Macrophage regulatory mechanisms in atherosclerosis

The interplay of lipids and inflammation

Neele, A.E.

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Chapter 5

Macrophage Kdm6b controls the pro-fibrotic transcriptome signature of foam cells

Annette E. Neele, Koen H.M. Prange, Marten A. Hoeksema, Saskia van der Velden, Tina Lucas, Stefanie Dimmeler, Esther Lutgens, Jan Van den Bossche, Menno P.J. de Winther

Abstract

Aim
In order to identify regulators of foam cells, we studied the H3K27 demethylase Kdm6b (also known as Jmd3), a known regulator of macrophages, in controlling the transcriptional profile of foam cells.

Material and Methods
Foam cells from Kdm6b deleted or Kdm6b wildtype mice were isolated and used for RNA-sequencing analysis.

Results
Pathway analysis revealed that pro-fibrotic pathways were strongly suppressed in Kdm6b deleted foam cells. Analysis of published datasets showed that foam cell formation induces these pro-fibrotic characteristics. Overlay of both data sets indicated that fibrotic genes which are induced upon foam cell formation, are reduced in the absence of Kdm6b. These data suggest that foam cell formation induces a pro-fibrotic gene signature in a Kdm6b-dependent manner.

Conclusion
We identified Kdm6b as a novel regulator of the pro-fibrotic signature of peritoneal foam cells.
**Introduction**

Foam cells are a key hallmark of atherosclerotic lesion formation. Atherosclerosis is a lipid-driven chronic inflammatory disorder of the arteries. Monocytes and macrophages play a central role in the initiation and progression of lesion formation and clinical outcome (1-3). Within the atherosclerotic lesion macrophages scavenge modified lipoproteins and thereby acquire their foam cell characteristics. Besides their foam cell phenotype, macrophages can have specific inflammation regulatory functions in atherosclerotic lesions (4). They can adopt different activation states based on local environmental triggers (5-7). Epigenetic pathways are crucial for monocyte to macrophage differentiation and activation (8, 9). Histone modifications are one of the epigenetic mechanisms of gene regulation and refer to posttranslational modifications of core histones and histone tails. Histone acetylation is in general associated with active gene transcription, whereas histone methylation can result in either gene transcription or repression depending on the site of methylation. While di- or trimethylation of histone H3 at lysine-4 marks promoters, and H3K-36 and -79 methylation is associated with gene activation, H3K9me2/3 and H3K27me3 are repressive marks. The methyltransferases Enhancer of the zeste homolog 1 (Ezh1) and Ezh2 act on H3K27 causing gene repression (10). H3K27 trimethylation can be removed by the lysine demethylase 6b (Kdm6b; Jmjd3), Kdm6a (Utx) and Ubiquitously-Transcribed Y Chromosome Tetratricopeptide Repeat Protein (Uty) (11). The role of H3K27 methylation has been extensively studied in developmental biology, but it is also important in macrophage activation (12). Kdm6b, kdm6a and Uty are induced upon Toll like receptor (TLR) stimulation (13). Kdm6b targets a large proportion of the lipopolysaccharide (LPS)-induced genes and Kdm6b deficiency suppresses a subset of these (14). The inflammatory response is significantly suppressed in human macrophages by a small molecule inhibitor acting on KDM6B and KDM6A (15). Besides pro-inflammatory responses, interleukin-4 (IL-4) stimulation also increases Kdm6b expression and Kdm6b affects helminth infection and responses to chitin (16, 17). Kdm6b is thus regulated in response to various triggers including LPS and IL-4, and regulates several modes of macrophage activation (13-18). Given the crucial role of macrophage foam cells in atherosclerosis, we here applied myeloid cell-specific Kdm6b deficient mice to perform RNA-sequencing analysis on peritoneal foam cells from *Kdm6b*<sup>wt</sup> and *Kdm6b*<sup>del</sup> mice to identify regulated pathways.
Material and Methods

Peritoneal foam cells

For our experiments we used C57BL/6 low density lipoprotein receptor knock out mice (Ldlr−/−) since macrophages from these mice accumulate lipids upon high fat diet. This is a well-known in vivo atherosclerosis model to study foam cell formation (19). Ldlr−/− mice were obtained from Jackson laboratories (ME, USA). A bone marrow transplantation (BMT) was performed with either LysMcre+ Kdm6bfl/fl mice (Kdm6bdel) or LysMcre- Kdm6bfl/fl littermates (Kdm6bwtt). Kdm6bfl/fl mice were described before (20). Crossbreeding with LysMcre was performed in our mice facility. Briefly, 10 (5 per group), 10-week old Ldlr−/− mice were divided over filter-top cages and provided antibiotics water (neomycin (100 mg/L, Sigma, Zwijndrecht, the Netherlands) and polymyxin B sulfate (60,000 U/L, Invitrogen, Bleiswijk, The Netherlands)) from 1 week pre-BMT until 5 weeks post-BMT. The animals received 2 x 6 Gy total body irradiation on two consecutive days. Bone marrow was isolated from 2 Kdm6bdel and 2 Kdm6bwtt mice, resuspended in RPMI1640 (Gibco, Breda, The Netherlands) with 5 U/ml heparin and 2 % iFCS (Gibco, Breda, the Netherlands), and 10⁷ cells were injected intravenously per irradiated mouse. Five weeks after the BMT, the mice were put on a high fat diet (0.15 % cholesterol, 16 % fat, Arie Blok Diets, The Netherlands) for 9 weeks. Blood samples were taken before the start of the diet and prior to sacrifice for immune cell flow cytometry. Four days before sacrifice all mice were intraperitoneally injected with thioglycollate medium (3%, Fisher, Bleiswijk, The Netherlands). Upon sacrifice, the peritoneum was flushed with 10 ml ice cold PBS and peritoneal macrophages (PEMs) were collected. Flushed thioglycollate-elicited macrophages were pooled per group and cultured at a density of 5*10⁵ cells/well in 24-well tissue culture plates in triplicate (Greiner Bio-One, alphen a/d Rijn, The Netherlands) in RPMI-1640 containing 25 mM HEPES, 2mM l-glutamine, 100 U/ml penicillin and 10 % FCS (all Gibco, Breda, the Netherlands). After 3 h adherence, non-adherent cells were washed away and the adherent cells (typically containing +95 % of CD11b+ F4/80+ macrophages) were either unstimulated or stimulated for 6 h with LPS (10 ng/ml) plus interferon gamma (IFNγ) (100 U/ml). All animal experiments were conducted at the University of Amsterdam and approved (permit: DBC10AD) by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam.

Flow cytometry

150 μl of blood was withdrawn from mice via tail vein incision before the start of the diet and 4 days prior to sacrifice and added to 20 μl of 0,5 M EDTA (Sigma-Aldrich, Gillingham, UK). Blood was drawn from mice which were restricted from food for at least 4 hours. The blood was centrifuged (10 min, 4 °C, 2000 rpm) to separate the plasma from blood cells. Red blood cells were lysed by adding 5 ml of erythrocyte lysis buffer (8.4 g of NH₄Cl, 0.84 g of NaHCO₃ and 0.37 g EDTA) for 15 min at RT. PBS was added to stop the reaction and cells were spun down. White blood cells were used for RNA isolation and quantitative PCR analysis. For our experiments we used C57BL/6 low density lipoprotein receptor knock out mice (Ldlr−/−) since macrophages from these mice accumulate lipids upon high fat diet. This is a well-known in vivo atherosclerosis model to study foam cell formation (19). Ldlr−/− mice were obtained from Jackson laboratories (ME, USA). A bone marrow transplantation (BMT) was performed with either LysMcre+ Kdm6bfl/fl mice (Kdm6bdel) or LysMcre- Kdm6bfl/fl littermates (Kdm6bwtt). Kdm6bfl/fl mice were described before (20). Crossbreeding with LysMcre was performed in our mice facility. Briefly, 10 (5 per group), 10-week old Ldlr−/− mice were divided over filter-top cages and provided antibiotics water (neomycin (100 mg/L, Sigma, Zwijndrecht, the Netherlands) and polymyxin B sulfate (60,000 U/L, Invitrogen, Bleiswijk, The Netherlands)) from 1 week pre-BMT until 5 weeks post-BMT. The animals received 2 x 6 Gy total body irradiation on two consecutive days. Bone marrow was isolated from 2 Kdm6bdel and 2 Kdm6bwtt mice, resuspended in RPMI1640 (Gibco, Breda, The Netherlands) with 5 U/ml heparin and 2 % iFCS (Gibco, Breda, the Netherlands), and 10⁷ cells were injected intravenously per irradiated mouse. Five weeks after the BMT, the mice were put on a high fat diet (0.15 % cholesterol, 16 % fat, Arie Blok Diets, The Netherlands) for 9 weeks. Blood samples were taken before the start of the diet and prior to sacrifice for immune cell flow cytometry. Four days before sacrifice all mice were intraperitoneally injected with thioglycollate medium (3%, Fisher, Bleiswijk, The Netherlands). Upon sacrifice, the peritoneum was flushed with 10 ml ice cold PBS and peritoneal macrophages (PEMs) were collected. Flushed thioglycollate-elicited macrophages were pooled per group and cultured at a density of 5*10⁵ cells/well in 24-well tissue culture plates in triplicate (Greiner Bio-One, alphen a/d Rijn, The Netherlands) in RPMI-1640 containing 25 mM HEPES, 2mM l-glutamine, 100 U/ml penicillin and 10 % FCS (all Gibco, Breda, the Netherlands). After 3 h adherence, non-adherent cells were washed away and the adherent cells (typically containing +95 % of CD11b+ F4/80+ macrophages) were either unstimulated or stimulated for 6 h with LPS (10 ng/ml) plus interferon gamma (IFNγ) (100 U/ml). All animal experiments were conducted at the University of Amsterdam and approved (permit: DBC10AD) by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam.
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RNA isolation and quantitative PCR analysis
RNA from peritoneal foam cells was isolated with High Pure RNA Isolation kits (Roche, Basel, Switzerland) from 500,000 cells. 400 ng of RNA was used for cDNA synthesis with iScript (BioRad, Veenendaal, The Netherlands). qPCR was performed with 4 ng cDNA using Sybr Green Fast on a ViiA7 PCR machine (Applied Biosystems, Bleiswijk, The Netherlands) for Kdm6b and normalized to the mean of the two housekeeping genes Ppia and Rplp0. Primer sequences are available on request.

RNA sequencing
RNA from unstimulated and 6h LPS (10 ng/ml) plus IFNγ (100 U/ml) stimulated Kdm6bwt and Kdm6bdel peritoneal foam cells was used for RNA sequencing. All samples were used in triplicate. However, one of the triplicates of the Kdm6bwtnstimulated cells was excluded as a result of poor RNA quality. Strand specific libraries were constructed from 100 ng total RNA using the the NuGen ‘ovation RNA-Seq system’ following manufacturer’s instructions (NuGen Technologies, Leek, The Netherlands). Samples were pooled, diluted to 10nM and were run on one flow cell lane of a HiSeq2500 with single-end reads of 50 base pairs.

Bioinformatic Analysis
Reads were aligned to the mouse genome mm10 by STAR 2.5.2b with default settings (21). BAM files were indexed and filtered on MAPQ > 15 with SAM Tools 1.3.1 (22). Raw tag counts and RPKM values per gene were summed using HOMER2’s analyzeRepeats.pl script (23) with default settings and the –noadj or –rpkm options for raw counts and RPKM reporting respectively. Differential expression was assessed using the DESeq2 (24) bioconductor package (25) in an R 3.3.1 environment (26).
Heatmaps were generated utilizing the pheatmap (27). Gene expression was called as differential with a multiple testing (Bonferonni) adjusted $P$-value < 0.05 and an average RPKM > 1 in at least one group. Gene lists of supplemental table 1 of the publication by Spann et al. were used to identify pathways upregulated upon foam cell formation (19). Overlap between groups was evaluated using a hypergeometric test. Pathway analysis was performed using the metascape webtool (www.metascape.org) (28) with default settings.

**Statistical Analysis**
For flow cytometry analysis and mRNA expression levels, data represent the mean ± standard error of the mean (SEM). Differences between $Kdm6b^{wt}$ and $Kdm6b^{del}$ transplanted mice are analyzed using a unpaired student’s t-test or a two-way ANOVA using Bonferroni post-hoc test analysis for grouped analysis. $P$-Values <0.05 were considered statistically significant. Data were analyzed using Prism version 5.0 (GraphPad software, La Jolla, California).

**Results**

**Kdm6b deficiency reduces the pro-fibrotic signature of peritoneal foam cells**
The blood leukocyte composition was not affected by myeloid Kdm6b deficiency since $Kdm6b^{wt}$ and $kdm6b^{del}$ transplanted $Ldlr^{-/-}$ mice showed similar percentages of monocytes, neutrophils, B cells and T cells after high fat feeding (Supplemental Figure S1A-B). Macrophage differentiation was also not affected as CD11b, F4/80 and CD11c surface expression on peritoneal foam cells was equal in both groups (Supplemental Figure S1C-E). $Kdm6b$ mRNA expression was reduced by approximately 70% indicating efficient deletion (Supplemental Figure S1F). Furthermore, different stimuli induced $Kdm6b$ expression after which deletion efficiency remained similar (Supplemental Figure S1F). RNA sequencing revealed that 43 genes were significantly upregulated in $Kdm6b^{del}$ peritoneal foam cells compared to $Kdm6b^{wt}$ cells and 40 genes were significantly downregulated (Figure 1A). We focused on genes downregulated in Kdm6b deficiency, since Kdm6b removes repressive histone marks. Interestingly, among the list of downregulated genes many genes involving fibrosis were affected in Kdm6b deficient foam cells including collagen genes ($Col1a1, Col1a2$), alpha smooth muscle actin ($Acta2$) and fibronectin-1 ($Fn1$) (Figure 1B). This was confirmed by qPCR analysis (Supplemental Figure S3).
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Results

$Kdm6b$ deficiency reduces the pro-fibrotic signature of peritoneal foam cells

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Figure 1. Myeloid $Kdm6b$ deficiency reduces the pro-fibrotic transcriptome signature of peritoneal foam cells. (A) Heatmap of significantly up- and downregulated genes in unstimulated $Kdm6b^{wt}$ and $Kdm6b^{del}$ foam cells. Bonferroni adjusted $P$-value < 0.05 and RKM > 1 in at least one group. (B) Significantly downregulated genes in $Kdm6b$ deficient foam cells compared to $Kdm6b^{wt}$ cells. (C) Top 20 pathways and GO-terms of downregulated genes from (B).
Pathway analysis on downregulated genes ($P$-value < 0.05) indicated that pathways involved in epithelial to mesenchymal transition (EMT) ($q$-value=$10^{-13}$) and extracellular matrix organization ($q$-value=$10^{-4}$) were significantly downregulated (Figure 1C). Additionally, we performed similar analysis on LPS plus IFNγ-stimulated Kdm6b deficient foam cells to characterize them under activated conditions. Also activated $Kdm6b^{del}$ cells showed clear suppression of fibrosis-related pathways (Hallmark epithelial mesenchymal transition, wound healing). Moreover, top affected pathways now also included cytokine signaling (Hallmark Tumor necrosis factor alpha (TNFA) signaling via NFκB, response to cytokine and Hallmark interferon gamma response) (Supplemental Figure S2A-C). Although inflammatory pathways are switched on, $Kdm6b^{del}$ cells thus still show a reduction in fibrotic pathways. These data show that deletion of Kdm6b activity in foam cells suppresses a pro-fibrotic gene signature.

**Peritoneal foam cells display a pro-fibrotic signature**

To our surprise fibrosis related genes were found to be differentially regulated in macrophages in the absence of Kdm6b. A previous study by Spann et al. (19) compared peritoneal foam cells to ‘normal’ peritoneal macrophages in both wild type and $Ldlr^{-/-}$ mice. Interestingly, pathways involved in extracellular matrix biology were upregulated upon foam cell formation, which was not further studied. Therefore we used this publicly available dataset to assess genes and pathways differently regulated upon foam cell formation in $Ldlr^{-/-}$ mice. Pathway analysis on upregulated genes ($P$-value < 0.05) identified pathways involved in blood vessel development, vasculature development and EMT to be induced (Figure 2A) (19). A significant overlap was found between pathways and genes upregulated upon foam cell formation and the ones downregulated upon Kdm6b deletion (Figure 2B-C, Supplemental Table 2). These data imply that foam cell formation induces a pro-fibrotic transcriptomic profile in macrophages, which is regulated by Kdm6b.
Discussion

We here studied the involvement of Kdm6b in the transcriptional profile of macrophage foam cells. The demethylase Kdm6b removes repressive trimethylation marks from H3K27, thereby allowing gene transcription (11). This suggests that Kdm6b-regulated genes will be repressed in the absence of Kdm6b. We found that Kdm6b deficiency downregulated 50% of all differently expressed genes. Lack of Kdm6b in other macrophage subsets reduced the expression of various responsive genes that are mainly inflammation-related (14, 17, 29). Interestingly, in addition to pathways related to inflammatory responses we found a set of genes involved in fibrosis to be downregulated upon Kdm6b deletion. Pathway analysis on these
downregulated genes showed pathways involved in matrix biology and EMT to be suppressed upon Kdm6b deficiency in foam cells. Foam cells are an unique activation state of macrophages specifically found in atherosclerotic lesions. We found that foam cell formation particularly enhanced the expression of pro-fibrotic genes and pathways. In agreement, Thomas et al. recently reported that foam cell formation in a subcutaneous granuloma model in apolipoprotein e deficient mice versus wildtype mice also switches macrophages to a pro-fibrotic phenotype (30). They found induction of platelet-derived growth factor and transforming growth factor-β pathways in foam cells which was linked to increased expression of collagen and proteoglycan genes. Interestingly, we found pro-fibrotic genes and corresponding pathways to be downregulated upon Kdm6b deletion, suggesting a role for Kdm6b in establishing the pro-fibrotic signature of foam cell macrophages. We propose that foam cell formation induces a pro-fibrotic signature of macrophages which is regulated by Kdm6b. We hypothesize that Kdm6b removes repressive histone marks at pro-fibrotic genes upon foam cell formation. Therefore, fibrotic genes remain silenced in foam cells in the absence of Kdm6b (Figure 3). Yet, Kdm6b is also described to regulate transcriptional gene programs in macrophages independent of H3K27me3 (14). The mechanism by which Kdm6b controls the pro-fibrotic gene signature of foam cells might therefore not solely be via H3K27 demethylation.

We thus here show a novel role for Kdm6b in the regulation of the pro-fibrotic signature of foam cells. The involvement of Kdm6b in the regulation of genes involved in matrix biology and collagen has been previously reported for other cell types. Osteoblast differentiation is impaired in the absence of Kdm6b and Col1a1, an early marker of osteoblast differentiation, is downregulated in the absence of Kdm6b in these cells (31). Col1a1 is also one of the genes highly regulated by Kdm6b in our macrophage foam cells. Chondrogenesis of mesenchymal stem cells (MSC) is also affected by Kdm6b. Treatment of MSCs with the Kdm6b and Kdm6a inhibitor GSK-J4 inhibited collagen production and expression of chondrogenic genes including COL2A1 (32). Besides pathways involved in matrix biology and collagen, we also found EMT pathways to be downregulated upon Kdm6b deficiency. EMT is the transdifferentiation of epithelial cells to mesenchymal cells. Three types of EMT are described depending on the tissue and context (33). Type 1 EMT is involved in embryonic and organ development, type 2 EMT contributes to wound healing and organ fibrosis, two processes in which macrophages also play a crucial role and which are of high relevance for atherosclerotic plaque stability (34) and type 3 EMT is associated to invasion and cancer progression. KDM6B is induced by TGF-beta in epithelial cells and promotes EMT. Knockdown of KDM6B in epithelial cells inhibits
EMT. Conversely, KDM6B overexpression induces the expression of mesenchymal genes and induces EMT (35). Foam cell formation mainly induce genes linked to type 2 EMT, like Col1a1, Acta2 and Fn1 which are all suppressed by Kdm6b deficiency. These data suggest that macrophages can activate mesenchymal gene programs involved in tissue fibrosis.

Figure 3. Kdm6b is involved in the pro-fibrotic signature of macrophage foam cells. Integrated model suggesting a role for Kdm6b in the regulation of pro-fibrotic genes via histone H3K27 demethylation.

**Conclusion**

We here demonstrate that peritoneal foam cells induce pro-fibrotic transcriptional changes and identified Kdm6b as novel regulator of this phenotype. Kdm6b deficient mice show reduced pro-fibrotic transcriptional changes in peritoneal foam cells.

**Summary points**

- Pro-fibrotic genes are upregulated in peritoneal foam cells.
- Pathways involved in collagen and matrix biology are upregulated in peritoneal foam cells.
- Myeloid Kdm6b deficiency reduces this pro-fibrotic signature of foam cells.
Chapter 5

References

Macrophage Kdm6b controls the pro-fibrotic transcriptome signature of foam cells

Supplemental Tables

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Supplemental Table 1: List of used antibodies

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Supplemental Table 2: Overlap of genes induced by foam cell formation and downregulated by Kdm6b deficiency.
Supplemental Figures

Supplemental Figure S1. Myeloid Kdm6b deficiency has no effect on leukocyte composition and macrophage foam cell differentiation. (A) Flow cytometry on whole blood of Kdm6bwtn and Kdm6bdeln transplanted mice after 8 weeks of high fat diet to study leukocyte composition. Leukocytes were gated as CD45+ singlets and the following subsets were divided: monocytes (CD45+, CD11b+, Ly6G-), neutrophils (CD45+, CD11b+, Ly6G+), B cells (CD45+, CD19+) and T cells (CD45+, CD3+). (B) Percentage of blood monocytes, neutrophils, B cells and T cells of Kdm6bwtn and Kdm6bdeln transplanted mice. (C) Flow cytometry on peritoneal foam cells of Kdm6bwtn and Kdm6bdeln transplanted mice. Peritoneal foam cells were gated as viable single CD11b+, F4/80+ cells (D) Percentage of macrophages of Kdm6bwtn and Kdm6bdeln transplanted mice. (E) Median fluorescence intensity (MFI) of CD11b, F4/80 and CD11c on CD11b+, F4/80+ cells. (F) Relative mRNA expression of Kdm6b in unstimulated or LPS (10 ng/ml), LPS plus IFNγ (100 U/ml) or IL-4 (20 ng/ml) stimulated cells. Gene expression was normalized to two housekeeping genes. n=5 per group, data represent mean ± SEM *P < 0.05; **P < 0.01; ***P < 0.001.
Supplemental Figure S2. Myeloid \textit{Kdm6b} deficiency reduces the inflammatory and pro-fibrotic transcriptome of activated foam cells. (A) Heatmap of significantly up- and downregulated genes in 6h LPS (10ng/ml) plus IFNγ (100U/ml) activated \textit{Kdm6b}^{wt} and \textit{Kdm6b}^{del} foam cells. Bonferroni adjusted $P$-value $< 0.05$ and PPKM $> 1$ in at least one group. (B) Significantly downregulated genes in activated \textit{Kdm6b} deficient foam cells compared to \textit{Kdm6b}^{wt} cells. (C) Top 20 pathways and GO-terms of downregulated genes from (B).
Supplemental Figure S2. Myeloid Kdm6b deficiency reduces the inflammatory and pro-fibrotic transcriptome of activated foam cells. (A) Heatmap of significantly up- and downregulated genes in 6h LPS (10ng/ml) plus IFNγ (100U/ml) activated Kdm6b wt and Kdm6b del foam cells. Bonferroni adjusted P-value < 0.05 and PPKM > 1 in at least one group. (B) Significantly downregulated genes in activated Kdm6b deficient foam cells compared to Kdm6bwt cells. (C) Top 20 pathways and GO-terms of downregulated genes from (B).

Supplemental Figure S3. q-PCR validation confirms that pro-fibrotic genes are downregulated in Kdm6b deficient mice. qPCR on Kdm6bwt and Kdm6bdel unstimulated or 6h LPS plus IFNγ stimulated foam cells for the pro-fibrotic genes (A) Col1a1 (B) Col1a2 (C) Acta2 (D) Fn1 (E) Sparc (F) Bgn (G) Serpine1 and (H) Serpinh1 which were significantly downregulated in the RNA-sequencing analysis. Relative mRNA expression is shown and gene expression was normalized to two housekeeping genes. Data represent mean ± SEM *P < 0.05; **P < 0.01; ***P < 0.001.