Reviewer #1:

Xing-Ya eT al report the effect of circRNA oo46366 on neutral lípid-loading in a cellular model of liver steatosis. Although the results are interesting, and the methods and experimental design appropriate, the manuscript should suffer fundamental changes before further consideration.

Steatosis is a descriptive word used to name the accumulation of lipids, mainly neutral lipids, in an organ of a living organism. The most frequent form of steatosis is liver or hepatic steatosis, often referred as fatty liver. In the present work, an in vitro experimental model is used to verify the lipid loading of hepatoma cells after incubating them with fatty acids. In this experimental setting, it is not correct to use terms such as liver steatosis, hepatic steatosis, hepatocyte steatosis, etc. to refer to hepatoma cells (not normal hepatocytes) loaded with triglycerides after a short term of incubation. Although this model allows to study molecular mechanism involved in the accumulation of lipids inside liver cells, it is not a true “hepatic steatosis” situation.

Reply: We are thankful for the reviewer’s comments. We have revised the title and text of manuscript in accordance to the reviewer’s suggestion.

It is very difficult to understand the meaning of the abstract, starting with the fact that nowhere in the text appears the experimental model used, culture HepG2 cells. Please, rewrite the abstract in a more concise, informative and understandable style. A similar situation happens with the Core tip section. . Although the English writing is generally correct, some sentences or words are difficult to understand. For example: What

means cerebral apoplexy (first paragraph in the introduction section). Are you talking about cerebrovascular diseases, such as occlusive ictus? miR-34a discriminates patients with hepatic steatosis from healthy controls with an area under the receiver operating characteristic curve of 0.781. This sentence is very confusing. circRNA_010567 in diabetic mice inactivate miR-26b-5p and miR-141, respectively, by the complementation between MREs and miRNAs. Are you referring to the fact that the physical interaction between the MRE domain of the circular RNA and the corresponding response element in the sequence of the miRNA inhibits its physiological activity? . Please, you do not administer anything to cultured cells, you administer to living organisms. .

Reply: We are thankful for the reviewer's comments. We have improved the expression as reviewer suggested.

The methods section should give more information. Which are the exact incubation protocol for loading hepatoma cells? Where is the description of the bioinformatic analysis? . PPARalpha controls a wide array of target gens in liver (apo AI, HMGCS, LPL, VLDL-R, Apo CIII, etc.). It will be interesting to know if at least several of these genes behave similarly to CPT-Ialpha.

Reply: We are thankful for the reviewer's comments. The methods section has been carefully revised so as to preset details concerning circRNA administration and bioinformatic analysis. The expression of multiple PPARalpha-targeted genes is also investigated.

Reviewer #2:

Recent discovery of a special class of RNAs has stimulated many investigators and has produced hundreds of new experimental studies. Circular RNAs are produced in the process of the transcription, and both ends of these are covalently closed. Most of these circular RNAs are expressed I cytoplasm. This report may have an interesting aspect of the regulation of PPARα.

Major 1. In the section of material method is insufficient, it hampers to confirm this result by other investigators. You should add detail condition of your experiment, especially administration of circ RNA. In the section of phenotypic evaluation, there is no definition of each subgroup.

Reply: We are thankful for the reviewer's comments. The section of material method has been carefully revised so as to provide sufficient details and definition.

2. Circ RNAs tend to be expressed weakly in usual. On the contrary, we can detect huge amount of miRNA-34 in the liver. Did you quantify the amount of the circ RNA in HepG2 cell ? If one to one binding between the miRNA and the circ RNA has regulatory effect of downstream signals, cric RNA should be huge amount.

Reply: We are thankful for the reviewer's comments. Revisions have been made as reviewer suggested.

miRNA has already been verified to function in a multiple-target manner [1]. Depending on this unique characteristics, has-mir-34, including miR-34a, miR-34b,

and miR-34c, exerts important impact on both physiological and pathological process of the liver [2-4]. In detail, recent experimental proofs reveal that miR-34a/miR-34c involve in hepatic fibrosis by targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) [2]. Nevertheless, miR-34a/miR-34c regulate the G1/S checkpoint in cells exposed to DNA damage or oxidative stress, mainly be linked to cyclin E/p53 [3-4]. Except for its roles in cell cycle/proliferation, fibrosis/cirrhosls, and carcinogenesis, miR-34a exhibits close association with hepatocyte steatosis, predominantly by the PPARα-targeted effect [5]. On the other hand, this multiple-target effect of different subtypes of miR-34 facilitates a subtle antagonism of steatosis-related miR-34a/PPARα interaction by relative limited circRNA on a basis of competitive binding. In our experiments, abundant circRNA_0046366 and miR-34a correlates to non-steatosis phenotype. Whereas insufficient circRNA_0046366 and augmented miR-34a characterizes the FFA-induced hepatocellular steatosis. The attenuated hepatosteatosis related to circRNA_0046366 upregulation further indicates the rebalancing of circRNA_0046366/miR-34a interaction. Moreover, the enzymatic resistance and long-term stability of circRNA_0046366 amplify its pharmacological action [6], which provides another explanation for the observations of our study.

1 **Djuranovic S,** Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science* 2012; **336**: 237-40 [PMID: 22499947 DOI: 10.1126/science.1215691]

2 **Li WQ,** Chen C, Xu MD, Guo J, Li YM, Xia QM, Liu HM, He J, Yu HY, Zhu L.

The rno-miR-34 family is upregulated and targets ACSL1 in dimethylnitrosamine-induced hepatic fibrosis in rats. *FEBS J* 2011; **278**: 1522-32 [PMID: 21366874 DOI: 10.1111/j.1742-4658.2011.08075.x]

3 **Koufaris C,** Wright J, Currie RA, Gooderham NJ. Hepatic microRNA profiles offer predictive and mechanistic insights after exposure to genotoxic and epigenetic hepatocarcinogens. *Toxicol Sci* 2012; **128**: 532-43 [PMID: 22584684 DOI: 10.1093/toxsci/kfs170]

4 **Pok S,** Wen V, Shackel N, Alsop A, Pyakurel P, Fahrer A, Farrell GC, Teoh NC. Cyclin E facilitates dysplastic hepatocytes to bypass G1/S checkpoint in hepatocarcinogenesis. *J Gastroenterol Hepatol* 2013; **28**: 1545-54 [PMID: 23574010 DOI: 10.1111/jgh.12216]

5 **Ding J,** Li M, Wan X, Jin X, Chen S, Yu C, Li Y. Effect of miR-34a in regulating steatosis by targeting PPARalpha expression in nonalcoholic fatty liver disease. *Sci Rep* 2015; **5:** 13729 [PMID: 26330104 DOI: 10.1038/srep13729]

6 **Hansen TB,** Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; **495:** 384-388 [PMID: 23446346 DOI: 10.1038/nature11993]

3. Why dose FAA reduce the expression of circ RNA 0046366? Show the reduction mechanism of circ RNA by FAA.

Reply: We have made the revision as reviewer suggested.

Being the products of alternative splicing, circRNA_0046366 and fatty acid synthase

(FASN) share the common precursor mRNA (pre-mRNA) [1-2]. The mRNA level of FASN, which serves as one of the major determinants in de novo lipogenesis, has been well described to experience significant upregulation during hepatic steatosis [3]. The circRNA_0046366 transcription is then inhibited on a basis of competitive effect in alternative splicing. Indeed, the steatosis group exhibits FASN mRNA level (7.64 ± 0.54) much higher than that of the normal group (0.98 ± 0.06, P <0.0001) in our experiments. Contrastively, expression loss of circRNA_0046366 features the HepG2 cells in steatosis group in comparison to those in normal group. This negative correlation between transcription levels of FASN and circRNA_0046366 highlights an evidence for the alternative-splicing-based mechanism of circRNA_0046366 downregulation on condition of FAA exposure.

1 **Salzman J,** Chen RE, Olsen MN, Wang PL, Brown PO. Cell-type specific features of circular RNA expression. *PLoS Genet* 2013; **9**: e1003777 [PMID: 24039610 DOI: 10.1371/journal.pgen.1003777]

2 **Glažar P,** Papavasileiou P, Rajewsky N. circBase: a database for circular RNAs. *RNA* 2014; **20**: 1666-70 [PMID: 25234927 DOI: 10.1261/rna.043687.113]

3 **Dorn C,** Riener MO, Kirovski G, Saugspier M, Steib K, Weiss TS, Gäbele E, Kristiansen G, Hartmann A, Hellerbrand C. Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol* 2010; **3**: 505-14 [PMID: 20606731]

4. Is the circ RNA is stable in cytoplasm of HepG2 cell? Add the stability data of the

circ RNA.

Reply: We have made the revision as reviewer suggested. With the linking of 3' and 5' ends, circRNA demonstrates resistant to enzymatic hydrolysis of RNase R and RNA exonuclease [1]. These characteristics confer the stable dynamics of circRNA. In the present study, expression level of hepatocellular circRNA_0046366 is subjected to dynamic evaluation in the circRNA group at different time points (24, 48, and 72 hour). Results of real-time RT-PCR confirm that intracellular circRNA_0046366 level remains constant until 72 hours after the transfection of circRNA-containing plasmid.

1 **Hansen TB,** Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J. Natural RNA circles function as efficient microRNA sponges. *Nature* 2013; **495:** 384-388 [PMID: 23446346 DOI: 10.1038/nature11993]