Time-restricted feeding downregulates cholesterol biosynthesis program via RORγ-mediated chromatin modification in porcine liver organoids

Kexin Zhang†, Hao Li†, Zimeng Xin†, Yanwei Li†, Xiaolong Wang†, Yun Hu†, Haoyu Liu* and Demin Cai†,2*

Abstract

Background: Time-restricted feeding (TRF) is a dieting strategy based on nutrients availability and diurnal rhythm, shown to improve lipid metabolism efficiency. We have demonstrated previously that retinoic acid-related (RAR) orphan receptor (ROR) γ is the primary transcription factor controlling cholesterol (CHO) biosynthesis program of animals. However, the functional role of RORγ in liver physiology of pigs in response to TRF has not been determined, largely due to the lack of functional models and molecular tools. In the present study, we established porcine liver organoids and subjected them to restricted nutrients supply for 10-h during the light portion of the day.

Results: Our results showed that TRF regimen did not alter hepatocyte physiology, including unchanged cell viability, caspase 3/7 enzyme activity and the gene signature of cell proliferation in porcine liver organoids, compared to the control group (P > 0.05). Furthermore, we found that TRF downregulated the hepatic CHO biosynthesis program at both mRNA and protein levels, along with the reduced cellular CHO content in porcine liver organoids (P < 0.05). Using unbiased bioinformatic analysis of a previous ChIP-seq data and ChIP-qPCR validation, we revealed RORγ as the predominant transcription factor that responded to TRF, amongst the 12 targeted nuclear receptors (NRs) (P < 0.05). This was likely through RORγ direct binding to the MVK gene (encoding mevalonate kinase). Finally, we showed that RORγ agonists and overexpression enhanced the enrichment of co-factor p300, histone marks H3K27ac and H3K4me1/2, as well as RNA polymerase II (Pol-II) at the locus of MVK, in TRF-porcine liver organoids, compared to TRF-vector control (P < 0.05).

Conclusions: Our findings demonstrate that TRF triggers the RORγ-mediated chromatin remodeling at the locus of CHO biosynthesis genes in porcine liver organoids and further improves lipid metabolism.

Keywords: Cholesterol biosynthesis program, Histone modification, Pig, Porcine liver organoids, RORγ, Time-restricted feeding
Background

Obesity is a major risk factor for chronic disorders such as nonalcoholic fatty liver disease (NAFLD), cardiovascular disease, and type II diabetes [1]. The etiology of obesity is complex, including nutrient imbalance and the disruption of multiple metabolic pathways in the liver [2, 3]. In addition to the dysregulation of glucose, lipid and cholesterol metabolism, it has been suggested that circadian rhythm is a major contributor to the pathophysiology of obesity [4–6]. The circadian rhythm is an evolutionarily conserved system in mammals that coordinates rhythms of behavior and physiology in response to predictable environmental changes in a 24-h solar day [7, 8]. Although the circadian clock is a ‘build-in’ system, it is entrained to the local environment by external cues, including light, temperature and feeding time [4, 5].

Time-restricted feeding (TRF), a defined daily period of feeding and fasting [9, 10], is increasingly recognized as a preventative intervention against nutritional challenges in animals and humans [4, 6]. Studies reported that TRF reduces fat depot and weight gain in mice under high-fat feeding and ameliorates metabolic disorders [11, 12]. It is reported that TRF can also reduce serum cholesterol (CHO) levels in obese mice [6, 11, 12]. Furthermore, TRF downregulates the master lipid regulator peroxisome proliferator-activated receptorα and enzymes involved in triglyceride metabolism in the liver [4], as well as controls hepatic transcriptome in both wild type and the clock-disrupted mice [8]. Remarkably, it has been demonstrated that around 10–15% of all liver mRNA are expressed in a rhythmic fashion. Many of these genes play a role in cholesterol and glucose metabolism [4, 5, 8] with one essential part being retinoic acid-related (RAR) orphan receptor (ROR) [4, 13, 14]. Both RORγ and RORα are involved in controlling hepatic circadian rhythmic expression of glucose genes, whereas mice deficient in RORγ showed improved insulin sensitivity and glucose tolerance, especially at daytime [14]. In addition, it is recently reported that RORγ dictates the entire CHO biosynthesis pathway in cancerous cells and overrides the classic transcription factor sterol regulatory element-binding protein (SREBP)-2 [13]. However, the role of RORγ in cholesterol biosynthesis in liver physiology of mammals and its relation to TRF remain unclear.

To date, most of the TRF studies were carried out using mice or Drosophila [5, 15, 16]. As light cycle impacts animal circadian phenotype, it is noteworthy that mice are nocturnal, in contrast to diurnal mammals such as humans and pigs [17]. In this regard, pigs (Sus scrofa) are increasingly used as an animal model since they share anatomical, physiological, and immunological similarities with human beings [18, 19]. Herein we developed a porcine liver organoid model, which combines porcine traits and the ease of genetic manipulation in basic and pharmacological research, as well as to evaluate dieting strategy in livestock management.

Indeed, liver organoid culture is becoming a popular alternative of primary cell culture to recapitulate tissues in a dish [20, 21] and to study liver physiology and disease pathogenesis in human and mice [22]. Using extra-cellular matrix (Matrigel), the unique system enables organoids to resemble architectural and functional properties of in vivo tissue more closely [22], though the establishment using 3D culture could affect cell proliferation, morphogenesis and survival [23]. Nevertheless, such an approach allows the removal of confounding effects and provides a reductionist model of in vivo tissue [20, 22], yet not well-established in large animals.

In the current study, the effects of 10-h TRF on liver tissues were investigated using transcriptomics and chro-matin immunoprecipitation, by applying temporal regulation of feeding cells nutrients in porcine liver organoids in vitro. We hypothesized that under normal healthy condition, TRF modifies the cyclical expression of metabolic regulators and associated cellular processes, thus improves metabolism.

Methods

Animals and the porcine liver organoid establishment

All animal procedures were in line with and approved by the Animal Ethical Committee of Yangzhou University (NSFC2020-DKXY-20). Liver tissues were obtained from 3 days old male piglets. Porcine organoids were established and cultured as previously described with modifications [24]. Briefly, dissected liver tissues of newborn piglet were finely minced and transferred to a 50-mL conical tube including a digestion mixture consisting of serum-free DMEM/F-12 medium (Gibco, basal medium) and 2.5 mg/mL collagenase D (Sigma), and were incubated for 1 h at 37 °C. Single cells were collected and mixed with 50 µL of Matrigel (BD Biosciences) and seeded in 24-well plates (Greiner bio-one) at a density of 1000 per well. When the matrix was solidified, 500 µL isolation medium (1:50 B27 supplement without vitamin A), 1:100 N2 supplement, 1 mmol/L N-acetylcysteine, 10% (vol/vol) Rspo1-conditioned medium, 10 mmol/L nicotinamide, 10 nmol/L recombinant human [Leu15]-gastrin I, 50 ng/mL recombinant human EGF, 100 ng/mL recombinant human FGF10, 25 ng/mL recombinant human HGF, 10 µmol/L Forskolin and 5 µmol/L A83-01, 25 ng/mL recombinant human Noggin or 5% (vol/vol) Noggin-conditioned medium, 30% (vol/vol) Wnt3a-conditioned medium and 10 µmol/L Rho kinase (ROCK) inhibitor were incubated for 4 d. Then the medium was replaced with normal liver expansion medium (1:50 B27 supplement without vitamin A, 1:100 N2 supplement, 1 mmol/L N-acetylcysteine, 10% (vol/vol) Rspo1-
conditioned medium, 10 mmol/L nicotinamide, 10 nmol/L recombinant human \([\text{Leu}^{15}]\) -gastrin I, 50 ng/mL recombinant human EGF, 100 ng/mL recombinant human FGF10, 25 ng/mL recombinant human HGF, 10 μmol/L Forskolin and 5 μmol/L A83-01). The medium was changed every 3–4 d.

**Dexamethasone synchronization and sample collection**

At day 15 from seeding, organoids of 12 wells were treated with 100 nmol/L (final concentration) of dexamethasone (DEX, Sigma-Aldrich) for 15 min to synchronize. The organoids were then washed three times with PBS (37 °C) and were incubated in expansion medium. Forty-eight hours after DEX treatment, organoids of 6 wells as control group were exposed to expansion medium for 14 h from 8:00 to 22:00 and to basal medium for 10 h from 22:00 to 8:00 (+1 d) in a 24-h cycle. Whereas organoids of the other 6 wells as TRF group were exposed to expansion medium for 10 h from 8:00 to 18:00 and to basal medium for 14 h from 18:00 to 8:00 (+1 d) in a 24-h cycle (Fig. 1). The exposure in the pattern of 24-h cycle was continued to 7 d and the organoids were harvested directly at 8:00 for fundamental testing. For the compounds/lentivirus treatment, the organoids were treated at 8:00 from the end of the 7th day of 24-h cycle for another 48-h period and then harvested for measurements.

**Cell viability and caspase 3/7 activity in organoids**

The organoids were seeded in 96-well plates at the density of 100 organoids in 10 μL Matrigel per well in a total volume of 100 μL expansion medium for 7 d exposure of the above 14/10 h feeding window and incubated with expansion medium for 48 h. Carefully aspirating the medium and adding 100 μL live/dead reagents (Thermo-fisher Scientific) for 30 min incubation at room temperature. Fluorescence microscopy was used to capture signals of a cell-permeant dye Calcein AM that represents live cells, and signals of ethidium bromide homodimer-1 to identify dead cells. Besides, Cell-Titer GLO reagents (Promega) were added and luminescence was measured on GLOMAX microplate luminometer (Promega) according to the manufacturer’s instructions. The caspase-3/7 activity was determined using a luminescent caspase-Glo 3/7 assay kit (Promega Corporation, Madison, USA) following the manufacturer’s instructions. The above assays were performed in triplicates and the entire experiments were repeated three times.

**qRT-PCR and western blotting analysis**

Total RNA of 2 μg was isolated from organoids in 24-well plates, and the cDNA was prepared, amplified and measured using SYBR green as previously described [13]. Briefly, the fluorescent values were collected, and a melting curve analysis was performed. Fold difference was calculated [13]. The primers are shown in Table S1. The experiments were performed at least three times with data presented as mean values ± SD. Organoids lysates were analyzed by western blotting with antibodies specifically recognizing the indicated proteins shown in Table S2.

**Ectopic lentivirus production**

For RORγ overexpression, porcine RORγ cDNA in pLX304 (DNASU) was amplified and cloned into a modified pLX304 vector as previously described [13]. Lentiviral particles were produced in 293 T cells after co-transfection of the above lentivirus vectors, psPAX2 and pMD2.G in 10-cm dishes.
Measurement of cholesterol contents
Organoids were washed three times with cold PBS and subjected to extraction with organic solvents (7:11:0.1, chloroform/isopropanol/Triton X-100). Free (3-OH) and total (3-OH and esters) sterol levels were measured using Amplex® Red Cholesterol Assay Kit (Thermo Fisher Scientific) and normalized to protein concentrations. All experimental points were set up as sextuplicate as biological replication and the entire experiments were repeated three times.

ChIP-qPCR analysis and ChIP-seq data analysis
Briefly, organoids of 24-well plates were pelleted in cold PBS and resuspended in fixing buffer (50 mmol/L Hepes-KOH, 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5 mol/L EGTA) before subject to crosslinking in 1% formaldehyde for 5 min followed by quenching with glycine for 5 min on ice. The pellets were collected by centrifugation and resuspended in lysis buffer (50 mmol/L HEPE S pH 8.0, 140 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 0.5% NP40, 0.25%. Triton X100). The pellets were then resuspended in washing buffer (10 mmol/L Tris pH 8.0, 1 mmol/L EDTA, 0.5 mmol/L EGTA, 200 mmol/L NaCl), washed and resuspended in shearing buffer (0.1% SDS, 1 mmol/L EDTA, pH 8, 10 mmol/L Tris HCl, pH 8) before sonication using Covaris E220 following manufacturer’s instructions. Chromatin fragments were precipitated using specific antibodies and protein G beads, washed, and treated with proteinase K and RNase A. Purified ChIP DNA was then used for ChIP-qPCR analysis. The forward and reverse primers for ChIP-qPCR analysis are “GCTCCATCCGGGAGACACACA” and “GCAGGGTGCAATGTGCAGTTTCT” respectively.

ChIP-qPCR analysis was performed as described previously [13]. The antibodies used for the RNAPII (Santa Cruz, sc-899); H3K4me1(Abcam, ab8895); H3K4me2 (Abcam, ab32356); H3K4me3 (Abcam, ab8580); H3K27ac (Abcam, ab4729); p300 (Abcam, ab10485); anti-RORγ rabbit serum was generated by Covance, (rabbit serum was generated by Covance, sc-2027). ChIPs were performed with each experimental point in triplicate, and each experiment was repeated three times.

Fastq files from previous datasets [13] were processed by the pipeline of AQUAS Transcription Factor and Histone (https://github.com/kundajelab/chipseq_pipeline). Briefly, sequencing tags were mapped against the reference genome using BWA 0.7.15 [25]. Uniquely mapped tags filtering and deduping were used for peak calling by model-based analysis for ChIP-seq (MACS; 2.1.0) to identify regions of ChIP-seq enrichment over background. Normalized genome-wide signal-coverage tracks from raw-read alignment files were built by MACS2, UCSC tools (bedGraphToBigWig/bedClip: http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/) and bedTools (https://github.com/arq5x/bedtools2). Visualization of ChIP-seq signal at enriched genomic regions (avgprofile and heatmap) was achieved by using deepTools (https://deeptools.readthedocs.io/en/develop/index.html).

Bioinformatic analyses using clinical dataset
METABRIC data sets were downloaded from cBioPortal website at http://www.cbioportal.org/study?id=brca_metabric#summary. The data were then Log2 transformed and quantile normalized before further analysis. Principal component analysis (PCA) was carried out with R ‘COMPADRE’ package [26]. After PCA transformation, the samples were visualized according to pathway activity score using ‘gplots’ R packages. Based on the pathway activity score and the gene profile across the samples, the Pearson correlation metric was computed between each gene by using the ‘cor’ function in R.

Statistics
Statistical analyses were performed by GraphPad Prism software 7.0. The data are presented as mean values ± SD from at least three independent experiments. Statistical analysis was performed using two-tailed Student’s t-tests or ANOVA with Tukey’s post hoc test to compare the means. P < 0.05 was considered significant.

Results
Time-restricted feeding does not affect cell growth and survival in porcine liver organoids
Given that organoids are more physiologically relevant than 2D monolayers cells, we developed the porcine liver organoids. Firstly, we evaluated the effects of TRF using live/dead regents (calcein AM/ethidium bromide homodimer-1). Immunofluorescent staining showed that TRF did not affect the hepatocytes viability (Fig. 2a), which was further confirmed by quantification of adenosine triphosphate (ATP) presence in cells using a cell-titer measurement, indicative of metabolically active cells (Fig. 2b, P > 0.05). In addition, there was no difference of the caspase 3/7 enzyme activity between the control and TRF treated organoids (Fig. 2c, P > 0.05). In line with this, TRF had no effects on the key proliferation and survival genes expression in the porcine liver organoids, compared to that in control (Fig. 2d, P > 0.05). These data demonstrated that the 10-h TRF regimen does alter hepatocytes physiology significantly in our established porcine liver organoids.
Time-restricted feeding downregulates cholesterol biosynthesis program

Previous studies have demonstrated that TRF resulted in lower CHO levels in circulation of mice [8, 27]. We thus examined both total and free CHO levels, and showed significantly decreased CHO content in organoids under TRF, compared to the control group (Fig. 3a, b, *P < 0.05*). Furthermore, the expression of key genes involved in CHO biosynthesis were investigated. Consistently, genes such as *MVK* (encoding mevalonate kinase), *FDPS* (encoding farnesyldiphosphate farnesyltransferase 1), *FDFT1* (encoding farnesyl-diphosphate farnesyltransferase), *SQLE* (encoding Squalene monooxygenase), *EBP* (encoding emopamil binding protein), *SC5D* (encoding sterol-c5-desaturase), *DHCR7* (encoding 7-Dehydrocholesterol reductase) and *DHCR24* were significantly downregulated in TRF group (Fig. 3c). In line with the mRNA levels, TRF resulted in strong downregulation of the CHO biosynthesis enzyme proteins including *MVK*, *FDFT1*, *SQLE*, *EBP* and *DHCR24* (Fig. 3d). These results indicated that the CHO biosynthesis program is responsive to TRF treatment.

**RORγ** is linked to TRF-induced CHO downregulation

Cholesterol biosynthesis pathway is under the tight regulation of major transcription NRs, such as liver X receptors and RORs [13, 28]. To identify potential drivers of the decreased CHO biosynthesis program in TRF treated organoids, we tested a panel of 20 small-molecule modulators targeting members of the NR family in liver
organoids (Fig. 4a). Intriguingly, the RORγ agonists SR0987 and desmosterol showed strongest capacity to rescue cellular CHO contents reduction induced by TRF (Fig. 4a, *P* < 0.05). Consistently, we found that the significantly downregulated expression of genes involved in CHO biosynthesis were restored to the levels that comparable to control (Fig. 4b). Furthermore, we analyzed the relevant clinical dataset and revealed a strong positive relationship between the expressions of RORγ gene RORC and MVK (*r* = 0.2383, *P* < 0.0001), FDPS (*r* = 0.1228, *P* = 0.0338), EBP (*r* = 0.2233, *P* < 0.0001) and DHCR24 (*r* = 0.4039, *P* < 0.0001), respectively. Together, these data suggested that RORγ is a key factor linked to the decreased CHO biosynthesis program in TRF treated organoids.  

**RORγ is required for TRF-induced CHO biosynthesis program downregulation**

Next, we investigated whether TRF downregulates CHO biosynthesis program via RORγ signaling. First, we examined the endogenous expression of RORγ in porcine liver organoids with qRT-PCR and western blotting, and

![Fig. 3 TRF reduces cholesterol biosynthesis program in porcine liver organoids.](image)
found that both mRNA ($P < 0.001$) and protein abundances were significantly decreased in TRF treated organoids, when comparing to the controls (Fig. 5a, b). To determine whether elevated ROR$\gamma$ alone is sufficient to promote the CHO biosynthesis program, we overexpressed ROR$\gamma$ in TRF treated organoids and confirmed its high expression compared to vector controls (Fig. 5c, $P < 0.001$). Similarly, the key CHO biosynthesis genes were significantly upregulated by overexpressed ROR$\gamma$, compared to the Vector-TRF group (Fig. 5e, $P < 0.05$). There was a trend that ROR$\gamma$ overexpression in the organoids caused higher expression of these CHO biosynthesis genes than the Vector-Ctrl group, although no statistical significance reached. Together, these results

![Fig. 4 ROR$\gamma$ agonist rescues TRF-induced CHO downregulation.](image)

Data were presented as means ± SD of at least three independent experiments, *$P < 0.05$, using two tailed Student's $t$-test. Agonists were in red; antagonists were in blue.
suggested that RORγ plays a direct role in the TRF regulation of CHO biosynthesis program.

Time-restriction feeding reduces RORγ enrichment on MVK gene promoter
To dissect molecular components of the RORγ pathway in the TRF downregulating CHO biosynthesis program, we examined the impact of TRF on RORγ recruitment to chromatin targets. Firstly, we performed the analysis of an available ChIP-seq data [13, 14, 29], and the results showed that RORγ peaks are present on MVK gene both in human (top) and mouse (bottom) (Fig. 6a). It is well-known that the specific sequence motifs of RORγ binding DNA including A(A/T)NTAGGTCA (the classic ROR element motif) or C(T/A)(G/A)GGNCA (the variant RORE motif) [30]. In consistent with the MVK-RORγ peak location in human or mouse, ChIP-qPCR of regions containing 12 putative ROREs across the MVK locus demonstrated that RORγ bound to a site around the transcription start site (TSS) region in porcine liver organoids (Fig. 6b). As shown in Fig. 6c, the site contains sequences that match the motif AGGTCA. When organoids were exposed to TRF, RORγ binding was reduced compared to control (Fig. 6d, P < 0.01). We next assessed the efficiency of RORγ agonist to restore RORγ binding, SR0987 or desmosterol treatment enhanced 2-
fold RORγ occupancy on MVK gene promoter in the TRF treated organoids (Fig. 6e, P < 0.01). Interestingly, RORγ enrichment was only increased 50% by RORγ overexpression in TRF treated organoids, compared to the vector-TRF group (Fig. 6f, P < 0.05). These data indicated that other factors may also contribute to the RORγ-mediated chromatin modifications in the TRF controlled CHO biosynthesis programming, than RORγ endogenous expression.

Time-restriction feeding modifies transcription-complex modifications on the loci of RORγ binding
Next, we investigated whether transcription co-factors or histone modifications facilitated the actions of RORγ in the regulation of CHO biosynthesis program in TRF treated organoids. The putative co-factors p300, SRC-1 and SRC-3 were predicted by STRING analysis from ELIXIR database (Fig. 7a). Of the three factors, only p300 occupancy was significantly reduced on the MVK
Fig. 7 (See legend on next page.)
gene in the TRF treated organoids, compared to that of control (Fig. 7b–d, P < 0.01). We then performed ChIP-qPCR to detect the transcriptional activation-linked histone marks H3K27ac, H3K4me1/2/3 at the locus of MVK. The results showed that TRF significantly decreased the enrichment of H3K27ac (Fig. 7e, P < 0.01), H3K4me1/2 (Fig. 7f, g, P < 0.01), but not H3K4me3 (Fig. 7h, P > 0.05). In line with the reduction of mRNA levels of CHO biosynthesis genes, promoter occupancies of RNA polymerase II (Pol-II) was also reduced at the target loci in the TRF treated organoids (Fig. 7i, P < 0.05). Furthermore, RORγ agonists enhanced the enrichments of p300, H3K27ac and H3K4me1 in the organoids exposed to TRF (Fig. 7j–l, P < 0.01). Taken together, these results implied that TRF triggers the RORγ-associated chromatin remodeling at the locus of CHO biosynthesis genes.

Discussion

Temporal regulation of feeding, i.e. TRF in animal husbandry, may offer a dieting strategy to modify metabolism through the oscillation of hepatic genes expression that are key metabolic regulators [4, 8]. In the current study, we used porcine liver organoids and showed that 10-h TRF regimen does not alter cell viability, proliferation, or apoptosis. Instead, TRF down-regulated hepatic cholesterol biosynthesis program involving MVK, FDFT1, SQLE, EBP and DHCR24 expressions at both mRNA and protein level, associated with the reduced CHO output in the TRF treated organoids. Subsequently, our search for responsible transcription factors focusing on NRs uncovered the predominant role of RORγ. In that RORγ agonist SR0987 and RORγ overexpression reprogrammed the CHO biosynthesis pathway induced by TRF in porcine liver organoids. Finally, we demonstrated that RORγ directly binds to MVK gene, whereas TRF downregulates CHO pathway via RORγ-mediated chromatin remodeling.

Interactions between circadian clock and metabolism can be affected by nutrition quality, quantity or daily eating pattern. Given the growing use of pigs in basic research, as well as in agriculture [18, 31], it is necessary to understand the extent to which circadian rhythms affect this species. By employing a 10-h TRF regimen, we observed a downregulation of CHO biosynthesis program, thus a TRF resultant decreased CHO output in the porcine liver organoids. In accordance, a substantial amount of studies demonstrate that various TRF strategies protect individuals from diet induced obesity and metabolic disorders [6, 10, 32]. For instance, Hatori et al. have shown that 8-h TRF reduced hepatic steatosis and hyperinsulinemia through cAMP-response element binding protein, mTOR (mammalian target of rapamycin) and AMP-activated protein kinase pathways in mice [11]. Recently, 10-h time-restriction eating has been applied to patients diagnosed with metabolic syndrome and showed positive effects including improved body weight, blood pressure and lowered cholesterol levels [10].

Although it is suggested that counteracting hypercholesterolemia is a general hallmark of TRF [6, 33], liver is the master regulator of cholesterol homeostasis of mammals. Our analysis based on clinical data revealed that hepatic expression of MVK, FDPS, EBP and DHCR24 are all positively correlated with the expression of NR family of transcription factor, RORγ. These genes, e.g., MVK encodes mevalonate kinase enzyme, catalyzing the conversion of CHO precursor [34], along with several others that were shown to participate in the TRF-reduced CHO biosynthesis program in our study. The link of CHO genes to RORγ is of great interest, as RORγ is involved in the direct regulation of circadian rhythm by binding to the main clock gene [35]. Studies have revealed that approximately 10% of all liver mRNA are expressed in a rhythmic fashion [8, 36]. We hypothesized that RORγ may represent the dominant respondent of liver oscillator in our TRF-treated porcine liver organoids. Indeed, we found that TRF decreased RORγ expression at both mRNA and protein level in porcine liver. While RORγ agonist rescued the TRF-resulted CHO downregulation, amongst 20 compounds targeting NRs. To support this, it is demonstrated in another study that hepatocyte-specific RORγ knockout mice exhibit improved insulin sensitive due to reduced gluconeogenesis, but also changed lipid metabolic genes [14]. Inversely, we showed that hepatocytes ectopic RORγ disrupted TRF-induced CHO biosynthesis genes downregulation and increased CHO end product in porcine liver organoids, pointing to the critical role of RORγ as the master transcription factor.

It has long been considered that SREBP-2 is the primary transcription factor for activation of genes involved in CHO biosynthesis [37–39], including MVK [40]. In contrast, our previous study has demonstrated that...
RORY plays a dominant function over that of SREBP-2 in controlling CHO biosynthesis program in cancerous cells [13], which is in line with our current demonstration. By further examining the downstream events, we identified a RORY binding site in the DNA sequence of MVK in porcine liver organoids. We have shown clearly that TRF reduces ROHY enrichment at the locus of MVK, involving the reduced enrichments of co-factor p300 and histone marks H3K27ac and H3K4me1/2. While RORY agonists enhanced the occupancies of p300, H3K27ac and H3K4me1 at target loci against TRF regulation. We therefore suggested that RORY is a targetable master regulator of CHO biosynthesis program during the temporal regulation of feeding and beyond.

**Conclusions**

In conclusion, we identified a novel connection between the regulator ROHY and the temporal regulation of hepatic CHO biosynthesis program in porcine organoids. Our findings showed the potential of organoids to be used as a platform for mechanistic studies and drug testing. More importantly, we contributed to the development of an optimal long-term organ culture and its application to animal husbandry. Challenges remain as to capture complex pathologies of liver diseases in a dish, such as inflammation and fibrosis [41], and further studies are warranted.

**Supplementary information**

**Supplementary information** accompanies this paper at https://doi.org/10.1186/s40104-020-00511-9.

**Additional file 1**: Table S1. Nucleotide sequences of specific primers used for real-time PCR.

**Additional file 2**: Table S2. Antibodies used.

**Abbreviations**

ATP: Adenosine triphosphate; CHO: Cholesterol; DEX: Dexamethasone; DHCR7: 7-Dehydrocholesterol reductase; EBP: Emopamil binding protein; FDFT1: Farnesyl-diphosphate farnesyltransferase 1; FDPS: Farnesyl pyrophosphate synthase; mTOR: Mammalian target of rapamycin; MVK: Mevalonate kinase; NAFLD: Nonalcoholic fatty liver disease; NRs: Nuclear receptors; PCA: Principal component analysis; Pol-II: Polymerase II; ROCK II: Rho kinase; ROHY: Retinoic acid-related (RAR)-related orphan receptor; RORE: ROR element; SCSD: Sterol-CS-desaturase; SQA: Squalene monooxygenase; SREBP-2: Sterol regulatory element-binding protein-2; TRF: Time-restricted feeding; TSS: Transcription start site.

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Not applicable.

**Authors’ contributions**

D.C. conceived the study. K.Z., H.L., Z.X., Y.L., X.W. and Y.H. performed the experiments. D.C. and H.Y.L. wrote the manuscript. D.C. supervised the study and approved the final version. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author upon request.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

*1* College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, PR China. *2* Institute of Epigenetics and Epigenomics, Yangzhou University, Yangzhou 225009, PR China.

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