Author's response to reviews

**Title:** Effects of hypoxia-inducible factor-1alpha on apoptotic inhibition and glucocorticoid receptor downregulation by dexamethasone in AtT-20 cells

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**Author's response to reviews:** see over
Response to Reviewer: Erica Gentilin’s comments:

Major Compulsory Revisions

Materials and Methods section

1) Page 4, "RNA extraction and Real time PCR" paragraph. Which is the calibrator sample? Which method did the Authors employ to analyze the real time PCR results? 28S primers do not match to 28S sequence.

The Authors should include in the manuscript the sentences they posted in this comment.

Response: We have put the comment sentences in the RNA extraction and real-time PCR section as following:

**RNA extraction and real-time PCR**

RNA was prepared using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA, 1 μL oligo(dT) 15 primer (Promega, USA) and DEPC water were added to a total volume of 10 μL, heated to 70°C for 5 min, and placed on ice for 5 min. Then a mixture of M-MLV RT 5× reaction buffer (5 μL), 100 mM dNTPs (0.5 μL), 1 μL M-MLV RT H(-) point mutant, and DEPC water in a final volume of 15 μL (all from Promega, Madison, USA) was added to each sample, followed by incubation at 40°C for 60 min and 70°C for 15 min. Real-time PCR was performed using the SYBR® Premix Ex Taq™ PCR kit (Takara, Japan) on the Applied Biosystems 7300 Real-Time PCR System (Foster, CA, USA). The 20-μL reaction of the SYBR Green assay contained 10 μL of 2× SYBR Premix Ex Taq, 0.4 μL PCR forward primers and 0.4 μL reverse primers, 0.4 μL ROX reference dye (50×), 2 μL cDNA, and 6.8 μL double-distilled H₂O. PCR was carried out as follows: one cycle of 95°C for 10 s (pre-denature) and 40 cycles of two steps (95°C for 5 s and 60°C for 31 s). At the end of the amplification, a dissociation curve (melting curve) was plotted in the temperature range 65–95°C. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical adhesive covers (Applied Biosystems). PCRs were performed in triplicate, and a reliable internal control under hypoxia, 28S rRNA, was co-amplified to normalize the amount of RNA added to the
reaction [14]. **Normoxia group in FIG.1B, 1% O₂-0 h without dexamethasone in FIG.3A and 3B, 1% O₂-0 h in FIG.4A and 4B were used as calibrator for relative quantitative PCR.** All data were analysed using the Applied Biosystems 7300 SDS Software (Applied Biosystems, CA, USA). \(^{-2^\Delta\Delta ct}\) method was used to analyze the real time PCR results.

Primer sequences were as follows:

- **HIF-1α:** forward 5’-ACCTTCATCGGAAACTCCAAAG-3’
  reverse 5’-CTGTTAGGCTGGGAAAAGTTAGG-3’;
- **GR:** forward 5’-AAGAGACAAACGAGAGTCCTTGG-3’
  reverse 5’-GTGTCCGGTAAAATAAGAGGCTT-3’;
- **28S rRNA:** forward: 5’-AATGCCTCGTCATCTAAT-3’
  reverse 5’-TTCGCTGGATAGTAGGTA-3’.

We designed the 28S rRNA primers online. The website was sigma.com/probedesignonline. Actually, we had searched many literatures about the 28S primers of mouse, but it was difficult to find a matched one.

2) Page 4, "Western blot analysis". Did the Authors employ the Bradford or BCA methods to detect protein quantification?

The Authors stated that "total proteins were quantified by the Bradford method using the BCA protein assay kit". Bradford and BCA assays are different methods to quantify protein concentration. The Authors should check the text in the manuscript and correct it.

**Response:** We have revised the text as following: Total proteins were quantified by the Thermo Scientific Pierce BCA protein assay kit according to its instruction.