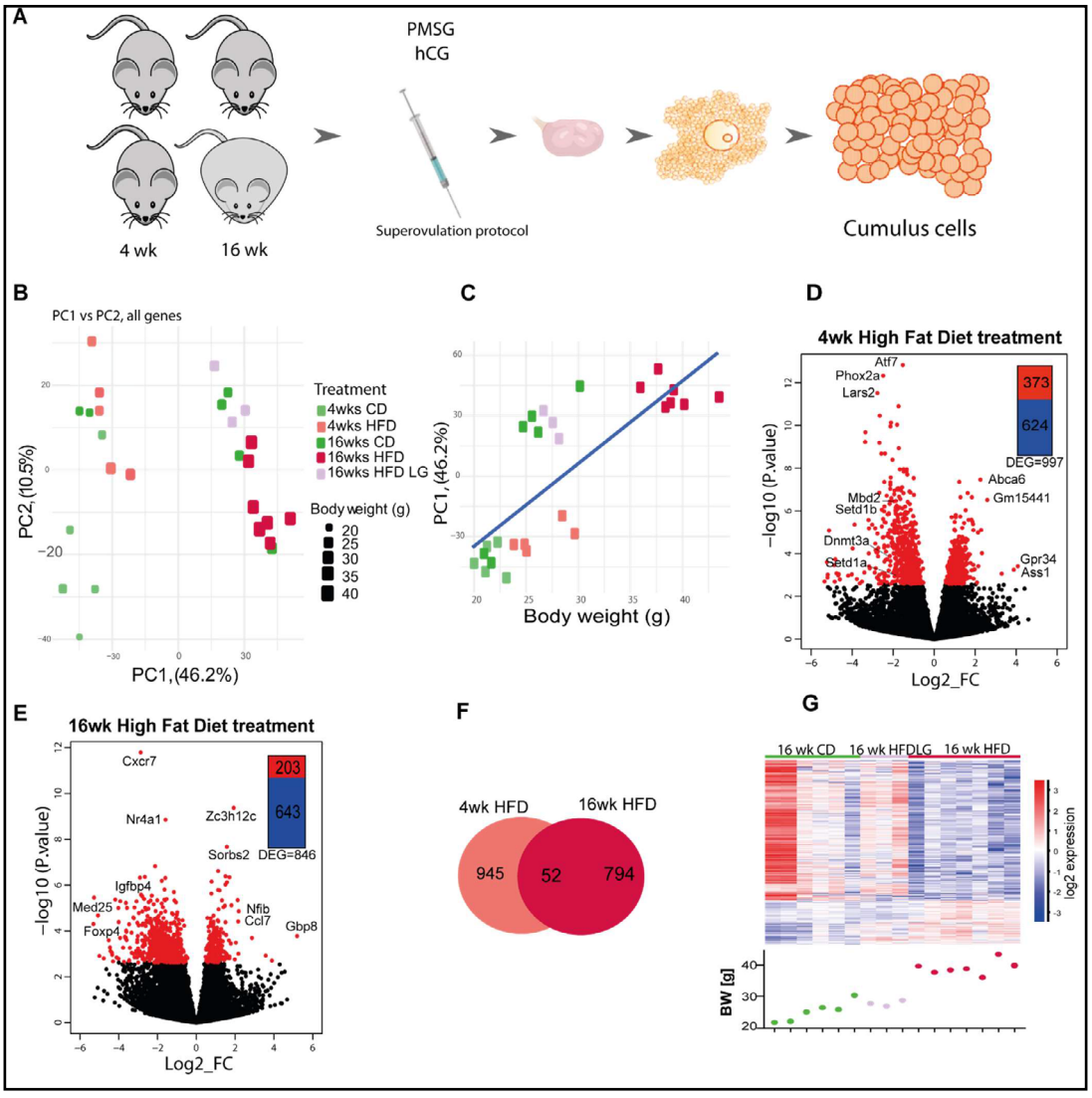

Fig. 2. Cumulus cell transcriptome analysis in diet induced-obese mice reveals strong correlation with body weight. (A) Experimental design: mice were subjected to the indicated dietary protocol, superovulated and cumulus cells were collected from cumulus-oophorus-complexes. RNA-seq analysis of gene expression in cumulus cells obtained from mice after 4 or 16 weeks (wk) on chow diet (CD), high fat diet (HFD) or low gainers on HFD (HFDLG). N= 3-7 mice per group. (B) Principal component analysis of global transcriptome shows samples cluster into 2 groups accordingly to their body weight (BW). (C) Correlation of Principal Component 1 (PC1) with BW; r=0.777, p=3.026e-06. Volcano plots showing distribution of differentially expressed genes in (D) 4 wk HFD and (E) 16 wk HFD; genes with false discovery rate (FDR) <0.05 colored red. (F) Venn diagram showing the number of genes differentially expressed at false discovery rate <0.05 between 4 wk and 16 wk groups. (G) Heatmap of 846 DEGs identified by DESeq2 analysis between 16 wk CD and HFD CCs, including data for HFDLG CC samples, with BW of mice at time of collection plotted below. Heatmap representing fold of change of gene expression. log2_FC of reads per million (RPM).

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].

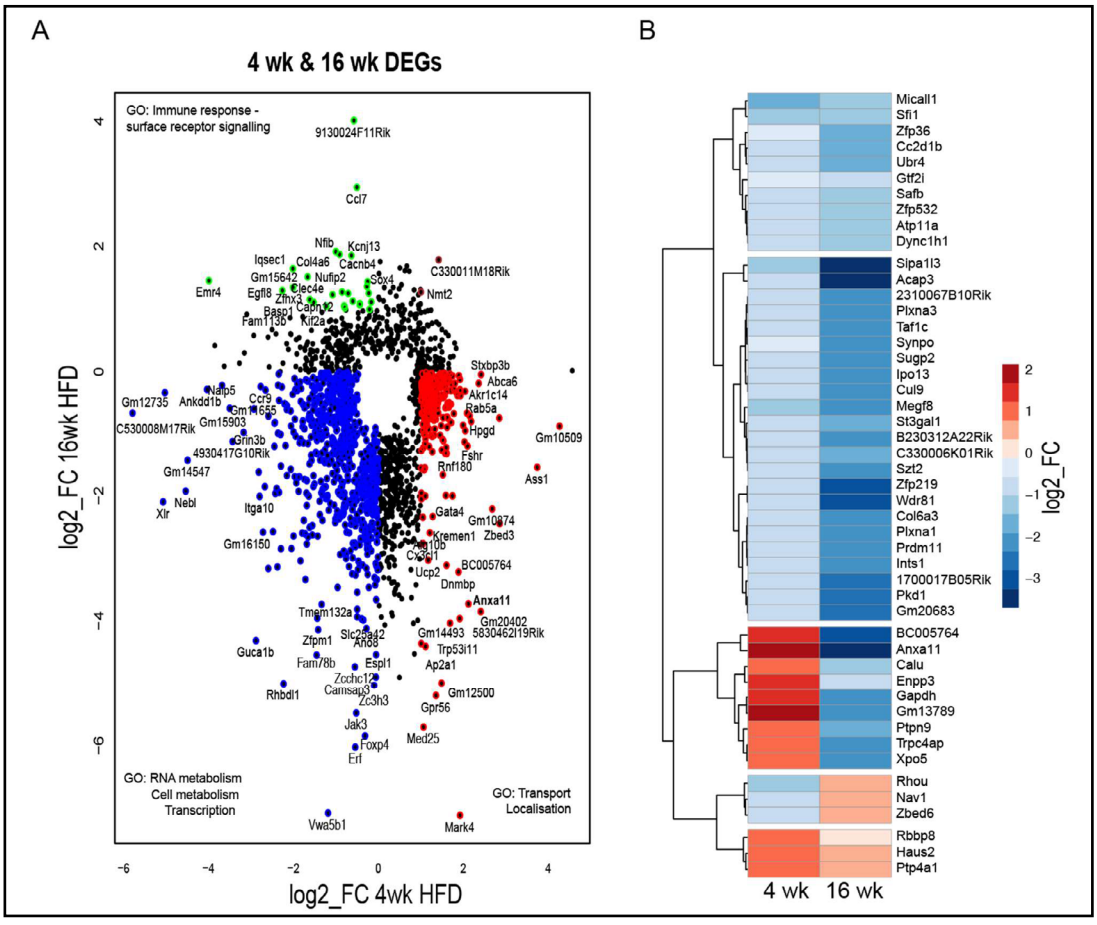

Fig. 3. Temporal changes in the transcriptome of cumulus cells during obesity progression. DESeq2 analysis of transcriptome data in cumulus cells (CC) of mice fed high fat diet (HFD) for 4 or 16 weeks (wk). N= 3-7 mice per group. (A) Scatter plot presents genes differentially expressed in at least one condition (false discovery rate <0.05) in CC after 4 wk (997 genes) or 16 wk HFD (846 genes). Genes coloured blue are downregulated after both 4 wk and 16 wk HFD; green downregulated after 4 wk HFD and upregulated after 16 wk HFD; brown upregulated after both 4 wk HFD and after 16 wk; red upregulated after 4 wk HFD and downregulated after 16 wk HFD. (B) Heatmap presents hierarchical clustering of genes significantly changed in both 4 and 16 wk HFD normalised by average control fed chow diet. Genes cluster in group downregulated after 4 wk and 16 wk HFD, group upregulated after 4 wk and downregulated after 16 wk HFD, group downregulated after 4 wk and upregulated after 16 wk HFD and group upregulated after both 4 wk and 16 wk HFD. Gene ontology analysis performed with Gene Ontology Enrichment Analysis and Visualisation Tool. log2_FC of reads per million (RPM).

Differential effects on gene expression in CCs early and late in obesity

We next sought to characterise how gene expression in CCs changes between the early and late stages of DIO. To do this, we evaluated the expression of the 997 DEGs from 4 wk and the 846 DEGs from 16 wk HFD at both time-points (Fig. 3A). Note that in this analysis, not all the DEGs attain a significant difference at both time points (as noted above in Fig. 2F), but we are aiming to identify the directionality of their expression changes throughout obesity. Only 3 DEGs were upregulated in both conditions (Fig. 3A), whereas we found 252 genes upregulated in 4 wk HFD but downregulated in 16 wk HFD, mainly linked to cell transport and localisation. Conversely, the 30 genes downregulated in 4 wk HFD, but upregulated in 16 wk HFD referred to immune response (Fig. 3A). Finally, a sum of 694 DEGs were downregulated in both 4 and 16 wk HFD, mainly involved in metabolism and transcription (Fig. 3A; Supplementary Table 6). This analysis revealed a large subset

of genes being downregulated throughout obesity, but also genes with opposite profile 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 the 52 DEGs in common between the two timepoints (Fig. 2F) identified 5 main clusters of DEGs. The first 2 clusters comprised 33 genes downregulated in both 4 wk and 16 wk HFD (Supplementary Table 6), with the most significantly deregulated gene at 4 wk HFD *MICAL Like* (*Micall) 1* (FDR = 0.0002), but other genes like *Dynein cytoplasmic 1 heavy chain* (*Dync1h*) or *Collagen* (*Col*) *6a3* (Fig. 3B; Supplementary Table 6) were also found. Clusters 3 and 4 revealed the most interesting set of genes concerning disease progression, due to their opposite profile between 4 wk and 16 wk treatment. In cluster 3 we found genes like *Annexin* (*Anxa) 11* or *Exportin* (*Xpo*) *5* strongly upregulated in 4 wk HFD, but inhibited in 16 wk HFD (Fig. 3B), whereas in cluster 4 we found genes like *Ras homology family member U* (*Rhou)* with opposing profiles at the two time points (Fig. 3B). Finally, cluster 5 comprised the 3 genes significantly upregulated throughout obesity (Fig. 3B). Therefore, in this analysis we identified gene expression profiles in CCs that represent a valuable tool to assess disease progression in the ovary of obese mice.

The contribution of leptin to changes in gene expression in CCs from obese mice

After identifying the major molecular changes in leptin signalling in the ovaries of DIO females, we aimed to establish an *in vivo* system that would expose the ovaries to the elevated levels of circulating leptin, a feature seen in obesity [7], but lacking all remaining traits of obesity. Thus, we conceived a model for pharmacological hyperleptinemia. Sixteen days of leptin treatment resulted in a consistent drop in BW and FM (Supplementary Fig. 2B, 2E; $p<0.01$) and increased incidence of oestrus (Supplementary Fig. 2G; $p<0.05$). The ovaries from animals in oestrus stage were collected for mRNA and protein analysis. Whereas injections of 100 µg leptin for 9 days did not change the protein expression of leptin signalling molecules (Supplementary Fig. 7A-J), ObR expression and phosphorylation of Tyr985 and STAT5 were decreased after 16 d (Supplementary Fig. 7A, 7B, 7G; $p<0.05$, $p=0.09$, $p<0.01$ respectively), together with increased SOCS3 (Supplementary Fig. 7I, 7J; $p<0.01$, $p<0.05$ respectively). Validation of a pharmacological hyperleptinemia model allowed us to access ovarian samples from mice with hyperactivation of ObRb, here indicated by increased SOCS3 expression, but lacking the remaining traits of obesity.

Next, we collected CCs from superovulated mice after LEPT treatment and analysed their transcriptome (Fig. 4A). After DESeq2 analysis (FDR <0.05), a total of 2026 differently expressed genes were found between LEPT and CONT samples (1212 genes upregulated and 814 downregulated) (Fig. 4B). Gene ontology analysis of the DEG lists showed that the upregulated genes for LEPT were associated primarily with cellular organisation, the cytoskeleton and immune responses, supporting the immune-mediating role of leptin as evidenced before [40–42]. Conversely, amongst the LEPT downregulated pathways were cell metabolism as well as chromatin organisation and histone modifications (Supplementary Fig. 8; Supplementary Table 7). Next, based on the hypothesis that early-onset obesity is followed by hyperactivation of leptin signalling in the ovary, we overlapped both 4 wk DIO model and LEPT transcriptome datasets, aiming to pinpoint the LEPT driven effects in the CC transcriptome during early obesity. PCA revealed the clustering of 4 wk DIO and CONT samples, and LEPT samples apart (Fig. 4C). Indeed, leptin treatment seemed to drive PC1. Next, we overlapped the DEGs from 4 wk HFD and LEPT protocols and found 144 genes upregulated in both LEPT and 4 wk HFD. These were related to response to toxins, transport and glucose metabolism (Fig. 4D; Supplementary Table 8). More specifically, the genes *Lipocalin* (*Lcn*) *2, Anxa11* and *Glucose-6-phosphate dehydrogenase x-linked* (*G6pdx*) were amongst the most significant (Fig. 4D). Conversely, the GO terms associated with the 177 downregulated genes in both protocols were metabolism and gene expression regulation (Fig. 4D; Supplementary Table 8). A number of downregulated genes were found to encode important epigenetic factors, such as *Dna segment, chr 14, abbott 1 expressed o* (*Tasor*), *Lysine (k)-specific methyltransferase 2d* (*Kmt2d/Mll2*), *Methyl-cpg binding domain protein* (*Mbd*) *2,*

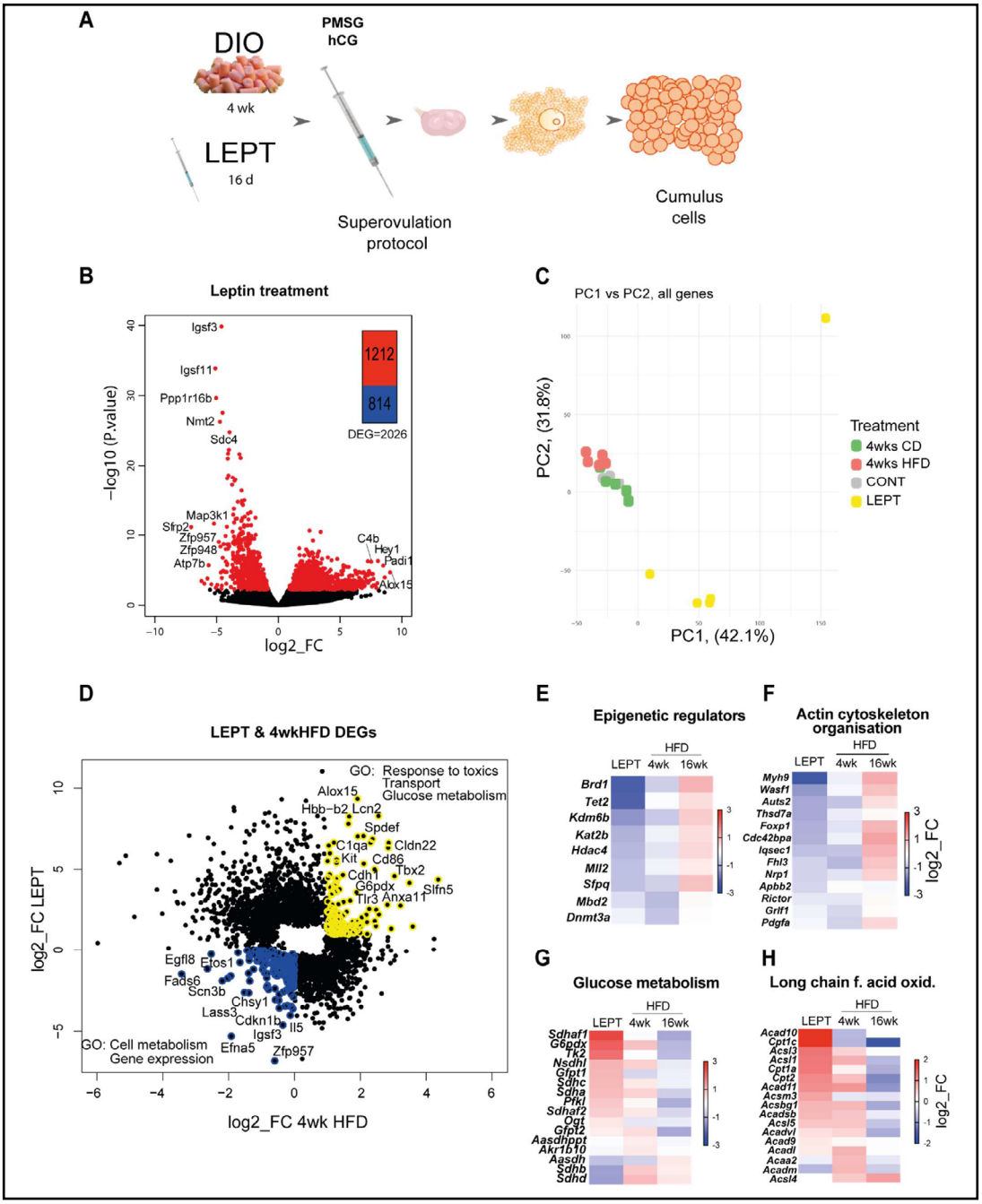

Fig. 4. Pharmacologically hyperleptinemic mouse model shows leptin effects in the transcriptome of cumulus cells during early obesity. (A) Experimental design: mice were fed chow diet (CD) of high fat diet (HFD) for 4 weeks (wk) (4 wk DIO) or injected with saline (CONT) or 100 µg of leptin (LEPT) for 16 days, followed by superovulation and collection of cumulus cells from cumulus-oophorus-complexes. RNA-seq analysis of gene expression in cumulus cells. N=3-7 mice per group. (B) Volcano plots showing distribution of differentially expressed genes in LEPT group; genes with False Discovery Rate <0.05 coloured red. (C) Principal component analysis of global transcriptome shows LEPT effect is the main source of variance in the data (first principal component, PC1). DESeq2 analysis of transcriptome data in cumulus cells. (D) Scatter plot presents genes differentially expressed in cumulus cells in LEPT or in 4 wk HFD, with False Discovery Rate <0.05. Those coloured blue are down-regulated both in response to leptin treatment and 4 wk HFD; those in yellow upregulated by both treatments. 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 Enrichment Analysis and Visualisation Tool. log2_FC of reads per million (RPM).

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 metabolism, and verified the role of leptin on glucose metabolism (Fig. 4G) and fatty acid oxidation (Fig. 4H), which was reflected in the similarities between LEPT and 4 wk HFD. 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.

Discussion

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 found in kidney [51] or heart [52] of obese humans, suggesting an organ-specific response. We confirmed here, for the first time, the establishment of leptin resistance in the ovary of obese female mice. This poses very important questions on the long-term effects of obesity on ovarian performance, concerning the known local roles of leptin on follicular growth [12], ovulation [13], and oocyte quality [53]. Leptin action in the ovary is highly intricate and its effects bimodal. Low leptin levels in circulation facilitate the transition from primary to secondary follicles [12], but leptin is also required for ovulation, possibly supporting CC expansion through cyclooxygenase (COX) 2 and hyaluronic acid synthase (HAS) 2 activity [13]. Indeed, *ob/ob* mice contain antral follicles in their ovaries (data not shown), but fail to ovulate. Thus, during obesity progression, altered leptin signalling in the ovary could lead to functional failure and infertility through different mechanisms, mainly characterised by the hyperactivation of ObRb in the onset of obesity and complete failure in signalling in late obesity.

Our first aim was to elucidate the molecular mechanisms leading to the establishment of leptin resistance in the obese ovary. The analysis of different ObRb Tyr domains highlighted the decrease in pTyr985 along with pJAK2 in ovaries from 16 wk HFD mice, concomitant with the increase in SOCS3 protein and decrease in pSTAT5. Functionally, STAT5 phosphorylation in the mouse ovary was shown to be crucial for prolactin signalling and cell proliferation during follicular growth [54], as well as corpus luteum formation [55]. Hence, reduced

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 was the striking correlation between BW and the global gene expression profile of CCs. On the other hand, other studies showed functional changes in the ovary, including depletion of primordial follicles and inflammation in HFD mice, irrespective of gain in BW [56]. Differences 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 analysis. Interestingly, when found that the expression profile of HFD-DEGs in the HFDLG CCs was similar to that in 16 wk CD, clearly demonstrating the impact of maternal BW, which probably largely reflects adiposity in this model, on gene expression in CCs.

Another major outcome of the transcriptome analysis of CCs was the identification of gene signatures altered in early vs late stages of obesity. After 4 wk HFD, mainly genes involved in glucose metabolism and cell membrane trafficking were differently expressed. The use of the pharmacologically hyperleptinemic model allowed us to dissect the contribution of hyperactivation of ObRb to the major changes taking place in CCs in early obesity. Increased activation of the JAK-STAT cascade seemed mainly to impair cellular trafficking and paracrine transfer of macromolecules. This is known to be a crucial process for the metabolic cooperation between the oocyte and somatic cells [23]. Cell trafficking and 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], *Claudine (Cldn) 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.

We also identified the metabolic gene *Arachidonate 15-Lipoxygenase* (*Alox15)*, and the transcription factor *Hes Related Family BHLH Transcription Factor With YRPW Motif* (*Hey) 1*, were amongst the most significantly upregulated genes in both 4 wk HFD and 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 detrimental role of increased ObRb activation during the onset of obesity in cell trafficking and immune response.

Amongst the downregulated signatures in both 4 wk HFD and LEPT we found genes that encoded for important epigenetic factors, such as *Tasor*, *Kmt2d*/*Mll2*, *Mbd2,* and *Dnmt3a* (Fig. 4D, Supplementary Table 8), which could indicate epigenetic dysregulation in these cells in early obesity being mediated by leptin. Another striking result was the coordinate downregulation of genes involved in cytoskeleton and actin-filament organisation again in 4 wk HFD and LEPT. As in axons, microtubules form the cytoskeletal core of granulosa cell transzonal projections (TZPs), which provide tracks for the polarized translocation of secretory pathway organelles [63]. Thus, by impairing the intrinsic stability of TZPs in granulosa cells, leptin could be affecting the paracrine exchanges between oocytes and

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 actually have beneficial effects, following the positive response on oocyte competence and GDF9 signalling (Supplementary Fig. 9D). However, at 16 wk HFD the inferred drop in CC metabolic fitness was paralleled by a decrease in the main paracrine mediators of oocyte maturation and responsiveness to GDF9 (Supplementary Fig. 9B-D), which invariably suggest 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 being highlighted in our transcriptome analysis, the role of leptin in the inflammatory 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.

The analysis of 16 wk HFD DEGs, as well as the profile of temporal changes revealed genes involved in cell trafficking as *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 regulates cell morphology [68]. Considering also the high expression level of inflammatory 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 regulation are followed by mounting inflammatory pathways and cellular rearrangement to accommodate the lipid surplus. Functional studies in CCs with variable levels of leptin and ObRb activity are needed to confirm the present observations.

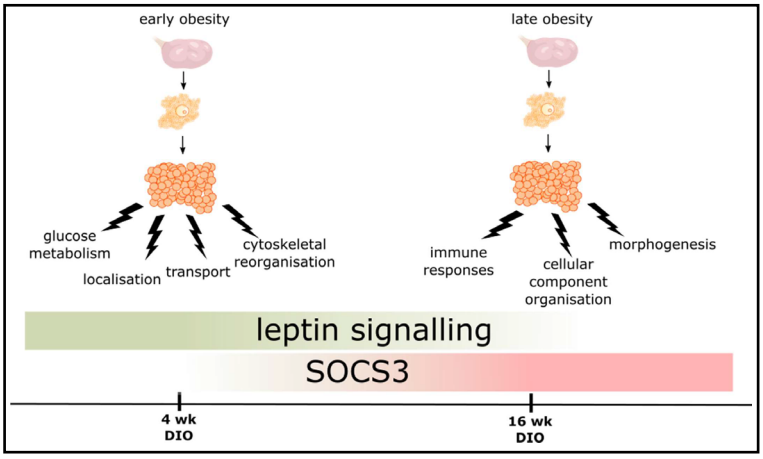

Fig. 5. Graphical representation of the main temporal changes in the ovary of obese mice. During early obesity (4 weeks of diet-induced obesity, DIO) increased leptin signalling affects the transcriptome of cumulus cells (CCs). RNA-seq analysis revealed mainly alterations in genes involved in membrane trafficking, 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 the activation of the inflammatory response and cellular anatomical morphogenesis, with inhibition of metabolism and transport.

Conclusion

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 linked to increased expression of genes for cell trafficking and cytoskeleton organisation, and inhibition of genes associated with epigenetic regulations in CCs. Conversely, in late obesity, altered gene signatures were mainly linked to inflammatory response and morphological rearrangement (Fig. 5). This analysis revealed for the first time the temporal changes in gene expression in CCs during obesity progression. The present study opens new avenues to better understand the impact of altered leptin signalling in obese ovary in both oocyte and granulosa cells function throughout its development, and potential implications for early embryo development.

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Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

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Authors Contribution

KW data acquisition, analysis and interpretation of the data, writing the manuscript; EW data acquisition, analysis and interpretation of the data; MA, data acquisition and analysis; JCF bioinformatic analysis and interpretation of data, revising the manuscript; GK supervision, funding, revising the manuscript; AG conception and design, funding acquisition, acquisition of data, bioinformatic analysis and interpretation of data, writing and revising of the manuscript.

Disclosure statement

The authors have no conflict of interest to declare.

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Supplemental Material

Leptin Resistance in the Ovary of Obese Mice is Associated with Profound Changes in the Transcriptome of Cumulus Cells

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Due to the size of the Supplementary Tables, please click on the following links to download and open the respective Excel-file.

Supplementary Table 1. Read Counts & BW.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 1.xlsx

Supplementary Table 2. BW & gene correlation.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 2.xlsx

Supplementary Table 3. DESeq values samples.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 3.xls

Supplementary Table 4. 4wks.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 4.xlsx

Supplementary Table 5. 16wks.
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Supplementary Table 6. 4wk_16wk.
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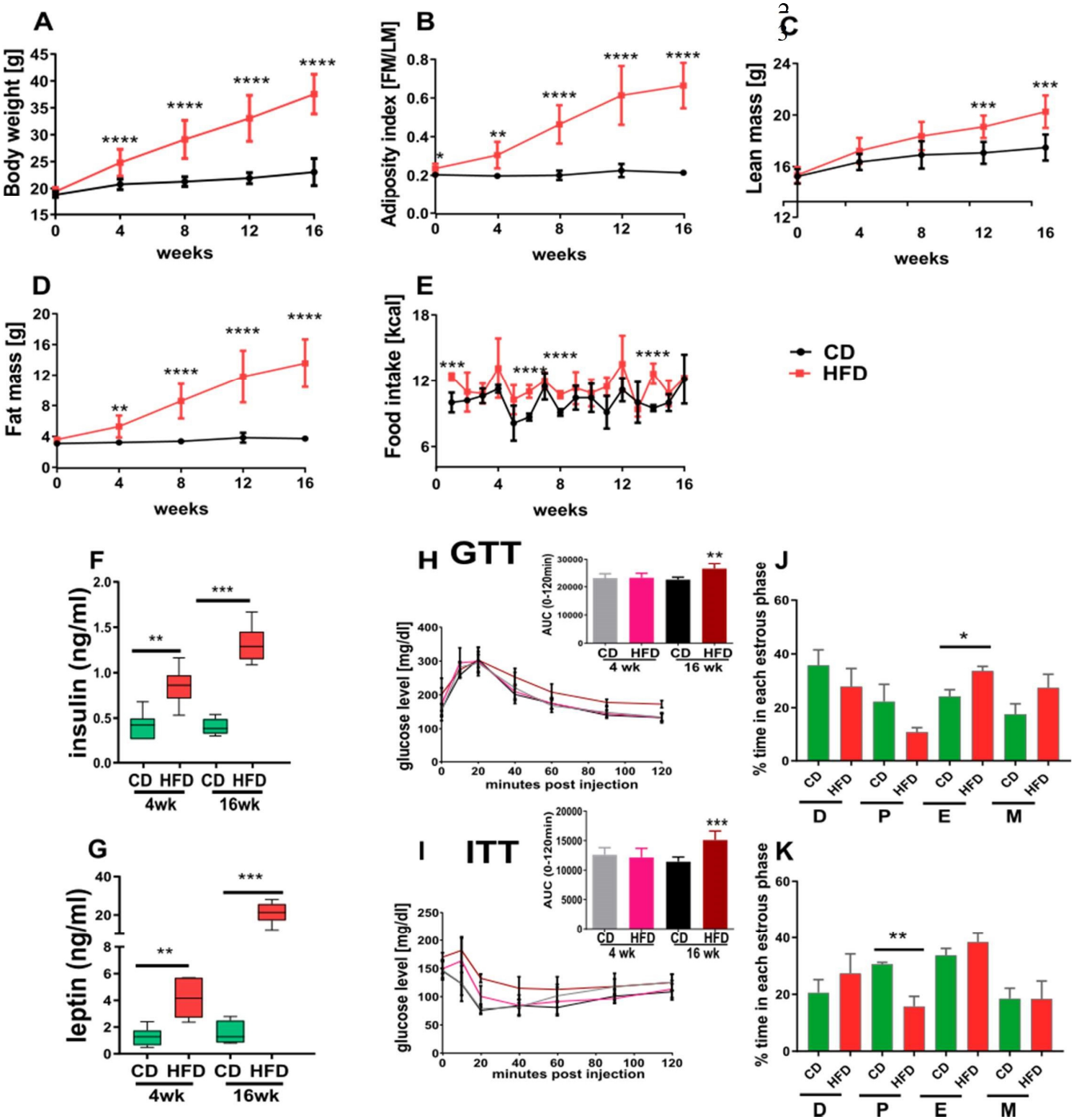
Supplementary Table 7. LEPT.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 7.xlsx

Supplementary Table 8. LEPT_4wks.
https://www.cellphysiolbiochem.com/Articles/000228/SM/Supplementary Table 8.xlsx

Supplementary Fig. 1. Phenotype characterisation of diet-induced obese mice.

Changes in (A) body weight, (B) adiposity index, (C) lean mass, (D) fat mass, (E) food intake in mice fed chow diet (CD, black line) and high fat diet (HFD, red line) for 4 or 16 weeks (wk). Plasma level of (F) insulin and (G) leptin in mice fed CD or HFD for 4 and 16 wk. Glucose tolerance test (GTT, H) and insulin tolerance test (ITT, I) present glucose levels at 0-120 min after glucose and insulin injection, respectively. Bar graphs in the upper right panel present area under the curve for each group. Grey, pink, black and brown line represent group maintained for 4 wk on CD, 4 wk on HFD, 16 wk on CD and 16 wk on HFD, respectively. Plasma collected from animals in oestrus phase. Proportion of time spent in each oestrous phase of mice subjected to CD or HFD for 4 wk (J) and 16 wk (K) monitored for 12 days. D, dioestrus; E, oestrus; M, metoestrus; P, pro-oestrus. Each bar represents the mean ± SD for n=12. Differences in phenotype characteristics and plasma hormone level between groups were analysed by Mann-Whitney test, oestrous cycle distribution analysed by unpaired t-test. * $p<0.05$; ** $p<0.01$; ***$p<0.001$.

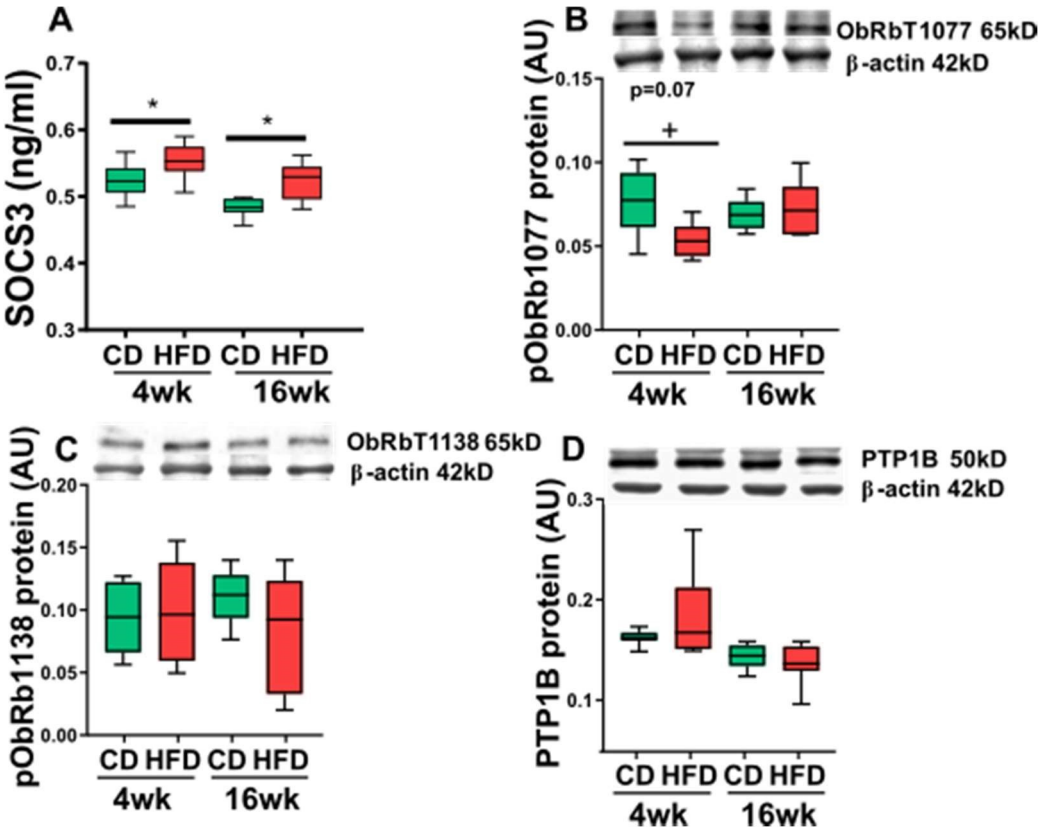
Supplementary Fig. 1.



Supplementary Fig. 2. Expression of leptin signalling components in the ovary of diet induced obese mice.

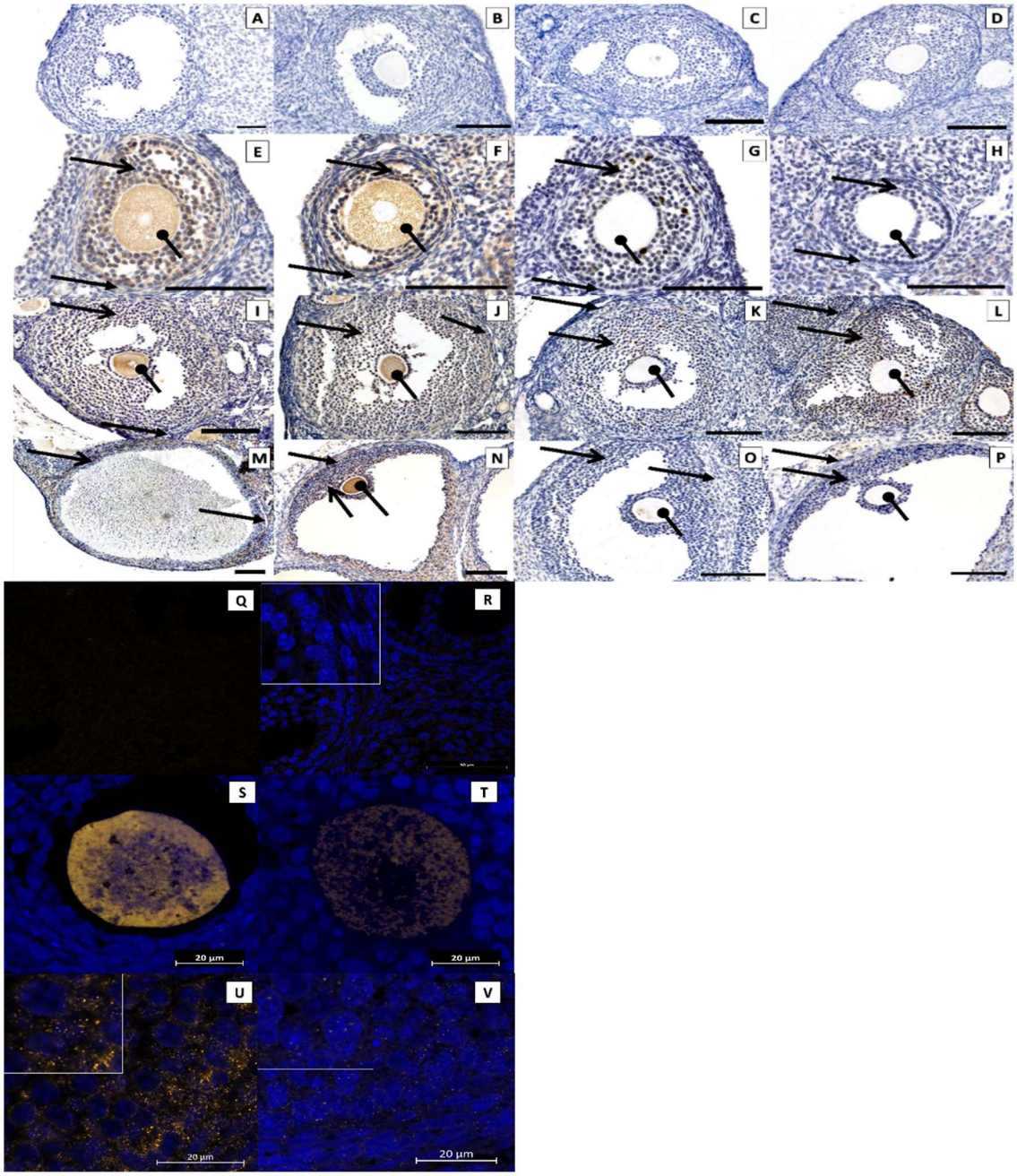
Protein abundance of components of the leptin signalling pathway in ovarian extracts analysed by Western blot or ELISA. Animals were maintained on chow diet (CD) or high fat diet (HFD) for 4 or 16 weeks (wk). (A) SOCS3 ovarian quantification in ELISA test. Phosphorylation of (B) tyrosine 1077 of leptin receptor, (C) tyrosine 1138 of leptin receptor, abundance of (D) PTP1B. Protein expression of β-actin were used to normalize the expression data. Each bar represents the mean ± SD. Differences between groups were analysed by Mann-Whitney test. N=4-8 for immunoblots and N=8 for ELISA. * $p<0.05$; ** $p<0.01$; ***$p<0.001$; + $p=0.07$.

Supplementary Fig. 2.



Supplementary Fig. 3. Immunolocalisation of SOCS3 and PTP1B protein in the ovary. Immunohistochemical localisation of SOCS3 and PTP1B protein during follicle development in mice fed chow diet (CD) or high fat diet (HFD) for 4 and 16 weeks (wk) and intraperitoneally injected with saline (C) or leptin (L) for 16 days (d). Positive staining in brown, counterstaining with heamatoxylin. Negative control stained with polyclonal rabbit IgG (A, B) 4 wk CD, (C, D) 4 wk HFD, localisation in secondary follicle SOCS3 (E) 4 wk CD and (F) 4 wk HFD, PTP1B (G) 4 wks CD, (H) 4 wks HFD, antral follicles SOCS3 (I) 16 wk CD, (J) 16 wk HFD, PTP1B (K) 16 wk CD, (L) 16 wk HFD, preovulatory follicle SOCS3 (M) 16 C, (N) 16 L, PTP1B (O) 16 C, (P) 16 L. The scale bar represents 100μm. The specificity of SOCS3 staining was confirmed by immunofluorescent localisation in *ob/ob* mice with genetic deficiency of leptin. Positive staining in orange, nuclear counterstaining with DAPI in blue. (Q-R) negative control 16 wk CD performed with polyclonal rabbit IgG, SOCS3 localised in (S, T) secondary follicle and (U,V) antral follicle from controls (*ob/ob.* +/+; S,U) and leptin deficient ovaries (*ob/ob* -/-; T,V). Images are representatives of 3 biological replicates. Inserts in left top corners are the amplifications of granulosa cells. Pictures are representatives of 3 biological replicates. The scale bar represents 20μm.

Supplementary Fig. 3.

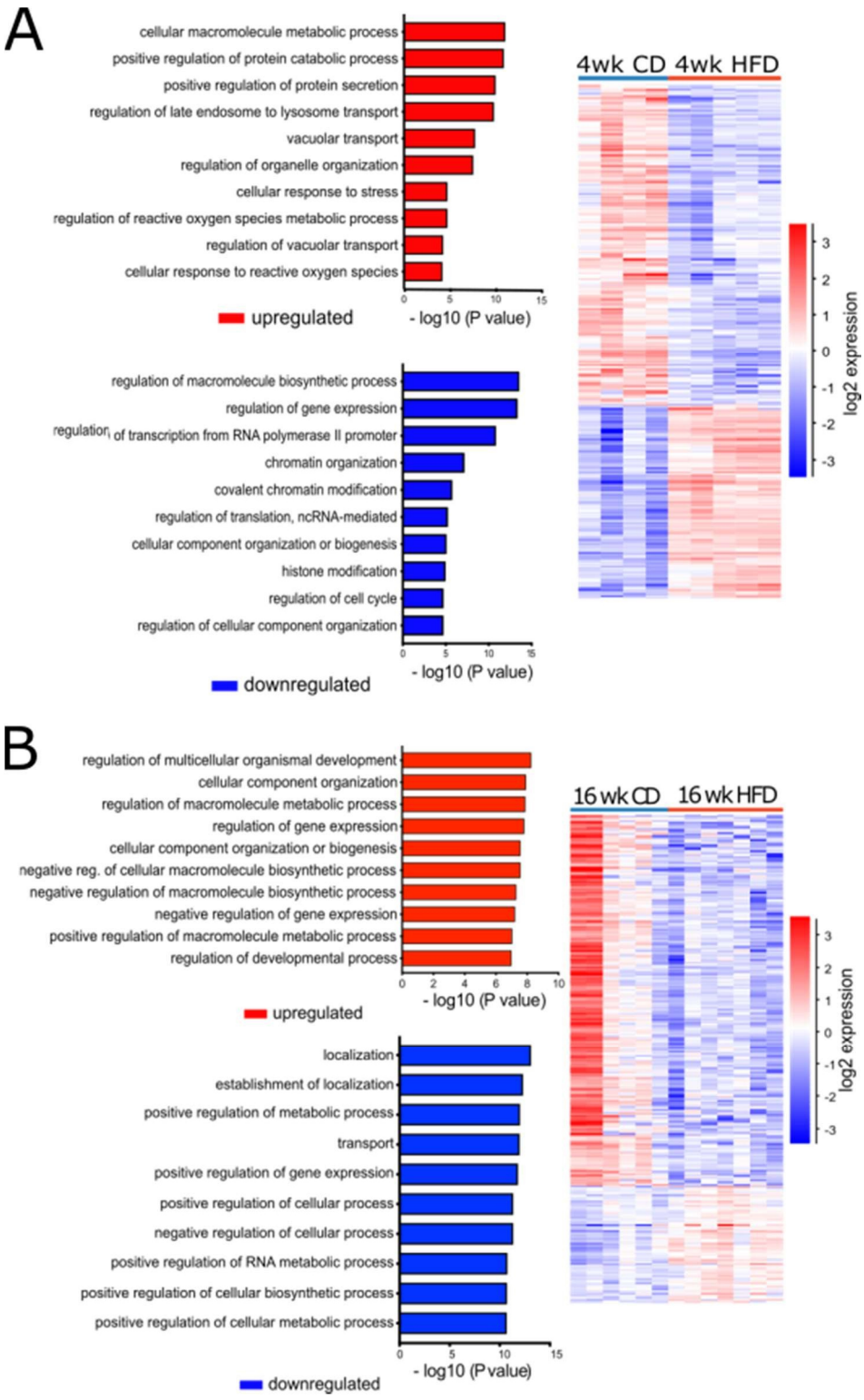


Supplementary Fig. 4. Differentially expressed genes and associated pathways in cumulus cells from diet- induced obesity protocol.

DESeq2 analysis of transcriptome data in cumulus cells obtained from mice after 4 or 16 weeks (wk) of chow diet (CD) or high fat diet (HFD). N= 3-7 mice per group.

On the right heatmap showing hierarchical clustering of (A) 997 differentially expressed genes after submitting mice to 4 weeks of CD and HFD, (B) 846 differentially expressed genes after submitting mice to 16 weeks of CD and HFD. On the left presentation of pathways of genes with the most significant enrichment after gene ontology analysis. Gene ontology analysis performed with Gene Ontology Enrichment Analysis and Visualisation Tool.

Supplementary Fig. 4.

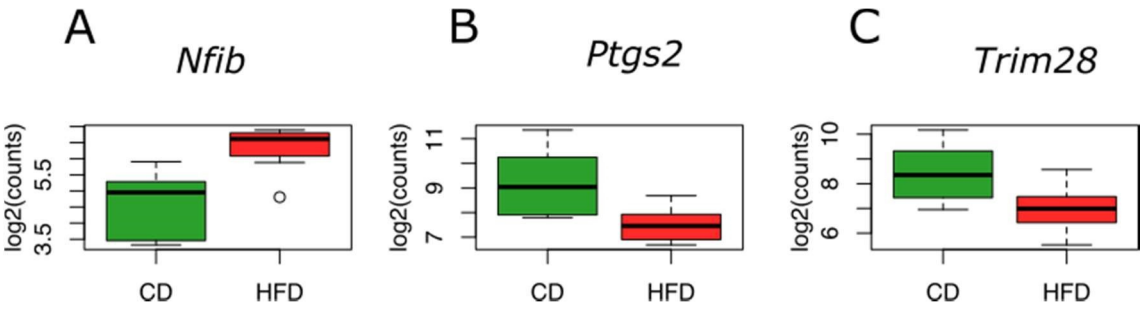


Supplementary Fig. 5. Oocyte competence and embryo quality markers differentially expressed in cumulus cells from mice with late obesity.

DESeq2 analysis of transcriptome data in cumulus cells obtained from mice after 16 weeks of chow diet (CD) or high fat diet (HFD). N= 3-7 mice per group.

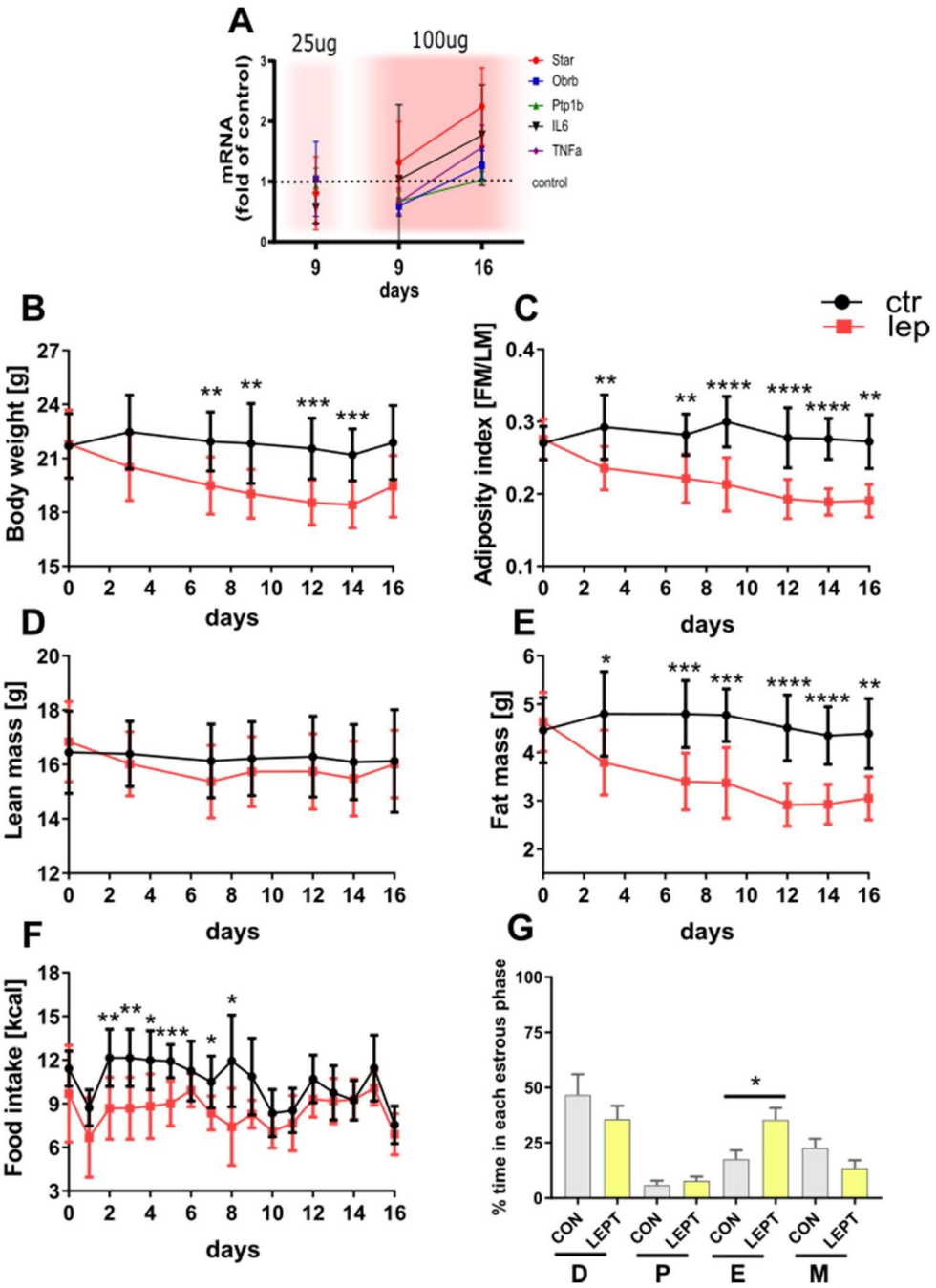
Expression of embryo quality markers (A) *nuclear factor I B (Nfib)*, (B) *cyclooxygenase 2 (Ptgs2)* and oocyte competence marker (C) *tripartite motif containing 28 (Trim28).* Log2 of counts.

Supplementary Fig. 5.



Supplementary Fig. 6. Pharmacologically hyperleptinemic mouse model validation.
In order to validate the length of the treatment and the dose of leptin, we analysed whole ovary mRNA from animals treated with 25 or 100 μg of leptin for 9 or 16 d. Injection of 100 μg for 16 days caused changes in the abundance of leptin-responsive transcripts [69–72] in ovarian extracts collected from animals in oestrous stage. Animals were injected with saline (C) or different doses of leptin (L) for 9 or 16 days (d). (A) mRNA level of *steroidogenic acute regulatory protein (Star), long isoform of leptin receptor Obrb, protein tyrosine phosphatase non-receptor type 1 (Ptp1b), interleukin 6 (Il6), tumor necrosis factor a (Tnfa)* expressed as fold of control after injecting animals for 9 or 16 days with 25μg or 100 μg of leptin. Changes in (B) body weight, (C) adiposity index, (D) lean mass, (E) fat mass, (F) food intake in mice intraperitoneally injected with saline (ctr, black line) or leptin (lep, red line) for 16 days. (G) Proportion of time spend in each oestrous phase of hyperleptinemic mice. D, dioestrus; E, oestrus; M, metoestrus; P, pro-oestrus. Each bar represents the mean ± SD. Differences in phenotype characteristics between groups were analysed by Mann- Whitney test, oestrous cycle distribution analysed by unpaired t-test. Data show mean values for n=10. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

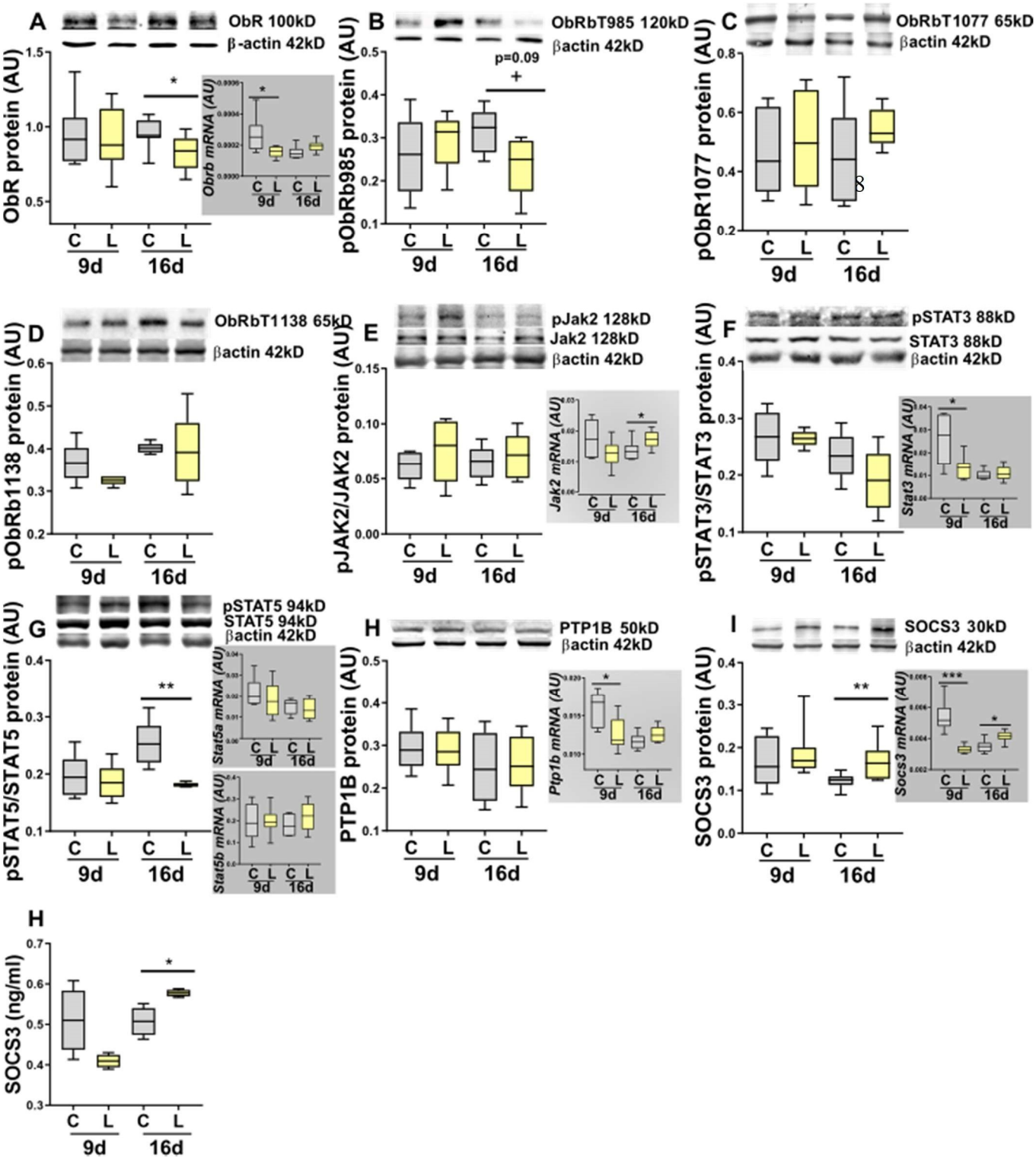
Supplementary Fig. 6.



Supplementary Fig. 7. Expression of leptin signalling components in the ovary of pharmacologically hyperleptinemic mice.

Abundance of mRNA (grey box) and protein of leptin signalling pathway components in ovarian extracts collected from animals injected with saline (C) or 100 μg of leptin (L) for 9 or 16 days (d) and sacrificed in oestrus stage. Expression of (A) leptin receptor (ObR), phosphorylation of (B) tyrosine 985 of leptin receptor, (C) tyrosine 1077 of leptin receptor, (D) tyrosine 1138 of leptin receptor, (E) Janus kinase 2 (JAK2), (F) signal transducer and activator of transcription 3 (STAT3), (G) STAT5, expression of (H) protein tyrosine phosphatase 1B (PTP1B) and (I) suppressor of cytokine signalling 3 (SOCS3) determined by real-time PCR and Western blot. (J) SOCS3 ovarian quantification in animals in oestrus stage determined by ELISA. mRNA expression of *Rpl37* and protein expression of β-actin was used to normalize the expression data. Each bar represents the mean ± SD. Differences between groups were analysed by Mann- Whitney test. N=4-8 for immunoblots and N=8 for RT PCR analysis and ELISA. * $p<0.05$; ** $p<0.01$; ***$p<0.001$; + $p=0.09$.

Supplementary Fig. 7.

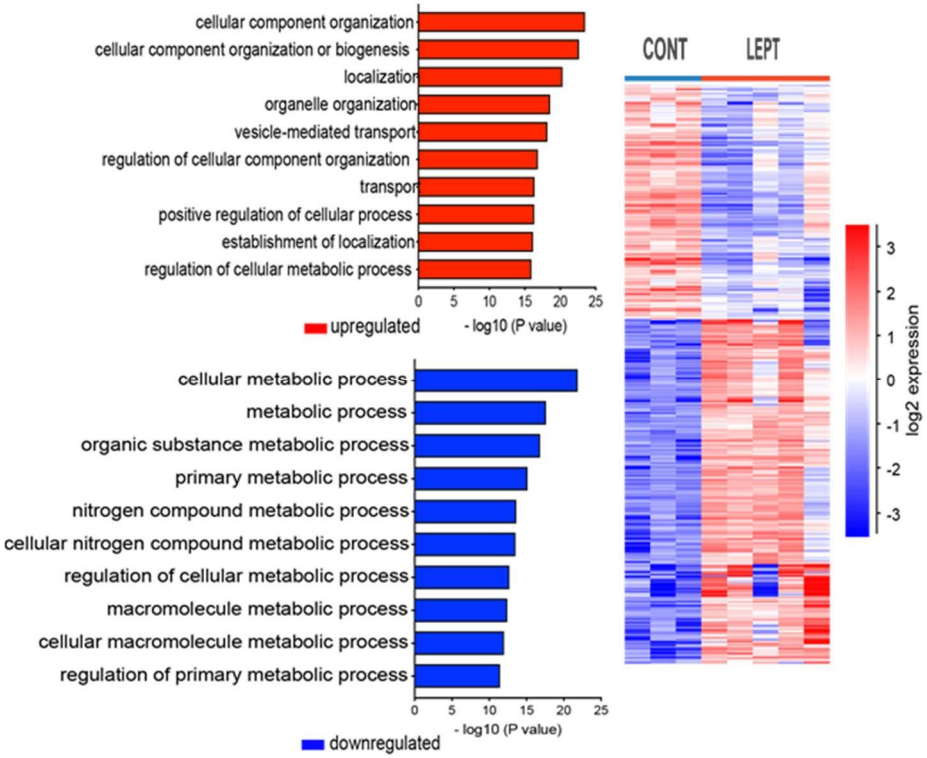


Supplementary Fig. 8. Differentially expressed genes and associated pathways in cumulus cells from hyperleptinemic mice.

DESeq2 analysis of transcriptome data in cumulus cells obtained from mice treated with saline (CONT) and leptin (LEPT). N= 3-7 mice per group.

On the right heatmap showing hierarchical clustering of 2026 differentially expressed genes after treating mice with leptin for 16 days. On the left presentation of pathways of genes with the most significant relevance after gene ontology analysis. Gene ontology analysis performed with Gene Ontology Enrichment Analysis and Visualisation Tool.

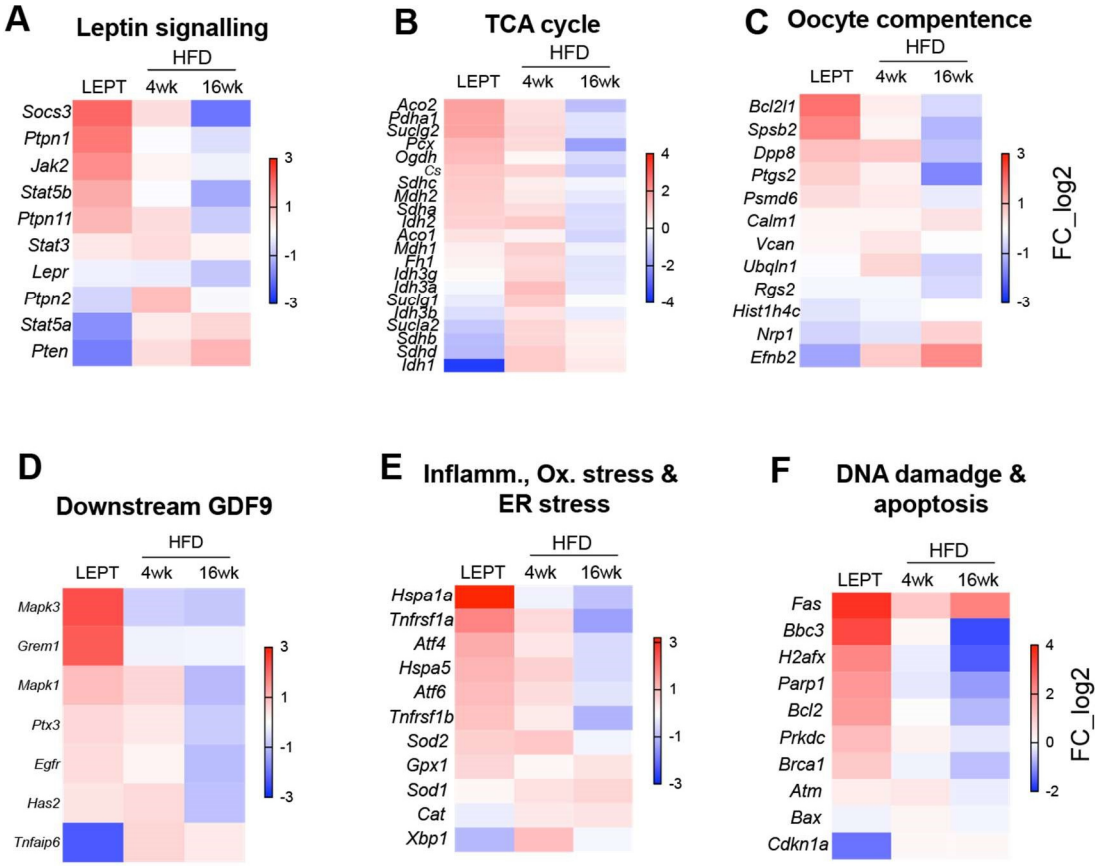
Supplementary Fig. 8.



Supplementary Fig. 9. Similarities between profiles of genes differentially expressed in cumulus cells in diet induced- obese mice and leptin treated mice.

DESeq2 analysis of transcriptome data in cumulus cells (CC) obtained from mice treated with saline (CONT) and leptin (LEPT) or after 4 or 16 weeks (wk) of chow diet (CD) or high fat diet (HFD). N= 3-7 mice per group. Heatmaps representing fold change in expression of genes associated with the following pathways or processes: (A) leptin signalling, (B) tricarboxylic acid (TCA) cycle, (C) oocyte competence, (D) genes regulated by oocyte-derived growth differentiation factor (GDF) 9, (E) inflammation, oxidative stress and endoplasmic reticulum stress, (F) DNA damage and apoptosis in CC. log2_FC of reads per million (RPM)

Supplementary Fig. 9.



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Review

Revisiting the Impact of Local Leptin Signaling in Folliculogenesis and Oocyte Maturation in Obese Mothers

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Abstract: The complex nature of folliculogenesis regulation accounts for its susceptibility to maternal physiological fitness. In obese mothers, progressive expansion of adipose tissue culminates with severe hyperestrogenism and hyperleptinemia with detrimental effects for ovarian performance. Indeed, maternal obesity is associated with the establishment of ovarian leptin resistance. This review summarizes current knowledge on potential effects of impaired leptin signaling throughout folliculogenesis and oocyte developmental competence in mice and women.

Keywords: leptin; obesity; ovary; folliculogenesis; oocyte

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1. Introduction

Obesity is a prevalent disease worldwide, usually associated with infertility. Studies in obese infertile females show the occurrence of systemic hyperestrogenemia, hyperinsulinemia, and associated ovarian dysfunction through premature follicular atresia and anovulation [1]. Indeed, the ovaries of obese mothers have been shown to accumulate lipids, high levels of reactive oxygen species [2], and inflammatory mediators [3]. Furthermore, obesity has been shown to hamper not only oocyte maturation and quality [4] but also embryo development [5], with reported long term effects and direct causality between obesity in the mother and prevalence of cardiovascular disease or cancer in the offspring.

We have recently identified striking links between gain in maternal body weight and global gene expression profile in cumulus cells [6], reiterating not only the importance of maternal metabolic state for ovarian function but also the dynamics of temporal alterations in the ovary throughout obesity progression [6]. In the same work, we demonstrated the establishment of leptin resistance in the ovary of diet-induced obese (DIO; abbreviations listed after the main text) mice after 16 weeks (wk) in comparison to 4 wk [6]. Hence, increased levels of leptin signaling inhibitor suppressor of cytokine signaling (SOCS) 3 were observed already after 4 wk DIO [6] with potential implications for ovarian pathogenesis during early obesity.

In the present narrative review, we revisit, first, physiological aspects governing folliculogenesis in mice and women, discussing the major molecular pathways preconizing developmental changes in both germ line and somatic cells. Second, we consider how maternal obesity and, particularly, local changes in leptin signaling can affect the regulation of such transitions, debating potential outcomes for oocyte quality and early embryo development.

2. Obesity and Ovarian Function

The worldwide epidemic of obesity has reached unprecedented levels and infertility is described as an associated comorbidity [1]. Indeed, obese women were linked to poor reproductive outcomes, such as anovulation or decreased conception rate [7]. Literature

has evidenced the link between peripheral insulin resistance and functional hyperandrogenism and hyperestrogenism [8], the main causes of anovulation and reduced endometrial receptivity [1]. Studies in mice have shown how obesity-associated hyperinsulinemia and diabetes can lead to delayed oocyte maturation in preovulatory follicles and apoptosis in granulosa cells (GC) [9]. Furthermore, ovaries of obese women were shown to present high levels of androstenedione and testosterone [8], responsible for premature follicular atresia. Therefore, the endocrine imbalance observed in obese mothers affects directly the follicular pool, reducing the number of primordial follicles and compromising fertility [10].

In recent years, studies, particularly in mouse models for obesity, have clearly revealed the main readouts in ovaries from obese mothers. Indeed, the accumulation of lipids in oocyte and surrounding GC or cumulus cells (CC) was shown to culminate with lipotoxicity and inflammation, GC apoptosis, endoplasmic reticulum (EnR) stress in cumulus–oocyte complexes (COCs) [11], and oocyte mitochondrial dysfunction [12]. Furthermore, we have also shown, through DIO protocols in mice, dramatic changes in the expression of genes regulating cytoskeletal organization and the formation of transzonal projections (TZPs), which play a key role mediating the crosstalk between somatic cells and oocyte during follicular growth [6]. Thus, maternal obesity has been linked to changes in local cytokine milieu and signaling mediators of follicle growth, maturation, steroidogenesis, and ovulation [3]. Ultimately, the oocyte quality was shown to be equally impaired, with delayed meiotic maturation, abnormal mitochondrial distribution, and oxidative stress [13]. Finally, Robker and collaborators reported altered follicular fluid levels of metabolites such as C-reactive protein and androgen activity in women [14], which have been associated with cellular stress and impaired oocyte nuclear maturation [4]. Undeniably, literature comprehensively describes the main features in the ovaries of obese mothers, but fewer studies have functionally addressed the mounting response leading to ovarian failure and hampered oocyte quality. Noteworthy, describing temporally the progression of events in the ovaries of obese mothers is fundamental to understanding the pathophysiology of ovarian failure and helping us delineate adequate treatments regarding disease progression.

2.1. Leptin—A Common Denominator between Ovarian Function and Obesity

During obesity progression, the ever-growing adipose tissue secretes large amounts of leptin, which causes systemic hormonal imbalance. Leptin is mainly known to regulate appetite at the central level [15] besides modulating the release of gonadotropin releasing hormone (GnRH) neuron activity and gonadotropins [16]. Nonetheless, leptin is also an important modulator of ovarian function [17]. Leptin long and short receptor isoforms were detected in most cell types in murine ovary, particularly in the oocyte [17]. Likewise, both isoforms of the leptin receptor (ObR) were previously detected in human GC and theca cells (TC) [18]; as well, leptin and leptin soluble receptor were detected in human follicular fluid [18,19]. Thus, leptin signaling components' heavy representation in the ovary makes the organ particularly vulnerable to the systemic hyperleptinemia observed in obese mothers [20].

When leptin signals through the long isoform of its membrane receptor, leptin receptor b (ObRb), a canonical cascade activates Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling. Conversely, noncanonical signaling results in insulin receptor substrate (IRS)/phosphatidylinositol 3 kinase (PI3K)/protein kinase B (Akt) and mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) signaling pathways activation (Figure 1). After binding the dimerized ObRb, leptin initially mediates the phosphorylation of JAK2 with further transfer of the phosphate group to three tyrosines within the BOX2 of ObRb. As a result: (i) SH2-domain containing protein tyrosine phosphatase (SHP-2) can be phosphorylated, subsequently binding to the adapter Grb-2 and activating downstream ERK1/2; (ii) STAT5 can be phosphorylated; and/or (iii) *STAT3* can be phosphorylated (Figure 1). Intriguingly, leptin can noncanonically regulate components of the insulin signaling cascade. After ObRb activation, IRSs can be phosphorylated by JAK2 with subsequent activation of PI3K pathway. As a result,

phosphatidylinositol-3,4,5-triphosphate (PIP_3) is generated with further activation of PIP_3-dependent serine/threonine kinases, such as phosphoinositide-dependent kinase (PDK) 1,2, a conserved activator of Akt, which in turn regulates downstream signaling [21]. With regard to the ERK signaling, leptin can activate the MAPK pathway mainly through two different ways: (i) first, after phosphorylation of ObRb tyrosine 985 by JAK2, SHP-2 is recruited and binds Grb-2, resulting in ERK activation [22]; or (ii) second, the short isoform of ObR, which possesses only BOX1 in the receptor domain and lacks tyrosine sites, can also activate SHP-2 and Grb-2 complex through JAK2 (Figure 1), followed by mitogen-activated protein kinase kinase (MEK) 1 and downstream phosphorylation of ERK1/2 [23]. It is noteworthy that, despite its effects on ERK activation, leptin can also interact with protein kinase C (PKC) and further activate ERK, playing both stimulatory and inhibitory effects on PKC.

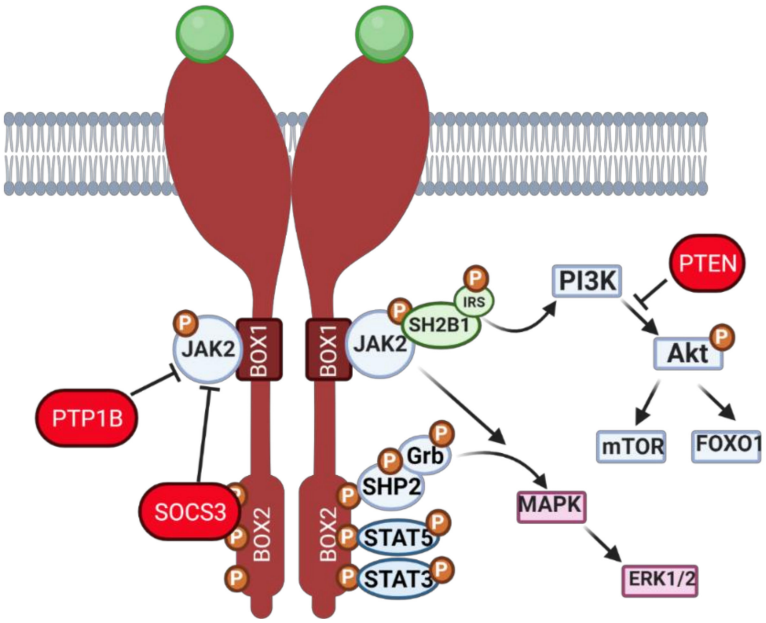

Figure 1. Schematic representation of leptin signaling pathway cascade. Leptin binds to its dimerized membrane receptor, and signal propagation starts. Janus kinase 2 (JAK2) phosphorylation causes transition of phosphate groups to three tyrosines within BOX2 of ObR and activation of (i) SH2-domain containing protein tyrosine phosphatase (SHP-2), which, in turns, binds its adapter molecule Grb-2 and activates downstream signaling, resulting in extracellular signal regulated kinase (ERK) 1/2 activation, (ii) signal transducer and activator of transcription (STAT) 5 activation and (iii) *STAT3* activation. The phosphorylation of JAK2 also activates the MAPK signaling pathway and promotes SH2B adaptor protein 1 (SH2B1) and insulin receptor substrate (IRS) binding, which initiates the phosphatidylinositol 3 kinase (PI3K) pathway, which leads to phosphorylation of protein kinase B (Akt), mammalian target of rapamycin (mTOR) and forkhead box O1 (FOXO1) activation. During hyperactivation of the leptin signaling pathway, two main inhibitors can be transcribed—protein tyrosine phosphatase (*PTP*) 1B, which dephosphorylates JAK2 and suppressor of cytokine signaling (SOCS) 3, which blocks tyrosine and JAK2 phosphorylation. Created with BioRender.com, accessed on 1 November 2020.

Concerning the role of leptin in the ovary, evidence gathered from studies with leptin or ObR deficient mice, as well as women, confirmed their infertility and altered pubertal development [24,25]. Functionally, leptin actions in the ovary were shown to have a bimodal nature. In vivo and in vitro studies in mouse ovarian explants [26] evidenced the dose-dependent effect of leptin on progesterone (P4) synthesis, with low doses stimulating and high doses inhibiting expression of enzymes involved in P4 synthesis [26]. Also, studies in other species corroborated the aforementioned observations as the in vitro treatment of equine luteal cells with lower doses of leptin supported P4 secretion, whereas

higher doses presented no effect [27]. Particularly regarding follicular dynamics, studies in mice showed that high levels of circulating leptin blocked folliculogenesis, but lower circulating levels of leptin supported the transition from primary to secondary follicle [28]. Conversely, the in vitro treatment of mouse follicles with mouse recombinant leptin once more revealed a dose-dependent response with higher treatment doses inhibiting follicular growth [29]. Presently, we used the data from Zhang and co-workers' recent report profiling the transcriptome of GC and oocytes isolated from different stages of folliculogenesis in women [30] and plotted the main components of the leptin signaling pathway in order to understand if their expression profile could be linked to a leptin concerted role during particular developmental stages. Indeed, we confirmed some leptin signaling components, such as *protein tyrosine phosphatase non-receptor type 2 (PTPN2)*, *protein tyrosine phosphatase (PTP) 1B*, or *STAT3*, were abundantly transcribed in both oocytes and GCs throughout folliculogenesis, whereas others, such as *SOCS3*, presented very low expression in both cell types under physiological conditions, particularly in later stages of folliculogenesis (Figure 2). Finally, we have recently shown that obesity progression in DIO mice alters leptin signaling in the ovary with increased leptin signaling in the ovary of 4 wk DIO mice, being followed by the establishment of leptin resistance in the ovaries of 16 wk DIO mice [6]. Indeed, expression levels of *SOCS3* in ovarian extracts were dramatically increased already at 4 wk DIO [6]. Therefore, our findings, as well as the observations on leptin signaling component expression in oocytes and GCs from women's follicles, clearly suggest that the impairment of leptin signaling in the ovaries of obese mothers may contribute to pathogenesis of ovarian failure, particularly given leptin's established role in ovarian function.

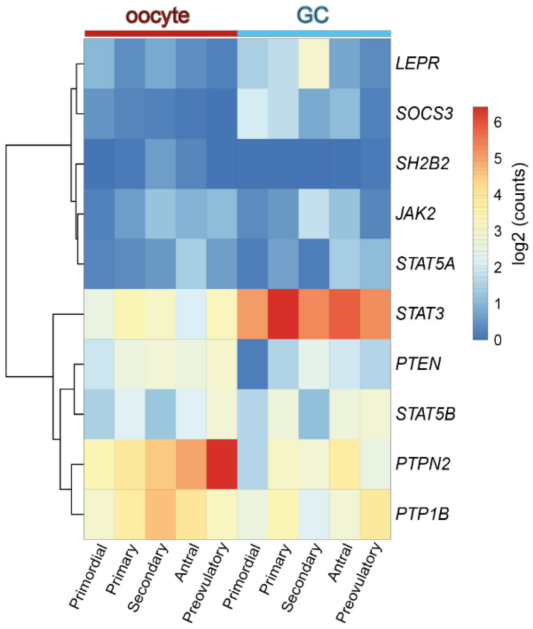

Figure 2. Heatmap representing the expression level of transcripts from the leptin signaling pathway components in human oocyte and granulosa cells (GC) throughout folliculogenesis. Primordial = primordial follicle; Primary = primary follicle; Secondary = secondary follicle; Antral = antral follicle; Preovulatory = preovulatory follicle. Color code from blue to red indicates the relative gene expression level from low to high, respectively. Data from Zhang et al. 2018 [30]. Leptin receptor (LEPR), suppressor of cytokine signaling 3 (*SOCS3*), SH2B Adaptor Protein 1 (SH2B1), Janus kinase 2 (JAK2), signal transducer and activator of transcription (STAT), protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), protein tyrosine phosphatase (*PTP*) 1B.

2.2. Other Adipokines and Ovarian Function during Obesity

Obesity and progressive expansion of white adipose tissue are largely associated with dramatic changes in the adipokine secretory profile, which ultimately lead to the

establishment of a proinflammatory, atherogenic, and diabetogenic systemic environment. Indeed, during obesity, the adipose tissue secretes adipokines other than leptin with potential consequences for ovarian function regulation. Making a full characterization of all adipokines and their potential involvement in ovarian function regulation is certainly beyond the scope of the present review. Nonetheless, literature highlights both local and central regulatory roles of major adipokines, such as adiponectin, visfatin, omentin, and resistin in folliculogenesis [31]. Therefore, in the present section, we briefly discuss potential outcomes for folliculogenesis associated with changes in the secretory profile of main adipokines other than leptin during obesity.

Adiponectin is known as the most abundant circulating adipokine in humans, and its receptors, adiponectin receptor 1 and 2, were shown to be ubiquitously expressed in female reproductive tissues, including the ovaries [32]. Adiponectin's main systemic roles encompass the increase in insulin sensitivity in both liver and muscle, suppression of hepatic gluconeogenesis, and promotion of fatty acid β-oxidation in the skeletal muscle [33]. Furthermore, adiponectin is also known to have a 'beneficial' role in reproduction [31]. Indeed, under physiological levels, a number of adipokines, such as visfatin, omentin and vaspin, or leptin itself, have been described as 'beneficial' for reproduction and ovarian function [31]. In this regard, leptin's prominent role in ovarian function was mainly evidenced by reports on anorectic or undernourished women [34], corroborating the importance of leptin's exact physiological amounts for ovarian homeostasis [34]. At physiological levels, adiponectin was shown to modulate steroidogenesis and promote oocyte maturation [35], as well as participating in ovulation and supporting early embryo development in various species [35,36]. Interestingly, a recent study has revealed adiponectin's role in preventing the hyperactivation of primordial follicles in mice treated with a high protein diet [37]. Therefore, decreased circulating levels of adiponectin observed in obese mothers might negatively contribute to ovarian function regulation.

Another particular adipokine, resistin, was shown not only to be increased in circulation during obesity, but also to synergize with leptin on *SOCS3* upregulation in various tissues [38]. Centrally, resistin was shown to lower hypothalamic ObRb transcript levels, contributing to central leptin resistance [38]. Indeed, resistin's role modulating the secretion of reproductive hormones at the pituitary level was previously evidenced in sheep [39]. As revisited elsewhere, resistin was shown to be widely expressed in the ovaries, particularly in rodent, bovine, porcine, and human TC, GC, and oocytes, modulating steroidogenesis and supporting androgen production [40]. Hence, resistin appears to be another major adipokine capable of modulating ovarian function in obese mothers. Therefore, the multiple contribution of different adipokines to ovarian failure during obesity shall not be neglected. Indeed, the difficulties of studying obesity and its comorbidities rely on its polygenic nature, very often largely simplified by the use of monogenic disease models.

3. Revisiting Folliculogenesis in Mice and Women: A Morphofunctional Characterization

The complex nature of folliculogenesis regulation certainly accounts for the vulnerability the ovaries present to the hormonal imbalance seen in obese mothers [7]. Generally, obesity may affect the oocyte and somatic cells at each single developmental stage of folliculogenesis (Figure 3) with the incidence of obesity earlier in life posing a greater threat for the quality of the gamete later in adulthood [41]. Hence, in this section, we revisit folliculogenesis, analyzing major cellular events taking place throughout the long journey the female gamete makes from primordial follicle until fertilization, based on lessons learnt from studies in mice and in humans (Table S1 summarizes the nature of the study presented in the text).

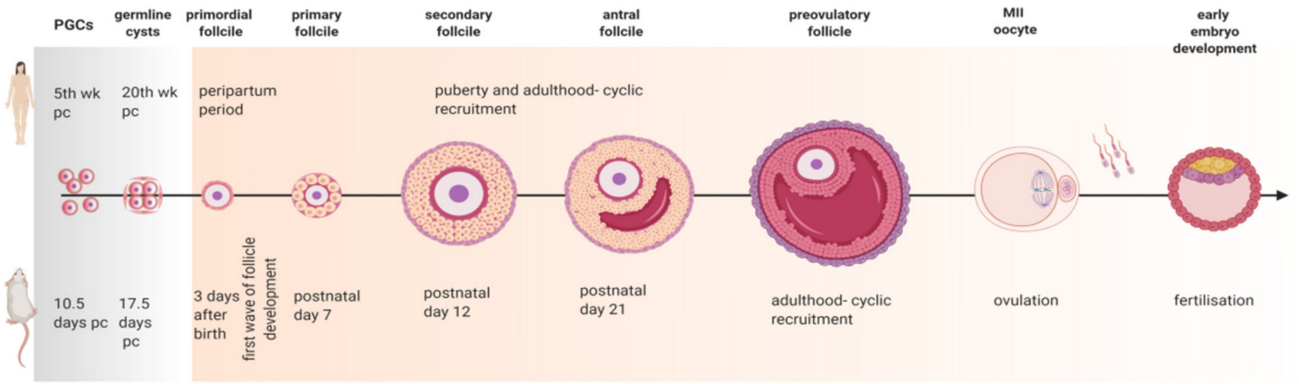

Figure 3. Diagram of folliculogenesis in mice and women. Approximately five weeks (wk) post coitum (pc) in women and 10.5 days (d) pc in mice, primordial germ cells (PGC) arrive at the genital ridge. Germline cysts start breaking 15 wk later in women and 7 days later in mice, creating the primordial follicles. During the peripartum period in woman, and within 3 days after birth in mice, primordial follicles are created. The process of follicle development in women is asynchronous, with menstrual cyclicity being started at puberty. In mice, the first wave of follicle development is detected around postnatal day 7, when the first primary follicles are originated, followed by secondary follicles detected at postnatal day 12 and early antral follicles around postnatal day 21. When mice reach sexual maturity, cyclic recruitment of follicles begins. After ovulation metaphase II (MII) oocyte is released into the oviduct where fertilization takes place, followed by early embryo development. Created with BioRender.com, accessed on 1 November 2020.

3.1. Primordial Follicle Assembly

In the mouse, primordial germ cells (PGC) arrive at the genital ridge on 10.5 days post coitum (dpc) and divide by mitosis with incomplete cytokinesis for the subsequent 3 days, originating the germline cysts (Figure 3) [42]. Next, oogonias enclosed in germline cysts enter the meiosis prophase and are named as oocytes. This is the beginning of a journey with two distinctive ends, the success of ovulation and eventual fertilization or the inevitable condemnation of follicular atresia. Oocyte progresses to the diplotene stage of prophase I of meiosis, remains arrested at this stage from 17.5 dpc, and resumes meiosis only after the surge of luteinizing hormone (LH) just before ovulation (Figure 3) [43]. Prior to cyst breakdown, mitochondria divide and reorganize in the cysts, suggesting a process of active mitochondrial selection [44]. Germline cyst breakdown starts at 17.5 dpc in the mouse [45], and follicles begin to form. The process of primordial follicle assembly is independent from gonadotropin action, being mainly regulated locally by factors like neurotrophin (NT) 4, brain-derived nerve factor (BDNF), folliculogenesis-specific basic helix–loop–helix (FIG-α). Within three days after birth, the cortex of the ovaries is replenished with primordial follicles, consisting of small oocytes surrounded by flattened GC, which remain dormant until awakened by local factors (Figure 3) [44]. In humans, follicular development starts during fetal life, and PGC first reach the gonadal ridge around the fifth wk of pregnancy [46]. They then divide mitotically until the fifth month of pregnancy, when PGC undergo the first meiotic division and become arrested in prophase I. At this stage, germ cells are surrounded by somatic cells, forming the primordial follicles (Figure 3) [46]. In primates, the first wave of primordial follicle activation starts during fetal development, and multiple preantral follicles can be found in the ovary at the sixth month of pregnancy in women, with antral follicles often developing during the next two months [47]. As a result, the ovary of a newborn is replenished with large antral follicles, which will invariably undergo atresia [47].

3.2. Primary Follicle Development and Growth

The process of early folliculogenesis encompasses important structural and molecular changes, with GC becoming cuboidal and the oocyte accumulating ribonuclease acid (RNA) and protein. Moreover, stromal/mesenchymal cells associated with the primordial follicle are presumably early stage precursors of TC in the primary follicle [48]. At last, primary

follicles are enclosed in basal lamina with one layer of GC (Figure 3). In secondary follicles, fibroblast-like cells from ovarian stroma form the TC layer that surrounds the follicle and, together with GC-derived aromatase, mediates the synthesis of steroid hormones essential for oocyte growth. Follicles then develop a fluid-filled antrum, being designated as antral follicles (Figure 3). Prior to ovulation, the surge of gonadotropins determines the resumption of oocyte meiosis, which progresses from prophase I to metaphase II (MII). Indeed, meiotic division is finished exclusively after fertilization [49]. In women, primordial follicles are formed during the peripartum period with around one to two million follicles being present at birth. These follicles form the follicular reserve, which determines reproductive potential throughout their lifespan. In women, it takes approximately one year for a primordial follicle to mature and reach the ovulation stage. The transition from resting pool into growing follicle is highly dependent on the game of forces between growth/differentiation and pro-apoptotic factors [46].

3.3. The Road to Ovulation

As in rodents, the progression from preantral to antral follicles in women determines the transition from gonadotropin-independent to gonadotropin-dependent follicular growth, with further selection of the dominant follicle prior to ovulation. At puberty, centrally released gonadotropins follicle-stimulating hormone (FSH) and LH promote the development of antral follicles and the onset of ovulation, with only one single follicle reaching the preovulatory stage during a menstrual cycle in humans (Figure 3) [46]. Conversely, in cycling mice, few follicles reach the preovulatory stage [50]. In women, approximately seven million oocytes initially start developing in the ovaries, with only two million oocytes present at birth [44] and approximately four hundred ovulating throughout reproductive lifespan [51]. Conversely, the mouse ovaries present great variation in oocyte number between strains, as well as rates of follicle loss [52], with approximately seven thousand oocytes being present in the ovaries at 3 days after birth [53]. Follicular response to the gonadotropin surge results in the rupture of the follicle wall. The dramatic expansion of the follicle volume is mainly associated with increased ovarian blood flow and enhanced capillary permeability, mediated by the activity of local proteolytic enzymes, steroids, and arachidonic acid metabolites [54]. After ovulation, COCs are released into the oviduct where, in the presence of sperm, fertilization takes place. Immediately after fertilization, meiosis is completed, followed by the zygotic first mitotic division and activation of transcription as the embryo develops until the blastocyst stage (Figure 3), the time of implantation in both mice and humans [55].

4. Molecular Mechanisms Regulating Primordial to Primary Follicle Transition

The rate of primordial follicle assembly and transition to primary follicle sets both the size and depletion rate of the primordial follicle pool. Therefore, its inadequate regulation culminates with premature follicle loss and sterility. The formation of the follicular pool and the first wave of primordial follicle activation is a synchronous process in rodents and takes place between days 3 and 7 postnatal (Figure 3). Nonetheless, due to the lack of gonadotropin activity, these follicles inevitably undergo atresia [56]. In primates, the process is asynchronous with some primordial follicles leaving the resting pool before others start meiosis [56], whereas remaining germ cells, mainly located peripherally, still undergo mitosis [53]. Asynchronous follicle activation in domestic animals and humans is notoriously difficult to study, hence, most of the studies on molecular regulation of primordial follicle activation were undertaken in rodents. In the present section, we discuss the molecular mechanisms controlling primordial to primary follicle transition, and how obesity and altered leptin signaling can jeopardize its regulation.

4.1. The PI3K Pathway and Primordial to Primary Follicle Transition

Mouse studies with three particular phenotypes of transgenic animals unraveled the main molecular pathways involved in primordial follicle activation. The first phenotype

consisted of mice with premature follicle activation, follicle loss, and sterility. The second phenotype presented follicles arrested in the primordial follicle stage, and in the third phenotype, follicles did not progress beyond the primary follicle stage. Studies on aforementioned models identified the PI3K pathway in the oocyte, together with the mammalian target of rapamycin (mTOR) pathway in pre-GC, as major regulators of primordial follicle activation (Figure 4). Indeed, ERK1/2 may regulate mTOR Complex (mTORC) 1 in pre-GC with subsequent activation of cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), which is known to promote the transcription of stem cell factor (SCF), a main activator of PI3K signaling in the oocyte [57]. Also, studies with CREB conditional knockdown in the ovary showed its regulatory role on pre-GC proliferation and oocyte apoptosis [57]. After PI3K activation in the oocyte, phosphatidylinositol-4,5-biphosphate (PIP_2) is phosphorylated into PIP_3, followed by Akt phosphorylation by PDK1. In fact, Akt may further interact with forkhead box O3 (FOXO3) or ribosomal protein S6 kinase beta-1 (S6K1) (Figure 4). Upon phosphorylation, pFOXO3 translocates from the nucleus to the cytoplasm, thereby losing transcriptional activity. Indeed, FOXO3 works as a molecular switch, ensuring, together with cyclin-dependent kinase inhibitor 1B (p27), follicle dormancy, and blocking follicular recruitment and oocyte growth [58]. Alternatively, Akt may also phosphorylate and inhibit tuberous sclerosis complex (TSC) 2, allowing for the activation of mTORC1 and S6K1/ribosomal protein S6 (rpS6), a pathway mainly responsible for protein translation and ribosome synthesis (Figure 4) [59]. Thus, Akt plays a key role in the primordial follicle fate, mediating survival during dormancy, as well as follicle activation or atresia [59].

Studies in bovines and baboons have linked PDK1 deficiency, and subsequent down-regulation of rpS6 in oocytes, to premature ovarian failure (POF) and loss of primordial follicles [59]. On the other hand, deficiency of phosphatase and tensin homolog (PTEN), a PI3K inhibitor, was seemingly associated with the overexpression of rpS6 and POF due to hyperactivation of primordial follicles and consequent follicular atresia [59]. Mutations in PTEN suggested its importance for maintenance of the primordial follicle pool [60]. Indeed, PTEN, together with regulators TSC1 and TSC2, negative regulators of mTOR signaling, maintain the quiescence of primordial follicles [61]. Furthermore, in vitro treatment of human ovarian cortical tissue with PTEN inhibitor resulted in primordial follicle development to preovulatory stage [62]. Finally, studies with porcine neonatal ovaries also showed the localization of PTEN, Akt, and FOXO3 in oocytes and GC of primordial, primary, and secondary follicles, suggesting their role in primordial follicle activation across species [63]. Literature describes additional factors involved in PI3K regulation and primordial follicle activation, particularly, growth differentiation factor (GDF) 9 [64] or SCF receptor (Kit). Mutations of the aforementioned factors resulted in folliculogenesis arrest at the primary follicle stage [65]. Finally, the involvement of insulin was also reported in primordial to primary follicle transition and its ability to inhibit FOXO3 through Akt signaling [66]. Taken together, PI3K and mTOR signaling pathways appear to be master regulators of primordial follicle activation. Precise control of these pathways is essential for maintenance of the female reproductive lifespan and the preservation of primordial follicles in quiescence.

Although the molecular mechanisms driving primordial to primary follicle transition in women remain much less clear than in the mouse, a recent study by Zhang et al. identified a number of genes differentially expressed at this stage [30]. As previously discussed in Section 2.1, the leptin signaling inhibitor *SOCS3* presented its highest expression level in GC from primordial follicles (Figure 2). Furthermore, functional pathways associated with differently expressed genes (DEGs) during primordial to primary transition included insulin signaling, GnRH, NT, and mTOR–PI3K, JAK–STAT pathways in both oocytes and GC [30]. Thus, components of leptin signaling appear to be amid the physiological factors regulating primordial to primary follicle transition in humans.

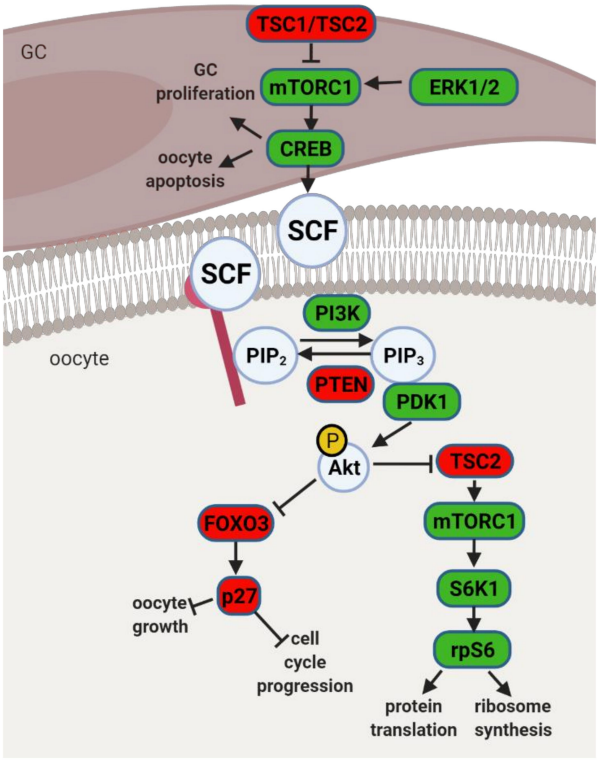

Figure 4. Schematic representation of mammalian target of rapamycin (mTOR) and phosphatidylinositol 3 kinase (PI3K) signaling pathway and its downstream regulators in the granulosa cells (GC) and oocyte. Extracellular signal-regulated protein kinase $\frac{1}{2}$ (ERK1/2) activates mTOR complex 1 (mTORC1) in pre-GC to initiate the activation of primordial follicles. mTORC1 activates cyclic AMP-response element binding protein (CREB), which promotes stem cell factor (SCF) transcription and stimulates PI3K signaling but also affects pre-GC proliferation and oocyte apoptosis. mTOR signaling negative regulators TSC1 and TSC2 suppress mTORC1 activity. PI3K signaling pathway is activated by SCF in the oocyte. PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) to phosphatidylinositol-3,4,5-triphosphate (PIP_3), which interacts with 3-phosphoinositide dependent protein kinase 1 (PDK1) for subsequent phosphorylation of protein kinase B (Akt) and forkhead box O3 (FOXO3) with its downstream mediator, cyclin-dependent kinase inhibitor 1B (p27). Upon phosphorylation, the inhibitory effect of FOXO3 and p27 on cell cycle progression and oocyte growth is inhibited, and the primordial follicle is recruited. Akt also activates ribosomal protein S6 kinase beta-1 (S6K1) through inhibition of TSC2 with subsequent mTORC1 activation and further ribosomal protein S6 (rpS6) phosphorylation, which leads to protein translation and ribosome synthesis. Factors indicated in red are associated with follicle dormancy; molecules indicated in green are associated with follicle activation. Created with BioRender.com, accessed on 1 November 2020.

In conclusion, studies in transgenic mice with oocyte and GC specific mutations helped us identify the major roles of PI3K and mTOR signaling mediating primordial follicle activation. More recently, a study in humans has revealed the involvement of JAK–STAT signaling and *SOCS3* in early folliculogenesis, highlighting the importance of the leptin signaling pathway in primordial to primary follicle transition.

4.2. Obesity, Leptin Signaling and Primordial Follicle Activation

Studies in rodents have highlighted the effects of maternal obesity on ovarian failure and, particularly, on PI3K dysregulation. A report in rats showed that DIO treatment led to POF through activation of mTOR and suppression of sirtuin (SIRT) 1 signaling [67], whereas the ovaries of mice fed a high-fat diet (HFD) presented aberrant expression levels of PI3K pathway components [68]. Indeed, the putative crosstalk between leptin and the PI3K pathway can be anticipated, mainly, through components of the insulin signaling

cascade (Figure 1). After ObRb activation, JAK2 can phosphorylate IRs, with subsequent activation of PI3K pathway [21]. This generates PIP_3, which further activates PIP_3-dependent serine/threonine kinases, such as PDK1,2, responsible for activation of Akt, as previously discussed (Figure 1) [21]. Indeed, a study in mice with a triple mutation in ObR tyrosines linked follicle loss to the activation of PTEN/PI3K/Akt/mTOR signaling [69]. Furthermore, Panwar and colleagues showed the direct effects of leptin on the follicular pool once passive immunization of prepubertal mice against leptin prompted the transition of primordial to primary follicles [28]. Therefore, adequate levels of leptin signaling in the ovary seem to prevent primordial follicle hyperactivation and help to maintain the follicular pool. Concordantly, these observations suggest the decrease in leptin signaling we have reported in the ovaries of 16 wk DIO mice [6] might accelerate the activation and depletion of the primordial follicle pool.

We have previously linked *SOCS3* overexpression in ovarian extracts of 16 wk DIO mice with the establishment of leptin resistance [6]. Despite no previous characterization of *SOCS3*'s putative role on follicular pool activation, its ability to inhibit the phosphorylation of JAK2 and ERK is well established. As a result, SCF signaling could be disturbed in GC (Figure 4), as well as PI3K activation in the oocyte, during primordial to primary follicle transition in obese mothers. After reanalyzing Zhang's data, we plotted the expression level of leptin signaling components across all stages of folliculogenesis (Figure 2). Indeed, we confirmed *SOCS3* mRNA levels remained very low in both oocyte and GC throughout folliculogenesis in women with regard to other components of the pathway (Figure 2). Therefore, our previous results showing a dramatic increase in *SOCS3* protein and mRNA levels in the ovaries of 16 wk DIO mice invite the speculation of PI3K putative dysregulation in primordial oocytes. Finally, the high mRNA level of *PTPN2* observed in the oocyte throughout folliculogenesis was also of particular relevance [30]. In fact, *PTPN2* has also been associated with the establishment of leptin resistance at central level [70] and, therefore, appears to be an important candidate in the pathophysiology of ovarian failure in obese mothers.

To conclude, previous associations between maternal obesity and decreased follicular count can be linked to changes in local leptin signaling. Effects of leptin resistance can be measured not only by lack of leptin action maintaining the follicular pool but also through excessive levels of *SOCS3*, potential dysregulation of PI3K signaling, and accelerated primordial follicle activation.

5. Molecular Regulation of Early Antral to Preovulatory Follicle Transition

The regulation of primary to secondary follicle transition is a process mostly paracrinally regulated that relies mainly on the communication between GC and oocyte. Morphologically, GC proliferate from a single monolayer to multiple layers with rapid expansion of the oocyte. Molecularly, the process is coordinated by local factors such as GC-derived anti-Mullerian hormone (AMH) [71], which was shown to regulate oocyte-derived GDF9 activity in follicular growth [64]. In addition, the factors Kit and SCF [65], as well as LIM homeobox protein (Lhx) 8, were shown to be involved in primary to secondary follicle transition [72]. Finally, NOTCH [73] and mTOR signaling were reported to control GC proliferation in primary follicle growth [74]. Conversely, after antrum formation, the follicles then become responsive to gonadotropins, responsible for orchestrating major events leading to preovulatory follicle formation, such as steroidogenesis, intercellular communication, GC differentiation, and oocyte expansion. In this chapter, we discuss the main pathways regulating preovulatory follicle formation, the particular involvement of leptin signaling, and the disruptive effects of obesity and impaired leptin signaling.

5.1. Preovulatory Follicle Formation—The Role of Estradiol

The systemic increase in pituitary-released FSH was shown to promote growth of early antral follicles as they became responsive to gonadotropins. In the mouse, after intensive GC proliferation and follicle growth, several follicles became dominant while

others underwent atresia. Follicular atresia is a highly regulated process of programmed cell death, or apoptosis, particularly through local levels of FSH- mediated estradiol (E2) and androgen activity [51]. Atresia is generally initiated in GC, which at first undergo apoptosis through the activity of factors such as tumor necrosis factor (TNF) α or Fas ligand [51]. Furthermore, androgens were identified to play a prominent role in atresia, increasing the number of pyknotic GC and degenerated oocytes [75]. Novel mediators of atresia have been recently characterized as micro RNA (miR) 26b [76] or miR146b [77], shown to promote GC apoptosis in atretic follicles in pig ovaries [78]. Conversely, in dominant follicles, FSH activity was shown to elevate E2 levels through GC aromatase cytochrome P450 (CYP) 19A1 [50] with the upregulation of pro-survival growth factors: insulin-like growth factor (IGF) 1, epidermal growth factor (EGF), and basic fibroblast growth factor (FGF) [79]. Indeed, lack of FSH activity and low levels of E2 were associated with increased androgen activity, apoptosis, and atresia [80].

Estradiol is a predominant circulating estrogen in humans [81]. Its synthetic pathway starts in TC through the activation of the LH receptor and production of androgens from cholesterol [82]. After being transferred to GC, androgens are further converted into E2 by aromatase *CYP19A1*, a step regulated by FSH receptor activity [82]. As previously stated, the newly synthesized E2 plays a major role in dominant follicle selection [83]. Two nuclear E2 receptors (ER) have been characterized as auto-, paracrine mediators of E2 local actions, the ERα and the ERβ. Interestingly, the expression of ERs was shown to be controlled by gonadotropins [84], with ERβ being mainly evidenced in GC and ERα detected in TC and interstitial cells [85]. Studies with double knockout mice for ERα and ERβ showed the arrest of folliculogenesis at the secondary follicle stage [86]. Generally, after activation of its nuclear receptors, E2 binds to specific regions of the DNA, the estrogens response elements (EREs) [81], further regulating the transcription of genes controlling oocyte maturation and growth [84]. More recently, ERβ was shown to mediate the expression of *Junction Adhesion Molecule Like (Jaml)*, a gene needed for the communication between GC and oocyte, as well as other genes *Polypeptide N-Acetylgalactosaminyltransferase (Galnt) 6* or *Egf receptor (Egfr)*, necessary for preovulatory follicle development [87]. It is noteworthy that detailed E2 genomic actions in antral follicle development have been described elsewhere [88]. Nonetheless, E2 is also known to exert non-genomic actions through the membrane receptor, the G protein-coupled estrogen receptor (GPER) 1 [81]. E2 non-genomic effects were shown to involve the activation of intracellular signaling pathways, such as the Phospholipase C (PLC)/PKC pathway, the Ras/Raf/MAPK pathway, the PI3K/Akt kinase cascade, and the cAMP/protein kinase A (PKA) signaling pathway [81], which culminate with the activation of transcription factors from the STAT family, nuclear factor kappa B (NFκB), and CREB. Thus, E2 plays a prominent role in preovulatory follicle formation, activating multiple pathways and mediating growth and expansion of both somatic cells and oocyte.

Connexins are a family of proteins responsible for the organization of intercellular membrane channels of gap junctions, which allow for the transport of molecules, ions, and metabolites between GC and the oocyte. They were shown to be particularly important for preovulatory follicle formation. Indeed, the ovaries of connexin (Cx) 37 knockout mice showed follicular arrest at the preantral stage due to lack of bidirectional communication between GC and oocyte [89]. Another Cx, the Cx43, was also shown to be highly expressed in large antral follicles and mainly induced by E2 [90] and FSH [91]. Furthermore, a morphofunctional process determinant for follicular expansion and antrum formation is the establishment of microtubule TZPs between germ cell and somatic cells. The TZPs originate from a microtubule core in GC projected towards the oocyte, providing tracks for polarized translocation of secretory pathway organelles [92]. Studies on FSHβ knockout mice evidenced the retraction of TZPs from the oocyte, with drastic changes in oocyte chromatin remodeling and meiotic competence acquisition. Thus, coordinated actions of FSH and E2 on TZPs formation [93,94] ensure adequate communication between GC and oocyte during preantral to antral follicle transition.

Research in humans has recently revealed a number of important genes regulating preantral to antral follicle transition [30]. Importantly, *CX40* was identified as the most abundant Cx in human GC of antral and preovulatory follicles. Concerning oocyte-derived factors, *Activating Transcription Factor (ATF) 2* and *Eomesodermin (EOMES)* were abundantly expressed in the oocytes of preovulatory follicles, as well as *Bone Morphogenic Protein (BMP) 15 [30].* Finally, particular relevance was given to the NOTCH pathway during preovulatory follicle formation in women, with *Delta Like Canonical Notch Ligand (DLL)3* and *Jagged Canonical Notch Ligand (JAG) 2* being predominantly expressed in the oocyte and their receptors, *NOTCH2, NOTCH3*, and downstream target gene *Hes Family BHLH Transcription Factor (HES) 1*, highly expressed in GC. These findings highlight the role of the NOTCH signaling pathway regulating oocyte-mediated GC proliferation and differentiation [30].

To conclude, preovulatory follicle formation requires concerted action of gonadotropins FSH and LH on the crosstalk between GC and TC during steroidogenesis and E2 signaling. As a result, the formation of junctional complexes and TZPs create routes for the exchange of metabolites and signaling molecules between somatic cells and oocyte, determining follicle expansion and formation of the Graafian follicle.

5.2. Preovulatory Follicle Formation—The Role of Leptin

Leptin's role in ovarian secretory activity is well established. In vitro studies with rat GC showed leptin treatment impaired E2 secretion [95], whereas the same effect was observed for human luteinized GC [96] or preovulatory follicles in swine [97]. Most importantly, experiments with GC collected from fertile woman showed that treatments with high doses of leptin had deleterious effects on FSH-mediated E2 secretion, mainly through IGF1 inhibition [98]. These results evidenced the detrimental effects of high leptin levels on E2 secretion by dominant follicles and consequent disruption of LH surge and impaired ovulation. Furthermore, a recent report has shown leptin interference with E2 secretion could be mediated by the induction of the neuropeptide cocaine- and amphetamine-regulated transcript (CART) at GC level [99]. Leptin negatively affected intracellular cAMP levels, MAPK signaling, and aromatase *Cyp19a1* mRNA expression with consequent downregulation of E2 synthesis [99]. Finally, we and others have also shown leptin effects on P4 synthesis, as leptin regulated, in a dose-dependent manner, the expression of cytochrome P450 side chain cleavage (P450 scc) and 3β hydroxysteroid dehydrogenase (HSD) [26,27]. Therefore, obesity and consequent hyperleptinemia can impair steroidogenesis in the growing antral follicle.

As previously stated, bidirectional communication between somatic cells and the gamete was revealed to be essential for oocyte maturation and follicular development progression. Interestingly, leptin was shown to modulate reorganization of actin, the main component of projections as TZPs, in systems like the hypothalamus [100] or nucleus pulposus cells in the intervertebral disks [101]. Recently, we have shown that pharmacological treatment of female mice with leptin altered the expression of genes associated with cytoskeleton organization in CC [6]. Moreover, the role of TZPs transferring leptin and *STAT3* from GC to oocytes has been previously reported [102]. Indeed, leptin was shown to regulate levels of Cx43 in the central nervous system (CNS) in vivo in mice [103], reiterating its potential role mediating intercellular communication between GC and the oocyte. On the other hand, the NOTCH pathway was shown by Zhang and co-workers to be upregulated in human preovulatory follicle [30]. Importantly, the crosstalk between leptin and NOTCH signaling was also shown in other systems, such as in vitro studies on glioblastoma cells [104]. Thus, disruption in ovarian leptin signaling during obesity can well affect steroidogenesis and the maintenance of channels for intercellular communication during preovulatory follicle formation.

5.3. Obesity and Leptin Signaling Disruption during Preovulatory Follicle Formation

Obesity has been strongly associated with increased circulating levels of both E2 and androgens, not only due to the ability of the adipose tissue to synthesize steroids [105]

but also to low circulating sex hormone binding globulin levels and suppression of gonadotropin release. Moreover, ovarian TC are known to respond to insulin during androgen synthesis [1]. Seminal work by Wu and co-workers clearly showed the link between hyperinsulinemia in DIO mice and increased ovarian androgen production through hyperactivation of CYP17 at TC level [106]. On the other hand, Souter et al. also correlated gain in body mass index (BMI) with E2 secretion by preovulatory follicles in obese women [107]. Finally, studies in rats evidenced the link between obesity and the lack of preovulatory surge of P4 and LH [108]. Hence, obesity clearly disrupts steroidogenesis, causing a predisposition to premature follicular atresia and anovulation.

The negative impact of obesity on preovulatory follicle formation can be also related to the expression of leptin signaling inhibitors *PTP1B* and *SOCS3*. Indeed, dephosphorylation of human estrogen receptor by *PTP1B* was shown to reduce E2 binding capacity [109]. In breast cancer cells, *PTP1B* was also shown to reduce aromatase activity when overexpressed [110]. Therefore, during leptin resistance, potential increased *PTP1B* activity [111] could impair aromatase activity and E2 levels in the follicle. Conversely, *SOCS3* was previously shown to block ERK1/2 signaling, an important kinase in the LH-mediated oocyte resumption of meiosis, ovulation, and luteinization [112]. Finally, we asked to what extent common DEGs previously identified in CCs collected from both leptin treated and early obesity mouse protocols (4 wk HFD) could be already expressed in GC from antral and preovulatory follicles. We used once more the data from human GC transcriptome throughout folliculogenesis [30] and plotted the DEGs from our study in mice (134 DEGs plus 10 leptin pathway genes—Table S2) [6]. Interestingly, most of the genes were actively transcribed in GC from human antral and preovulatory follicles (Figure 5) as *PTPN2* and *PTP1B*, suggesting this way that previously observed changes in gene expression in CC in early obesity, driven by increased leptin activity, could be seemingly important for GC from preantral and antral follicles. To conclude, dysregulation of leptin signaling inhibitors, through hyperactivation of ObRb and activity of downstream leptin genes, can equally affect preovulatory follicle formation in obese mothers.

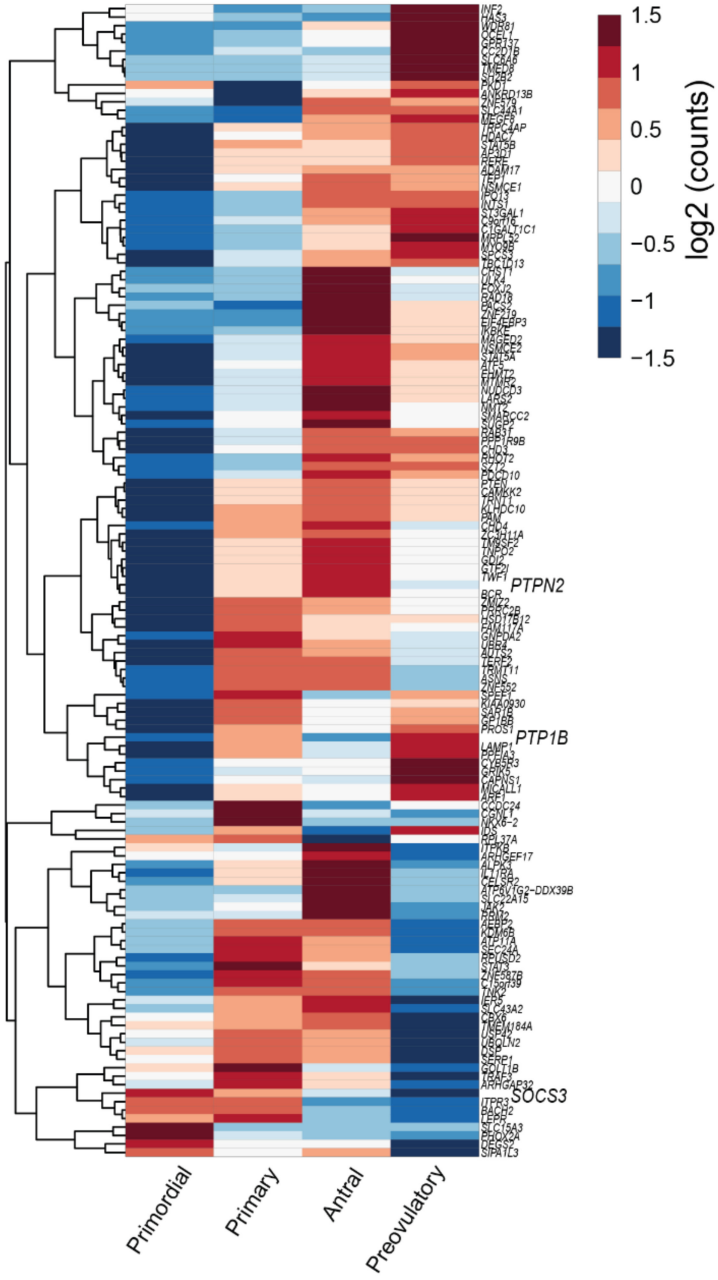

Figure 5. Heatmap representing transcription profile of genes identified as differentially expressed in cumulus cells of mice both fed high fat diet for 4 wk and treated with leptin (for details please see [6]) in human granulosa cells throughout folliculogenesis. Primordial = primordial follicle; Primary = primary follicle; Antral = antral follicle; Preovulatory = preovulatory follicle. Color code from blue to red indicates the relative gene expression level from low to high, respectively. Data from Zhang et al. 2018 [30]; 134 DEGs plus 10 leptin pathway genes—Table S2. Suppressor of cytokine signaling (SOCS) 3, protein tyrosine phosphatase non-receptor type 2 (*PTPN2*), protein tyrosine phosphatase (*PTP*) 1B.

6. Oocyte Maturation: The Last Step before Fertilization

The acquisition of developmental competence by the oocyte is a prerequisite for fertilization and sustained embryo development. Oocyte maturation involves nuclear and cytoplasmic transformation, which largely determine the quality of the female gamete. Oocyte meiotic division remains blocked until hormonal signals unlock the process. The nuclear transformation encompasses dramatic changes in chromatin organization, which

evolves from a decondensed and transcriptionally active state in preantral follicles with a non-surrounded nucleolus (NSN) oocyte [113] into condensed and silenced chromatin in antral follicles with a surrounded nucleolus (SN) oocyte [113]. The cytoplasmic maturation culminates with the reorganization of organelles and preparation for completion of meiosis. After fertilization, meiosis is completed and maternal transcripts are degraded, with the zygote becoming transcriptionally independent and undergoing intense cell division. In the present section, we describe the signaling pathways leading major events orchestrating oocyte maturation and early embryo development, particularly, highlighting potential links to leptin signaling and its disruption during obesity.

6.1. Regulation of Oocyte Maturation

Oocyte nuclear maturation is key for meiosis accomplishment and fertilization [113]. Both human and mouse oocytes from preovulatory follicles are capable of re-entering meiosis; nonetheless, they remain arrested in prophase I until exiting the follicle. This allows for the accumulation of transcripts and proteins, chromatin reorganization, and cytoplasm maturation. Meiotic arrest was shown to be sustained by high cAMP concentrations in the oocyte through the activity of constitutive G protein coupled receptor (GPR) 3 and/or GPR 12 [114]. Hence, cAMP was shown to ensure high levels of PKA activity, leading to inactivation of cell division cycle 25 homolog B (CDC25B) and maturation promoting factor (MPF) and, therefore, enforcing meiotic arrest [115]. Receptors GPR3/12 can be activated by multiple lysophospholipids, such as sphingosylphosphorylcholine (SPC) and sphingosine 1-phosphate (S1P), which were shown to delay spontaneous oocyte maturation [116]. Moreover, cAMP degradation by phosphodiesterase 3A (PDE3A) in the oocyte was also shown to be prevented by exogenous cyclic guanosine monophosphate (cGMP), which appears to be transferred from GC via gap junctions in both mice [117] and humans [118]. The GC-derived cGMP is generated by guanylyl cyclase activity after activation of natriuretic peptide receptors (NPR) by their ligands natriuretic peptides (NPP). The predominant receptor in GC is NPR2 with its cognate ligand NPPC [116]. Interestingly, the expression of the NPR2/NPPC system was shown to be regulated by E2 signaling [84]. Another recent study showed that transforming growth factor (TGF) β upregulated the expression of NPPC [119]. Furthermore, sex steroids were linked to meiosis resumption in a transcription independent manner, with testosterone shown to induce oocyte maturation [120]. After the LH surge, oocyte nuclear maturation and meiosis resumption started with germinal vesicle (GV) breakdown (BD), morphologically characterized by the dissolution of the oocyte nuclear envelope. Indeed, LH was shown to decrease cGMP levels through suppression of NPR2 activity and inhibition of NPPC transcription in GC [116]. Finally, after LH surge, cGMP production was stopped, Cx43 phosphorylated and gap junctions closed, and PDE3A activated in the oocyte with rapid drop in cAMP levels [116]. As a result, MPF could be activated and meiosis resumed.

Another pivotal step during oocyte maturation is the chromosome condensation, generally followed by meiosis progression through prometaphase I, metaphase I, anaphase I, and extrusion of first polar body (PB) in telophase I. Finally, adequate microtubule polymerization is also determinant for oocyte maturation and a prerequisite for correct spindle assembly and chromosome trafficking. The process of microtubule nucleation and subsequent spindle assembly is considered chromosome-dependent in human oocytes [121]. In contrast, in mice, the spindle formation was shown to be driven by the organization of microtubule organizing centers (MTOCs). Once the first PB is extruded, oocyte reassembles the MII spindle with chromosomes aligned in equatorial plate, ready to extrude a second PB upon fertilization.

Cytoplasmic oocyte maturation starts during the GVBD and comprises reorganization of the cytoplasm, remodeling and repositioning of intracellular organelles, movement of vesicles like Golgi and EnR, but also mitochondria repositioning around spindle [122]. Oocytes from most species, including humans, contain Balbiani bodies, which are unique transient cytoplasmic structures for RNA storage and control of translation [123]. Addi-

tionally, rodent oocytes contain also cytoplasmic lattices to store ribosomes. Studies with transgenic mice showed the involvement of peptidyl arginine deiminase 6 (PADI6) [124] in the organization of such structures, as well as the maternal effect gene maternal antigen that embryos require (Mater) [125]. Indeed, during oocyte maturation, a tightly regulated program of maternal mRNA synthesis, degradation, and storage, particularly mRNAs related to ribosome and mitochondria biogenesis, is determinant for acquisition of developmental competence and early embryo development before zygotic genome activation.

The three previously described steps for oocyte maturation (oocyte nuclear maturation and meiosis resumption, chromatin condensation, and cytoplasm maturation) are entirely dependent on oocyte metabolism. It provides energy for meiotic progression, balancing intracellular redox and osmotic potential, and provides building blocks for oocyte growth [122]. Indeed, it was shown that oocytes collected from prepubertal cows had reduced ability to generate viable embryos due to altered metabolic profile with regard to adult animals [126]. Furthermore, genes controlling metabolism were shown to be upregulated in mouse MII oocytes compared to GV oocyte [127]; also, the expression of metabolic genes differed between in vitro and in vivo maturation in bovine oocytes [128]. Mouse oocytes mainly use pyruvate as an energy source for oxidative phosphorylation as pyruvate can exclusively support maturation, maintain viability, and promote cleavage of zygote in the absence of CC [129]. Glucose is mostly metabolized by CC, and together with glucose-6-phosphate, lactate or pyruvate can be transported into the oocyte via gap junctions. Two main oocyte- secreted factors, BMP15 and GDF9, were shown to control CC metabolism [130]. Furthermore, mitochondria significantly contribute to cellular metabolism, and deficiencies in aerobic mitochondrial metabolism have been linked to impaired oocyte competence [131]. Finally, lipid metabolism also plays a role in energy production in oocyte and embryo in mice, supporting its maturation and development. In response to LH surge, fatty acid oxidation takes place in the oocyte, with long chain fatty acid metabolism being a source of acetyl coenzyme A (acyl-CoA), which enters tricarboxylic cycle and electrons for nicotinamide adenine dinucleotide phosphate (NADPH) production in the electron transport chain for adenosine triphosphate (ATP) production [132]. On the other hand, leptin is known to promote lipid oxidation and regulation of triglyceride cellular homeostasis [133]. Therefore, changes in leptin levels can affect oocyte fatty acid oxidation and energy provision during oocyte maturation.

In conclusion, major steps for oocyte maturation encompass dramatic structural changes at the nuclear and cytoplasmic level, which rely on the metabolic performance of both oocyte and CC. Indeed, oocyte quality is known to affect embryo development, being, therefore, the integrity of oocyte maturation and metabolic performance determinant for early embryo development and implantation.

6.2. Leptin Effects on Oocyte Maturation

Leptin involvement in oocyte maturation and early embryo development was previously documented. Intriguingly, leptin was shown to support meiotic progression and developmental competence of bovine oocytes, as well as fertilization and blastocyst development [134]. Furthermore, leptin promoted nuclear and cytoplasmic maturation via MAPK pathway activation in porcine oocytes, as in vitro studies showed increased proportion of oocytes reaching MII stage, upregulation of cyclin B1 protein expression, and enhanced embryo development [135]. In another in vitro study, the supportive effects of leptin on GVBD formation in oocytes from preovulatory follicles were demonstrated, enhancing first PB extrusion and development of preimplantation embryos, mainly through MEK1/2 signaling [136]. Reports in pancreatic beta cells revealed leptin's ability to stimulate adenylate cyclase and increase cAMP cellular levels [137] and activate PDE3B [138]. Moreover, leptin treatment increased intracellular cAMP level and PKA activity in murine macrophages [139]. Indeed, in different cellular contexts, leptin was also shown to modulate factors, such as cAMP and PKA activity, known to have a prominent role in meiosis reactivation and maturation of the gamete. Finally, the role of leptin on glucose homeostasis

systemically is well described through the modulation of the IRS/PI3K pathway [140]. Leptin was also shown to attenuate the deleterious impact of high glucose levels in oocyte, promoting glycolysis and oocyte maturation [141]. Thus, changes in leptin signaling during obesity can produce expected changes in oocyte metabolism.

6.3. Oocyte Maturation and Early Embryo Development in Obese Mothers

Obesity leads to severe systemic hormonal imbalance with drastic consequences for oocyte maturation and embryo development. Firstly, maternal obesity was shown to alter insulin, glucose, and free fatty acid concentration in follicular fluid, directly affecting oocyte metabolism and reducing oocyte maturation [142]. Secondly, insulin-stimulated glucose uptake was shown to be impaired in CC isolated from mice treated with HFD, suggesting the establishment of insulin-resistance [143]. Indeed, activation of the polyol pathway during hyperglycemia was shown to negatively affect metabolism and CC–oocyte communication [144]. As a result, in obese mothers, oocyte maturation, fertilization rate, and embryo quality were significantly decreased [145]. Moreover, oocytes derived from obese mice after in vitro fertilization and culture presented reduced development [145]. Furthermore, studies in mice clearly linked maternal obesity with oocyte and zygote increased mitochondrial potential, mitochondrial DNA content and biogenesis, and generation of reactive oxygen species (ROS) [2]. Importantly, the absence of mitophagy was presented as the main cause of mitochondrial dysfunction in oocytes and early embryos from obese mothers [12]. Another recent report also linked lack of expression of Stella in oocytes from obese mice with increased hydromethylation in the zygote and DNA instability [5]. Thus, obesity can drastically affect both oocyte and embryo quality. Finally, with regard to direct effects of altered leptin signaling components in oocyte maturation, *SOCS3* mRNA was shown to be decreased in human CC collected from patients with polycystic ovary syndrome (PCOS) [146]. Indeed, *SOCS3* was suggested as a biomarker of oocyte or embryo competence in CC of PCOS patients. In summary, maternal obesity leads to disruption in mitochondrial dynamics, excessive free fatty acids, and cellular damage, which affect not only the oocyte but may also hamper embryo development.

7. Conclusions

Folliculogenesis regulation is a complex process that depends on the crosstalk between local and systemic factors, accounting, therefore, for its vulnerability to maternal physiological fitness. Indeed, leptin, an established local regulator of folliculogenesis, presents increased circulating levels in obese mothers with major consequences for follicular activation, recruitment, and growth. In addition to increased ovarian ObRb activation and altered leptin signaling, deleterious effects of systemic hyperleptinemia during obesity can also result from local overexpression of mediators of leptin resistance, such as *SOCS3*. As a result, leptin's prominent role as suppressor of follicular pool activation can be affected in obese mothers, with consequent POF. During preovulatory follicle formation, altered leptin signaling affects not only steroidogenesis but the communication between GC and oocyte, which is known to be key for antrum formation and oocyte growth. Finally, changes in leptin signaling and impaired metabolism may also incur drastic consequences for oocyte maturation, hampering meiosis resumption and cytoplasmic maturation (Figure 6). Hence, advances in our understanding of the role of leptin on ovarian pathophysiology during obesity should unravel innovative tools to monitor the quality of the oocyte during disease progression, potentially preventing pregnancy failure and ensuring the birth of a healthy offspring.

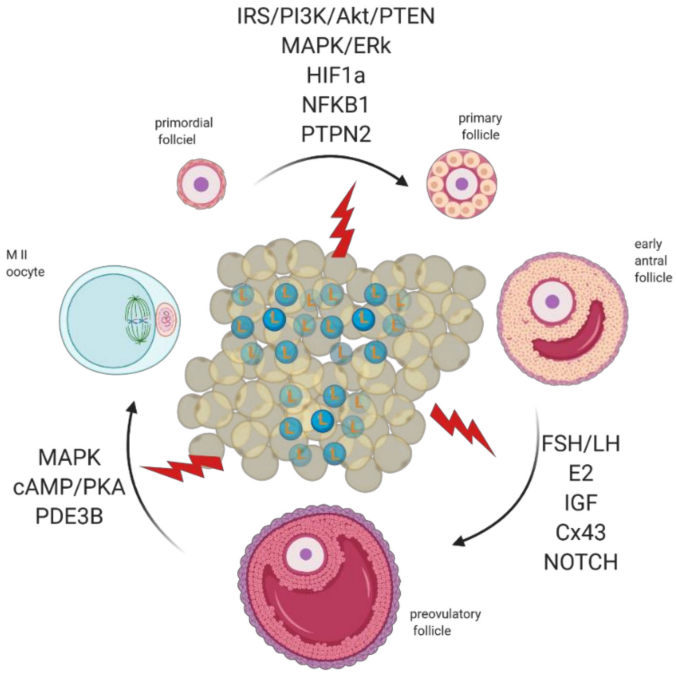

Figure 6. Adipose tissue secretes excessive amounts of leptin (L) during obesity. Leptin signaling in the ovaries of obese mothers is altered, culminating with the establishment of leptin resistance. As a result, signaling pathways governing primordial follicle activation, preovulatory follicle formation, and oocyte maturation can be affected. Created with BioRender.com, accessed on 1 November 2020.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22084270/s1, Table S1: Summary of the studies cited in the present review, regarding species, nature of study (in vivo or in vitro), type of report (basic study, clinical report, review/book chapter); Table S2: List of differentially expressed genes (A) The overlap between differentially expressed genes in cumulus cells collected from mice after 4 weeks of diet-induced obesity or 16 days of leptin treatment [6]. (B) The list of components of leptin signaling pathway.

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Abbreviations

acyl-CoA	acetyl coenzyme A
Akt	Protein kinase B
AMH	Anti-Mullerian hormone
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BDNF	Brain-derived nerve factor
BMI	Body mass index
BMP	Bone morphogenic protein
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CC	Cumulus cells
CDC25B	Cell division cycle 25 homolog B
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
COCs	Cumulus–oocyte complexes
CREB	Activates cyclic AMP-response element binding protein
Cx	Connexin
CYP	Cytochrome P450
DEGs	Differently expressed genes
DIO	Diet-induced obesity
DLL	Delta like canonical notch ligand
dpc	Days post coitum
E2	Estradiol
EGF	Epidermal growth factor
Egfr	Epidermal growth factor receptor
EOMES	Eomesodermin
EnR	Endoplasmic reticulum
ER	Estradiol receptor
EREs	Estrogens response elements
ERK	Extracellular signal regulated kinase
FGF	Basic fibroblast growth factor
FIGa	Folliculogenesis-specific basic helix–loop–helix
FOXO	Forkhead box O
FSH	Follicule-stimulating hormone
Galnt	Polypeptide N-acetylgalactosaminyltransferase
GC	Granulosa cells
GDF	Growth differentiation factor
GnRH	Gonadotropin releasing hormone
GPER	G protein coupled Estrogen receptor
GPR	G protein coupled receptor
GVBD	Germinal vesicle breakdown
HES	Hes family BHLH transcription factor
HFD	High-fat diet
HSD	Hydroxysteroid dehydrogenase
IGF	Insulin-like growth factor
IRS	Insulin receptor substrate
JAG2	Jagged canonical notch ligand
JAK	Janus kinase
Jaml	Junction adhesion molecule like
Kit	Stem cell factor receptor
LH	Luteinizing hormone
Lhx8	LIM homeobox protein
MAPK	Mitogen-activated protein kinase

Mater	Maternal antigen that embryos require
MEK	Mitogen-activated protein kinas
MII	Metaphase II oocyte
miR	Micro RNA
MPF	Maturation promoting factor
MTOCs	Microtubule organizing centers
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NFKB	Nuclear factor kappa B
NPP	Natriuretic peptides
NPR	Natriuretic peptide receptors
NSN	Non-surrounded nucleolus oocyte
NT	Neurotrophin
ObR	Leptin receptor
ObRb	Leptin receptor isoform b
p27	Cyclin-dependent kinase inhibitor 1B
P4	Progesterone
P450scc	Cytochrome P450 side chain cleavage
PADI	Peptidyl arginine deiminase
PB	Polar body
PCOS	Polycystic ovary syndrome
PDE3A	Phosphodiesterase 3A
PDK	Phosphoinositide-dependent kinase
PGC	Primordial germ cells
PI3K	Phosphatidylinositol 3 kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POF	Premature ovarian failure
PTEN	Phosphatase and tensin homolog
PTP1B	Protein tyrosine phosphatase
PTPN2	Protein tyrosine phosphatase non-receptor type 2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpS6	Ribosomal protein S6
S1P	Sphingosine 1-phosphate
S6K1	Ribosomal protein S6 kinase beta-1
SCF	Stem cell factor
SHP-2	SH2-domain containing protein tyrosine phosphatase
SIRT	Sirtuin
SN	Surrounded nucleolus oocyte
SOCS3	Suppressor of cytokine signaling 3
SPC	Sphingosylphosphorylcholine
STAT	Signal transducer and activator of transcription
TC	Theca cells
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TSC	Tuberous sclerosis complex
TZPs	Transzonal projections
wk	Weeks

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