In Reply:

Thank you for giving us the opportunity to respond to the comments about our work[1] raised by Dr. Hirota. We would like to address these points one by one.

First, hypoxia-inducible factor (HIF) protein was barely expressed in renal cell carcinoma-4 cells under controlled conditions in our study. Indeed, our Western blots indicated a baseline level of HIF, but no marked overexpression. In searching the literature, the expression level that we detected is comparable with the expression level in findings for renal cell carcinoma-4 cells reported in numerous publications.[2–4] Thus, although this cell line is Von Hippel–Lindau tumor suppressor deficient and HIF proteins are somewhat stabilized, it is not unusual to find that this constitutive expression level is detected at low levels with Western blot. Regarding the second issue that was raised, namely that hypoxia does not increase HIF expression in renal cell carcinoma-4 cells, an elegant previous work clearly demonstrated a marked increase in HIF expression in renal cell carcinoma-4 under hypoxic conditions.[4]

The need to examine isoflurane under hypoxic conditions is a highly valid point with clear relevance to tumor resection surgery. However, our initial study was exploring a proof of concept, and as a first step, we therefore decided to investigate a one-factor system before adding the second variable of hypoxia. Nonetheless, it is also important to note that the effects we are describing would affect "circulating" cancer cells remaining in the body posttumour resection and thus would not be in a hypoxic environment as such.

In terms of the discrepancy between Dr. Hirota's work and our findings, it seems likely that the impact of anesthetics on HIF induction is influenced by both anesthetic type and target tissue. For instance, halothane suppressed hypoxia-induced HIF induction in Hep3B cells. However, the opposite effect has been reported using isoflurane in hypoxic Hep3B cells.[5] Similarly, examining the HIF-target gene erythropoientin demonstrated that isoflurane suppressed hypoxia-induced HIF-2 and erythropoientin in mouse brain. Interestingly, these effects were not observed for renal tissue, where hypoxia-induced erythropoientin activity (evidence of HIF activation) was not inhibited by isoflurane.[6] Furthermore, isoflurane-induced HIF-1α up-regulation has been demonstrated in a number of *in vivo* studies, for example, in the brains of neonatal rats[7] and of adult rats after ischemic shock.[8] Clearly, this warrants further study.

Moving on to our observation that increased cellular proliferation was associated with greater HIF induction, we have not claimed HIF to be an oncogene; we just specified that the activation of the phosphatidylinositol 3-kinase (PI3K)–Akt–HIF pathway correlated with increased cell proliferation in these cells, as shown by both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and ki67 staining; an effect that was abrogated when the PI3K–Akt was blocked. As HIF was associated with proliferation in already cancerous cells, there is no requirement for an "oncogene" *per se*.

We acknowledge that the current study does not determine whether the induction of HIF1-α protein is essential to these effects; however, if one reads closely, it was stated that the cell proliferation could be attributed to the induction of PI3K–Akt–mammalian target of rapamycin (mTOR) pathway.[1]

Although we did not perform any studies on the reversibility of isoflurane in our initial study, it is noteworthy that this proliferative effect was lost when the PI3K–Akt pathway was blocked.[1] We therefore conclude that this may represent an important avenue down which to explore the impact of anesthesia on postoperative cancer recurrence.

Finally, LY294002 is a PI3K inhibitor. However, it is widely reported that it is a PI3K–Akt inhibitor as well. Nevertheless, because PI3K is upstream of Akt, once this inhibitor is applied, the whole system will be blocked. That is to say, our conclusion remains valid. However, we acknowledge this could have been further clarified in the text.

Competing Interests

The authors declare no competing interests.

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References

1. Benzonana LL, Perry NJ, Watts HR, Yang B, Perry IA, Coombes C, Takata M, Ma D: Isoflurane, a commonly used volatile anesthetic, enhances renal cancer growth and malignant potential *via* the hypoxia-inducible factor cellular signaling pathway *in vitro*. Anesthesiology 2013; 119:593–605
2. Razorenova OV, Finger EC, Colavitti R, Chernikova SB, Boiko AD, Chan CK, Krieg A, Bedogni B, LaGory E, Weissman IL, Broome-Powell M, Giaccia AJ: VHL loss in renal cell carcinoma leads to up-regulation of CUB domain-containing protein 1 to stimulate PKC{δ}-driven migration. Proc Natl Acad Sci U S A 2011; 108:1931–6
3. Perier A, Fregni G, Wittnebel S, Gad S, Allard M, Gervois N, Escudier B, Azzarone B, Caignard A: Mutations of the von Hippel-Lindau gene confer increased susceptibility to natural killer cells of clear-cell renal cell carcinoma. Oncogene 2011; 30:2622–32
4. Bienes-Martínez R, Ordóñez A, Feijoo-Cuaresma M, Corral-Escariz M, Mateo G, Stenina O, Jiménez B, Calzada MJ: Autocrine stimulation of clear-cell renal carcinoma cell migration in hypoxia *via* HIF-independent suppression of thrombospondin-1. Sci Rep 2012; 2:788
5. Li QF, Wang XR, Yang YW, Su DS: Up-regulation of hypoxia inducible factor 1α by isoflurane in Hep3B cells. Anesthesiology 2006; 105:1211–9
6. Tanaka T, Kai S, Koyama T, Daijo H, Adachi T, Fukuda K, Hirota K: General anesthetics inhibit erythropoietin induction under hypoxic conditions in the mouse brain. PLoS One 2011; 6:e29378
7. Jiang H, Huang Y, Xu H, Sun Y, Han N, Li QF: Hypoxia inducible factor-1α is involved in the neurodegeneration induced by isoflurane in the brain of neonatal rats. J Neurochem 2012; 120:453–60
8. Fang Li Q, Xu H, Sun Y, Hu R, Jiang H: Induction of inducible nitric oxide synthase by isoflurane post-conditioning *via* hypoxia inducible factor-1α during tolerance against ischemic neuronal injury. Brain Res 2012; 1451:1–9

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