In renal cell carcinoma, cancerous phenotypes linked to hypoxia-inducible factors are insensitive to the volatile anesthetic isoflurane

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Abstract

The possibility that anesthesia during cancer surgery may affect cancer recurrence, metastasis, and patient prognosis has become one of the most important topic of interest in cancer treatment. For example, the volatile anesthetic isoflurane was reported in several studies to induce hypoxia-inducible factors, and thereby enhance malignant phenotypes *in vitro*. Indeed, these transcription factors are considered critical regulators of cancer-related hallmarks, including "sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, invasion, and metastasis." We have now investigated the impact of isoflurane on the growth and migration of derivatives of the renal cell line RCC4. We confirmed that hypoxia-inducible factors do promote tumor growth and migration, but found that isoflurane does not affect cancerous phenotypes due to such factors.

Introduction

The hypothesis that anesthesia during cancer surgery may affect tumor recurrence, metastasis, and patient prognosis [1,2] is gaining increasing importance at present [3]. Accordingly, there is a growing body of in vitro, in vivo, retrospective, and translational studies on the effect of anesthetics on perioperative immunity and cancer metastatic potential. For example, isoflurane was reported in several studies to induce hypoxia-inducible transcription factors (HIFs), and thereby enhance malignant phenotypes *in vitro* [4-6]. HIF-1 was originally cloned as a driver of erythropoietin expression [7-10], but was linked shortly thereafter to tumor grade in various cancers [11]. Indeed, HIFs are now well-known as critical regulators of cancer hallmarks, including "sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, invasion, and metastasis" [12,13]. Moreover, tumor suppressors such as TP53 and PTEN also regulate HIFs. Another striking example of the physiological significance of HIFs is von Hippel-Lindau (VHL) disease, a hereditary cancer syndrome that predisposes to highly angiogenic tumors, in which the constitutive overexpression of vascular endothelial growth factor and glucose transporter 1 can be corrected by functional VHL protein, a tumor suppressor that targets HIFs for degradation. In this study, we investigated the impact of the volatile anesthetic isoflurane on growth and migration of derivatives of the renal cell line RCC4 that express (RCC-VHL) or do not express (RCC4-EV) VHL [14]. We demonstrate that HIFs significantly influence growth and migration, but isoflurane does not affect HIF-dependent phenotypes.

Results

HIF-1 and HIF-2 expression and activity in RCC4-EV and RCC4-VHL cells

HIF-1α and HIF-2α protein were detected at 20 % O_2 in RCC4-EV cells, but not in RCC4-VHL cells (Fig. 1a). Similar levels of expression were observed in the former at 1 % O_2 (Fig. 1a). In addition, expression in RCC4-EV cells was insensitive to 2 % isoflurane at 20 % O_2 (Fig. 1b). In contrast, HIF-1α and HIF-2α were barely detectable in RCC4-VHL cells even after exposure to 20 % O_2 for 8 h with or without 2 % isoflurane (Fig. 1b). However, HIF-1α and HIF-2α were induced in RCC4-VHL cells at 1 % O_2 (Fig. 1a), but were suppressed by isoflurane (Fig. 1c). To investigate possible protocol-dependent effects, HIF-1α was also quantified in RCC4-EV and RCC4-VHL cells exposed to isoflurane for 2 h and then incubated for 6 h at either 20 % or 1 % O_2 (Figs. 1d and 1e). In RCC4-EV cells, HIF-1α was insensitive to isoflurane regardless of subsequent exposure to 20 % or 1 % O_2, but was suppressed in RCC4-VHL cells subsequently exposed to 1 % O_2. Similar trends were observed in cells exposed to isoflurane for 8 h at 20 % and 1 % O_2 (Figs. 1d and 1e). In any case, HIF-1β expression was stable in both cells regardless of isoflurane treatment.

As quantified by semi-quantitative RT-PCR, *SLC2A1* (glucose transporter 1) and *VEGFA* (vascular endothelial growth factor A) were more abundant in RCC4-EV cells than in RCC4-VHL cells, but were induced in the latter at 1 % O_2 (Figs. 2a and b). However, expression in RCC4-VHL cells at 1 % O_2 was suppressed by isoflurane. Interestingly, *HIF1A* and EPAS1 (HIF-2α) mRNAs were less abundant in RCC4-EV cells, but were insensitive to isoflurane (Figs. 2c and d).

Effect of isoflurane on cell proliferation

Cell proliferation, as assessed by MTS assay, was higher in RCC4-EV cells than in RCC4-VHL cells, but was insensitive to isoflurane (Fig. 3a). Similarly, cellular ATP was more abundant in the former than in the latter, and was also insensitive to isoflurane (Fig. 3b).

Effect of isoflurane on cell migration

RCC4-EV cells migrated significantly faster than RCC4-VHL cells over 12 h (Fig. 4a), although exposure to isoflurane for 2 h significantly suppressed migration in both cells (Fig. 4b).

Effect of isoflurane on glucose metabolism

In comparison to normal cells, cancer cells exhibit the Warburg effect, and thus preferentially metabolize glucose by glycolysis, producing lactate as an end product, despite availability of oxygen. Using an Extracellular Flux Analyzer™, the mitochondrial oxygen consumption rate was found to be lower in RCC4-EV cells in comparison to RCC4-VHL cells (Fig. 5a), but was insensitive to isoflurane (Fig. 5b). On the other hand, extracellular acidification rate was higher in RCC4-EV cells relative to RCC4-VHL cells (Fig. 5c), but was also insensitive to isoflurane (Fig. 5d). Key parameters that determine the mitochondrial oxygen consumption rate, including basal oxygen consumption rate, maximum respiration, proton leak, and nonmitochondrial respiration, were also calculated from Cell Mito Stress Test™ data (Fig. 6). These parameters were significantly different between RCC4-EV and RCC4-VHL cells, but were insensitive to isoflurane.

Effect of isoflurane on global gene expression

Clustering of RNA sequencing data (Supplementary Information Table1) indicated that transcriptomic bias due to isoflurane was smaller than transcriptomic variations due to VHL expression (Fig. 7a). Indeed, more than 200 genes were differentially expressed between RCC4-EV and RCC4-VHL cells, as inferred from Wilcoxon signed rank test of FPKM values at significance level 0.05 (Fig. 7b). However, only one gene was differentially expressed in RCC4-VHL cells exposed to isoflurane, while no such gene was identified in RCC4-EV cells. Pairwise scatter plots comparing $\log_{10}$[FPKM+1] values from four experiments confirmed this result (Fig. 7c). Enrichment analysis also revealed that GO:0001666 (response to hypoxia), GO:0010035 (response to inorganic substance), hsa05230 (central carbon metabolism in cancer), GO:003198 (extracellular matrix organization), and GO:0097190 (apoptotic signaling pathway) were significantly enriched in RCC4-EV cells (Fig. 7d) regardless of isoflurane exposure (Fig. 7e). Finally, only 42 genes annoted to cancer hallmark gene ontologies were sensitive to isoflurane, although the effects were negligible (Fig. 8). Indeed, only CITED1 was strongly responsive to isoflurane.

Discussion

Collectively, the data suggest that clinically relevant doses of isoflurane suppress cell mobility, HIF-dependent intracellular signaling, and expression of genes associated with cancer hallmarks and phenotypes. We also confirmed that HIFs sustain cancer-associated gene expression, metabolism, cell proliferation, and cell motility.

The hallmarks of cancer were originally proposed by Hanahan and Weinberg in 2000 [12], and included sustained proliferative signaling, evasion of growth suppressors, resistance to cell death, replicative immortality, angiogenesis, invasion, and metastasis. Subsequent conceptual progress has added another two emerging hallmarks, namely reprogramming of energy metabolism and evasion of immune destruction [13]. Accordingly, we investigated the impact of isoflurane and HIF on these hallmarks based on global gene expression. Noting that HIF has been extensively investigated in the context of cancer biology [4,11], we used RCC4-EV cells, which are derived from human renal cell carcinoma. As these cells are VHL-deficient, both HIF-1 and HIF-2 are activated even under normoxic conditions, but are suppressed by forced expression of VHL, as in RCC4-VHL cells [14]. Accordingly, RCC4-EV cells proliferate and migrate faster than RCC4-VHL cells, and exhibit metabolic reprogramming from oxidative phosphorylation to glycolysis. These results clearly indicate that HIFs are critically involved in cancerous phenotypes, as previously reported.

However, isoflurane treatment did not affect HIF-1α and HIF-2α expression in RCC4-EV cells. In contrast, isoflurane suppressed HIF-1α expression in RCC4-VHL cells at 1 % O2. Accordingly, isoflurane also suppressed *SLC2A1* and *VEGFA*, which are downstream of HIF-1, under normoxic or hypoxic conditions, although this effect is far smaller than that of VHL expression. Further, isoflurane also slowed growth and

migration, but also to a smaller extent than VHL expression. Strikingly, exposure to isoflurane for 2 h was also demonstrated to boost HIF-1α and HIF-2α protein expression within 6 h in RCC4 cells, and in a PTEN/Akt-dependent manner [15]. Indeed, HIF-1α and HIF-2α were barely detectable in that study without isoflurane treatment, contradicting for unknown reasons several other reports, including the first to link isoflurane to HIFs [14,16-18]. Hence, we investigated potential protocol-dependent effects, but found that neither exposure to isoflurane for 8 h, nor for 2 h followed by culture for another 6 h, induced HIF-1 and HIF-2 in RCC4-EV cells.

In 2006, Exadaktylos et al. [2] proposed that anesthesia and analgesia during cancer surgery may affect tumor recurrence or metastasis, a hypothesis that was subsequently supported by several clinical studies [1]. Potential underlying mechanisms include direct cellular effects, as well as indirect effects on patient immunity and on cancer metastasis. On the contrary, we found that isoflurane clearly has adverse effects against cancer cells, isoflurane suppressing migration but not proliferation, both of which are linked to HIFs, and which may determine the fate of cancer cells. In addition, metabolism and global gene expression appeared to be sensitive to HIFs but not to isoflurane. We note, however, that our data are entirely from *in vitro* experiments in established cell lines, while xenografts may be required to elucidate the impact of anesthetics on cancer progression *in vivo*.

In summary, we demonstrated that isoflurane does not affect HIF activity in renal carcinoma cells, nor the expression of genes associated with cancer hallmarks. However, we confirmed that HIFs help maintain cancerous phenotypes.

Materials and methods

Cell culture and reagents

Renal cell carcinoma cell lines stably transfected with pcDNA3-VHL (RCC4-VHL) or empty pcDNA3 (RCC4-EV) were kindly provided by Dr. Hiroshi Harada (Kyoto University) [19]. These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Purified mouse antibodies to human HIF-1α (Clone 54/HIF-1α) were purchased from BD Biosciences (San Jose, CA), while rabbit monoclonal antibodies to HIF-1β/ARNT (D28F3) XP were purchased from Cell Signaling Technology (Danvers, MA). Antibodies to HIF-2 α/EPAS1 were obtained from Novus Biologicals (Littleton, CO). Isoflurane and mouse monoclonal antibodies to α-tubulin were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan) [19-21] (Table 1).

Hypoxic and isoflurane treatment

Cells were maintained in an airtight chamber or workstation (AS-600P; AsOne, Osaka, Japan) perfused with air (MODEL RK120XM series; Kofloc, Kyotanabe, Japan) mixed with or without test anesthetic delivered by a specialized vaporizer within the open circuit. Gases and anesthetics were monitored during the experiment on an anesthetic gas monitor (Type 1304; Bluël & Kjær, Nærum, Denmark) calibrated with a commercial standard gas of 47 % O_2, 5.6 % CO_2, 47 % N_2O, and 2.05 % sulfur hexafluoride [22,23].

Immunoblotting

Whole-cell lysates were prepared by incubating cells for 30 min in cold radioimmune

precipitation assay buffer with cOmplete™ Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Tokyo, Japan). Samples were then centrifuged at 10,000 ×*g* to sediment cell debris, and 35 μg protein from the resulting supernatant was resolved on 7.5 % sodium dodecyl sulfate-polyacrylamide, transferred to membranes, probed with 1:2,000 of indicated primary antibodies, labeled with 1:8,000 of donkey anti-rabbit IgG (GE Healthcare, Piscataway, NJ) or sheep anti-mouse IgG (GE Healthcare) conjugated to horseradish peroxidase, and visualized with enhanced Chemi-Lumi One Super (Nacalai Tesque, Kyoto, Japan). Bands were quantified by densitometry in Image Studio Lite (LI-COR Biosciences, Lincoln, NE, USA) [19,24,25]. Experiments were repeated at least three times, and representative blots are shown. Images have been cropped for presentation for HIF-1α and HIF-2α. Full size images are presented in Supplementary Figure 1.

Semi-quantitative real-time reverse transcriptase-polymerase chain reaction analysis (*q*RT-PCR)

Total RNA was extracted from cells using RNeasy™ Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand synthesis and RT-PCR were performed using QuantiTect™ Reverse Transcription Kit (Qiagen) and Rotor-Gene™ SYBR Green PCR Kit (Qiagen), following the manufacturer's protocol. Targets were amplified and quantified in Rotor-Gene Q™ (Qiagen), and change in expression of each target mRNA was calculated relative to β-actin. *SLC2A1* was quantified with the QuantiTect Primer Assay QT00068957, while *VEGFA* was quantified with forward primer AGCCTTGCCTTGCTGCTCT and reverse primer

TCCTTCTGCCATGGGTGC. HIF1A was quantified using the forward and reverse primers ACACACAGAAATGGCCTTGTGA and CCTGTGCAGTGCAATACCTTC, while *EPAS1* was quantified with the forward and reverse primers ATGGGACTTACACAGGTGGAG and GCTCTGTGGACATGTCTTTGC.

Assessment of cell growth

Cell growth was assessed using Proliferation Assay™ Kit (Promega, Madison, WI, USA) [19,25,26]. Briefly, cells were seeded in 96-well plates (2×10^3 cells/well), cultured overnight, treated with or without 2 % isoflurane for 2 h, and then incubated in 5 % CO_2 and 95 % air for indicated times. Wells were then reacted for 30 min at 37 °C with 20 μL of CellTiter 96 AQueous One Solution™, and the resulting absorbance at 490 nm was measured on an iMark™ microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated relative to that of cells incubated without isoflurane, which was defined as 100 %. All samples were tested in triplicate or quadruplicate per experiment.

ATP assay

Intracellular ATP was quantified with CellTiter-Glo™ Luminescent Cell Viability Assay Kit (Promega, Madison, WI) [19,26]. Briefly, cells were seeded in 96-well plates (2×10^3 cells/well), cultured overnight, treated with or without 2 % isoflurane for 2 h, incubated in 5 % CO_2 and 95 % air for indicated times, reacted for 10 min with 100 μL CellTiter-Glo reagent, and assayed on an EnSpire™ Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). In this reaction, luminescence is generated by luciferase, which requires ATP to oxidize luciferin.

Cellular oxygen consumption and extracellular acidification rate

Cellular oxygen consumption rate and extracellular acidification rate were determined with XF Cell Mito Stress Test™ and XF Glycolysis Stress Test™, respectively, using an XFp Extracellular Flux Analyzer™ (Seahorse Bioscience, USA) [19,25,26]. RCC4-EV and RCC4-VHL cells (1×10^4 cells/well) were seeded in an XFp cell culture microplate, cultured overnight, treated with or without 2 % isoflurane for 2 h, and incubated in 5 % CO_2 and 95 % air for 6 h. Oxygen consumption was then assessed in glucose-containing XF base medium according to the manufacturer's instructions, using a sensor cartridge hydrated at 37 °C in a non-CO_2 incubator the day before use. Injection port A was loaded with 1.0 μM oligomycin (complex V inhibitor), port B was loaded with 1.0 μM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, and port C was loaded with 0.5 μM rotenone/antimycin A (inhibitors of complex I and complex III). The sensor was calibrated using cells incubated at 37 °C in a non-CO_2 incubator and in 180 μL of assay medium (XF base medium with 25 mM glucose, 1 mM pyruvate, and 2 mM L-glutamine, pH 7.4). The plate was immediately assayed following calibration. Assay parameters were calculated as follows: basal oxygen consumption rate = last rate before oligomycin injection − minimum rate after rotenone/antimycin-A injection; maximal oxygen consumption rate = maximum rate after FCCP injection − minimum rate after rotenone/antimycin A injection; non-mitochondrial oxygen consumption rate = minimum rate after rotenone/antimycin A injection; proton leak = minimum rate after oligomycin injection – non-mitochondrial respiration. To measure extracellular acidification rate, injection port A was loaded with

10 mM glucose, and the sensor was calibrated with cells incubated at 37 °C in a non-CO_2 incubator and in 180 μL of assay medium (XF base medium with 2 mM L-glutamine, pH 7.4). The plate was immediately assayed following calibration and loading with oligomycin (1 μM) and 50 mM 2-deoxy-D-glucose. Extracellular acidification rate was normalized to total protein/well and calculated as extracellular acidification rate (glycolysis) = maximum rate after glucose injection − last rate before glucose injection.

Cell migration assay

Cell migration was analyzed using Oris™ Cell Migration Assay (Platypus Thechnologies, Madison, WI). Cells (2×10^4) were seeded in wells plugged with stoppers to restrict seeding to outer areas only. Cells were then exposed for 8 h to 21 % oxygen and 5 % carbon dioxide balanced with nitrogen with or without 2 % isoflurane. Stoppers were then removed to expose unseeded sites, into which cells could migrate during subsequent incubation at 37 °C in 5 % CO_2 and 95 % air for indicated times. Cell migration was imaged on a BZ-9000 Fluorescence Microscope (KEYENCE, Itasca, IL), and colonized areas were quantified in pixels in ImageJ 1.51 (National Institutes of Health), corrected for total unseeded area, and expressed as percentage of colonized areas in reference wells.

RNA sequencing

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) [19,25], and processed using TruSeq Stranded mRNA Sample Prep Kit (Illumina, San

Diego, CA, USA). Poly(A) RNA libraries were then constructed using TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, USA), and sequenced at 100 bp paired-ends on an Illumina HiSeq 2500 platform. Sequencing data in FASTQ format were deposited in the DDBJ Sequence Read Archive under accession numbers DRR100656 (RCC4-EV cells), DRR100657 (RCC4-VHL cells), DRR111123 (RCC4-EV cells treated with isoflurane), and DRR111124 (RCC4-VHL cells treated with isoflurane). FASTQ files were evaluated as described previously [19,25]. In brief, gene lists for Metascape analysis were generated from the Cuffdiff output file of significantly differentially expressed genes ($p < 0.05$; Table S1). Gene ontology annotations were extracted in Ensembl Biomart [27], and sorted by the common logarithms of ([FPKM of RCC4-EV] + 1) / ([FPKM of RCC4-VHL] + 1), which were calculated from the same Cuffdiff output file (Supplementary Information Table S1). We added 1 to FPKM values because it is not possible to calculate the logarithm of 0. Histograms were generated in TIBCO Spotfire Desktop v7.6.0 with the "Better World" program license (TIBCO Spotfire, Palo Alto, CA, USA) (http://spotfire.tibco.com/better-world-donation-program/).

Statistical analysis

Experiments were repeated at least twice with triplicates of each sample. Data are mean ± SD. Groups were compared in Prism 7™ (GraphPad Software, Inc. La Jolla, CA) by one-way analysis of variance or Dunnett's test for multiple comparisons. p values < 0.05 indicated statistical significance.

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Competing interests

All authors declare that there are no competing interests.

Author contributions

C.S., Y.M., T.I., H.B., and K.H. conceived and designed experiments. C.S. M.K, T.S., T.U., and K.H. acquired data. H.B. contributed reagents/materials/analysis tools. C.S. and K.H. prepared figures and/or tables and wrote the paper.

Additional information

The datasets analyzed in the current study available were from Supplementary Infomations and the corresponding author on reasonable request.

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Table 1 KeyResourcesTable

Reagents	Identifier	Source
Dulbecco's modified Eagle's medium	11965-092	Thermo Fischer Scientific, Waltham, MA, USA
fetal bovine serum	SH30910	GE Healthcare, Little Chalfont, UK
Anti-Mouse IgG, HRP-Linked Whole Ab Sheep	NA931	
Anti-Rabbit IgG, HRP-Linked Whole Ab Donkey	NA934	
ECL prime enhanced chemiluminescence reagent	RPN2232	
penicillin-streptomycin	09367-34	Nacalai Tesque, Kyoto, Japan
Chemi-Lumi One Super	02230-14	
RIPA Buffer	16488-34	
10%-SDS Solution	30562-04	
sodium pyruvate	06977-34	
2-deoxy-D-glucose	040-06481	FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan
Anti-α-tubulin mouse monoclonal antibody	017-25031	
Isoflurane	099-06571	
oligomycin	O4876	SIGMA, St Louis, MO, USA
carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP)	C2920	
rotenone	R8875	
antimycin A	A8674	
CellTiter 96™ AQueous One Solution Cell Proliferation Assay	G3582	Promega, Madison, WI, USA
CellTiter-Glo™ luminescent cell viability assay kit	G7570	
RNeasy™ Mini Kit	74104	Qiagen, Hilden, Germany
QuantiTect™ Reverse Transcription Kit	205311	
Rotor-Gene™ SYBR Green PCR Kit	204074	
anti-human HIF-1α antibody Clone 54/HIF-1α	610959	BD Biosciences, San Jose, CA, USA
HIF-1β/ARNT (D28F3) XP rabbit monoclonal antibody	5537	Cell Signaling Technology, Danvers, MA, USA
cOomplete™ Protease Inhibitor Cocktail	4693116001	Roche Diagnostics, Tokyo, Japan
HIF-2 α/EPAS1 antibody was purchased from Novus Biologicals	NB100-122	Novus Biologicals, Littleton, CO
Oris™ Cell Migration Assembly-FLEX Kit	CMAUFL4	Platypus Technoplogies, Madison, WI

Figure legends

Figure 1 Expression of HIFs under isoflurane

(a) HIF expression after 8 h at 20 % and 1 % O_2, (b) after 8 h at 20 % O_2 with or without 2 % isoflurane, (c) and after 8 h at 1 % O_2 with or without 2 % isoflurane. Cells were harvested, and 35 μg whole-cell lysates were immunoblotted using primary antibodies to indicated proteins. (d and e) Cells were exposed to 2 % isoflurane by two different protocols as indicated. HIF-1α expression was analyzed by densitometry and normalized to that in RCC4-VHL cells at 20 % O_2, which was considered 100 %. Data are mean ± SD; #, $p < 0.05$; NS, not significant.

Figure 2 Expression of HIF-1 target genes under isoflurane

RCC4-EV and RCC4-VHL cells were exposed for 8 h to 20 % or 1 % O_2 with or without 2 % isoflurane. Cells were then harvested and mRNA levels quantified by semi-quantitative qRT-PCR. Fold expression was calculated based on expression in RCC4-EV cells at 20 % O_2. Data are mean ± SD. *, $p < 0.05$ vs cells at 20 % O_2 and no isoflurane; #, $p < 0.05$ for the indicated comparison; NS, not significant; *SLC2A1*, solute carrier family 2 member 1; *VEGFA*, vascular endothelial growth factor A; *HIF1A*, hypoxia-inducible factor 1 α subunit; *EPAS1*, endothelial PAS domain protein 1.

Figure 3 Cell proliferation under isoflurane

RCC4-EV and RCC4-VHL cells were grown for 48 h with or without exposure to 2 % isoflurane for 2 h. (a) Cell growth at indicated time points, as evaluated by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetraz olium] assay. (b) Cellular ATP at indicated time points. Data are mean ± SD. *$p < 0.05$,

for the comparison between isoflurane treatment (+) and (-) groups in RCC4-EV and RCC4-VHL cells, #$p < 0.05$, for the comparison between RCC4-EV and RCC4-VHL with or without isoflurane treatment.

Figure 4 Cell migration under isoflurane

RCC4-EV and RCC4-VHL cells were allowed to migrate for 12 h with or without 2 % isoflurane treatment for 2 h. Migration rate was assessed as described in Materials and Methods. (a) Cell migration at 0 h and 12 h. (b) Data are mean ± SD. *$p < 0.05$, for the comparison between isoflurane treatment (+) and (-) groups in RCC4-EV and RCC4-VHL cells, #$p < 0.05$, for the comparison between RCC4-EV and RCC4-VHL with or without isoflurane treatment.

Figure 5 Oxygen metabolism in RCC4-EV and RCC4-VHL cells

(a) Cell Mito Stress Test™ at 20 % O_2. (b) Oxygen consumption rate with or without 2 % isoflurane treatment for 2 h. (c) Glycolysis test at 20 % O_2. (d) Extracellular acidification rate with or without 2 % isoflurane treatment for 2 h. #, $p < 0.05$ for the indicated comparison.

Figure 6 Reprogrammed oxygen metabolism in RCC4-EV and RCC4-VHL cells

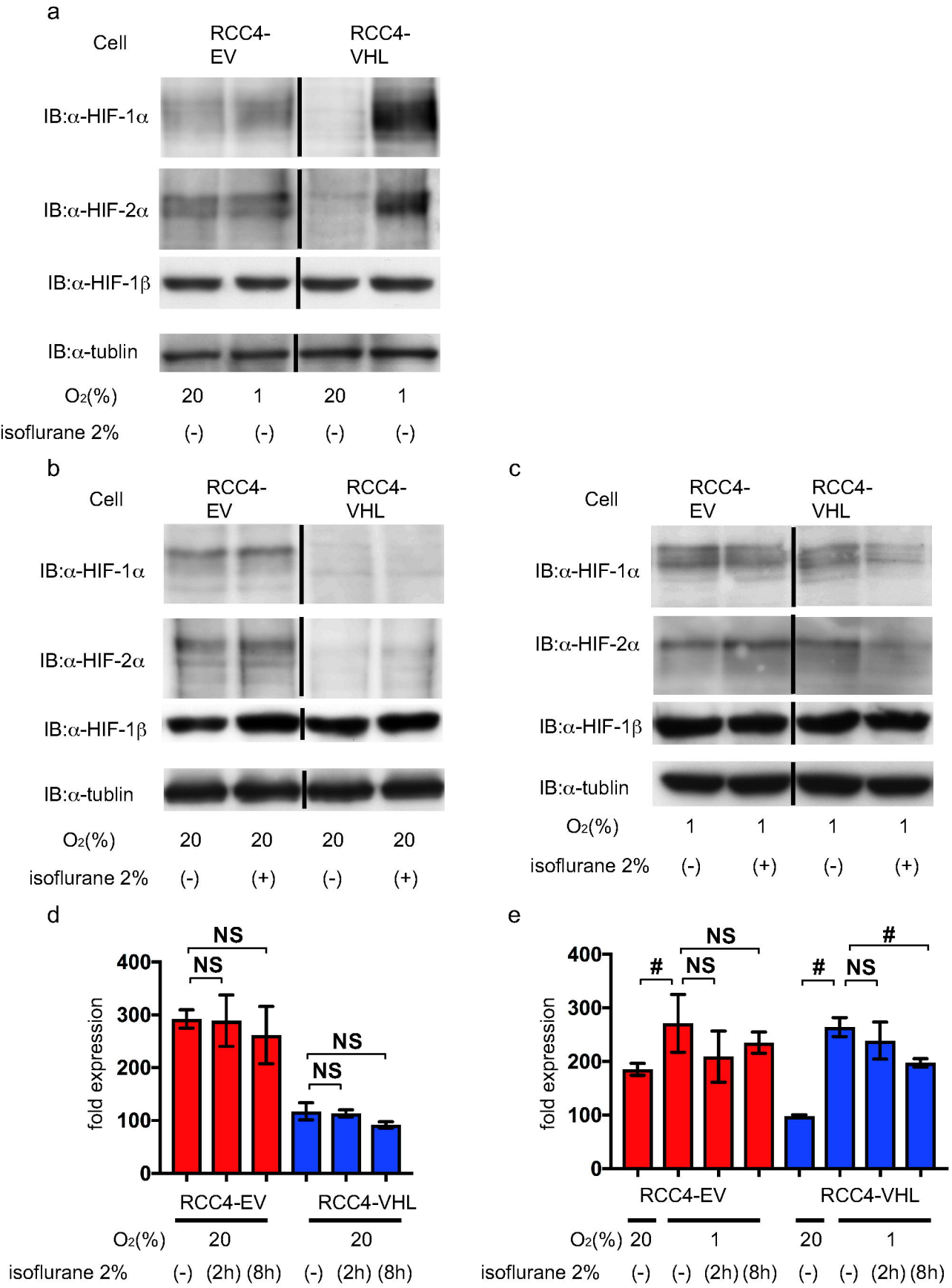
(a) Cell Mito Stress Test™ profile of key parameters that determine mitochondrial oxygen consumption rate. (b) Basal oxygen consumption rate, (c) maximal oxygen consumption rate, (d) proton leakage, and (e) nonmitochondrial respiration rate with or without 2 % isoflurane for 2 h. #, $p < 0.05$ by *t* test vs control.

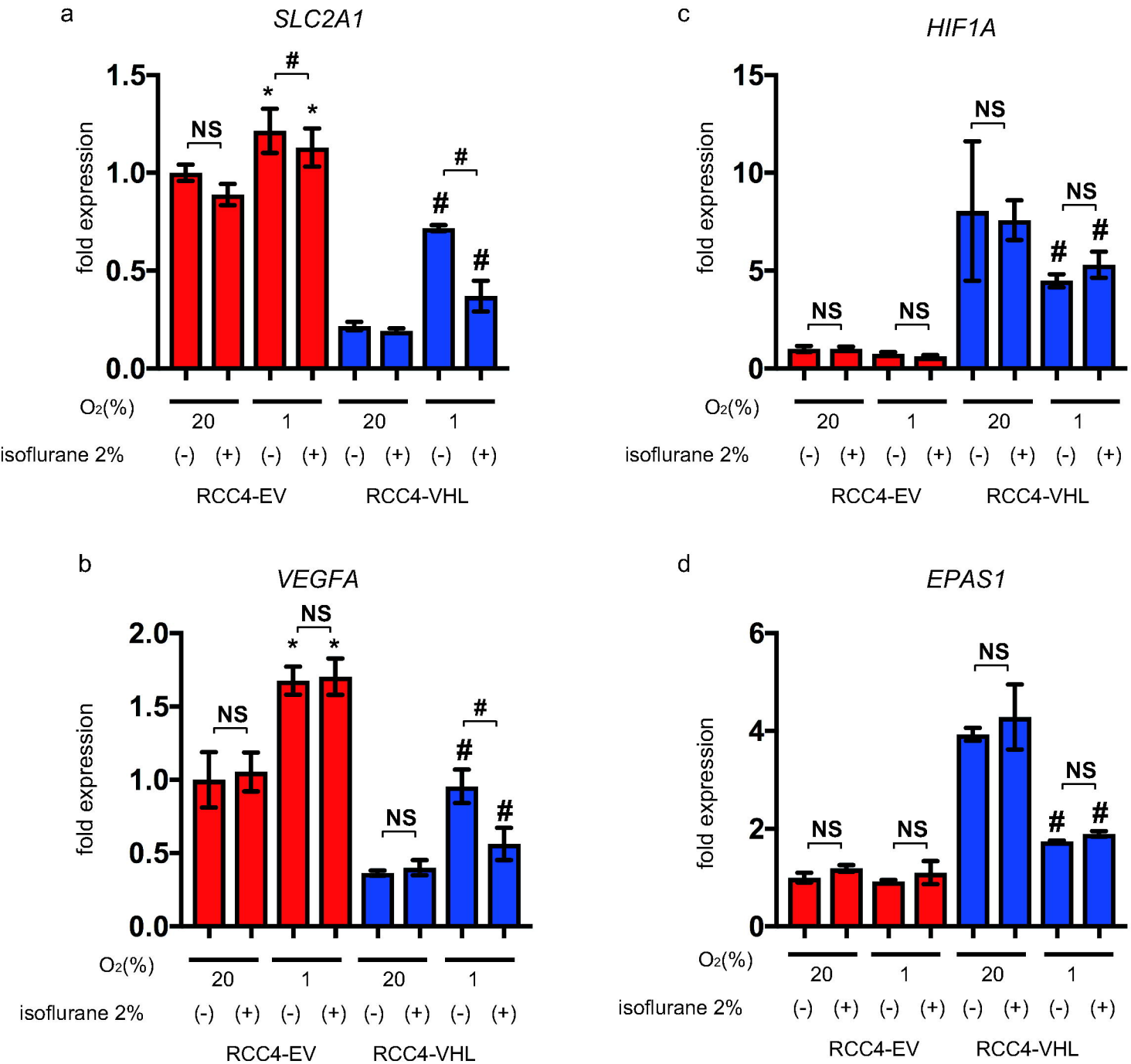
Figure 7 Global gene expression in RCC4-EV and RCC4-VHL cells

(a) Hierarchical clustering and (b) number of differentially expressed genes identified by pairwise Wilcoxon signed rank test at $p < 0.05$. (c) Scatter plots below the diagonal are pairwise comparisons of $\log_{10}$[FPKM+1]. Histograms at the diagonal show the distribution of genes by level of expression, and numbers above the diagonal are pairwise correlation coefficients. (d and e) Heatmap of enriched terms in differentially expressed genes, colored by *p* values and as inferred by Metascape analysis (http://metascape.org/) from the Cuffdiff output file (gene_exp.diff).

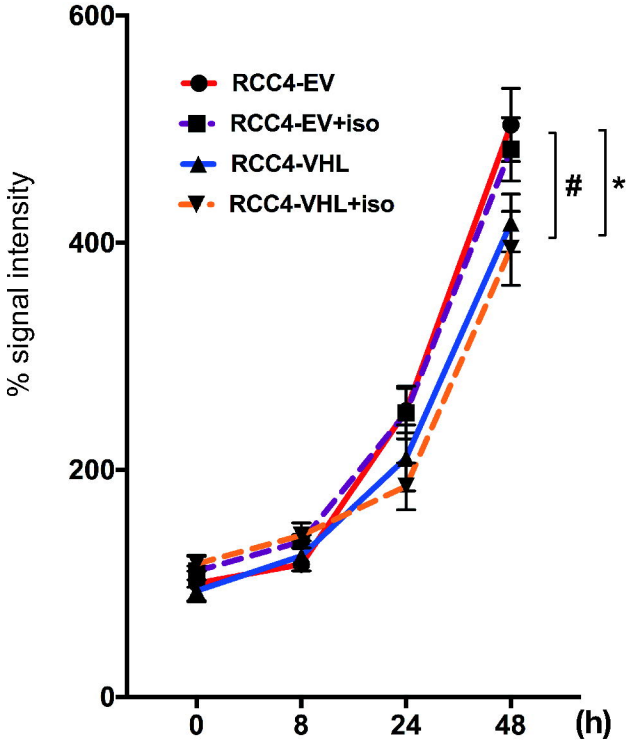
Figure 8 Transcriptomic variations in gene ontologies related to cancer hallmarks

Logarithms of ([FPKM of RCC4-EV] + 1) / ([FPKM of RCC4-VHL] + 1) were plotted for cells treated with or without isoflurane on the Y and X axis, respectively. Data were calculated from the same Cuffdiff output file described in Fig. 7. (a) GO:0071456 cellular response to hypoxia, (b) GO: 0061621 canonical glycolysis, (c) GO: 0030334 regulation of cell migration, (d) GO: 0001558 regulation of cell growth, (e) GO: 0030198 extracellular matrix organization, and (f) GO: 0060231 mesenchymal to epithelial transition.

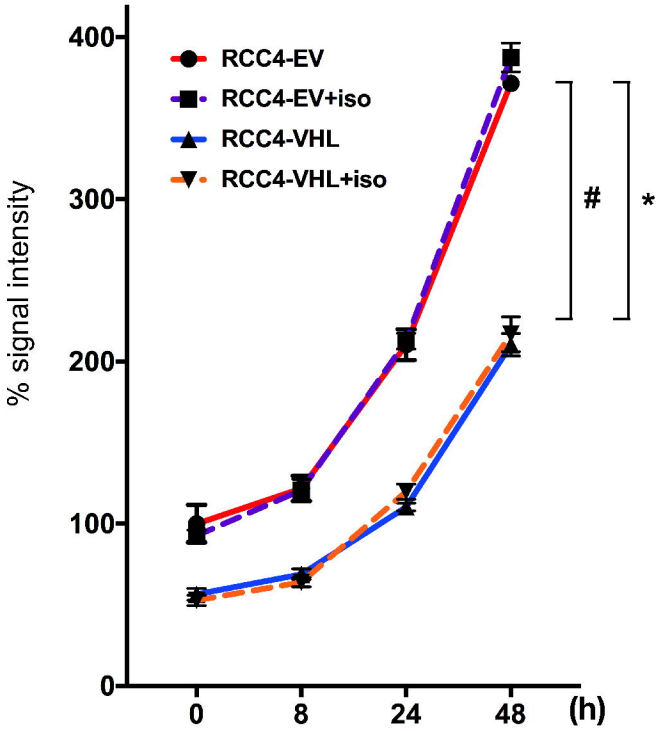

a
Cell
RCC4-EV
RCC4-VHL
IB:α-HIF-1α
IB:α-HIF-2α
IB:α-HIF-1β
IB:α-tublin
O2(%) 20 1 20 1
isoflurane 2% (-) (-) (-) (-)
b
Cell
RCC4-EV
RCC4-VHL
IB:α-HIF-1α
IB:α-HIF-2α
IB:α-HIF-1β
IB:α-tublin
O2(%) 20 20 20 20
isoflurane 2% (-) (+) (-) (+)
c
Cell
RCC4-EV
RCC4-VHL
IB:α-HIF-1α
IB:α-HIF-2α
IB:α-HIF-1β
IB:α-tublin
O2(%) 1 1 1 1
isoflurane 2% (-) (+) (-) (+)
d
fold expression
400
300
200
100
0
NS
NS
NS
NS
RCC4-EV
RCC4-VHL
O2(%) 20 20
isoflurane 2% (-) (2h) (8h) (-) (2h) (8h)
e
fold expression
400
300
200
100
0
#
NS
NS
#
NS
#
RCC4-EV
RCC4-VHL
O2(%) 20 1 20 1
isoflurane 2% (-) (-) (2h) (8h) (-) (-) (2h) (8h)


a
SLC2A1
fold expression
1.5
1.0
0.5
0.0
NS
*
*
#
#
#
O2(%)
20
1
20
1
isoflurane 2%
(-) (+) (-) (+) (-) (+) (-) (+)
RCC4-EV
RCC4-VHL
c
HIF1A
fold expression
15
10
5
0
NS
NS
NS
NS
#
#
O2(%)
20
1
20
1
isoflurane 2%
(-) (+) (-) (+) (-) (+) (-) (+)
RCC4-EV
RCC4-VHL
b
VEGFA
fold expression
2.0
1.5
1.0
0.5
0.0
NS
NS
NS
*
*
#
#
#
O2(%)
20
1
20
1
isoflurane 2%
(-) (+) (-) (+) (-) (+) (-) (+)
RCC4-EV
RCC4-VHL
d
EPAS1
fold expression
6
4
2
0
NS
NS
NS
NS
#
#
O2(%)
20
1
20
1
isoflurane 2%
(-) (+) (-) (+) (-) (+) (-) (+)
RCC4-EV
RCC4-VHL

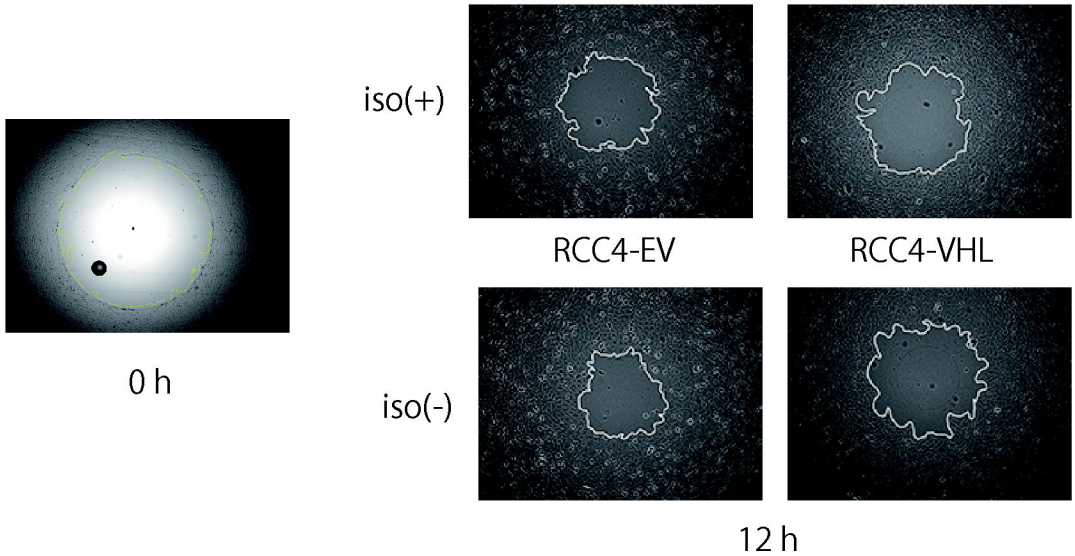
a


600
400
200
0
% signal intensity
RCC4-EV
RCC4-EV+iso
RCC4-VHL
RCC4-VHL+iso
#
*
0
8
24
48
(h)

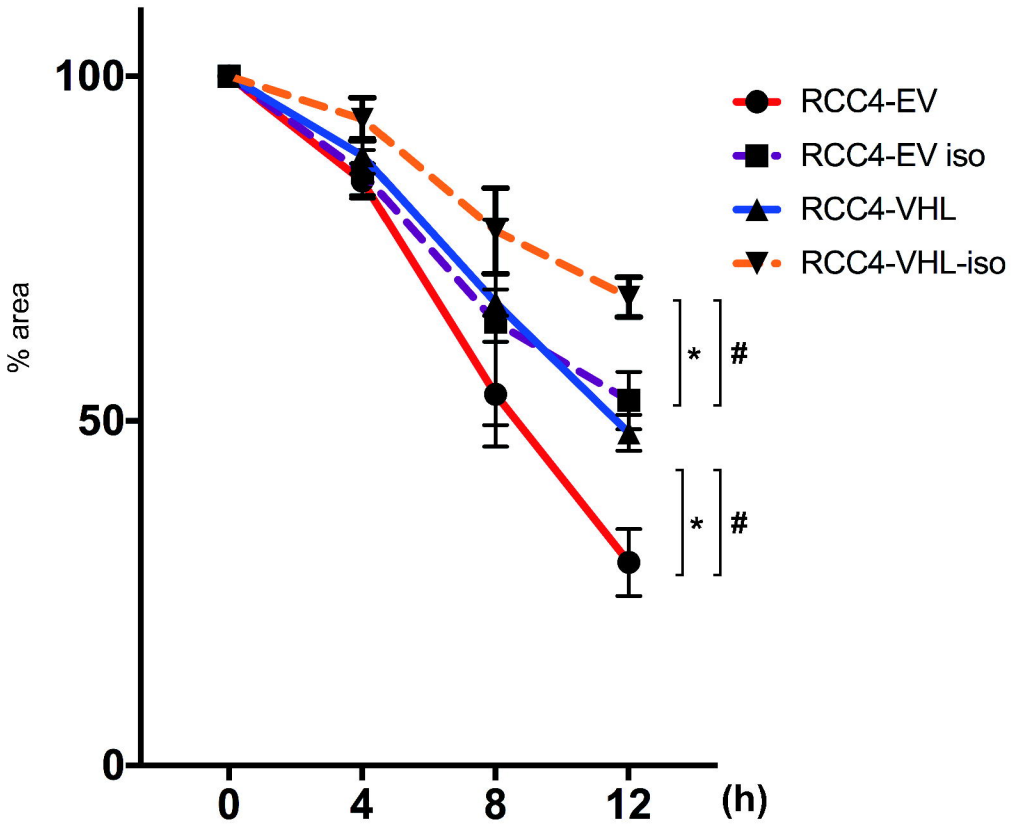
b


400
300
200
100
0
% signal intensity
RCC4-EV
RCC4-EV+iso
RCC4-VHL
RCC4-VHL+iso
#
*
0
8
24
48
(h)

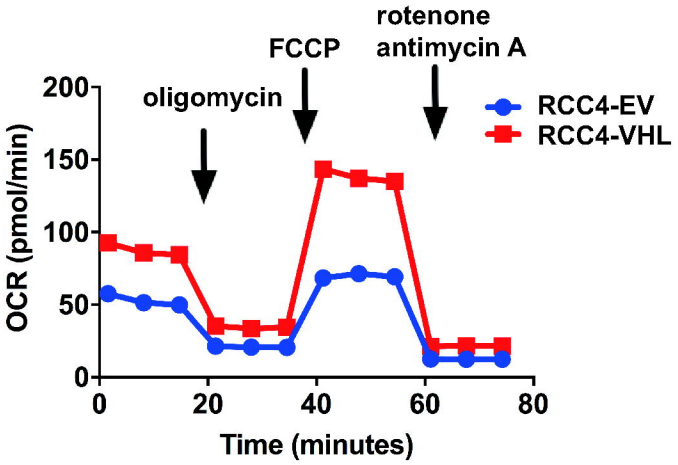
a


iso(+)
RCC4-EV
RCC4-VHL
0 h
iso(-)
12 h

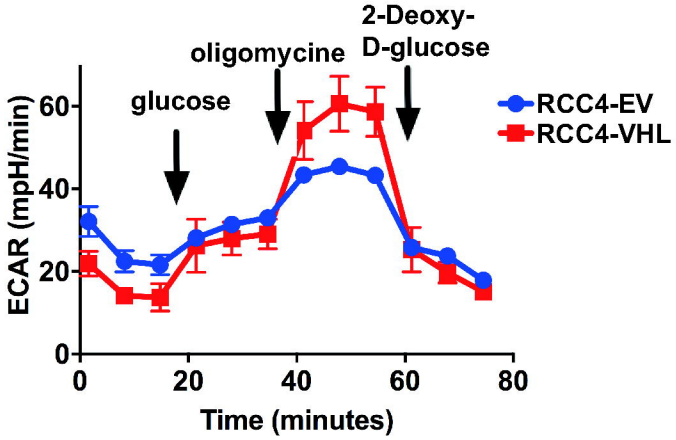
b


100
50
0
% area
0
4
8
12
(h)
RCC4-EV
RCC4-EV iso
RCC4-VHL
RCC4-VHL-iso
*
#
*
#

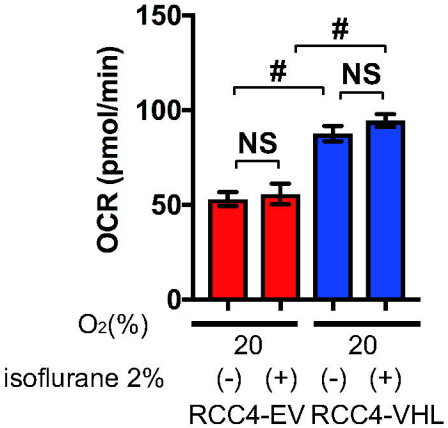
a


rotenone
antimycin A
FCCP
oligomycin
RCC4-EV
RCC4-VHL
OCR (pmol/min)
200
150
100
50
0
0
20
40
60
80
Time (minutes)

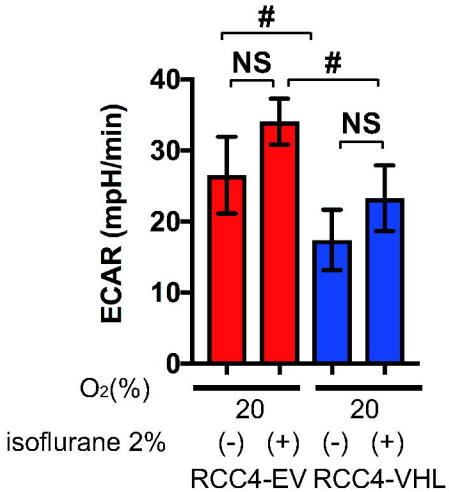
c


2-Deoxy-
D-glucose
oligomycine
glucose
RCC4-EV
RCC4-VHL
ECAR (mpH/min)
60
40
20
0
0
20
40
60
80
Time (minutes)

b

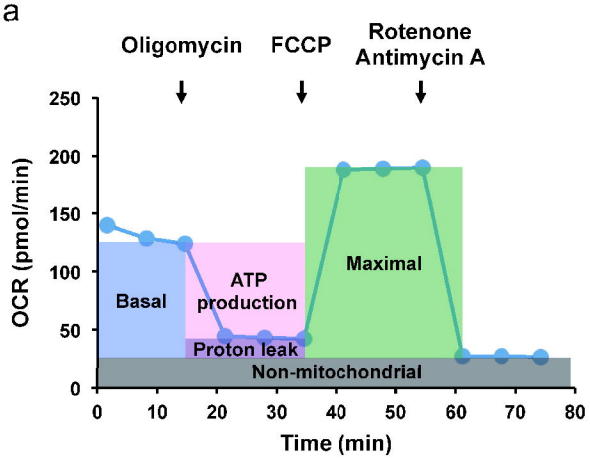

OCR (pmol/min)
150
100
50
0
#
#
NS
NS
O2(%)
20
20
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

d

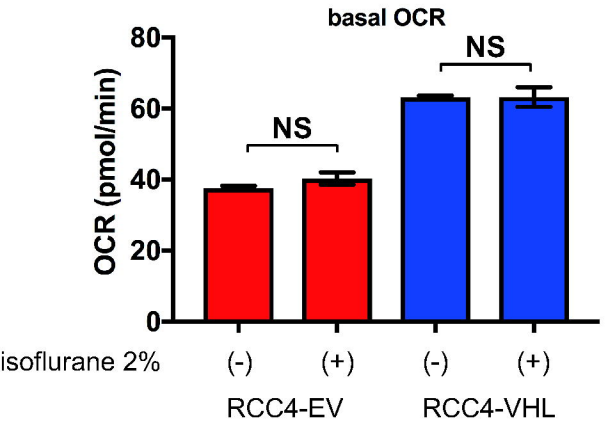

#
NS
#
NS
ECAR (mpH/min)
40
30
20
10
0
O2(%)
20
20
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

NS

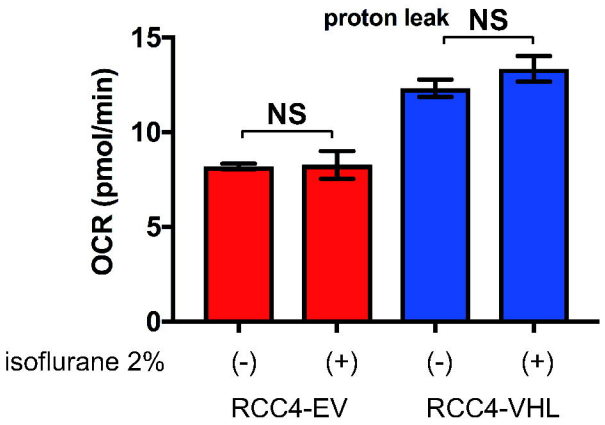
a


Oligomycin
FCCP
Rotenone
Antimycin A
OCR (pmol/min)
250
200
150
100
50
0
Basal
ATP
production
Proton leak
Maximal
Non-mitochondrial
0
10
20
30
40
50
60
70
80
Time (min)

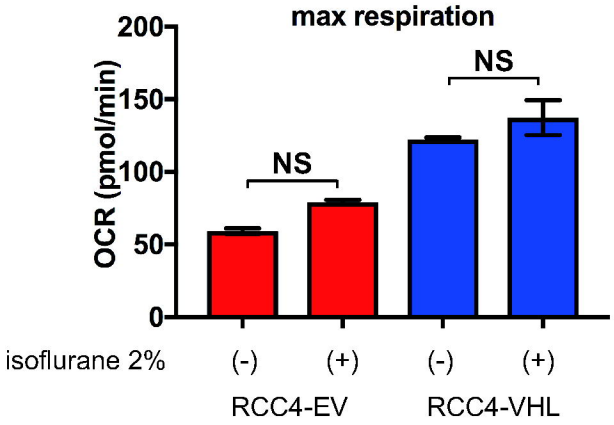
b


basal OCR
OCR (pmol/min)
80
60
40
20
0
NS
NS
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

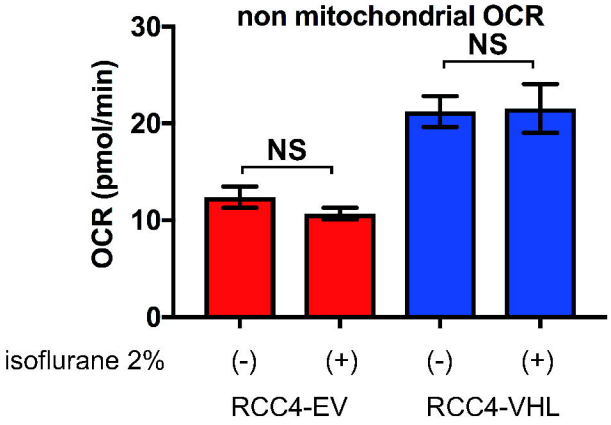
d


proton leak
NS
OCR (pmol/min)
15
10
5
0
NS
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

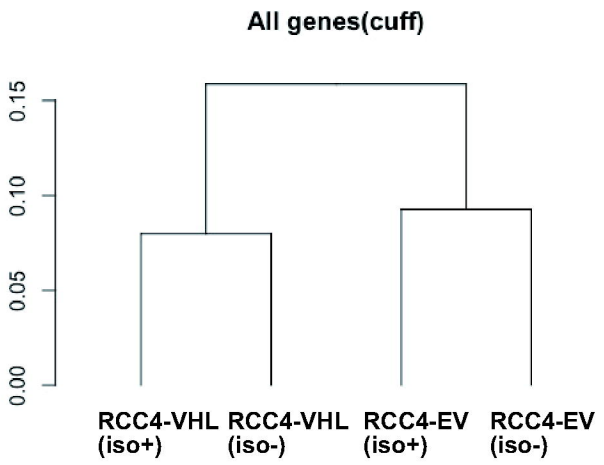
c


max respiration
OCR (pmol/min)
200
150
100
50
0
NS
NS
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

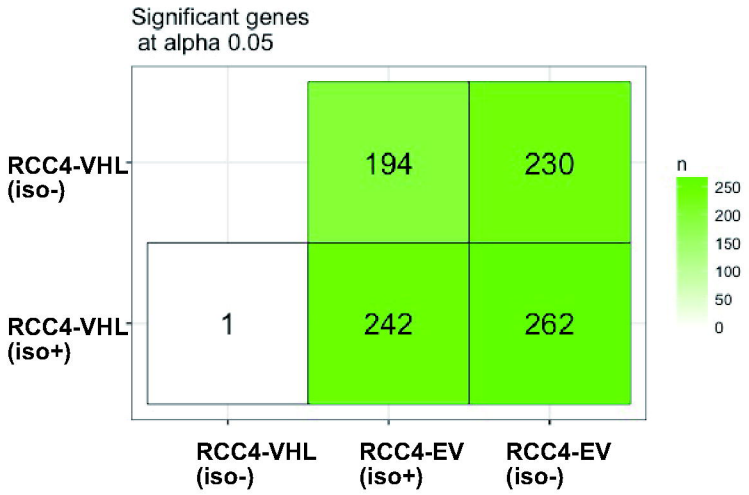
e


non mitochondrial OCR
NS
OCR (pmol/min)
30
20
10
0
NS
isoflurane 2%
(-)
(+)
(-)
(+)
RCC4-EV
RCC4-VHL

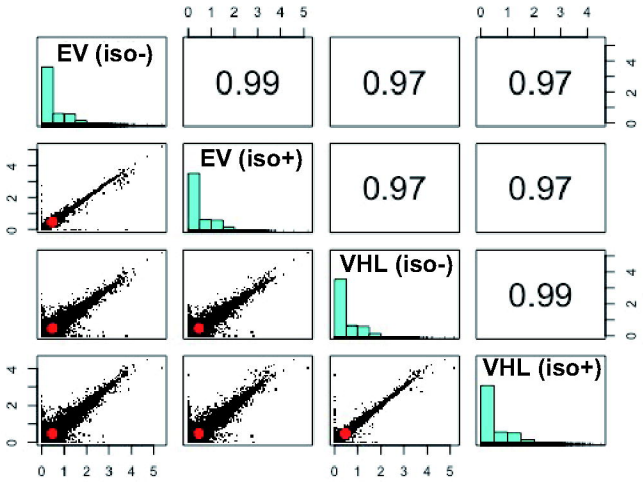
a


All genes(cuff)
0.15
0.10
0.05
0.00
RCC4-VHL (iso+)
RCC4-VHL (iso-)
RCC4-EV (iso+)
RCC4-EV (iso-)

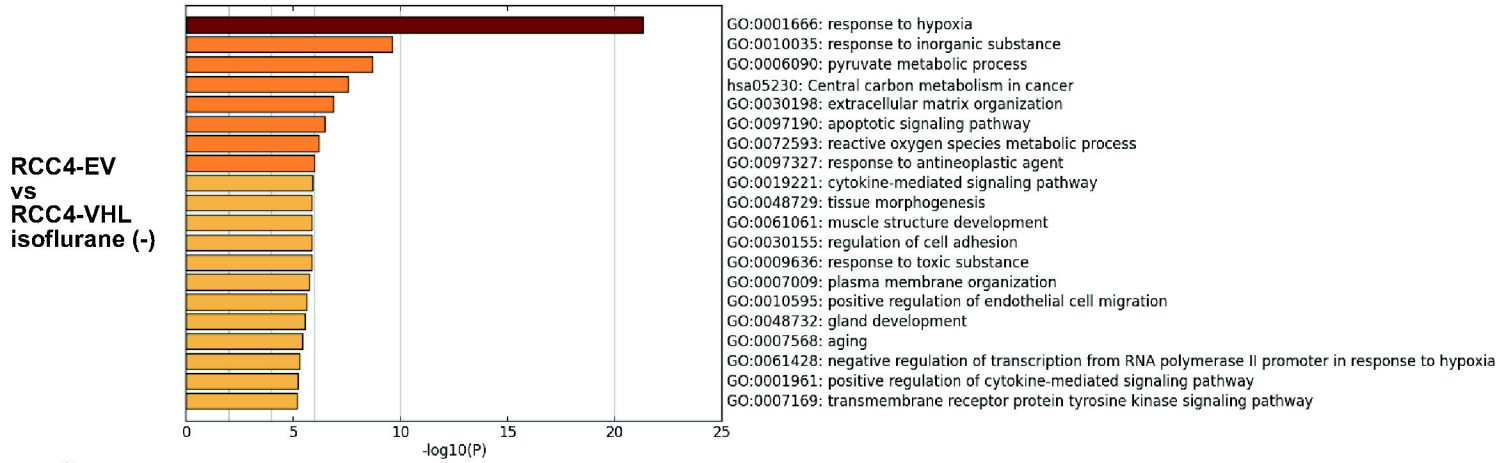
b


Significant genes at alpha 0.05
RCC4-VHL (iso-)
RCC4-VHL (iso+)
194
230
1
242
262
n
250
200
150
100
50
0
RCC4-VHL (iso-)
RCC4-EV (iso+)
RCC4-EV (iso-)

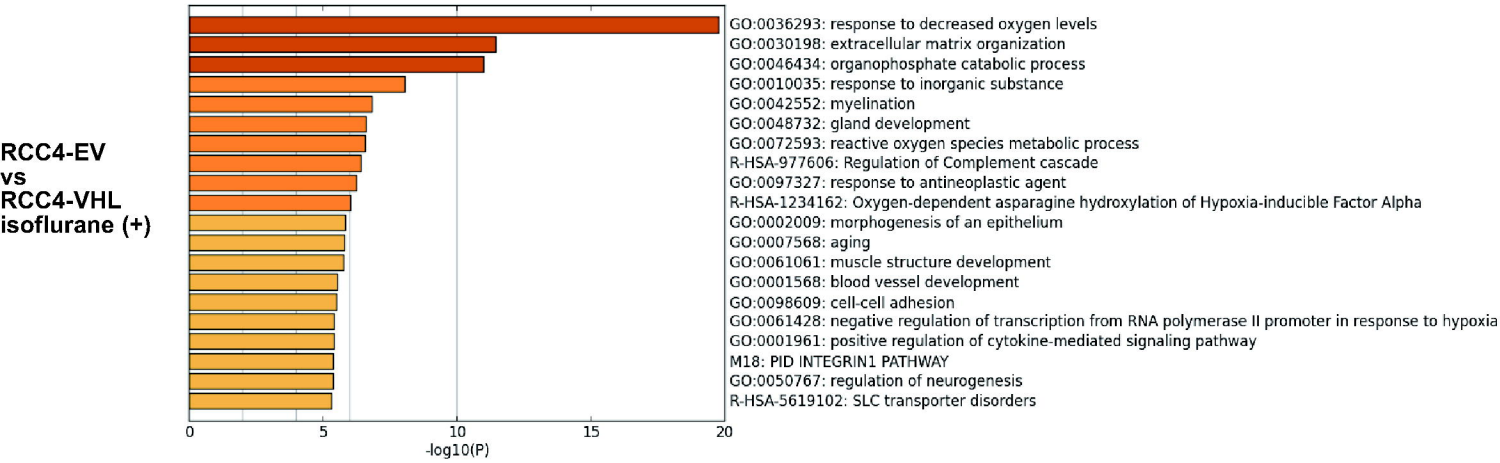
c

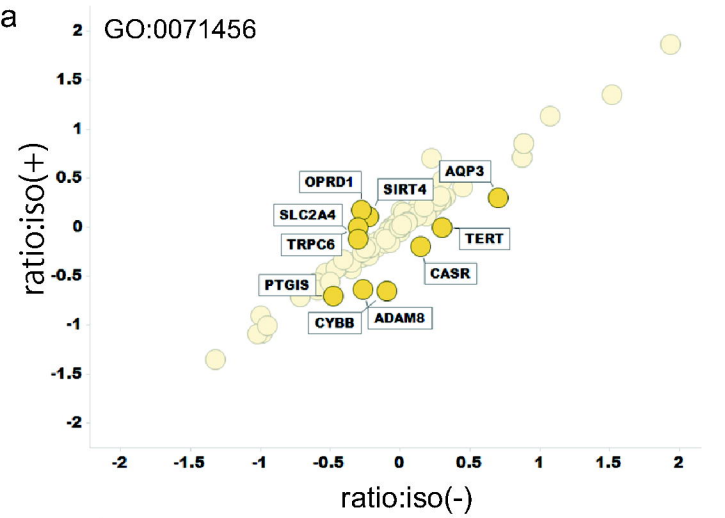

EV (iso-)
0.99
0.97
0.97
EV (iso+)
0.97
0.97
VHL (iso-)
0.99
VHL (iso+)
0 1 2 3 4 5

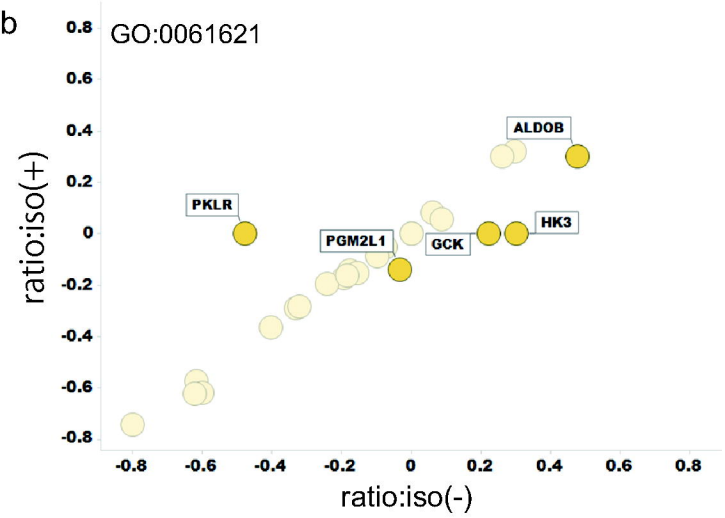
d

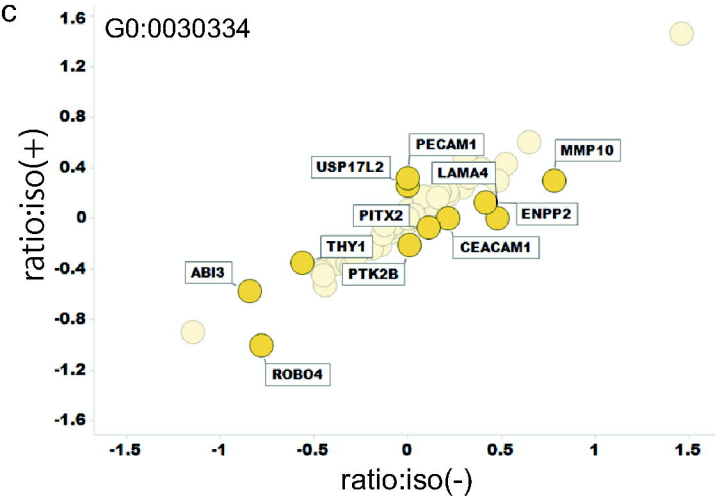

RCC4-EV vs RCC4-VHL isoflurane (-)
GO:0001666: response to hypoxia
GO:0010035: response to inorganic substance
GO:0006090: pyruvate metabolic process
hsa05230: Central carbon metabolism in cancer
GO:0030198: extracellular matrix organization
GO:0097190: apoptotic signaling pathway
GO:0072593: reactive oxygen species metabolic process
GO:0097327: response to antineoplastic agent
GO:0019221: cytokine-mediated signaling pathway
GO:0048729: tissue morphogenesis
GO:0061061: muscle structure development
GO:0030155: regulation of cell adhesion
GO:0009636: response to toxic substance
GO:0007009: plasma membrane organization
GO:0010595: positive regulation of endothelial cell migration
GO:0048732: gland development
GO:0007568: aging
GO:0061428: negative regulation of transcription from RNA polymerase II promoter in response to hypoxia
GO:0001961: positive regulation of cytokine-mediated signaling pathway
GO:0007169: transmembrane receptor protein tyrosine kinase signaling pathway
0
5
10
15
20
25
-log10(P)

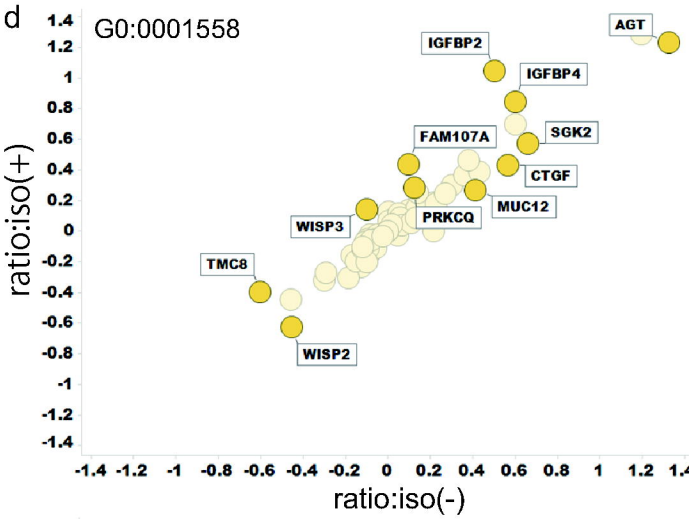
e

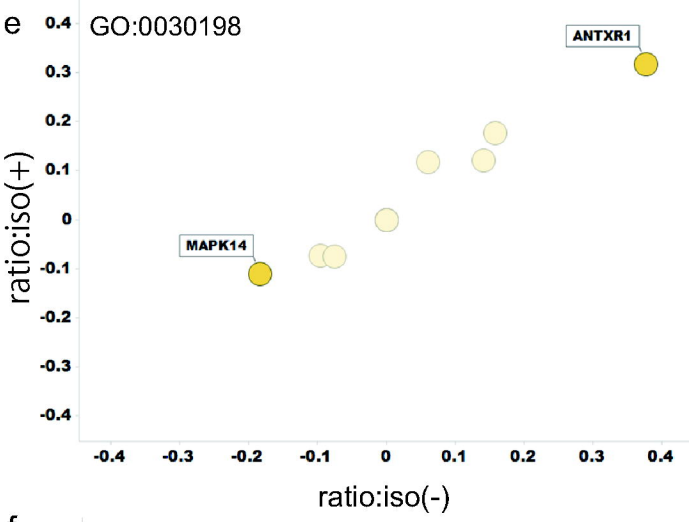

RCC4-EV vs RCC4-VHL isoflurane (+)
GO:0036293: response to decreased oxygen levels
GO:0030198: extracellular matrix organization
GO:0046434: organophosphate catabolic process
GO:0010035: response to inorganic substance
GO:0042552: myelination
GO:0048732: gland development
GO:0072593: reactive oxygen species metabolic process
R-HSA-977606: Regulation of Complement cascade
GO:0097327: response to antineoplastic agent
R-HSA-1234162: Oxygen-dependent asparagine hydroxylation of Hypoxia-inducible Factor Alpha
GO:0002009: morphogenesis of an epithelium
GO:0007568: aging
GO:0061061: muscle structure development
GO:0001568: blood vessel development
GO:0098609: cell-cell adhesion
GO:0061428: negative regulation of transcription from RNA polymerase II promoter in response to hypoxia
GO:0001961: positive regulation of cytokine-mediated signaling pathway
M18: PID INTEGRIN1 PATHWAY
GO:0050767: regulation of neurogenesis
R-HSA-5619102: SLC transporter disorders
0
5
10
15
20
-log10(P)

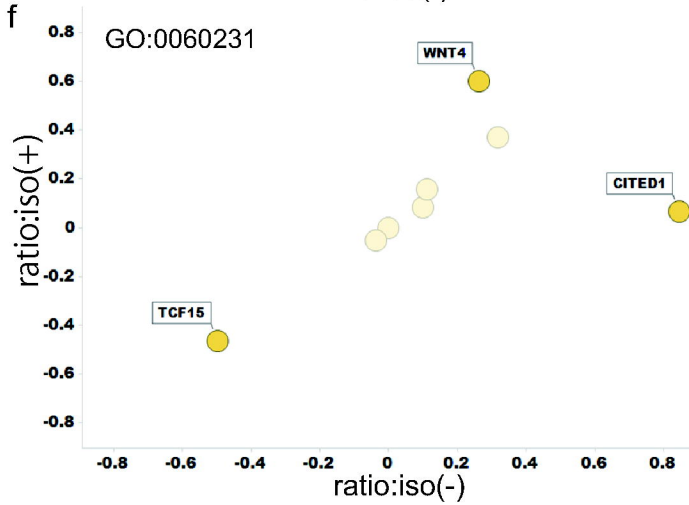

a
GO:0071456
OPRD1
SIRT4
AQP3
SLC2A4
TRPC6
TERT
CASR
PTGIS
CYBB
ADAM8
ratio:iso(+)
ratio:iso(-)


b
GO:0061621
ALDOB
PKLR
HK3
PGM2L1
GCK
ratio:iso(+)
ratio:iso(-)


c
G0:0030334
PECAM1
MMP10
USP17L2
LAMA4
PITX2
ENPP2
THY1
CEACAM1
ABI3
PTK2B
ROBO4
ratio:iso(+)
ratio:iso(-)


d
G0:0001558
AGT
IGFBP2
IGFBP4
FAM107A
SGK2
CTGF
MUC12
PRKCQ
WISP3
TMC8
WISP2
ratio:iso(+)
ratio:iso(-)


e
GO:0030198
ANTXR1
MAPK14
ratio:iso(+)
ratio:iso(-)


f
GO:0060231
WNT4
CITED1
TCF15
ratio:iso(+)
ratio:iso(-)