Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells

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Organogenesis of the ovary is a highly orchestrated process involving multiple lineage determination of ovarian surface epithelium, granulosa cells and theca cells. Although the sources of ovarian surface epithelium and granulosa cells are known, the origin(s) of theca progenitor cells have not been definitively identified. Here we show that theca cells derive from two sources: \textit{Wt1}+ cells indigenous to the ovary and \textit{Gli1}+ mesenchymal cells that migrate from the mesonephros. These progenitors acquire theca lineage marker \textit{Gli1} in response to paracrine signals Desert hedgehog (\textit{Dhh}) and Indian hedgehog (\textit{Ihh}) from granulosa cells. Ovaries lacking \textit{Dhh}/\textit{Ihh} exhibit theca layer loss, blunted steroid production, arrested folliculogenesis and failure to form corpora lutea. Production of \textit{Dhh}/\textit{Ihh} in granulosa cells requires growth differentiation factor 9 (GDF9) from the oocyte. Our studies provide the first genetic evidence for the origins of theca cells and reveal a multicellular interaction critical for the formation of a functional theca.
Ovarian morphogenesis is a highly orchestrated process in which follicles, the basic unit of the ovary, form through an intricate communication between the oocyte, granulosa cells immediately surrounding the oocyte, and theca cells in the mesenchyme. Defects in this process have dire consequences for female reproductive health and fertility. The process of folliculogenesis starts with the breakdown of germ cell nests, in which an individual oocyte becomes encased by somatic granulosa cells. As the follicle continues to grow, it recruits precursors for the theca cell lineage and completes the process of follicle assembly. Theca and granulosa cells communicate through epithelial–mesenchymal crosstalk during the course of follicle development. Theca cells produce androgens, which is subsequently converted to estrogens by granulosa cells. Granulosa cell-derived estrogens in turn provide a local feedback loop in regulating androgen production in the theca cells. This unique interaction lays the foundation of the two-cell lineage specification in many organs. In adult ovaries, Indian hedgehog (Ihh) ligand, a morphogen responsible for epithelial–mesenchymal crosstalk, is expressed in the ovary before birth and becomes apparent in the adult ovary after birth. Ihh expression was restricted to the mesenchyme surrounding the FOXL2-positive granulosa cells (Fig. 1d,h). This pattern persisted in the adult ovary (Fig. 1i,j).

Mesonephros-derived Gli1+ cells are a source of theca cells. The close association of Gli1-positive cells in the mesonephros and the ovary suggests that Gli1-positive cells from the mesonephros could be a source of theca progenitors. To test this hypothesis, we utilized a tamoxifen-induced Rosa-LSL-tdTomato lineage-tracing model, in which Gli1-positive cells in the mesonephros were labelled exclusively during embryogenesis. We administered a single tamoxifen injection to pregnant mice carrying Gli1-CreERT2; Rosa-LSL-tdTomato embryos at E12.5, when Gli1 expression was restricted to the mesonephros. The specificity of this model was confirmed by the lack of fluorescein in control embryos (Supplementary Fig. 3). During fetal life, the tdTomato-positive cells were present specifically in the mesonephros but absent in the ovary (Fig. 2a,b), consistent with endogenous Gli1 expression pattern (Fig. 1a,e). The tdTomato-positive cells first appeared in the ovary just before birth (Fig. 2c) and a significant number of these cells were observed in the ovary at birth (Fig. 2d and Supplementary Fig. 4). This result demonstrates that the tdTomato-positive cells in the neonatal ovary were derived from the Gli1-positive cells in the fetal mesonephros. At 2 months of age, the mesonephros-derived Gli1-positive cells in the ovarian interstitium became steroidogenic theca cells positive for 3βHSD (Fig. 2e–i).

When comparing the Gli1-positive cells in the ovary (Fig. 1d,h) with the contribution of Gli1-positive cells from the mesonephros (Fig. 2e–i), it became apparent that the mesonephros-derived Gli1-positive cells represent a small fraction of the ovarian Gli1-positive cell population. The majority of these cells seem to come from the ovarian mesenchymal cells that become Gli1-positive (Fig. 1g,h). This hypothesis was supported by the findings that lineage-labelled ovarian Gli1-positive cells at P2 (Fig. 1c,g) became 3βHSD-positive cells in the theca compartment (Fig. 2n). Notably, ovarian Gli1-positive cells at P2 gave rise to nearly all the cells in the theca layer in the adult ovary (Fig. 2j). These results suggest that a small percentage of the theca cells in the adult ovary are derived from the mesonephros, whereas the majority of the theca cells must come from progenitor cells that originated in the ovarian mesenchyme.

Ovary-derived Wt1+ cells are the main source of theca cells. The progenitor cells in the gonadal primordium are thought to be the bona fide source of gonadal somatic cells. When the somatic cell progenitors first appear in the gonad, they express Wilms’ tumour 1 (Wt1), a transcription factor essential for gonadal formation. To determine whether the gonad-derived Wt1-positive cells contribute to the theca cell lineage, we performed similar tamoxifen-induced lineage-tracing experiments by labelling the Wt1-positive cells in Wt1-CreERT2; Rosa-LSL-tdTomato gonads at the onset of sex determination (E10.5). Twenty-four hours after tamoxifen treatment, the lineage-labelled Wt1-positive cells were present in the somatic compartment of fetal ovaries overlapping with endogenous WT1 protein (Fig. 3a,d). At 1 month of age, the ovary-derived Wt1-positive cells were located in the ovarian interstitium surrounding the follicles and positive for 3βHSD (Fig. 3c,f). These results indicate that Wt1 marks a specific pool of steroidogenic precursors in the indifferent gonad before sexual differentiation occurs. To further investigate if these fetal ovary-derived Wt1-positive cells...
become Gli1-positive theca progenitor cells after birth (Fig. 1g, h), we examined the induction of Gli1-LacZ expression in the Wt1-positive cells. When the fetal ovary-derived Wt1-positive cells began to surround the primary follicles in the medulla of the ovary, they acquired the expression of Gli1, indicating that they have committed to the theca cell lineage (Fig. 3b,e).

Transcriptomes of ovary- and mesonephros-derived Gli1+ cells. Fetal ovary-derived Wt1-positive cells and mesonephros-derived Gli1-positive cells appear to represent two different populations of theca cells. Fetal ovary-derived Wt1-positive cells were found in the entire theca in the adult ovary, whereas mesonephros-derived Gli1-positive cells were located preferentially to the basal lamina (Supplementary Fig. 5), a region that contains predominantly steroidogenic cells. This observation leads to the hypothesis that these two cellular sources of progenitors represent unique populations in the theca. We isolated mesonephros-derived Gli1-positive cells (Fig. 2e) and neonatal ovary-derived Gli1-positive cells (Fig. 2j) by fluorescence-activated cell sorting from 2-month-old mice, and compared their transcriptomes (Fig. 3g). The microarray analysis indicated that mesonephros-derived theca cells exhibit a transcriptome distinctly different from that of the ovary-derived Gli1 population. Many of the genes enriched in the mesonephros-derived theca cells were associated with steroidogenesis, including Star, Cyp11a1, Cyp17a1 and Lhcgr (Fig. 3h). The steroidogenic activity of these cells was also confirmed by immunostaining with 3βHSD (Supplementary Fig. 6). In contrast, the ovary-derived Gli1-positive cells exhibited higher expression of Esr1 (Estrogen receptor 1), Wt1 and genes implicated in cell growth and proliferation (Supplementary Table 1), consistent with their ubiquitous distribution within the theca layer. These results demonstrate that ovary-derived Gli1-positive cells represent a broad mesenchymal cell population(s), whereas the mesonephros-derived Gli1-positive cells contribute to the steroidogenic cell population in the theca.

Granulosa cells are sources of Hh ligands Dhh and Ihh. The identification of Gli1 as a theca cell lineage marker raises the question: what signal(s) specifies theca cell lineage and induces Gli1 expression? Gli1 is a known downstream target of the Hh pathway. We first examined whether activation of the Hh
pathway is responsible for inducing \textit{Gli1} expression by culturing fetal ovaries in the presence or absence of the Hh inhibitor cyclopamine\textsuperscript{23}. Cyclopamine treatment inhibited \textit{Gli1} expression in the ovaries compared with the controls (Fig. 4a–c), indicating that \textit{Gli1} expression is induced by the canonical Hh pathway. In search of the Hh ligands that may be responsible for activating the Hh pathway, we examined mRNA expression of \textit{Dhh}, \textit{Ihh} and Sonic hedgehog (\textit{Shh}) in the ovary before (E17.5) and after (P3)
the appearance of Gli1. Expression of Dhh and Ihh was low in the ovary before birth and significantly increased at P3, corresponding to the onset of Gli1 expression (Fig. 4d). Expression of Shh was undetectable in ovaries at both stages (Supplementary Fig. 7). To identify which somatic cell type(s) produce Dhh and Ihh, we isolated theca progenitor cells (Gli1-CreERT2; Rosa-LSL-tdTomato) and granulosa cells (Foxl2-CreERT2; Rosa-LSL-tdTomato) from perinatal ovaries by fluorescence-activated cell
Loss of Dhh and Ihh abolishes theca cell differentiation. Female Dhh-deficient mice are fertile and exhibit normal ovarian development, suggesting that the other Hh ligands, such as Ihh, may compensate for the loss of Dhh during folliculogenesis. Most of the Ihh global deficient embryos die before birth, therefore precluding the analysis of the ovary in the adult. To investigate whether Dhh and Ihh together regulate theca cell development, we generated Dhh/Ihh double knockout mice (hereafter referred as Dhh/Ihh DKO), in which both Dhh and Ihh were ablated from Sfl-positive gonadal somatic cells (Sfl-Cre; Ihh\textsuperscript{+/−}; Dhh\textsuperscript{−/−})\textsuperscript{26}. Although Sfl-Cre is known to be active in the pituitary, we do not expect that it affects pituitary functions based on the fact that Dhh and Ihh are not expressed in the pituitary\textsuperscript{16,27}. Ovaries deficient in either Dhh or Ihh alone exhibited normal folliculogenesis (Supplementary Fig. 9). However, Dhh/Ihh DKO ovaries were significantly smaller in size and irregular in shape compared with the control (Fig. 5a, b). In contrast to the normal progression of folliculogenesis and the presence of corpora lutea in adult control ovaries, Dhh/Ihh DKO ovaries lacked corpora lutea, and follicles failed to progress beyond preantral follicle stage, suggesting that ovulation did not occur (Fig. 5c–f). Antral follicles were rarely found in the DKO ovaries and if they were present, they appeared cystic and haemorrhagic (Fig. 5g, h). In addition to a loss of theca, as determined by α-SMA, a marker for smooth muscle cells in the theca layer (Fig. 6a,b)\textsuperscript{28}, CYP17A1-positive androgen-producing theca cells were also absent in the mesenchyme of DKO ovaries (Fig. 6c,d). Furthermore, steroidogenic genes, including Nr5a1, Star, Cyp11a1 and Hsd3b1 (Fig. 6e), were significantly downregulated along with a decrease in serum levels of dehydroepiandrosterone (DHEA), testosterone and progesterone in the DKO mice (Fig. 6f). These results altogether support the model that Dhh and Ihh from granulosa cells are responsible for the establishment of the theca cell lineage. In the absence of Dhh and Ihh, theca cell layer failed to form and preantral follicles were unable to develop further.

**Oocyte-derived GDF9 regulates Dhh/Ihh in granulosa cells.** Next, we investigated what signal induces the production of Hh ligands in the granulosa cells. Functions of granulosa cells are regulated not only systemically by hormone signals from the pituitary, but also locally via factors produced in the oocytes\textsuperscript{1}. Without the oocyte, follicle formation never occurs\textsuperscript{29}. To test if oocytes play a role in Hh ligand production, we treated pregnant females with busulfan, a chemotherapeutic drug known to induce oocyte death in the embryos\textsuperscript{30,31}. The specificity of in utero busulfan treatment on oocytes was confirmed by a complete abolishment of oocytes, coincident with normal establishment of FOXL2-positive granulosa cell population in ovaries of busulfan-treated pups (Supplementary Fig. 10). In the oocyte-depleted ovaries, expression levels of Dhh, Ihh and Gli1 were significantly downregulated (Fig. 7a and Supplementary Fig. 10), suggesting that oocyte-derived factor(s) contributes to Dhh and Ihh production in granulosa cells. One potential candidate of such factor is Gdf9, an oocyte-specific factor essential for folliculogenesis\textsuperscript{1,32}. In neonatal Gdf9 knockout ovaries, expression of Dhh and Ihh was significantly reduced, and Gli1 expression was also decreased (Fig. 7b). When recombinant GDF9 was supplemented to oocyte-depleted ovaries (busulfan-treated) in culture, it increased mRNA expression of Ihh, Dhh and subsequent Gli1 (Fig. 7c), further supporting the role of oocyte-derived GDF9 in stimulating Dhh and Ihh production in granulosa cells and subsequent appearance of Gli1-positive theca progenitor cells (Fig. 7d). To exclude the possibility that sorting (Supplementary Fig. 8). We found that the Hh downstream targets Gli1 and Ptch1 were highly enriched in theca progenitor cells, whereas Hh ligands Dhh and Ihh were found predominantly in granulosa cells (Fig. 4e). These results implicate a novel regulation of theca cell differentiation by granulosa cells through the Hh signalling pathway.
GDF9 might directly act on theca cells, we examined the expression of Gdf9 in the DKO ovaries and it was not different from those of control animals (Supplementary Fig. 11).

**Discussion**

We provide the first genetic evidence for the origins of theca progenitor cells: Wt1-positive cells intrinsic to ovary and Gli1-positive cells in the mesonephros. It is not clear why two sources of theca cell progenitors are required for follicle development and we speculate that this phenomenon may relate to temporal and spatial characteristics of follicle development. Two classes of primordial follicles are present in the ovary: the primordial follicles (first wave) that are activated immediately after birth in the medulla of the ovary, and the primordial follicles (second wave) that are gradually activated later in adulthood in ovarian cortex. Granulosa cell precursors that comprise each primordial follicle appear to be heterogeneous populations.

**Figure 5** | Loss of Dhh and Ihh results in defective folliculogenesis in the ovary. (a–h) Whole mount light-field microscopic images (a,b) and histological analysis of the ovaries (c–h) from control (Sf1-Cre; Ihhf/++; Dhh+/−) and Dhh/Ihh DKO mice (Sf1-Cre; Ihhf−/−; Dhh−/−) at 2 months of age. (c,d) Whole ovarian sections with different stages of follicle development. (e,f) The most advanced stage of follicle development in control (corpus luteum) and DKO ovaries (Preantral follicle). (g,h) Antral follicles from control and DKO ovaries (haemorrhagic). Asterisks indicate the presence of corpora lutea. n = 7 for all control specimens and n = 3 for the Dhh/Ihh DKO specimens. Scale bar, a–b, 1 mm; c–h, 200 μm.
The pre-granulosa cells that are specified during fetal stage contribute to the first wave of primordial follicles in the medulla of the ovary immediately after birth. On the other hand, the granulosa cells in the cortex of postnatal ovaries participate in the second wave of primordial follicles later in adulthood. Along this line of evidence, our finding demonstrated that theca cells also arise from two distinct sources. It is possible that the two sources of theca progenitor cells are involved in the two waves of follicular development. The mesonephros-derived Gli1-positive cells are first seen in the medulla of the neonatal ovary where the first wave of follicular development occurs. This observation suggests that the mesonephros-derived theca progenitor cells could be implicated in the initiation of the first wave of folliculogenesis. It is not clear whether the GDF9/Hh signalling is controlling both classes of follicles, or a certain population. However, if GDF9/Hh signalling controls only one class of follicles but not the other, one would assume that the origin of follicles should develop normally with the formation of a functional theca in the absence of GDF9 or Hh signalling. In fact, in the Dhh/Ihh DKO ovaries, the theca layer failed to differentiate in all follicles, indicating that both classes of follicles are affected by the loss of Dhh and Ihh.

Although Dhh and Ihh appear to be downstream targets of GDF9, the ovarian phenotypes of Gdf9 KO ovaries differ from those observed in Dhh/Ihh dKO ovaries. The follicles in the Gdf9 KO ovaries arrest at primary stage, whereas follicles in Dhh/Ihh dKO ovaries develop beyond the primary stage and arrest at the preantral stage. The difference in phenotypes reveals two important findings: first, DHH/IHH signalling pathway plays a specific role in theca cell differentiation and second, GDF9, the factor responsible for the production of Dhh and Ihh, has a broader function in follicle development. The identities of other players that act downstream of GDF9 remains to be identified.

In contrast to the long-standing assumption that theca cells derive exclusively from within the ovarian stroma, we reveal an extra-ovarian source of Gli1-positive cells from the mesonephros that contributes to the adult theca cell lineage. In addition, we demonstrate that Wt1 marks a specific pool of steroidogenic precursors in the bipotential gonad before sexual differentiation occurs. These theca progenitor cells commit to theca cell lineage via signals from oocyte (GDF9) and granulosa cells (DHH and IHH). Oocyte-specific factor GDF9 stimulates Hh ligand production in granulosa cells, which in turn induces the differentiation of Gli1-positive theca progenitor cells. Our discovery of the origins of theca cells and the mechanism underlying their appearance not only fill a critical void in basic ovarian biology, but also serve as novel entry points to understand how theca cell-related pathology affects female reproductive health.

**Methods**

**Animals breeding.** Gli1-LacZ (#008211), Gli1-CreERT2 (#007913), Wt1-CreERT2 (#009192), Foxl2-CreERT2 (#015854), Rosa-LSL-tdTomato (#007905), Dhh+/– (#002784), Ihh+/– (#004290) mice were purchased from the Jackson Laboratory. Sf1-Cre and Ihh flox/flox mice were kind gifts from Dr Keith Parker (UT Southwestern Medical Center) and Dr Francesco DeMayo (Baylor College of Medicine), respectively. Female mice were housed with male mice overnight and checked for the presence of vaginal plug the next morning. The day when the vaginal plug was detected was considered embryonic day (E) 0.5. The day of birth was considered postnatal day 1 (P1). All animal procedures were approved by the National Institute of Environmental Health Sciences (NIEHS) Animal Care and

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**Figure 6 | Dhh and Ihh are required for theca cell development in the ovary.** (a–d) Immunofluorescence of α-SMA in red (a, b), CYP17A1 in magenta (c, d) and FOXL2 in green in Dhh/Ihh DKO (n = 3) and control ovaries (n = 7). α-SMA is a marker for smooth muscle cell. CYP17A1 is a marker for androgen-producing theca cells and FOXL2 marks granulosa cells. Scale bar, 200 μm. (e) Quantitative PCR analysis of gene expression for Hedgehog signalling components (Dhh, Ihh and Gli1), and genes associated with steroidogenesis (Nr5a1, Star, Cyp11a1 and Hsd3b1) in control (n = 4) and DKO ovaries (n = 3). Two-tailed Student’s t-test was used. *P < 0.05; **P < 0.01. (f) Serum levels of DHEA, testosterone and progesterone in control (n = 7) and Dhh/Ihh DKO female mice (n = 3). *P < 0.05. Two-tailed Student’s t-test was used and values in all graphs are presented as means ± s.e.m.
Student’s t-test. Values in all graphs are presented as means ± s.e.m. (d) Proposed model for the origins and establishment of theca cell lineage in the mouse ovary.

Use Committee and are in compliance with a NIEHS-approved animal study proposal. All experiments were performed on at least three animals for each genotype.

Busulfan treatment. Pregnant CD-1 females were injected intraperitoneally (IP) at E10.5 with 40 mg kg⁻¹ of busulfan (Sigma) dissolved in 50% dimethyl sulphoxide, or an equivalent volume of 50% dimethyl sulphoxide as the vehicle control.

Tamoxifen treatment. CreER<sup>T2</sup> activity was induced by IP injection of 1 mg tamoxifen (T5648, Sigma-Aldrich) per mouse in corn oil, respectively. For the vehicle control, an equivalent volume of corn oil was injected. No overt teratological effects were observed after tamoxifen administration under these conditions.

Immunohistochemistry and histological analysis. For immunohistochemistry on frozen sections, ovaries were fixed in 4% paraformaldehyde in PBS at 4 °C overnight, dehydrated through a sucrose gradient, embedded and cryosectioned at 10 μm increments. After preincubating with 5% normal donkey serum in PBS for 1 h, the sections were then incubated with either anti-FOXL2 (1:500, a gift from Dr Dagmar Wilhelm, Monash University, Australia), anti-PECAM-1 (1:500, BD Biosciences), anti-3β-hydroxysteroid dehydrogenase (1:500, CosmoBio Co. Ltd), anti-β-galactosidase (1:1,000, Abcam), anti-TRA98 (1:1,000, MBL International) or anti-WT1 (1:300, Abcam), primary antibodies in PBS-Triton X-100 solution with 5% normal donkey serum at 4 °C overnight. The sections were then washed and incubated in the appropriate secondary antibody (1:500; Invitrogen) before mounting in Vector Mount with haematoxylin/eosin or PAS/haematoxylin, and were scanned using an Aperio ScanScope XT Scanner (Aperio Technologies, Inc.) for digital image analysis.

LacZ staining. The LacZ staining solution was made by dissolving X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Roche) and 1 mg ml⁻¹ of 4-β-D-galactosidase (β-gal) in 100 mM Tris/100 mM NaCl/1 mM MgCl₂, pH 7.4. Slides were immersed in the stain solution for 4–18 h at 37 °C, counterstained with Fast Red (Sigma) and mounted with mounting medium (Vector Laboratories). Samples were examined under a Leica DM2500 confocal microscope. For histological analysis, the samples were fixed in 4% paraformaldehyde in PBS at 4 °C overnight, dehydrated through an ethanol gradient and embedded in paraffin wax. Sections were stained with haematoxylin/eosin or PAS/haematoxylin, and were scanned using an Aperio ScanScope XT Scanner (Aperio Technologies, Inc.) for digital image analysis.

Gene expression analysis. Total RNA was isolated from ovaries using the PicoPure RNA isolation kit (Arcturus) according to the manufacturer’s protocol. The cDNA preparation was synthesized using random hexamers and the

Figure 7 | **Gli1** expression in the theca progenitor cells is induced by oocyte-derived factor GDF9 through Hedgehog signalling in granulosa cells.** (a) Quantitative PCR (qPCR) analysis of Gli1, Ihh and Dhh mRNA expression in the control (n = 4) and busulfan-treated ovaries (n = 3). Two ovaries were pooled as n = 1 and three times independent experiments were repeated. (b) qPCR analysis of Gli1, Ihh and Dhh mRNA expression in control (n = 3) and Gdf9 knockout ovaries (n = 3). (c) qPCR analysis of Gli1, Ihh and Dhh mRNA expression in the E18.5 oocyte-depleted ovaries (busulfan-treated) cultured with (n = 5) or without (n = 5) recombinant mouse GDF9 protein (60 ng ml⁻¹). The expression level in oocyte-depleted ovaries without GDF9 (Control) was set as 1. Two ovaries were pooled as n = 1 and two times independent experiments were repeated. *P < 0.05; **P < 0.01; ***P < 0.001; Two-tailed Student’s t-test. Values in all graphs are presented as means ± s.e.m. (d) Proposed model for the origins and establishment of theca cell lineage in the mouse ovary.
Superscript II cDNA synthesis system (Invitrogen Corp.) following the manufacturer’s instruction. Gene expression was analysed by real-time PCR using Bio-Rad CFX96 Real-Time PCR detection system. Taqman gene-expression probes (Gli1: Mm00494656_m1, Ptc1: Mm00436206_m1, Ihh: Mm00439613_m1, Shh: Mm00436528_m1, Dhh: Mm01310203_m1, Fox2: Mm00483454_m1, Star: Mm00441558_m1, Cyp1a1: Mm00490735_m1, Cyp1a2: Mm00484040_m1, Lhcg: Mm00496087_m1, Hsd3b1: Mm00490735_m1, Gdf9: Mm00436565_m1, Nr5a1: Hs01018738_m1, Gli1: Mm00484849_m1) were used to detect fold changes of the transcripts. Fold changes in gene expression were determined by quantification of cDNA from target (treated) samples relative to a calibrator sample (control). All real-time PCR analyses were performed in duplicate, and the results were reported from at least three independent experiments. The relative fold change of transcript was calculated using the mathematical model of Pfaffl (ref. 38) and was normalized to 18S rRNA (Mm9328990_g1, Gapdh (Mm99999995_g1) or Gata4 (Mm00484689_m1) as an endogenous reference.

Microarray analysis. Gene expression analysis was conducted using Affymetrix Mouse Genome 430 2.0 GeneChip arrays (Affymetrix). Four microarrays of total RNA (due to varying concentrations among samples) were amplified as directed in the WT-Ovation Pico RNA Amplification System protocol, and labelling with biotin following the Environ Biotin Module. 4.6 µg of amplified biotin-aRNAs were fragmented and hybridized to each array for 18 h at 45 °C in a rotating hybridization oven. All arrays were hybridized with streptavidin/phycoerythrin utilizing a double-antibody staining procedure and then washed for antibody amplification before-antibody staining procedure and then washed for antibody amplification and then scanned using the Affymetrix GeneChip MS 500 scanner. GeneChip Command Console Software was used to perform data analysis. The probe set expression summaries were generated, and data were obtained using the GeneChip Command Console Software by two-tailed Student’s t-test. Values are presented as mean ± s.e.m. A minimal of three biological replicates was used for each experiment.

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Additional information
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