Suppression of miR-199a maturation by HuR is crucial for hypoxia-induced glycolytic switch in hepatocellular carcinoma

Ling-Fei Zhang, Jia-Tao Lou, Min-Hua Lu, Chunfang Gao, Shuang Zhao, Biao Li, Sheng Liang, Yong Li, Dangsheng Li and Mo-Fang Liu

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Anne Nielsen

1st Editorial Decision 19 May 2015

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all three referees express interest in the findings reported in your manuscript, although they also raise a number of constructive criticisms and concerns that you will have to address before they can support publication of the manuscript here.

For the revised manuscript I would particularly ask you to focus your efforts on the following points:

-> Please expand the analysis of both mechanism and specificity of HuR-dependent control of miRNA processing, as pointed out by refs #1 and #3. It will also be important for you to address the potential contribution from miR199a in both miRNA biogenesis and functional assays as brought up by ref#1.

-> Please include further cell lines in the analysis for miR199 target regulation (ref#1 and #2)

-> Please provide quantification of data for the nuclear to cytoplasmic translocation of RNA and HuR during hypoxia (ref#3). In addition, I would ask you to strengthen/clarify the data on a
transcriptional effect via HIF1 regulation (and to tone down the conclusions here if these experiments should prove inconclusive)

Given the referees' overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

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Referee #1:

Starting with the analysis of miRNA expression during hypoxia of HCC cells, the authors describe a role for miR-199 in the regulation of hepatocellular carcinoma metabolism and tumorigenesis. The authors propose a novel regulatory axis by which hypoxia-induced binding of HuR protein to pri-miR-199a inhibits miRNA processing, which in turn increases the levels of two key regulators of glucose metabolism, Hk2 and Pkm2. In general the authors provide with abundant cellular and in vivo experiments supporting the model, but the following issues bellow need to be addressed.

1) Down-regulation of miR-199a during hypoxia
   The authors analyzed the expression of 38 miRNAs in two HCC cell lines upon hypoxia and found a prominent downregulation of miR-199a. Unfortunately, material and methods about miRNA analysis is missing, which precludes the evaluation of these experiments. Authors should correct this issue and clarify the following questions:
   Why did the authors choose those 38 miRNAs? An unbiased analysis of miRNA expression would have provided with a better picture of miRNA regulation during hypoxia. This is required.
   The expression of miR-199b, the other member of the miR-199 family of miRNAs, has not been analyzed, although it has been recently linked to hypoxia (Shang et al, Int J Mol Sci, 2013; Jiang et al, Int J Neurosci, 2014). Given the sequence similarity, can the authors discard cross-amplification of miR-199b in the miRNA expression analysis? Which method has been used for miRNA quantification?
   Likewise, closely related miRNAs sharing the same seed sequence may have overlapping targets and functions. Can the authors discard that the impact on hypoxia of miR-199a overexpression compensates a hypothetical downregulation of miR-199b?

2) Hypoxia selectively inhibits processing of pri-miR-199a
   Strikingly, while the pri-miRNA of miR-199a/214 is overexpressed during hypoxia, the authors claim that the levels of miR-199a are specifically downregulated by HuR protein.
   An alternative hypothesis could be that the observed downregulation of miR-199a is due to a cross-amplification of miR-199b, as commented above. This could also explain why miR-214 is not affected. Can the authors discard this hypothesis?
   On the other hand, the authors show a modest upregulation of miR-214 in hypoxia conditions. However, Duan et al, PLoS One, 2012, reported downregulation of miR-214 upon CoCl2 induced hypoxia in HepG2 cells. How can the authors explain this discrepancy?
   Studies about pri-miR-199a/214 are confusing. The authors distinguish between pri-miR-199a and pri-miR-214, when they are supposed to be the same molecule, originated from antisense transcription from an intron of Dynamin-3 (Lee et al, NAR, 2009). Which criteria were used to define those two pri-miRNAs? What is the size of the pri-miR-199a detected by Northern-blot in figure 2B? Does it fit with previously published data? The authors should clarify these issues, and show a scheme of the miR-199a/214 transcript (or transcripts), indicating the probes used in the different experiments and the amplicons for pri-miR overexpression analysis.

3) HuR binding represses miR-199a processing
   Methods used to predict HuR binding elements in pri-miRNAs are not described.
   Again, the authors distinguish two different primary miRNAs for miR-199a and miR-214 to show different regulation by HuR. Unless the authors provide with evidences of the existence of two different pri-miRNAs, experiments in figure 2H should be repeated with the full length pri-miRNA,
and both the levels of miR-199a and miR-214 should be determined to prove the specific effect on miR-199a.

4) miR-199a directly targets Hk2 and Pkm2
Luciferase assays with mutant miR-199a sites are compelling, but experiments with endogenous Hk2 and Pkm2 in figure 3C and 3D should be performed in the same cell line. If miRNA inhibition is challenging for SMMC-7721 because of high levels of miR-199a, the authors can use the Hep3B cell line also for miR-199a mimics, since it has been shown in figure 1 that miRNA precursors affect glucose metabolism at very low concentration. Are blots shown in figure 3D and 3E representative images of different experiments? If so, a side plot showing the statistics of band intensities from the different repetitions should be included. If not, the authors should repeat those experiments to support their conclusions.

5) miR-199a:Hk2/Pkm2 axis regulation of glycolysis
As commented above, the authors could use the Hep3B cell line for both overexpression and inhibition of miR-199a to further demonstrate how the manipulation of miR-199a affect glucose metabolism also in xenograft experiments. Using different cell lines for functional experiments raises doubts about the model. The involvement of HuR in the miR-199a:Hk2/Pkm2 axis has not been investigated in further experiments. Did the authors try to recapitulate the effect of miR-199a inhibition on tumorigenesis in HCC cells by HuR knockdown? If feasible, this experiment would reinforce the proposed model.

Referee #2:
The current manuscript by Zhang et al. describes a novel regulatory axis in hepatocellular carcinoma (HCC). This axis comprises the hypoxia-induced repression of miR199a in hepatoma cells, miR199a repression in turn led to the induction of key glycolytic enzymes in hepatoma cells, thereby contributing to the malignant phenotype and metabolic reprogramming in both in vitro and in vivo systems. Analysis of human HCC specimens revealed a consistent regulation of miR199a in human tumors. Given the aggressiveness of HCC and the absence of effective therapeutic options, the identification of novel mechanisms in HCC pathogenesis clearly represents an important topic in biomedical research. In particular, metabolic reprogramming has gained increasing attention in the cancer field as it turned out to be a powerful target in the development of novel therapeutic options. In this regard, the current manuscript by Zhang et al employs a huge variety of technical approaches to pinpoint the importance of the novel miR199a axis in this context. The manuscript is clearly written, well structured and employs state-of-the art technology. The findings are novel and of importance for the field. Two points need attention by the authors: Main findings in figure 2 should be duplicated/verified in a second HCC cell line, i.e. SMMC-7721. The same holds true for the rescue effects of Hk2 or Pkm2 in terms of tumorigenesis, i.e. use Hep3B to verify the functional importance of these two enzymes for malignant phenotypes. The second issue concerns the question whether the miR199 axis is particularly important for a distinct HCC etiology, e.g. NASH- or virus-driven HCC. The authors should at least discuss the different possibilities.

Referee #3:
In this paper Zhang et al. examined the role of miR199a on two glycolytic genes, HK2 and PKM2 that play pivotal roles in the Warburg effect, a cancer cell-specific feature induced by hypoxia. The main claims of the paper are novel and provide important new information on miRNA-dependent regulation of cancer metabolic pathways. The key novel findings that are well supported by the data include: i. miR-199a efficiently regulates glucose uptake and lactate production in human HCC cell lines via regulating HK2 and Pkm2, which are bona fide targets of miR-199a. ii. miR-199a-dependent regulation of glucose metabolism also takes place in xenografts and there is a clear correlation between miR-199 and Hk2 and Pkm2 in human tumors. iii. Mechanistic insight into the regulation is provided, suggesting that HuR binding under hypoxic conditions blocks miR-199a processing.
The above results are timely and important for the field. Some (minor) issues should be addressed before publication. These include:

1. How common is the HuR-mediated block of miRNA processing? Did the authors check HuR association of the other miRs (34-a, 301, 181a, 181c) in hypoxic conditions? What about miR-155, which was found up-regulated during hypoxia? The authors have previously shown in breast cancer cells that this miRNA also regulates indirectly Hk2 expression. Does upregulation of miR155 in HCC have a compensatory role to the effect exerted by miR-199a?

2. The nuclear to cytoplasmic translocation of pri-miR-199a and HuR is not evident from the data in Figure 2 C-D and S2E. The cytoplasmic signals in Fig 2D and S2E are marginal, while the data in the northern blot are not convincing as one should determine the total amount of pri-miR-199a in nucleus + cytoplasm and then compare these ratios in normoxic and hypoxic conditions. Because demonstrating the cytoplasmic translocation of unprocessed miR-199a is not central to the conclusions, these data could be omitted from the paper or replaced by a more thorough quantitation.

3. While the mechanism that involves direct targeting of Hk2 and Pkm2 by miR-199a is clearly demonstrated, the role of a second - transcriptional - mechanism is not that obvious. The authors claim that miR-199a also targets HIF1a and as a consequence of it Hk2 and PKM2 transcription could also be affected. This claim is not well supported by the data. First of all, data showing that HIF1a is a target for miR-199a in HCC cell lines are missing. The results showing restoration of Hk2 and Pkm2 mRNAs in hypoxic cells by co-transfection of HIF1a (Figure 3E) are puzzling since they are not correlating with similar restoration of their protein levels (Figure 3E western blot). Therefore the transcriptional effect even if true, physiologically is not very relevant in this case. If miR-199a-mediated regulation of HIF1a was significant, one would expect a more general effect on many genes during hypoxia.

4. The toxicity data in Figure S8 are largely negative, but they lack positive controls. Eg. a positive F4/80 staining assay and baselines, above which they are considered positive for the other measurements should be included.

5. The text and figures should be screened for inconsistencies. Eg. Figure 1C or Figure 6B are mislabeled. Explanations of mut3 and mut4 in Figure S2F are needed. The pimonidazole hydroxychloride staining and principle should be described in the Methods section. The source of antibodies should be indicated.

1st Revision - authors’ response 20 July 2015

Referee #1:
Starting with the analysis of miRNA expression during hypoxia of HCC cells, the authors describe a role for miR-199 in the regulation of hepatocellular carcinoma metabolism and tumorigenesis. The authors propose a novel regulatory axis by which hypoxia-induced binding of HuR protein to pri-miR-199a inhibits miRNA processing, which in turn increases the levels of two key regulators of glucose metabolism, Hk2 and Pkm2. In general the authors provide with abundant cellular and in vivo experiments supporting the model, but the following issues bellow need to be addressed.

The reviewer has concisely summarized key discoveries presented in our manuscript.

1) Down-regulation of miR-199a during hypoxia
The authors analyzed the expression of 38 miRNAs in two HCC cell lines upon hypoxia and found a prominent downregulation of miR-199a. Unfortunately, material and methods about miRNA analysis is missing, which precludes the evaluation of these experiments. Authors should correct this issue and clarify the following questions:
Why did the authors choose those 38 miRNAs? An unbiased analysis of miRNA expression would have provided with a better picture of miRNA regulation during hypoxia. This is required.
We thank the reviewer for this comment and suggestion. We have now described the method for miRNA analysis in Material and Methods section (Pages 20, the last paragraph). We used TaqMan® MicroRNA Assay kits (Applied Biosystems by Life Technology) to quantify miRNA expression levels in HCC cells. The main aim of this work is to gain new insights into hypoxia-mediated regulation of cancer metabolism. Thus, we chose to examine the expression of miRNAs that have been shown to possess important function in cancer cells, as summarized by Garzon et al. (Nat Rev Drug Discov 2010, 9:775-789) and Liu et al. (Physiological and pathological functions of mammalian microRNAs. In: McQueen CA eds. Comprehensive Toxicology: Cellular and Molecular Toxicology 2nd Edition. Oxford: Elsevier 2010; 427-446). We have now included the references in the revised manuscript.

The expression of miR-199b, the other member of the miR-199 family of miRNAs, has not been analyzed, although it has been recently linked to hypoxia (Shang et al, Int J Mol Sci, 2013; Jiang et al, Int J Neurosci, 2014). Given the sequence similarity, can the authors discard cross-amplification of miR-199b in the miRNA expression analysis? Which method has been used for miRNA quantification? Likewise, closely related miRNAs sharing the same seed sequence may have overlapping targets and functions. Can the authors discard that the impact on hypoxia of miR-199a overexpression compensates a hypothetical downregulation of miR-199b?

This is a good point. We used TaqMan miRNA assays, which can discriminate miRNAs that differ by as little as a single nucleotide (Chen et al., Nucleic Acids Res 2005; 33:e179) to quantify the expression levels of miRNAs in HCC cells. As a matter of fact, we have previously analyzed miR-199b expression but not included the result in Figure 1A due to a low expression level of this miRNA in both Hep3B and SMMC-7721 cell lines. Here, we provided the result in this response letter for inspection by the Reviewer [see the appended Figure R1].

During the manuscript revision, we performed pre-miR-199 cloning experiments as previously described by Xuetao Cao’s laboratory (Hou et al., Cancer Cell 2011, 19:232-43) to further compare expression of miR-199b to miR-199a in Hep3B cells. Sequencing pre-miR-199 clones showed that pre-miR-199b was much less abundant than pre-miR-199a in Hep3B cells [see the appended Table R1], indicating that miR-199a is the major miR-199 family member present in HCC cells. Of note, the link of miR-199b to hypoxia pointed by the Reviewer has been shown in brain tissue (Jiang et al., Int J Neurosci 2014, 24:1-29) or prostate cancer cells (Shang et al, Int J Mol Sci 2013, 14:8422-36).

![Figure R1 qRT-PCR analyses of miR-199a and miR-199b expression in SMMC-7721 (left) and Hep3B cells under normoxic or hypoxic conditions. The average values ± s.d. of three separate experiments were plotted. ***P<0.001.](image-url)
Table R1 Comparison of expression of miR-199a to miR-199b in Hep3B cells by pre-miR-199 cloning. The experiments were carried out as described previously (Hou et al., Cancer Cell 2011, 19:232-43). In brief, based on similar “stem” sequences for pre-miR-199a and pre-miR-199b, we used two forward primers, with the two nucleotides differences and equally mixed, and one reverse primer (also worked as RT primer) to amplify pre-miR-199 in Hep3B cells by RT-PCR. The amplified pre-miR-199 cDNA fragments were cloned to T vector and sequenced. Shown is the distribution of miR-199a or miR-199b clones in a total of 60 sequenced clones.

2) Hypoxia selectively inhibits processing of pri-miR-199a

Strikingly, while the pri-miRNA of miR-199a/214 is overexpressed during hypoxia, the authors claim that the levels of miR-199a are specifically downregulated by HuR protein. An alternative hypothesis could be that the observed downregulation of miR-199a is due to a cross-amplification of miR-199b, as commented above. This could also explain why miR-214 is not affected. Can the authors discard this hypothesis?

We believe this hypothesis could be excluded given that mir-199a-2/214 is the major source of miR-199 expression in HCC cells (see our above response to Question #1 and Supplemental Figure S2A).

On the other hand, the authors show a modest upregulation of miR-214 in hypoxia conditions. However, Duan et al, PLoS One, 2012, reported downregulation of miR-214 upon CoCl2 induced hypoxia in HepG2 cells. How can the authors explain this discrepancy?

For hypoxia stimulation in our experiments, we cultured HCC cells in a hypoxia chamber with 1% oxygen. Indeed, CoCl2 treatment has also been used to mimic hypoxia due to CoCl2 functioning as a chemical inducer of HIF-1, the oxygen-sensitive transcription factor (Piret et al., Ann N Y Acad Sci 2002, 973:443-7). Nevertheless, the CoCl2 method is not equivalent to hypoxic induction by modular incubator chamber, given that activation of HIF-1 cannot fully represent cellular responses to hypoxia. Moreover, the possibility that CoCl2 may alter cell behavior independently of oxygen tension cannot be formally excluded. To address the discrepancy between our observation and that of Duan et al. (PLoS ONE 2012, 7: e31518), we examined the levels of mature miR-214 in HepG2 cells cultured under normoxia, hypoxia or CoCl2 treatment, respectively. Our qRT-PCR showed that miR-214 expression was modestly increased in hypoxia chamber-cultured HepG2 cells but slightly reduced in CoCl2-treated cells, compared to that in normoxic cells. This suggests that such discrepancy is likely caused by different experimental methods. Due to space limitation of the manuscript, we have now provided the result in this response letter for inspection by the Reviewer [see the appended Figure R2].
Studies about pri-miR-199a/214 are confusing. The authors distinguish between pri-miR-199a and pri-miR-214, when they are supposed to be the same molecule, originated from antisense transcription from an intron of Dynamin-3 (Lee et al., NAR, 2009). Which criteria were used to define those two pri-miRNAs? What is the size of the pri-miR-199a detected by Northern-blot in figure 2B? Does it fit with previously published data? The authors should clarify these issues, and show a scheme of the miR-199a/214 transcript (or transcripts), indicating the probes used in the different experiments and the amplicons for pri-miR overexpression analysis.

Which criteria were used to define those two pri-miRNAs? What is the size of the pri-miR-199a detected by Northern-blot in figure 2B? Does it fit with previously published data?

We thank the Reviewer for this comment and suggestion. Accordingly, we draw a schematic diagram (as shown in Figure 2A) to indicate the qRT-PCR primer sets, northern blot probes, predicted HuR binding sites, and Drosha binding sites in DNM3os (miR-199a/214 cluster) transcript, and to denote the construction of pri-miR199a/214 expression vector. To quantify the expression levels of pri-miR-199a and pri-miR-214a, the primers for qRT-PCR were designed proximal to their precursor forms, respectively. Additionally, we labeled the size of blotted bands in the revised Figure 2C based on the RNA size markers co-resolved on the same gels.

We agreed with the Reviewer that pri-miR-199a and pri-miR-214 are likely to be highly similar in sequences and sizes. Indeed, our previous northern blot showed a ~6 kb pri-miR-199a transcript (see the revised Figure 2C), which is close to the size of full-length of DNM3os. A previous study showed that the binding sites for Drosha in DNM3os are juxtaposed to both ends of pre-miR-199a and pre-miR-214 (Fukuda et al., Nat Cell Biol 2007, 9:604-11). Thus, we used the terms "pri-miR-199a" and "pri-miR-214" to denote the partially processed forms of DNMos-3 transcripts in which the regions corresponding to pre-miR-214 and pre-miR-199a have been removed by Drosha-mediated cleavage, respectively. Given that the pre-miR-199a and pre-miR-214 sequences are respectively located near the 5’ and 3’ ends of DNM3os transcript (see Figure 2A), cleaving either of the pre-miRNAs by Drosha would not dramatically decrease the size of remaining transcripts. In other words, the sizes of "pri-miR-199a" and "pri-miR-214" are indeed close to the full-length size of DNM3os (pri-miR-199a/214) transcript. To confirm this, we used Drosha siRNA-transfection as a positive control and repeated the northern blot experiments for probing pri-miR-199a and pri-miR-214. In theory, Drosha knockdown would result in accumulation of full-length of DNM3os transcript in cells, which could be simultaneously detected by pri-miR-199a and pri-miR-214 probes; and we found this is indeed the case (see the revised Supplemental Figure S2D, lanes 2 and 5). Our results support that pri-miR-214 is rapidly processed in hypoxic HCC cells whereas pri-miR-199a is not. This is likely due to the lack of HuR binding near the pre-miR-214 region (also see our response to Question #3 below).

In addition, according to our knowledge, the expression levels of pri-miR-199a and pri-miR-214 were examined by qRT-PCR but not northern blot assays in previous studies (Fukuda et al., Nat Cell Biol 2005, 9:604-11; Davis et al., Nature 2008, 454: 56-61; Hou et al., Cancer Cell 2011, 19:232-
43; Chen et al., Sci Rep 2014, 4:6413). Thus, the size of "pri-miR-199a" or "pri-miR-214" has not been experimentally shown before.

3) HuR binding represses miR-199a processing
Methods used to predict HuR binding elements in pri-miRNAs are not described. Again, the authors distinguish two different primary miRNAs for miR-199a and miR-214 to show different regulation by HuR. Unless the authors provide with evidences of the existence of two different pri-miRNAs, experiments in figure 2H should be repeated with the full length pri-miRNA, and both the levels of miR-199a and miR-214 should be determined to prove the specific effect on miR-199a.

We thank the Reviewer for this comment and suggestion. We have now provided a brief description for prediction of HuR binding sites along with a reference in the revised text (page 7, the last paragraph). In brief, we searched HuR binding motifs as shown previously by Ray et al. [Nat Biotechnol 2009, 27:667-70] in DNMsos transcript. In addition, as indicated in our above response to Question #2, we used the terms "pri-miR-199a" and "pri-miR-214" to denote the partially processed forms of DNMs-3 transcripts in which the regions corresponding to pre-miR-214 and pre-miR-199a have been removed by Drosha-mediated cleavage, respectively.

According to the Reviewer’s suggestion, we inserted ~2 kb mir-214 sequences downstream of the mir-199a-2 in the previous pri-miR-199a expression vector to construct a pri-miR-199a/214 expression vector, and repeated the experiments that were shown in previous Figure 2H (now the new Figure 2G). Compared with control vector, transfecting this pri-miR-199a/214 vector into Hep3B cells led to about a 4-fold increase of mature miR-214 under either normoxic or hypoxic conditions. As expected, miR-214 overexpression was barely altered by HuR knockdown or depletion of HuR-binding sites in the expression vector. These results further support that the processing of pri-miR-214 is not regulated by hypoxia or HuR. These new data are now described in the revised text (Page 7, the 1st paragraph; Page 8, the 2nd paragraph) and illustrated in the revised Figures 2D and 2G, and Supplemental Figure S2J.

4) miR-199a directly targets Hk2 and Pkm2
Luciferase assays with mutant miR-199a sites are compelling, but experiments with endogenous Hk2 and Pkm2 in figure 3C and 3D should be performed in the same cell line. If miRNA inhibition is challenging for SMMC-7721 because of high levels of miR-199a, the authors can use the Hep3B cell line also for miR-199a mimics, since it has been shown in figure 1 that miRNA precursors affect glucose metabolism at very low concentration.

We have performed the suggested experiments. We found that miR-199a overexpression significantly repressed Hk2 and Pkm2 expression in Hep3B cells, too. The newly obtained results are now provided in the revised Figure 3D, while the previous results in SMMC-7721 cells are now illustrated as Supplemental Figure S4B.

Are blots shown in figure 3D and 3E representative images of different experiments? If so, a side plot showing the statistics of band intensities from the different repetitions should be included. If not, the authors should repeat those experiments to support their conclusions.

Indeed, results shown are representative of three independent experiments. According to the Reviewer’s suggestion, we have now indicated the average values ± s.d. of band intensities in the images of Figures 3D and 3E in parentheses.

5) miR-199a:Hk2/Pkm2 axis regulation of glycolysis
As commented above, the authors could use the Hep3B cell line for both overexpression and inhibition of miR-199a to further demonstrate how the manipulation of miR-199a affect glucose metabolism also in xenograft experiments. Using different cell lines for functional experiments raises doubts about the model.

The involvement of HuR in the miR-199a:Hk2/Pkm2 axis has not been investigated in further experiments. Did the authors try to recapitulate the effect of miR-199a inhibition on tumorigenesis in HCC cells by HuR knockdown? If feasible, this experiment would reinforce the proposed model.
We agree with the Reviewer’s points and have performed the suggested experiments, as summarized below.

(1) We examined the effect of miR-199a overexpression on glucose metabolism in cultured Hep3B cells and engrafted Hep3B tumors. The newly obtained results are now provided in the revised Figures 4A and 4B, while the previous results in SMMC-7721 cells and tumors are now illustrated as Supplemental Figure S5.

(2) We repeated the rescue experiments, and confirmed that restoration of Hk2 or Pkm2 expression also overrode the inhibitory effects of miR-199a on glucose metabolism in Hep3B cells and tumors. The newly obtained results are now provided in the revised Figures 4E and 4F, while the previous results in SMMC-7721 cells and tumors are now illustrated as Supplemental Figure S7.

(3) We examined the effect of miR-199a overexpression on tumorigenesis in Hep3B cells in vivo and in vitro. The newly obtained results are now provided in the revised Supplemental Figure S8A, while the previous results in SMMC-7721 cells and tumors are now illustrated as Supplemental Figure S8B.

(4) We repeated the rescue experiments, and confirmed that restoration of Hk2 or Pkm2 expression also overrode the inhibitory effects of miR-199a on tumorigenesis in Hep3B cells in vivo and in vitro. The newly obtained results are now provided in the revised Figure 5, while the previous results in SMMC-7721 cells and tumors are now illustrated as Supplemental Figure S10.

(5) We examined the effects of HuR knockdown on HCC tumorigenesis in hypoxic Hep3B cells and engrafted tumors. The results are now described in the revised text (Page 9, the 2nd paragraph) and illustrated as Supplemental Figure S3B.

Taken together, these results demonstrate that miR-199a overexpression similarly reduced glucose metabolism and tumorigenesis in cultured Hep3B cells and engrafted Hep3B tumors. In line with our previous data showing that the HuR:miR-199a axis is critical to regulating the glycolytic switch under hypoxia, the axis is also important for HCC tumorigenesis in vitro and in vivo.

Referee #2:
The current manuscript by Zhang et al. describes a novel regulatory axis in hepatocellular carcinoma (HCC). This axis comprises the hypoxia-induced repression of miR199a in hepatoma cells. miR199a repression in turn led to the induction of key glycolytic enzymes in hepatoma cells, thereby contributing to the malignant phenotype and metabolic reprogramming in both in vitro and in vivo systems. Analysis of human HCC specimens revealed a consistent regulation of miR199a in human tumors. Given the aggressiveness of HCC and the absence of effective therapeutic options, the identification of novel mechanisms in HCC pathogenesis clearly represents an important topic in biomedical research. In particular, metabolic reprogramming has gained increasing attention in the cancer field as it turned out to be a powerful target in the development of novel therapeutic options. In this regard, the current manuscript by Zhang et al employs a huge variety of technical approaches to pinpoint the importance of the novel miR199a axis in this context. The manuscript is clearly written, well structured and employs state-of-the art technology. The findings are novel and of importance for the field.

We appreciate the Reviewer’s high appraisal of our work.

Two points need attention by the authors: Main findings in figure 2 should be duplicated/verified in a second HCC cell line, i.e.SMMC-7721.

We thank the Reviewer for this suggestion. Accordingly, we repeated the main experiments in SMMC-7721 cells and verified that HuR was also required for hypoxia-mediated blockade of pri-miR-199a processing in these cells. The results are now illustrated in Supplemental Figures S2B, S2G and S2H).
The same holds true for the rescue effects of Hk2 or Pkm2 in terms of tumorigenesis, i.e. use Hep3B to verify the functional importance of these two enzymes for malignant phenotypes.

We performed the suggested experiments. The results are now illustrated in the revised Figure 5 (also see response to Question #5 by Reviewer #1). In brief, miR-199a overexpression similarly inhibited tumorigenesis in cultured Hep3B cells and engrafted Hep3B tumors, while ectopic expression of Hk2 or Pkm2 in miR-199a-transfected Hep3B cells substantially overrode the effects of miR-199a on tumorigenesis in vitro and in vivo.

The second issue concerns the question whether the miR199 axis is particularly important for a distinct HCC etiology, e.g. NASH- or virus-driven HCC. The authors should at least discuss the different possibilities.

We thank the Reviewer for this suggestion and have now discussed this point in the revised text (Page 18, the 2nd paragraph).

Referee #3:
In this paper Zhang et al. examined the role of miR199a on two glycolytic genes, HK2 and PKM2 that play pivotal roles in the Warburg effect, a cancer cell-specific feature induced by hypoxia. The main claims of the paper are novel and provide important new information on miRNA-dependent regulation of cancer metabolic pathways. The key novel findings that are well supported by the data include:

i. miR-199a efficiently regulates glucose uptake and lactate production in human HCC cell lines via regulating Hk2 and Pkm2, which are bona fide targets of miR-199a. ii. miR-199a-dependent regulation of glucose metabolism also takes place in xenografts and there is a clear correlation between miR-199 and Hk2 and Pkm2 in human tumors. iii. Mechanistic insight into the regulation is provided, suggesting that HuR binding under hypoxic conditions blocks miR-199a processing. The above results are timely and important for the field. Some (minor) issues should be addressed before publication. These include:

We appreciate the Reviewer’s high appraisal of our work.

1. How common is the HuR-mediated block of miRNA processing? Did the authors check HuR association of the other miRs (34a, 301, 181a, 181c) in hypoxic conditions? What about miR-155, which was found up-regulated during hypoxia? The authors have previously shown in breast cancer cells that this miRNA also regulates indirectly Hk2 expression. Does upregulation of miR155 in HCC have a compensatory role to the effect exerted by miR-199a?

We thank the Reviewer for this suggestion. Accordingly, we examined the effect of HuR knockdown on expression of all miRNAs that are substantially down-regulated in hypoxic Hep3B cells, including miR-34a, miR-101, miR-181a, miR-301, miR-181b, miR-376a, miR-181c, miR-345, miR-125a, and miR-99a. Our qRT-PCR showed that HuR knockdown only effectively attenuated the repressive effect of hypoxia on miR-181b. Moreover, by searching HuR binding motifs [Ray et al., Nat Biotechnol 2009, 27:667–70], we found a potential HuR-binding site at the stem region of pre-miR-181b in human pri-miR-181b. These results together suggest that HuR is also involved in hypoxia-induced down-regulation of miR-181b in HCC cells. Due to space limitation of the manuscript, we have now provided the result in this response letter for inspection by the Reviewer [see the appended Figure R3].

Additionally, our previous study indicates that miR-155 activates Hk2 and promotes glycolysis in breast cancer cells (Jiang et al., EMBO J 2012, 31: 1985-1998). Based on these findings, miR-155 up-regulation in hypoxic HCC cells should have a complimentary role to miR-199a down-regulation in regulating glucose metabolism in HCC cells. We have now discussed this point in the revised text (Page 16, the last paragraph).
Figure R3 HuR is involved in hypoxia-induced down-regulation of miR-181b in Hep3B cells. (A) qRT-PCR analyses of the effect of HuR knockdown on expression of indicated miRNAs in hypoxic Hep3B cells. (B) Schematic representation of predicted HuR binding site in human pri-miR-181b. The average values ± s.d. of three separate experiments were plotted. **P<0.01.

2. The nuclear to cytoplasmic translocation of pri-miR-199a and HuR is not evident from the data in Figure 2 C-D and S2E. The cytoplasmic signals in Fig 2D and S2E are marginal, while the data in the northern blot are not convincing as one should determine the total amount of pri-miR-199a in nucleus + cytoplasm and then compare these ratios in normoxic and hypoxic conditions. Because demonstrating the cytoplasmic translocation of unprocessed miR-199a is not central to the conclusions, these data could be omitted from the paper or replaced by a more thorough quantitation.

We agreed with the Reviewer that cytoplasmic translocation of unprocessed miR-199a is not central to the conclusions in this work. Accordingly, we have now deleted these data in the revised Figure 2 and Supplemental Figure S2.

3. While the mechanism that involves direct targeting of Hk2 and Pkm2 by miR-199a is clearly demonstrated, the role of a second - transcriptional - mechanism is not that obvious. The authors claim that miR-199a also targets HIF1a and as a consequence of it Hk2 and Pkm2 transcription could also be affected. This claim is not well supported by the data. First of all, data showing that HIF1a is a target for miR-199a in HCC cell lines are missing. The results showing restoration of Hk2 and Pkm2 mRNAs in hypoxic cells by co-transfection of HIF1a (Figure 3E) are puzzling since they are not correlating with similar restoration of their protein levels (Figure 3E western blot). Therefore the transcriptional effect even if true, physiologically is not very relevant in this case. If miR-199a-mediated regulation of HIF1a was significant, one would expect a more general effect on many genes during hypoxia.

We have previously shown in Figure 3E that transfection of miR-199a mimics into Hep3B cells markedly reduced HIF-1α protein expression under hypoxic condition (left panel; lane 4). This indicates HIF-1α as a target for miR-199a in HCC cells. We have now described this result in the revised text (Page 10, the last paragraph). According to the Reviewer’s suggestion, we further examined whether miR-199a targets HIF-1α in Hep3B cells. Given that HIF-1α is lowly expressed in normoxic cells, we thus examined the effect of miR-199a on HIF-1α expression in Hep3B cells under hypoxic condition. Indeed, both qRT-PCR and western blot assays showed that expression of HIF-1α were markedly reduced in miR-199a mimic-transfected cells [see the appended Figure R4], indicating that HIF-1α is targeted by miR-199a in HCC cells.

It’s true that co-transfection of HIF-1α expression vector did restore Hk2 and Pkm2 mRNA expression but not their protein levels in hypoxic cells. This is because Hk2 and Pkm2 are targets of miR-199a and thus they are also post-transcriptionally repressed by miR-199a overexpressed in the cells. To test the significance of miR-199a-mediated regulation of HIF-1α, we further examined the effect of miR-199a overexpression on expression of the HIF-1 downstream genes that are not targeted by miR-199a, including Glut1, Pdk1, and Ldha. As expected, miR-199a overexpression
substantially reduced the expression levels of these genes in hypoxic Hep3B cells with a concomitant decrease of HIF-1α (see the appended Figure R5, left, lane 4). Ectopic HIF-1α expression completely restored both of their mRNA (right, green column) and protein levels in cells overexpressing miR-199a (left, lane 5), indicating that targeting HIF-1α represents a major mechanism for miR-199a in regulating these genes. Due to space limitation of the manuscript, we have now provided these results in this response letter for inspection by the Reviewer [see the appended Figures R4 and R5].

Figure R4 Transfection of miR-199a mimics attenuates hypoxia-induced HIF-1α expression in HCC cells. qRT-PCR (left) or western blot analyses (right) of HIF-1α expression in Hep3B cells under indicated conditions. The average values ± s.d. of three separate experiments were plotted. *P<0.05.

Figure R5 Targeting HIF-1α is responsible for miR-199a in regulating GLUT1, PDK1, and LDHA in hypoxic HCC cells. Western blot (left) and qRT-PCR analyses (right) of GLUT1, PDK1 and LDHA expression were performed in Hep3B cells under indicated conditions. The average values ± s.d. of three separate experiments were plotted. *P<0.05, **P<0.01.

4. The toxicity data in Figure S8 are largely negative, but they lack positive controls. E.g. a positive F4/80 staining assay and baselines, above which they are considered positive for the other measurements should be included.

We thank the Reviewer for this suggestion. Accordingly, we used Cyclophosphamide (CP) treatment as a positive control for liver toxicity analyses. CP is an antineoplastic and immunosuppressive agent that possesses hepatotoxicity in animals [Capel et al., Clinical Chemistry 1979, 25: 1381-3; Fraiser et al., Drugs 1991, 42:781-95]. Same as our therapeutic protocols, the mice received 20 mg/kg CP intravenously by tail veins with one injection every 3 days for seven cycles before they were sacrificed for immunohistochemistry and clinical blood chemistry assays. The results are now illustrated in the revised Supplemental Figures S12C and S12D. In brief, we found a substantial increase in Kupffer cell macrophages in liver sections from CP-treated mice, along with elevated activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and significant increases of serum levels of urea, cholesterol total (CHO),
and total bilirubin (TBIL) in CP-treated animals. These results confirm that CP treatment induces liver damage in mice.

5. The text and figures should be screened for inconsistencies. Eg. Figure 1C or Figure 6B are mislabeled. Explanations of mut3 and mut4 in Figure S2F are needed. The pimonidazole hydroxychloride staining and principle should be described in the Methods section. The source of antibodies should be indicated.

We thank the Reviewer for pointing out these errors. We have now corrected the mis-labeling in Figure 1C and Supplemental Figure S2F, and provided the method for intratumoral hypoxia detection by pimonidazole hydroxychloride in Method and Material section (Page 22, the last paragraph).

2nd Editorial Decision 06 August 2015

Thank you for submitting the revised version of your manuscript and my apologies for the slightly extended duration of the re-review process here. Your manuscript has now been seen by the original referees and their comments are shown below.

As you will see they find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept your manuscript there are a few editorial issues that I need you to address. In addition, and following our communication a few weeks back on a related study reporting on miR199a, I would ask you to refer to refer to this other study in the manuscript discussion.

I would thus invite you to submit a final revision of the manuscript including the following points:

-> Please fill out and include an author checklist as listed in our online guidelines (http://emboj.embopress.org/authorguide)

-> Since we can only accommodate up to 5 figures as expanded view, we would recommend keeping all supplemental data in a single pdf (ie as currently displayed). However, I would ask you to relabel these figures as appendix figures and to refer to them as such in the main manuscript text (please see our online Guide to Authors for details).

-> We generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to your revision.
REFEREE COMMENTS

Referee #2:

The authors have adequately addressed all previous concerns and significantly improved the manuscript.

Referee #3:

In the revised version the authors provide adequate experimental evidence and explanations that properly address the initial concerns. The miR-199a-dependent inhibition of the Warburg effect is a nice contribution to the HCC research field.