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High-fat Medium and Circadian Transcription Factors *(Cryptochrome* and *Clock)* Contribute to the Regulation of Cholesterogenic *Cyp51* and *Hmgcr* Genes in Mouse Embryonic Fibroblasts

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Abstract

The aim of our research was to investigate how cholesterol, unsaturated fatty acids and circadian genes affect the expression of cholesterogenic genes, *Cyp51* and *Hmgcr*, in somatic and in embryonic fibroblast cell lines. We found that in immortal Hepa1–6 cells cholesterol represses the transcription of *Hmgcr* and *Cyp51* for 80%, while unsaturated fatty acids have different effects: *Hmgcr* was repressed for 50%, but *Cyp51* was unaffected by the presence of linoloeic acid. In embryonic fibroblasts the abundance of cholesterol in the media did not repress the expression of *Cyp51* and *Hmgcr,* while the presence of linoleic acid repressed transcription of both genes for 40%. Mutation of the *Clock* gene activated the basal transcription of *Cyp51* and *Hmgcr* and also reconstituted the cholesterol feedback loop, that was lacking in the wild type embryonic fibroblasts. Deletion of repressor genes *Cry1* and *Cry2* resulted in activated transcription of cholesterogenic genes after addition of linoleic acid, while response to cholesterol was unchanged compared to wild type embryonic fibroblasts. Our results indicate that cholesterol, unsaturated fatty acids and the circadian transcription factors participate in the regulation of cholesterogenesis through different molecular mechanisms, presumably using different SREBP transcription factors and their coregulatory proteins.

Keywords: Cholesterol biosynthesis, circadian rhythm, *Cyp51, Hmgcr,* mouse embryonic fibroblasts

1. Introduction

Cholesterol is a prerequisite for the animal life, because it is a component of membranes. Its biosynthetic pathway is not destined only to the production of the end product, but several other physiologically important molecules, such as vitamin D, heme, isopentenyl-tRNA, dimethylalyl pyrophosphate, ubiquinone, dolichol, farnesyl pyrophosphate, originate from the pathway. The homeostasis of cholesterol is precisely regulated. The disturbances can lead to hypercholesteremia, one of the leading causes of death in the developed countries. On the other hand, hypocholesteremia is linked to the higher risk for aggression, depression and even suicide[1]. Cholesterol is important also during the embryonic development,[2–4] where the main roles are likely its covalent binding to the Sonic Hedgehog proteins which act as signaling molecules.[2,5] Shortage of cholesterol can

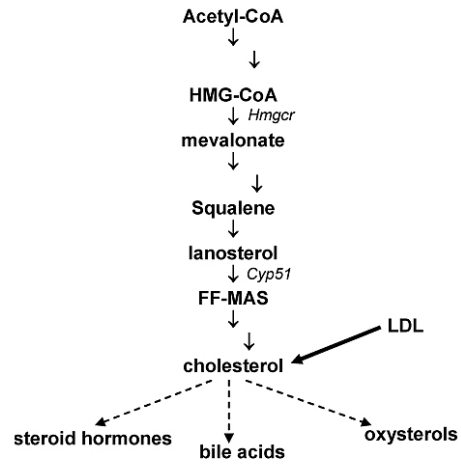

Figure 1: Shematic representation of cholesterol biosynthesis and homeostasis.

be caused by inhibition of enzymes participating in its biosynthesis or by the mutations of cholesterogenic genes.[3] Lack of cholesterol during embryogenesis leads to serious developmental abnormalities.[6] Cellular cholesterol requirements are met through *de novo* synthesis and/or uptake of plasma lipoproteins (Fig 1).

Cholesterol is synthesized *via* isopreonid biosynthetic pathway (Fig 1) starting from acetyl-coenzyme A (Ac–CoA), which is transformed into hydroxymethyl-glutaryl coenzyme A (HMG–CoA). HMG–CoA is reduced into mevalonate with HMG–CoA reductase (HMGCR), the rate-limiting enzyme of the cholesterol biosynthetic pathway. Mevalonate is transformed into squalene, leading to lanosterol and the cholesterol. Lanosterol is reduced into 4,4-dimethylcholesta-8(9),14,-24-trien-3β-ol (FF-MAS) by the lanosterol 14α-demethylase (CYP51).

In somatic cells the homeostasis of cholesterol is regulated *via* several signaling pathways, such are cholesterol feedback loop, the cAMP-dependent signaling pathway, etc,[7–11] the cholesterol feedback loop being seemingly most important and best understood. Cholesterogenesis is also under the circadian control, although the underlying mechanisms are poorly understood.[12,13]

Dietary polyunsaturated fatty acids and cholesterol suppresses transcription of hepatic genes involved in the metabolism of fatty acids and cholesterol through the action of sterol responsive element binding proteins (SREBP) transcription factors. Newly synthesized SREBPs are embedded into nuclear and endoplasmic reticulum membranes and are transcriptionally inactive in sterol/fatty acids loaded conditions. In lipid deprived physiological conditions, the N-terminal part of SREBPs are released from membranes and transferred to the nuclei.[14] SREBP-1 isoforms (SREBP-1a and SREBP-1c) primarily regulate fatty acid synthesis, while SREBP-2 is mainly involved in the regulation of cholesterol homeostasis.[15] Contrary to the animal tissues where SREBP-1c and SREBP-2 are highly expressed, SREBP-1a is mainly expressed in immortal cell lines. SREBP-1 seems to be mainly involved in energy metabolism including fatty acid and glucose/insulin metabolism, whereas SREBP-2 is specific to cholesterol synthesis,[16] although all types of SREBPs can modulate both cholesterol and fatty acids metabolism *in vitro*. The proteolytic activation of SREBP-1 and SREBP-2 can be regulated individually. For example, in rodents statins upregulate SREBP-2 activity and reduce SREBP-1 activity, while polyunsaturated fatty acids inhibit SREBP-1, but contrary to the oxysterols they have no effect on SREBP-2 maturation and activity, indicating that SREBP-1c is a key target for PUFA-dependent suppression of *de novo* lipogenesis.[17–19]

Circadian rhythm is a biological rhythm with the period of approximately 24 h. It is daily entrained by environmental cues (e.g. light, temperature), and it is generated by the transcription-translation negative feedback loop composed of a set of circadian genes. Circadian proteins are transcriptional activators and repressors binding to several promoter-regulatory elements such as E-box, c-AMP-responsive elements, DBP/E4BP4 and REV-ERBα /ROR. Among the most investigated mammalian circadian genes are also activators *Clock* and *Bmal a*nd repressors of the chryptochrome (*Cry1, Cry2*) and period (*Per1, Per2, Per3*) families.[20–22] Molecular mechanisms of the circadian regulation are explained elsewhere.[23–27]

The goal of our work was to investigate for the first time the effects of lipids (cholesterol and fatty acids) on the expression of model cholesterogenic genes (*Hmgcr* and *Cyp51*) in mouse embryonic fibroblasts and in immortal liver Hepa 1–6 cells. The role of mutation/deletion of circadian genes *Clock* and *Cryptochrome 1* and *2* to the process of cholesterogenesis has also been investigated.

2. Methods and Materials

2. 1. Cell culture Conditions

We used human choricarcinoma (Jeg3) cells, mouse hepatocarcinoma cells (Hepa 1–6), wild type mouse embryonic fibroblast cells (Mef3), mouse embryonic fibroblast cells with the mutated *Cry1* and *Cry2* genes (*Cry1, 2 -/-)* and mouse embryonic fibroblast cells with the mutated *Clock* gene (*Clock -/-)*. Jeg3 and Hepa 1–6 cells were grown in DMEM media (Sigma, Taufkirchen, Germany) containing 5% bovine calf serum and 1% L-glutamine in a 5% CO_2 incubator at 37°C, while the three mouse embryonic fibroblast cell lines were cultured in similar conditions, only that 5% serum bovine calf serum was exchanged with 10% fetal calf serum. All used mouse embryonic fibroblast cells were obtained from P. Sassone-Corsi laboratory and maintained by previously published protocols.[28] Mice with deleted *Cry1, 2* genes were prepared as published,[29] while *Clock* mutated animals originate from the laboratory of J. Tahakashi.[30]

In experiments the following media were used: (1) COPUFA – lipid rich medium (DMEM with 1% bovine serum albumin (BSA), cholesterol (10 μg/ml), 25-hydroxycholesterol (1 μg/ml), linoleic acid (0.15 mM), (2) CO – COPUFA without linoleic, (3) PUFA – COPUFA media without cholesterol and 25-hydroxycholesterol and normal medium (NM). Cells were grown to 90% confluency in T75 tissue culture flasks.

For RNA isolation cells (Hepa 1–6, Mef3, *Cry1,2 -/-, Clock -/-)* were split 1:3 24 h before the beginning of the experiment and were put into 6-well microtiter plates into normal media. At the beginning of the experiment, media was changed into normal, COPUFA, CO or PUFA, as indicated. RNA was isolated 24 h after media have been changed. For the transfections only Jeg3 cells were used. After splitting cells were put directly into experimental medium (normal, COPUFA, CO, PUFA). At the beginning of the experiment, medium was replaced with fresh and was changed every 24 h.

2. 2. RNA Isolation

Total cellular RNA was isolated form Hepa 1–6, Mef3, *Cry 1,2* -/- and *Clock* -/- cell lines. After 24 h the media were aspirated. Cells were washed twice with RNAse-free PBS buffer and 0,5 ml of TRI reagent (Sigma, Taufkirchen, Germany) was added directly to the cells. After 5 min the solution of TRI reagent with lysed cells was transferred into RNAse-free microcentrifuge tubes and total RNA was isolated. RNA concentration and quality were determined by RNA 6000 Nano Assay with Agilent 2100 Bioanalyzer (Agilent Technologies). At least two different samples of each RNA sample have been isolated and investigated for each experimental condition.

2. 3. Quantitative RT-PCR

For Q-RT-PCR analysis 1 μg of total RNA was converted into cDNA in a 20 μl reaction mixture using a SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CI) with random primers (Promega, Madison, WI). Reaction mixture was treated with DNase I (Sigma, Taufkirchen, Germany) to remove the contaminating DNA. The quality of each cDNA was tested in PCR reaction with β-actin primers. Q-RT-PCR analysis was preformed using SybrGreen technology on an Applied Biosystems Prism 7900 HT sequence detection system. Specific primers for *Cyp51* and *Hmgcr* were published previously.[31,32] Primers were validated by the analysis of template titration and dissociation curves. PCR products were analyzed by melting curve analysis. All reactions were performed in triplicate, control 18S rRNA or investigated Cyp51 and Hmgcr primers were amplified as follows: after incubation at 50 °C for 2 min and denaturating at 95 °C for 10 min, 40 cycles were performed at 95 °C for 15 s, and 60 °C for 1 min. The relative amounts of all mRNAs were calculated by the comparative Ct (cycle number at threshold) method (User Bulletin No.2, Applied Biosystems, PE) using 18S rRNA as the internal control. Relative mRNA levels were determined by expressing the mRNA amount of cells grown in normal media comparable to the expression of investigated genes in cells grown in lipid rich media (COPUFA, CO, PUFA). Expression level in embryonic fibroblast lines with deleted / mutated *Cry 1, 2* and *Clock* genes was normalized to the expression in wild type Mef3 cells grown in the normal media. Data were processed with $2^{-\Delta\Delta CT}$ method as described previously.[33] The average value, SEM and p values from two-tailed t test were calculated with the Excel program (Microsoft Corp., Redmond, WA).

2. 4. Transient Transfection Assays and Determination of Reporter Gene Activity

One day prior transfections Jeg3 cells were plated into 12-well plates. On the day of transfection, the media was replaced with fresh media and transfection was performed with Ca^{2+} precipitate method with the *firefly* luciferase human *CYP51* D7 reporter (–334/+316). Cells were transfected with 500 ng of luciferase reporter, 125 ng of *pSV β-galactosidase* and 100 ng of pCMV SREBP-2, pCMV BMAl1, pCMV CLOCK, pCMV Per1 and pCMV CRY1 expression plasmids. In the Clock/Bmal1 titration experiment quantities of expression plasmid are indicated in the Fig 4B. *pCAT basic* plasmid was used as the carrier DNA to 1 μg. β-galactosidase was used for normalization of the transfection efficiency. Media was changed every 24 h. 48 h after transfection cells were harvested with Promega passive lysis buffer (Promega, Madison, WI). The assay for determining the β-galactosidase activity was performed as described.[34] Activity of the *firefly* luciferase was analyzed with the commercial kit for luciferase (Promega, Madison, WI) and Turner TD-20/20 luminometer. All transfection experiments were performed at least three times in duplicates for each experimental condition. In all transfection experiments, reporter activity has been calculated by the formula: reporter activity = (normalized reporter gene activity) / (reporter activity in non-treated cells). Reporter activity in non-treated cells represents unit 1 and is shown in column 1 of diagram. The average value, SEM and p values from two-tailed t test were calculated with the Excel program (Microsoft Corp., Redmond, WA). Preparation of human *CYP51* luciferase reporter construct (–334/+316) was published previously.[11]

SREBP- 2 expression plasmid originates from the lab of J. Horton (University of Texas Southwestern Medical Center, Dallas, TX, USA) while Bmal1, Clock, Per1 and Cry1 expression plasmids were prepared in P. Sassone-Corsi group (IGBC, Strasbourg, France).

3. Results

Cholesterol and unsaturated fatty acids affect expression of Cyp51 and Hmgcr in mouse hepatoma and in embryonic fibroblasts.

Cholesterol biosynthesis is a housekeeping process, which is in completely differentiated somatic cells primarily regulated by the negative feedback loop, where transcription factors of SREBP family are used as major mediators. SREBPs in such physiological conditions activate the transcription of cholesterogenic genes in lipid deprived conditions, but in lipid loaded conditions SREBPs are not present in cell nuclei.[8,35,36] In embryonic cells the cholesterol feedback loop is not functional probably due to the increased need for cholesterol.[5] We wanted to reveal whether the same molecular mechanisms participate in the regulation of the two model cholesterogenic genes (*Hmgcr* and *Cyp51*) in somatic cells and embryonic fibroblasts. Figures 2A and 2B show that lipid rich medium (COPUFA) represses the transcription of cholesterogenic *Cyp51* and *Hmgcr* in mouse Hepa 1–6 cells. The repressor effect of linoleic acid and cholesterol is additive in the case of *Hmgcr* (Fig 2B). Presence of linoleic acid alone in

the media repressed *Hmgcr* expression for 50%, but *Cyp51* transcription was almost unaffected, repression was only 10% (Fig 2A). *Cyp51* expression was downregulated only with cholesterol and 25-hydroxycholesterol (Fig 2A white and grey bars). *Cyp51* and *Hmgcr* expression in wild type mouse embryonic fibroblasts (Mef3) was unaffected by the addition of cholesterol and 25–hydroxycholesterol (CO) to the media (Fig 2A, B), while polyunsaturated fatty acids (PUFA) have the repression effect (the striped bars in Mef3, Fig 2A, B).

by COPUFA in Mef3 cells with deleted *cryptochrome* genes (Fig 3A, white bars in Cry 1, 2 -/- column), while addition of cholesterol alone repressed transcription of *Cyp51* for 50% (Fig 3A) but did not affected the expression of *Hmgcr* (Fig 2B). Growing mouse embryonic cells with deleted *Cry1, 2* genes in the medium with linoleic acid activated the expression of *Cyp51* and *Hmgcr* genes 2,5 fold (Fig 3A, B).

Mutation of *Clock* activated transcription of *Cyp51* for 6-fold, while *Hmgcr* was induced 2-fold (Fig 3A, B. Addi-

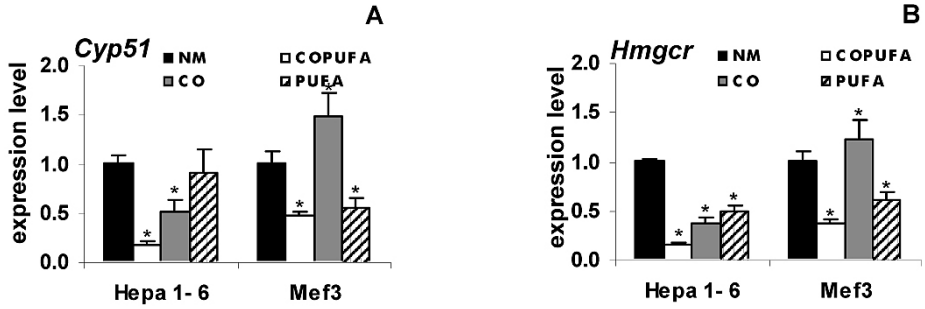

Figure 2: *Role of lipid rich media on the Cyp51 and Hmgcr expression in Hepa 1–6 cells and wild type Mef3 cells.* A; *Cyp51*. B; *Hmgcr*. Total RNA was isolated from cells grown in indicated media for 24 h. Normal media (NM) – black bars, COPUFA (10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol, 0,15 mM linoleic acid) – white bars, CO – (10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholestero) – grey bars or PUFA (0.15 mM linoleic acid) – striped bars. * – significantly modulated expression ($p < 0.05$) compared to the expression in normal media (black bars).

The second question was how the deletion/mutation of three circadian genes (*Clock, Cry1, Cry2*) changes the response of *Hmgcr* and *Cyp51* to cholesterol and unsaturated fatty acids in mouse embryonic fibroblasts. Deletion of both *cryptochrome* (*Cry1* and *Cry2*) genes led to a weak repression (30–35%) of *Hmgcr* compared to expression in the wild type mouse embryonic fibroblasts (compare black bars in Fig 2 B and 3 B) in normal media, while basal expression of *Cyp51* was unaffected under the same (normal) conditions (Fig 3B). Media change into the lipid rich medium (COPUFA) led to the diminished repression

tion of linoleic acid to the media did not cause changes in the expression of *Cyp51* and *Hmgcr* in mouse embryonic cells with mutated *Clock* (Fig 3A, B, column *Clock* -/-, striped bars). Loading media with cholesterol and 25-hydroxycholesterol repressed expression of *Cyp51* and *Hmgcr* for 90% and 70% compared to level in normal media (Fig 3A, B, column *Clock* -/-, grey bars).

To check if the proximal promoter is responsible for the effects of mutation/deletion circadian genes seen in Fig 3A to the *Cyp51* transcription, the transfections of human *CYP51 luciferase* reporter construct (–334/+316) in-

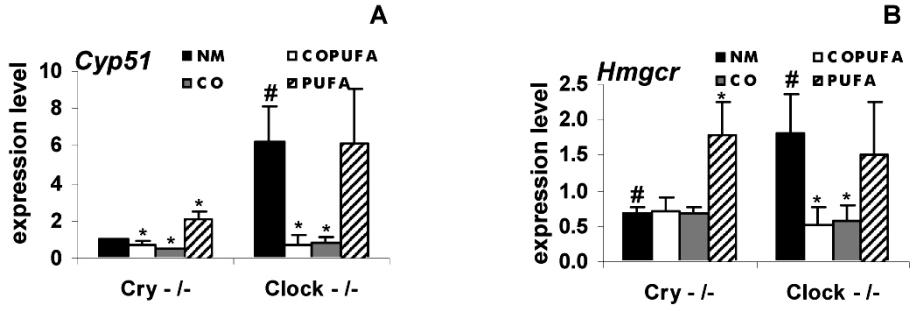

Figure 3: *Role of lipid rich media in the Cyp51 and Hmgcr gene expression in Mef3 cells with deleted/ mutated Cry1, 2 and Clock genes.* A; *Cyp51* expression, B; *Hmgcr* expression. Total RNA was isolated from cells grown in indicated media for 24 h. Relative expression unit 1 is expression in wild type Mef3 cells in normal media (Fig 2, black bars in Mef3 column). Normal media (NM) – black bars, COPUFA (10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol, 0.15 mM linoleic acid) – white bars, CO – (10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol) – grey bars or PUFA (0.15 mM linoleic acid) – striped bars. * – significantly modulated expression ($p < 0.05$) compared to the expression in normal media (black bars), # significantly modulated expression ($p < 0.05$) compared to the expression in wild type Mef3 (black bars in figure 2).

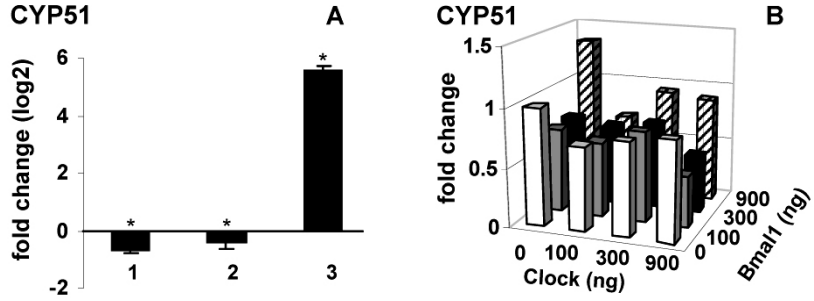

Figure 4: *Circadian transcription factors Clock and Bmal1 do not transactivate the proximal promoter of human CYP51 gene in Jeg3 cells.* A; Transient transfections of Jeg3 with human *CYP51* proximal promoter/reporter construct with the overexpression of SREBP-2 and circadian transcription factors. Overexpression of 1; Clock + Bmal1, 2; Cry1 + Per1, 3; SREBP-2. B; Transfections of human *CYP51* proximal promoter luciferase reporter with different amouns of Clock and Bmal1 overexpression vectors. * – significantly modulated expression ($p < 0.05$).

to Jeg3 cells were performed. Figure 4 shows that in *ex vivo* conditions the proximal promoter of human *CYP51* gene is mainly responsive to the cholesterol feedback loop and transcription factor SREBP-2 (Fig 4A, column 3) and is almost unresponsive to the overexpression of circadian transactivators CLOCK and BMA1, irrespectively of their quantity (Fig 4A column 1 and Fig 4B) or repressors PER1 and CRY1 (Fig 4A, column 2).

4. Discussion

Cholesterol is a molecule with the diverse and indispensable functions during prenatal and postnatal life of animals. Different molecular mechanisms are involved in regulation of cholesterol homeostasis in the cells, tissues and whole organism in different physiological and pathophysiological conditions. The major pathway regulating cellular cholesterol level in normal physiological conditions in adult somatic cells is the SREBP pathway, which represses transcription of genes involved in the biosynthesis and uptake of cholesterol and fatty acids in the conditions of abundance of lipids. However, when cells lack lipids, the SREBP signaling pathway is activated and genes are actively transcribed leading to a higher level of biosynthesis and uptake of lipids.[8,35,36]

Hepa 1–6 and Jeg3 immortal cell lines are frequently used as *ex vivo* models for research of cholesterol homeostasis in completely differentiated somatic tissues. Our earlier research of *CYP51* was done principally in Jeg3 cells and it was determined that the COPUFA medium represses *CYP51* transcription for 70%–90% in 24 h.[31] In the present study we investigated how loading of cells with unsaturated fatty acids (linoleic acid) and/or cholesterol affect the transcription of two model cholesterogenic genes *Hmgcr* and *Cyp51* in different physiological conditions when the circadian activators or repressors are depleted from the experimental system.

In some tissues, such as germ cells and embryonic tissues, the cholesterol feedback loop does not work properly and cholesterol is synthesized although there is abundance of lipids. The role for such regulation is not well understood, but it is proposed that the cholesterol and intermediates are important for the development of fetus and mature germ cells.[5,37,38] The differences in responsiveness of sterol synthesis rates between adult and fetal tissues are at least partially due to the constitutive processing of SREBP-2.[5] Both model cholesterogenic genes *Hmgcr* and *Cyp51* are in Hepa 1–6 cells 85–90% down-regulated by the presence of cholesterol. On the other hand, the addition of linoleic acid to the media repressed for 50% only transcription of *Hmgcr*, while *Cyp51* expression was reduced for only 10% (Fig 2A, B). This indicates that *Hmgcr* and *Cyp51* might be differently regulated by SREBP-1 and SREBP-2, although they are both a part of the same cholesterol biosynthetic pathway. We propose that *Cyp51* is regulated mostly by SREBP-2 in completely developed cells and that unsaturated fatty acids acting through SREBP-1 are not important for *Cyp51* transcription. *Hmgcr,* the rate limiting enzyme, of the early phase of the cholesterol biosynthesis, which is not destined only for the cholesterol synthesis, is regulated much more precisely by the participation and interactions of several other signaling pathways. One of these is probably mediated by SREBP-1 and fatty acids.

We also investigated how lipids affect expression of *Hmgcr* and *Cyp51* in embryonic fibroblasts (Mef3). The presence of cholesterol and 25-hydroxycholesterol in the media did not repressed *Cyp51* and *Hmgcr* genes (Fig 2A, B). Lower expression of *Cyp51* and *Hmgcr* in COPUFA media is only due to the repression by linoleic acid (Fig 2A, B). Experiments with animals with deleted *Srebp* genes showed the urgent presence of SREBP-2 as a principal activator of cholesterogenesis during embryonic development, because deletion of *Srebp-2* is 100% embryonic lethal, while *Srebp-1* knock-out is in 15–50% of mice compatible with life.[39] The repression of *Cyp51* and *Hmgcr* by linoleic acid in wild type Mef3 cells may be explained by the suggestion of Woollett et al, that polyunsaturated fatty acids (PUFA) alter lipid metabolism within fetus *via*

SREBP-1 and PPAR signaling pathways.[40] Embryonic uptake of exogenous maternal cholesterol was previously demonstrated to rescue the drug induced Smith-Lemli-Opizt syndrome,[40] suggesting a dispensable action of cholesterol feedback loop in the embryo. It was proposed that in fetal tissues, the ability to suppress and activate sterol synthesis may not be advantageous since the need for cholesterol is high and constant. This apparent dysregulation of sterol synthesis in fetal and other rapidly growing tissues might be the result of the changed regulation of SREBP-2 activity, which can occur at different levels, such constitutive processing of SREBP-2 and its decreased degradation.[5]

Cholesterol homeostasis is also a circadian process. Mutation of the *Clock* gene is involved in the development of metabolic syndrome, characterized by obesity, hyperlipidemia, hyperglycemia and hypoinsulinemia in the mouse C57BL/6J strain, while the ICR strain is almost unaffected.[41–43] In our experiments mutation of *Clock* activated the basal expression of *Cyp51* and *Hmgcr* genes (Fig 3, black bars) indicating a key role of the *Clock* protein even in early embryonic development. This observation can be explained by the report of Doi,[44] that CLOCK acts as a histone acetyltransferase and in this way regulates expression of target genes. Addition of cholesterol repressed the transcription of *Cyp51* in both investigated embryonic fibroblast cell lines with mutations/deletions of circadian genes for 50% (*Cry1,2* -/-) and 90% (*Clock* -/-) (Fig 3A), similarly to the effect observed in liver cells. Expression of *Hmgcr* in cells with deleted *Cry1* and *Cry2* genes was not changed in cholesterol loaded conditions, but was repressed for 70% in cells with mutated *Clock* gene, indicating that the mutation of *Clock* might reconstitute the cholesterol feedback of embryonic fibroblasts missing in wild type embryonic fibroblast cells. The effect of linoleic acid was opposite to the effect of cholesterol. Addition of linoleic acid to the media lead to a 2-fold induction of *Cyp51* and *Hmgcr* expression in *Cry 1,2* -/- cells while mutation of *Clock* did not affect transcription of cholesterogenic genes (Fig 2A, B, striped rows in *Cry1,2* -/- and *Clock* -/-). Proposed explanation is that deficiency of circadian activator *Clock* leads to the loss of circadian expression pattern of cholesterogenic genes and consequently to the activation of cholesterogenic gene transcription. This is in accordance with our unpublished results with mice with the deleted *Crem* gene, where the expression of most cholesterogenic genes is upregulated (Ačimovič et al, unpublished) and with the observations in *Clock* mutant mice that develop metabolic syndrome with hypoinsulinemia and hyperlipidemia.[41] We propose that there is a possibility of development of hyperlipidemia in this mouse strain as a consequence of the obesity and disturbed energy metabolism through the activation of SREBP-2 system and inhibition of SREBP-1 proteins.

Deletion of *cryptochrome* genes had almost no effect on the transcription of *Cyp51* and *Hmgcr* mediated by cholesterol (Fig 3, grey lines), while addition of linoleic acid surprisingly activated expression of both cholesterogenic genes, indicating a novel role of repressor cryptochrome proteins in the regulation of cholesterogenic genes through the SREBP-1 system. The effects of CRY proteins might be indirect, because these proteins do not interact directly with DNA but through protein – protein interactions. We propose that in the case of cholesterol homeostasis the role of activating CLOCK protein is greater because the lack of *Clock* leads also to the depletion of cryptochrome repressors. In our research mutation of *Clock* completely changed the response of embryonic fibroblast to the addition of lipids by inhibition of the SREBP-1 pathway and activation of the SREBP-2 pathway. On the other hand, deletion of *cryptochrome* genes left the SREBP-2 system unchanged and repressed only the SREBP-1 pathway.

The results shown in Fig 3 clearly demonstrate that the expression of *Hmgcr* and *Cyp51* is closely connected with the circadian proteins. The target DNA regulatory element of circadian activators CLOCK and BMAl1 is E-box with SRE as a variant and we wanted to know if the proximal promoter region, which is responsible for SREBP-dependent expression of *CYP51,* is also responsible for the circadian regulation. Transfections of proximal promoter *luciferase* reporter construct of human *CYP51* gene suggested that the circadian transcription factors likely act through distal promoter regulatory elements or they participate in the regulation of cholesterogenic genes through interactions with other signaling pathways.

Further investigations are needed for elucidation of the proposed hypothesis. Our results are in accordance with the results of Oishi et al. in the ICR mice strain, reporting that diet with high cholesterol significantly decreased expression of *Hmgcr*, *Ldl* and *Cyp7a1* in livers of *Clock* mutant mice.[42] Repressed transcription of cholesterogenic genes prevented the development of the metabolic syndrome. Although the overexpression of circadian transcription factors CLOCK, BMAl1, PER1 and CRY1 did not affect the expression of human *CYP51* proximal promoter/reporter construct (Fig 4) in *ex vivo* conditions (also hamster HMGCR – data not shown), bioinformatic analysis of human and mouse *CYP51* distal promoter regions showed the presence of several circadian regulatory elements (E-Box, CRE, ROR-REW) in the distal promoters of both mammalian *CYP51* genes (Španinger, unpublished). This suggests that the circadian regulation of cholesterogenic genes might be mediated through distal and not proximal promoters.

5. Conclusion

Lipid metabolism is an important factor during development and is also significant for maintaining the body energy homeostasis. Lipid homeostasis is regulated by se-

veral signaling pathways although the molecular mechanisms are not fully understood. We showed for the first time, that 1. Cholesterol homeostasis is regulated in a different manner in embryonic cells and in completely differentiated cells. 2. Circadian genes are directly involved in the regulation of cholesterol homeostasis: mutation of *Clock* gene activates the expression of cholesterogenic *Cyp51* and *Hmgcr* and reconstitutes the cholesterol feedback loop while deletion of *cryptochromes* extinguishes the repression effect of unsaturated fatty acids to the cholesterol biosynthesis. Deletion/mutation of circadian activators and repressors has different effects to the expression of cholesterogenic *Hmgcr* and *Cyp51* probably due to their different response to SREBP-1 and SREBP-2 transcription factors. Further research is needed to elucidate the role of individual signaling pathway and the interactions between different factors in different physiological/pathophysiological conditions.

6. Acknowledgments

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7. Abbreviations

BSA	bovine serum albumin
CO	lipid rich medium containing cholesterol and 25-hydroxycholesterol
COPUFA	lipid rich medium containing cholesterol, 25-hydroxycholesterol and linoleic acid
CRE	cAMP response element
Cry	chryptochrome
Cyp7a1	cytochrome P450 7a1
Cyp51	lanosterol 14α-demethylase
Hmgcr	3-hydroxy-3-methylglutaryl coenzyme A reductase
Mef	mouse embryonic fibroblast
P450	cytochrome P450
Per	period
PUFA	lipid rich media with linoleic acid, SREBP, sterol regulatory element-binding protein
SRE	sterol regulatory element

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Povzetek

Namen našega dela je študij vpliva holesterola, nenasičenih maščobnih kislin in cirkadialnih genov na izražanje holesterogenih genov *Cyp51* in *Hmgcr* v nesmrtnih celičnih linijah tkivnih celic in zarodnih fibroblastov. Ugotovili smo, da v nesmrtni celični liniji Hepa1-6 prisotnost holesterola za 80 % zavre prepisovanje genov *Cyp51* in *Hmgcr,* medtem ko je dodatek nenasičenih maščobnih kislin privedel do drugačnega odziva obeh genov. Prisotnost linolenske kisline v mediju ni vplivala na izražanje *Cyp51*, prepisovanje *Hmgcr* pa je bilo zavrto za približno 50 %. V mišjih zarodnih fibroblastih (Mef3) izobilje holesterola ni vplivalo na izražanje *Cyp51* in *Hmgcr*, medtem ko je dodatek linolenske kisline zavrl izražanje obeh genov za približno 40 %. Mutacija cirkadialnega gena *Clock* je aktivirala osnovno prepisovanje *Cyp51* in *Hmgcr* ter ponovno vzpostavila negativno povratno zanko holesterola, ki je v nespremenjenih celicah zarodnih fibroblastov nismo opazili. Delecija genov zaviralnih transkripcijskih faktorjev CRY1 in CRY2 je aktivirala prepisovanje holesterogenih genov ob dodatku linolenske kisline, medtem ko je bil odziv celic na izobilje holesterola enak kot v zarodnih fibroblastih divjega tipa. Naši rezultati nakazujejo, da holesterol, nenasičene maščobne kisline in cirkadialni ritem sodeljujejo pri uravnavanju homeostaze holesterola preko različnih molekularnih mehanizmov, ki vključujejo tudi kombinacije transkripcijskih faktorjev družine SREBP in njihovih koregulatornih proteinov.