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Prof. Dr. rer. nat. Aymelt Itzen

All Three AKT Isoforms Can Upregulate Oxygen Metabolism and Lactate Production in Human Hepatocellular Carcinoma Cell Lines

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Lingyu Tian
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Ling-Yu Tian [1,2], Daniel J. Smit [1], Nadezhda V. Popova [1], Stefan Horn [3], Lis Noelia Velasquez [4,5], Samuel Huber [4,5] and Manfred Jücker [1,*]

1 Institute of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; l.tian.ext@uke.de (L.-Y.T.); d.smit@uke.de (D.J.S.); npopova@gmail.com (N.V.P.)
2 Beijing Key Surgical Basic Research Laboratory of Liver Cirrhosis and Liver Cancer, Department of Hepatobiliary Surgery, Peking University People's Hospital, Beijing 100044, China
3 Research Department Cell and Gene Therapy, Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; s.horn@uke.de
4 I. Department of Medicine, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; l.velasquez@uke.de (L.N.V.); s.huber@uke.de (S.H.)
5 Hamburg Center for Translational Immunology, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany
* Correspondence: juecker@uke.de; Tel.: +49-(0)-40-7410-56339

Abstract: Hepatocellular carcinoma (HCC), the main pathological type of liver cancer, is related to risk factors such as viral hepatitis, alcohol intake, and non-alcoholic fatty liver disease (NAFLD). The constitutive activation of the PI3K/AKT signaling pathway is common in HCC and has essential involvement in tumor progression. The serine/threonine kinase AKT has several downstream substrates, which have been implicated in the regulation of cellular metabolism. However, the contribution of each of the three AKT isoforms, i.e., AKT1, AKT2 and AKT3, to HCC metabolism has not been comprehensively investigated. In this study, we analyzed the functional role of AKT1, AKT2 and AKT3 in HCC metabolism. The overexpression of activated AKT1, AKT2 and AKT3 isoforms in the human HCC cell lines Hep3B and Huh7 resulted in higher oxygen consumption rate (OCR), ATP production, maximal respiration and spare respiratory capacity in comparison to vector-transduced cells. Vice versa, lentiviral vector-mediated knockdowns of each AKT isoform reduced OCR in both cell lines. Reduced OCR rates observed in the three AKT isoform knockdowns were associated with reduced extracellular acidification rates (ECAR) and reduced lactate production in both analyzed cell lines. Mechanistically, the downregulation of OCR by AKT isoform knockdowns correlated with an increased phosphorylation of the pyruvate dehydrogenase on Ser232, which negatively regulates the activity of this crucial gatekeeper of mitochondrial respiration. In summary, our data indicate that each of the three AKT isoforms is able to upregulate OCR, ECAR and lactate production independently of each other in human HCC cells through the regulation of the pyruvate dehydrogenase.

Keywords: AKT signaling; AKT isoforms; oxygen metabolism; lactate metabolism; extracellular acidification; glycolysis; Warburg effect; HCC metabolism; HCC treatment

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1. Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death in East Asia and the sixth leading cause of cancer death in Western countries. The similarity between incidence (906,000 per year) and mortality (830,000 deaths per year) underlines the dismal prognosis associated with this disease [1,2]. The leading risk factors of HCC are hepatitis, virus infection, auto-immune diseases, drug- and non-drug-related toxicity, as well as non-alcohol fatty liver disease (NAFLD) [3]. Increasing evidence suggests the strong

association between metabolic factors and HCC prevalence [3]. Metabolic reprogramming is one of the most substantial tumor hallmarks [4]. Recently, tremendous attention has been brought to the implication of deregulated metabolism on HCC carcinogenesis [5].

Frequently, the alteration of intracellular signaling pathways has been observed in HCC and has been associated with tumor progression. The phosphoinositide 3-kinase (PI3K), AKT and mammalian target of rapamycin (mTOR) pathway are among these oncogenic signal transduction pathways in HCC [6]. The PI3K/AKT/mTOR signaling pathway regulates crucial cellular processes, including cell survival, metastasis and metabolism. It can be aberrantly activated through various mechanisms, including genomic alterations and mutations of PIK3CA, AKT and mTOR or a loss of the tumor suppressor phosphatase and tension homolog (PTEN) [7]. Cancer with activated PI3K/AKT signaling has been demonstrated to present as an aggressive phenotype, and the activation of PI3K/AKT signaling has been indicated as a significant risk factor for early recurrence and poor prognosis in liver cancer patients [8–10]. The PI3K/AKT signaling networks have multifarious downstream influences on cellular metabolism, via both the direct regulation of nutrient transporters and metabolic enzymes as well as the regulation of transcription factors that control the expression of key elements of metabolic pathways [11]. Some downstream molecules of the PI3K/AKT/mTOR signaling pathway are particularly increased in HCC patients with elevated 2[18F]fluoro-2-deoxy-d-glucose (FDG) uptake, implying that the activation of this pathway might regulate the HCC metabolism [12].

The AKT family has three members (AKT1/PKBα, AKT2/PKBβ and AKT3/PKBγ) sharing a high structural homology, but studies on AKT isoform-specific knockout mice suggest that AKT signaling diversity might in part be due to different functions of the three AKT family members [13]. The AKT1 knockout (KO) mice are growth-restricted but have no metabolic irregularities and AKT2 KO mice are glucose intolerant and show systemic insulin resistance, whereas AKT3 KO mice have decreased brain size but regular glucose homeostasis [14]. Recently, several studies showed functional roles of the three AKT isoforms in multiple solid tumors, demonstrating that different isoforms have distinct functions in tumors [15–17]. In tumor metabolic studies, AKT1 activation was associated with the accumulation of aerobic glycolysis metabolites in prostate cancer [18]. Moreover, the AKT2 phosphorylation of hexokinase 2 has been demonstrated to enhance hexokinase activity and lactic acid production in colon cancer [19]. Furthermore, for AKT3 knockdown, it has been demonstrated to cause mitochondrial dysfunction in human lung cancer cells [20].

The liver plays a crucial role in the body's metabolic homeostasis and is significantly involved in the regulation of various metabolic processes. Nevertheless, the functional role of the three AKT isoforms in metabolic regulation has never been simultaneously investigated in HCC [21]. Therefore, we aimed to clarify the metabolic roles of three AKT isoforms, AKT1, AKT2 and AKT3, in human HCC cell lines and to provide a rationale for AKT inhibition in HCC patients. Our data demonstrate that all three AKT family members can upregulate oxygen metabolism and extracellular acidification in human HCC cell lines.

2. Results

2.1. Generation of Stable Knockdowns and Ectopic Expression of Activated AKT1, AKT2 and AKT3 Mutants in Human HCC Cell Lines

Our previous findings have shown that Hep3B and Huh7 cells express AKT1 and AKT2, but AKT3 expression is restricted to Hep3B cells [15]. Therefore, stable knockdowns of AKT1, AKT2 and AKT3 were introduced in Hep3B cells (Figure 1A) as well as AKT1 and AKT2 knockdowns in Huh7 cells (Figure 1B). The knockdown efficiency in Hep3B is 98.9%, 95.3% and 98.9% for AKT1, AKT2 and AKT3, respectively. For Huh7, the knockdown efficiency of AKT1 and AKT2 is 91.4% and 98.2%, respectively (Supplementary Figure S1). In addition, constitutively activated AKT isoforms were strongly overexpressed in Hep3B and Huh7 cells by the lentiviral transduction of vectors with constitutively activated AKT by amino acid exchange to aspartic acid (i.e., AKT1 T308D/S473D, AKT2 T309D/S474D,

AKT3 T305D/S472D) (Figure 1C,D). AKT1 DD, AKT2 DD and AKT3 DD displayed 4.4, 8.2 and 21.8-fold higher expression in comparison to the Hep3B parental cell line, respectively. In Huh7 cells, AKT1 DD, AKT2 DD and AKT3 DD displayed 15.5, 7.5 and 101.4-fold higher expression than the parental HuH7 cell line, respectively (Supplementary Figure S2).

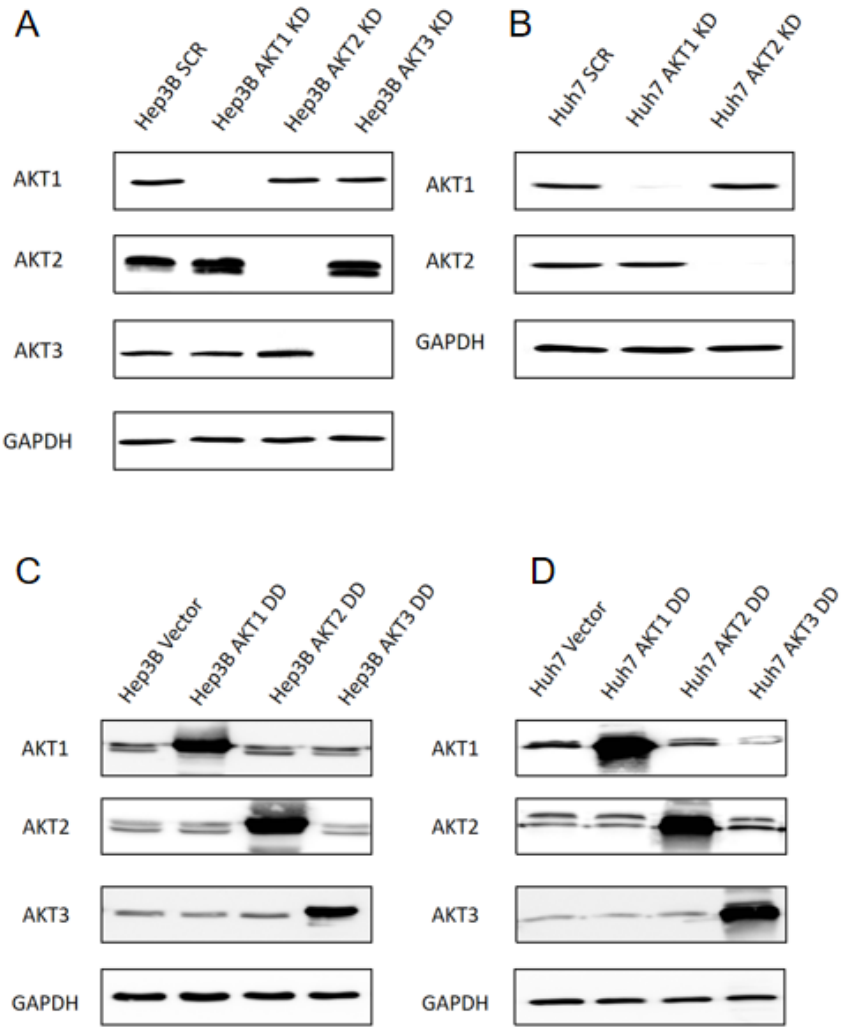

Figure 1. Stable knockdown or overexpression of AKT isoforms in HCC cell lines. Knockdown of AKT isoforms was conducted by lentiviral transduction with AKT-isoform-specific shRNAs in Hep3B (**A**) or Huh7 (**B**) cells. Overexpression of AKT1, AKT2 and AKT3 isoforms upon transduction with constitutively activated AKT lentiviral vectors in Hep3B (**C**) or Huh7 (**D**) cells. Protein expression levels of AKT isoforms were examined by Western blot analysis using AKT-isoform-specific antibodies. The experiments were repeated three times and the representative results are shown.

2.2. The Knockdown of AKT1, AKT2 and AKT3 Inhibits the Oxygen Consumption Rate of HCC Cells

We first determined the proliferative behavior of HCC cells with AKT isoform knockdowns using the IncuCyte Zoom live cell imaging system. The knockdown of AKT1 and knockdown of AKT2 decreased the proliferation of both HCC cells, i.e., Hep3B and HuH7, whereas the knockdown of AKT3 did not show a significant alteration in proliferation in comparison to the SCR control in Hep3B cells (Supplementary Figure S3). We determined the real-time state of the oxygen metabolism profiles of AKT1, AKT2 and AKT3 knockdown of HCC cell lines using the Seahorse XF Cell Mito Stress Test. We observed that Hep3B cell lines with AKT1, AKT2 and AKT3 knockdown exhibited lower oxygen consumption rates (OCR) than Hep3B cells after transduction with the scrambled control vector (Figure 2A). A detailed data analysis further showed that the AKT1, AKT2 and AKT3 knockdown cells

had a significantly lower basal OCR, maximal respiration and ATP production compared to the control cells. Moreover, AKT1, AKT2 and AKT3 knockdown decreased the spare respiratory capacity, which means the percentage of total oxygen consumptive capacity that is not utilized and can be reserved in reacting to raised metabolic demands (Figure 2B–E). Similarly, significantly lower basal OCR, maximal respiration and ATP production as well as spare respiratory capacity were found in Huh7 cells after the knockdown of AKT1 or AKT2 in comparison to the control Huh7 cells (Figure 3). In conclusion, the OCR parameters (basal respiration, maximal respiration, spare respiratory capacity and ATP production) were significantly reduced after each individual AKT isoform knockdown in both HCC cell lines.

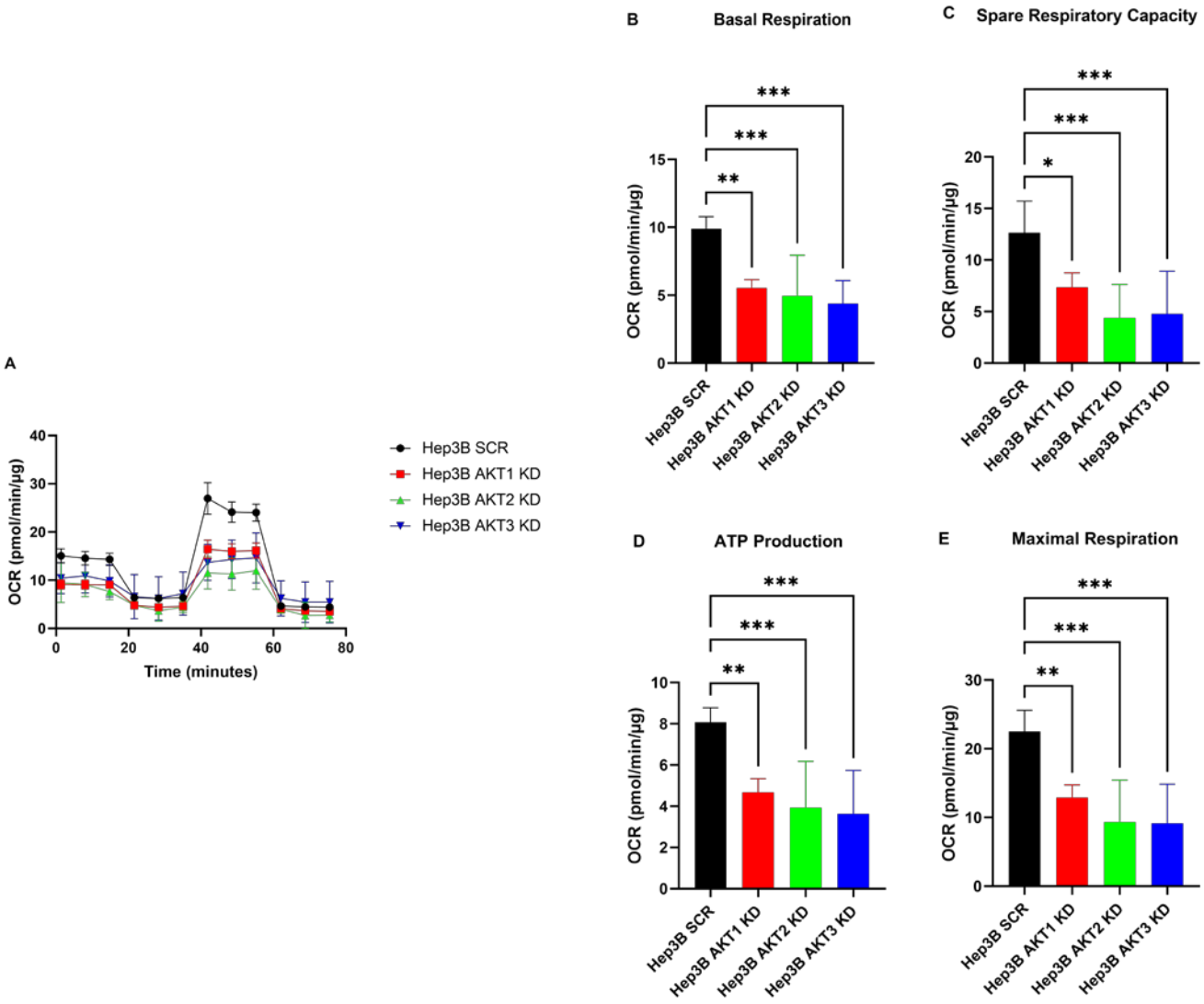

Figure 2. Effect of AKT1, AKT2 and AKT3 silencing on oxygen metabolism in the Hep3B cell line. (**A**) Knockdown of AKT1, AKT2 and AKT3 reduced the oxygen consumption rate (OCR) in HCC cell lines Hep3B. (**B**) Summary statistics of Basal rate of respiration. (**C**) Summary statistics of spare respiratory capacity. (**D**) Summary statistics of oligomycin-sensitive respiration (ATP production). (**E**) Summary statistics of uncoupled respiration (maximal respiratory capacity). The experiments were repeated six times and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

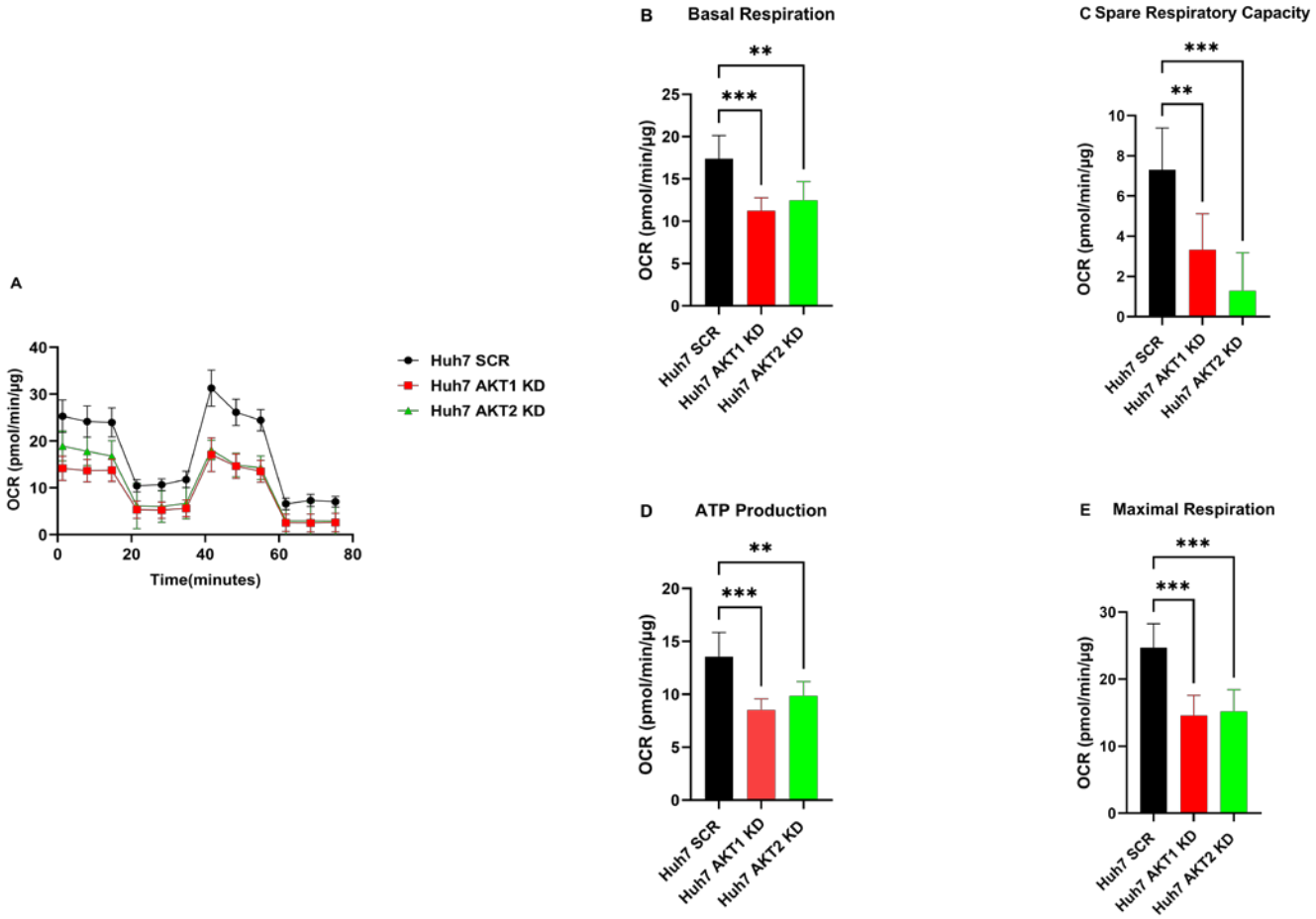

Figure 3. Effect of AKT1 and AKT2 silencing on the oxygen metabolism in Huh7 cell lines. (**A**) Knockdown of AKT1 and AKT2 reduced the oxygen consumption rate (OCR) in the HCC cell line Huh7. (**B**) Summary statistics of Basal rate of respiration. (**C**) Summary statistics of spare respiratory capacity. (**D**) Summary statistics of oligomycin sensitive respiration (ATP production). (**E**) Summary statistics of uncoupled respiration (maximal respiratory capacity). The experiments were repeated six times and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test. ** $p < 0.01$, *** $p < 0.001$.

2.3. AKT1, AKT2 and AKT3 Promote OCR in Hep3B and Huh7

Next, we analyzed whether the observed inhibitory effect on OCR by AKT isoform knockdowns can be reversed by the expression of activated AKT isoforms. We observed that Hep3B cells with activated AKT1, AKT2 and AKT3 variants exhibited higher OCR than control cells (Figure 4A). Moreover, basal OCR, maximal respiration, ATP production and spare respiratory capacity (Figure 4B–E) were significantly higher in mutant AKT1-, AKT2- and AKT3-overexpressing cells than in corresponding control cells. Likewise, we found significantly higher basal OCR, maximal respiration and ATP production, as well as spare respiratory capacity, in the constitutively activated AKT1-, AKT2- and AKT3-overexpressing Huh7 cells compared to the corresponding vector control cells (Figure 5). These data indicate that the activation of AKT1, AKT2 and AKT3 can stimulate all mitochondrial parameters, which contributes to the activation of mitochondrial oxidative phosphorylation in HCC cells.

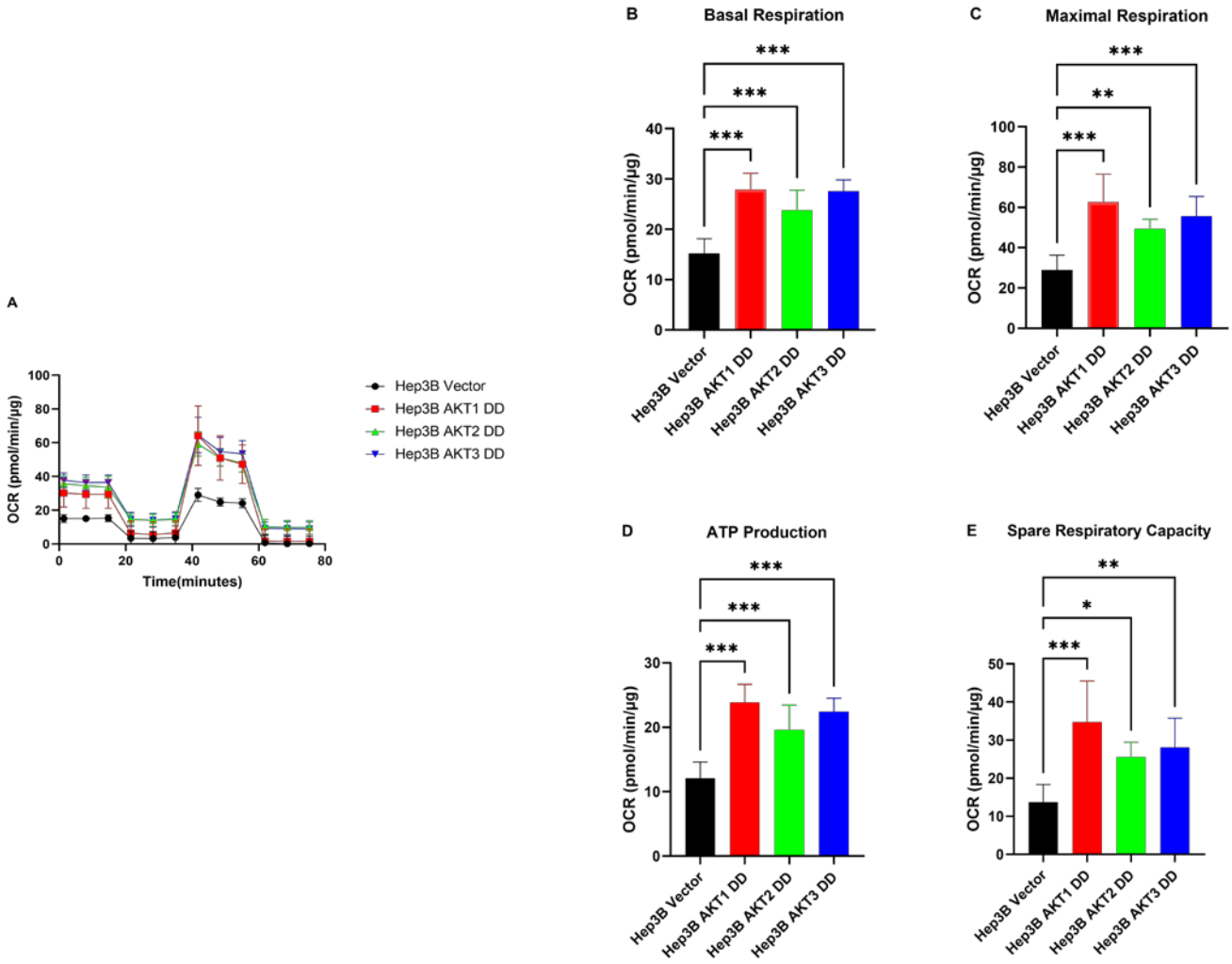

Figure 4. Effect of AKT1, AKT2 and AKT3 activation on the oxygen metabolism of the Hep3B cell lines. (**A**) Overexpression of constantly activated AKT1, AKT2 and AKT3 increased the oxygen consumption rate (OCR) in HCC cell lines Hep3B. (**B**) Summary statistics of Basal rate of respiration in Hep3B. (**C**) Summary statistics of spare respiratory capacity in Hep3B. (**D**) Summary statistics of oligomycin-sensitive respiration (ATP production) in Hep3B. (**E**) Summary statistics of uncoupled respiration (maximal respiratory capacity) in Hep3B. The experiments were repeated six times in Hep3B cells and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4. AKT Isoforms Regulate the Phosphorylation of Pyruvate Dehydrogenase

To gain mechanistic insight into the regulation of AKT-mediated oxygen consumption in HCC cells, we examined the phosphorylation of the pyruvate dehydrogenase (PDH) complex, which is a major regulator of the mitochondrial tricarboxylic acid cycle (TCA) that produces the precursors for the oxidative phosphorylation, i.e., NADH and $FADH_2$. As shown in Figure 6, the overexpression of activated AKT1, AKT2 and AKT3 mutants in Huh7 cells resulted in decreased inhibitory phosphorylation at Ser232 (Figure 6A). Conversely, the knockdown of AKT1, AKT2 and AKT3 in Hep3B cells resulted in an increase in Ser232 phosphorylation of PDH (Figure 6B).

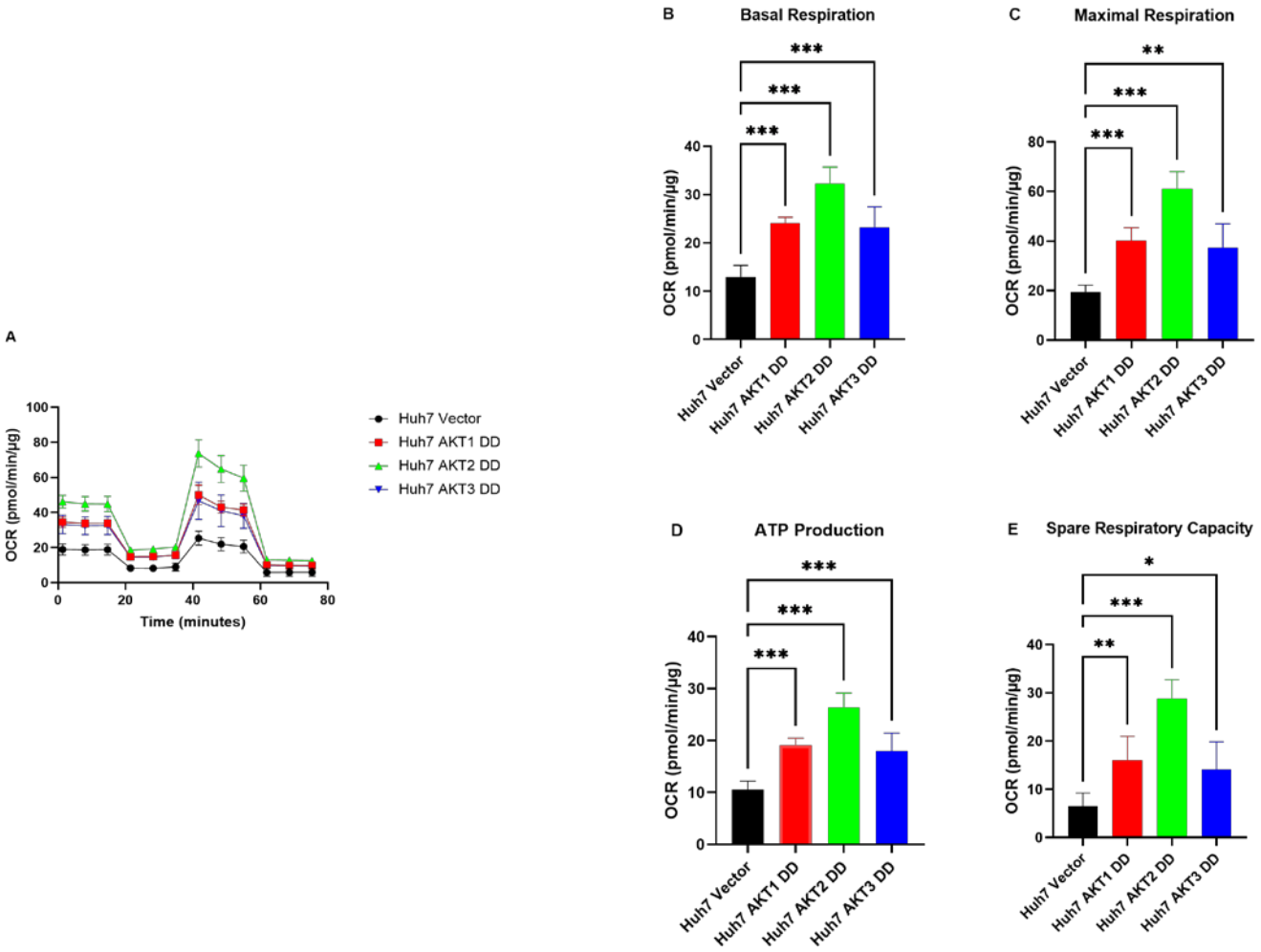

Figure 5. Effect of AKT1, AKT2 and AKT3 activation on the oxygen metabolism of the Huh7 cell lines. (**A**) Overexpression of constantly activated AKT1, AKT2 and AKT3 increased the oxygen consumption rate (OCR) in HCC cell lines Huh7. (**B**) Summary statistics of Basal rate of respiration in Huh7. (**C**) Summary statistics of spare respiratory capacity in Huh7. (**D**) Summary statistics of oligomycin sensitive respiration (ATP production) in Huh7. (**E**) Summary statistics of uncoupled respiration (maximal respiratory capacity) in Huh7. The experiments were repeated five times in Huh7 cells and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.5. The Effects of Three AKT Isoforms on ECAR

To further clarify the detailed differences in cell metabolism caused by the AKT isoforms, extracellular acidification rates (ECARs) were assessed by a cell flux analyzer. To generate a stressed metabolic phenotype that demonstrates the maximal metabolic potential of the cells, ECARs were estimated before (basal) and after (stressed) the injection of the ATP synthetase inhibitor oligomycin and the mitochondrial de-coupler FCCP. In Hep3B cells, basal and stressed ECAR were significantly lower in the AKT1, AKT2 and AKT3 knockdown as compared to the corresponding control cells (Figure 7A–C). In line with our findings in Hep3B, the knockdown of AKT1 and AKT2 decreased both basal and stressed ECAR in Huh7 cell lines (Figure 7D–F). Taken together, we demonstrated that the knockdown of AKT isoforms decreased both ECAR and OCR in HCC cell lines.

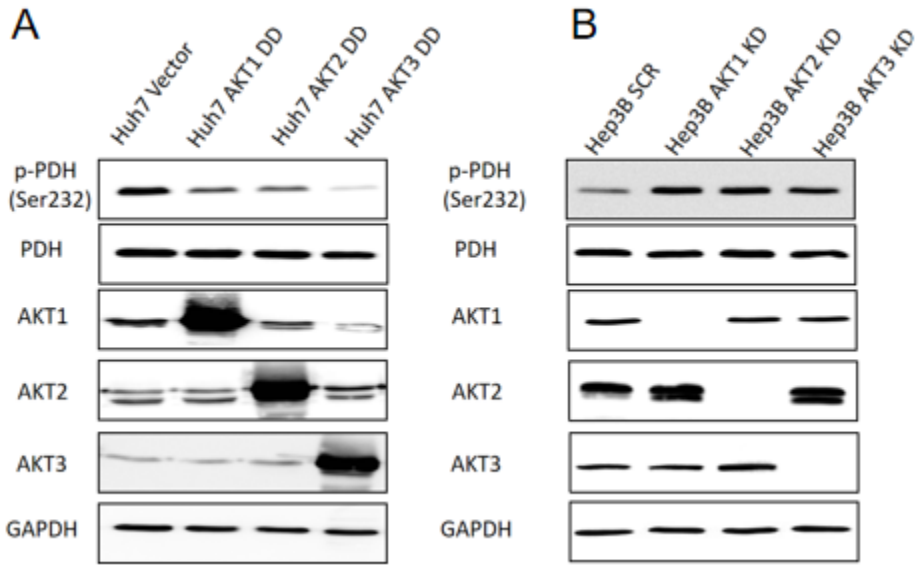

Figure 6. The impact of single AKT isoforms on the inhibitory phosphorylation of PDH at Ser232 in HCC cells. Expression of AKT isoforms, levels of pyruvate dehydrogenase (PDH) phosphorylated at the inhibitory residue Ser232 (p-PDH) as well as total PDH expression were analyzed by Western blot (**A**) after the expression of constitutively activated AKT isoforms in Huh7 cells and (**B**) following the knockdown of individual AKT isoforms in Hep3B cells. The experiments were repeated three times and the representative results are shown.

2.6. The Knockdown of AKT Isoforms Is Associated with Decreased Extracellular Lactate Production

As lactate contributes to the ECAR, we analyzed the possibility that the observed AKT-mediated effect on the ECAR is due to an AKT-dependent regulation of lactate. Therefore, lactate secretion was measured in the culture medium of Hep3B and Huh7 cells with AKT isoform knockdowns after culturing the cells for 8, 24, 48 and 72 h in fresh medium without lactate. A significant reduction in lactate secretion was found in Hep3B cells after AKT1, AKT2 and AKT3 knockdown (Figure 8A). Consistently, a significant decrease in the extracellular lactate was observed after the knockdown of AKT1 and AKT2 in Huh7 cells (Figure 8B).

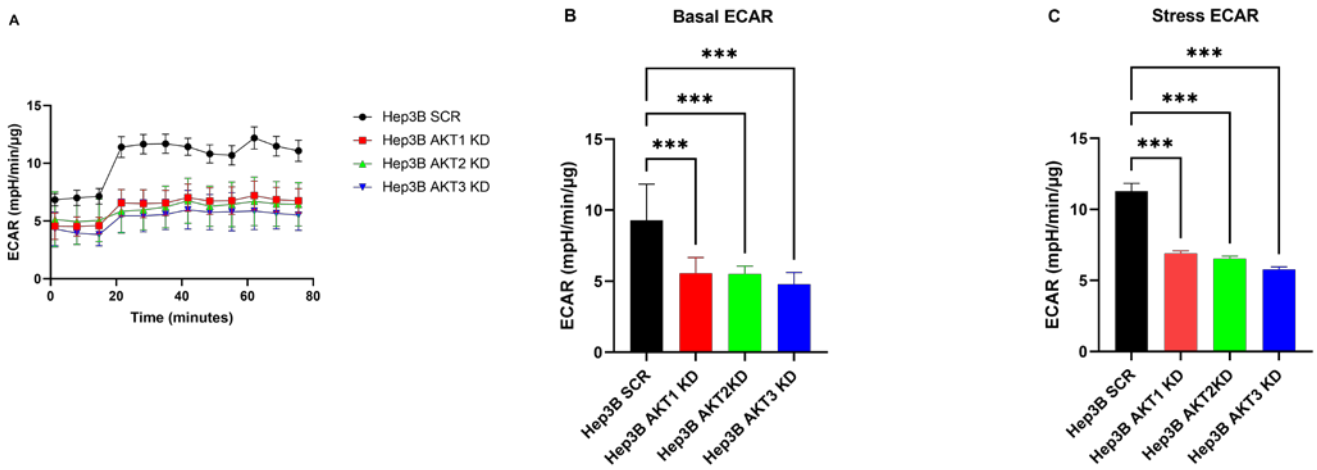

Figure 7. *Cont.*

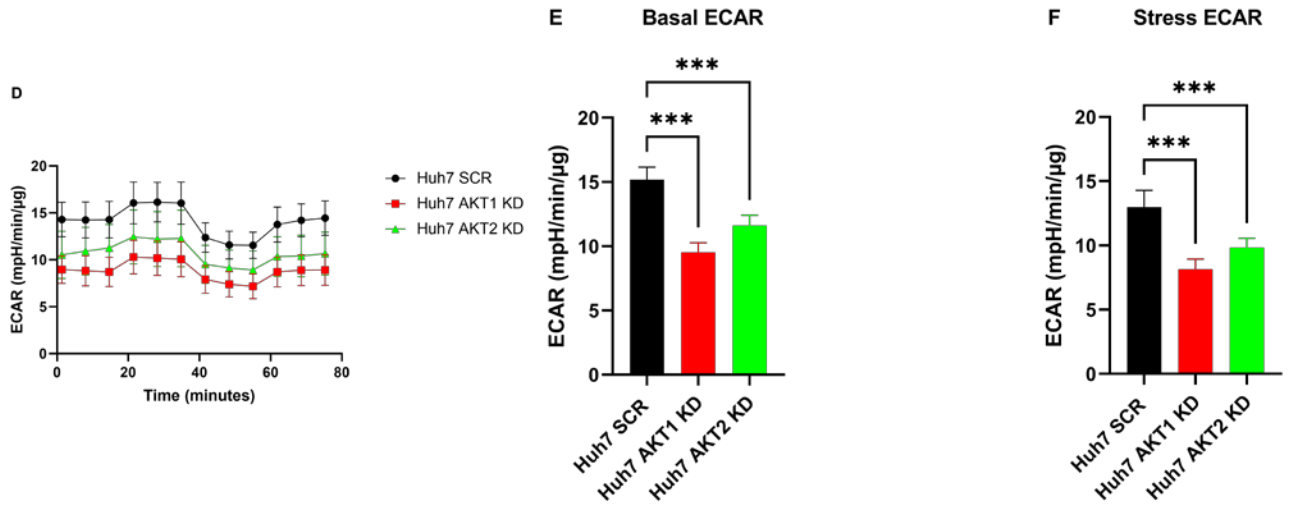

Figure 7. Knockdown of AKT1, AKT2 and AKT3 suppresses ECAR in HCC cell lines. (**A**,**D**) Extracellular acidification rate (ECAR) measured by extracellular flux analysis. (**B**,**E**) Summary statistics of basal ECAR. (**C**,**F**) Summary statistics of stress ECAR. The experiments were repeated six times and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test *** $p < 0.001$.

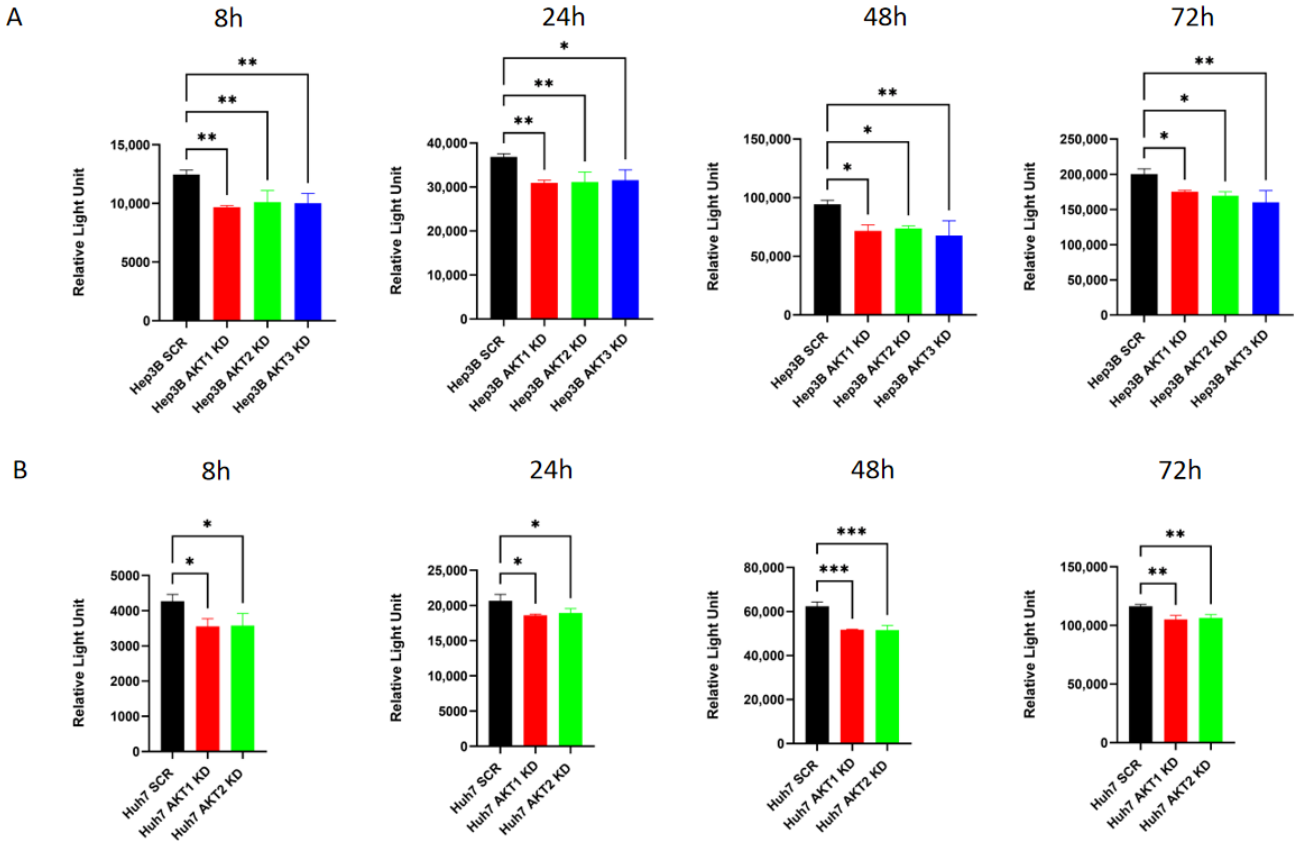

Figure 8. The effects of AKT1, AKT2 and AKT3 on lactic acid production in HCC cells were evaluated using the lactate assay. (**A**) Stable knockdowns of AKT1, AKT2 and AKT3 were generated in (**A**) Hep3B and (**B**) Huh7 cells. Thereafter, the cells were cultured in lactate-free medium for the indicated times and lactate levels were determined. The experiments were repeated three times and the pooled results are shown. *p*-values were calculated with ordinary one-way ANOVA with Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3. Discussion

In this work, we provide evidence that each of the three AKT isoforms is involved in the regulation of oxygen consumption, extracellular acidification and lactate production in human HCC cells. The overexpression of activated AKT1, AKT2 and AKT3 isoforms resulted in a higher oxygen consumption rate (OCR), ATP production, maximal respiration and spare respiratory capacity in both HCC cell lines examined. Vice versa, lentiviral vector-mediated knockdowns of each AKT isoform reduced OCR, which was associated with reduced extracellular acidification rates (ECAR) and reduced lactate production. Consistent with our findings, lactate production was inhibited by blocking AKT1/GSK3β (glycogen synthase kinase 3 beta) signaling in ovarian cancer cells after treatment with the VEGFR2 kinase inhibitor apatinib [22]. In colon cancer, it has been demonstrated that AKT2 can interact with and phosphorylate hexokinase 2 (HK2), the rate-limiting enzyme in glycolysis. The phosphorylation of HK2 at T473 was observed to raise hexokinase activity and lactic acid production [19]. Osteosarcoma cell glycolysis, proliferation, migration and invasion were suppressed by decreasing glycolysis-related proteins and migration-related proteins via the inhibition of c-MET and AKT3/mTOR [23]. In addition, the knockdown of AKT1 and AKT2 reduced lactate production in the prostatic adenocarcinoma cell line PC3 [24]. Furthermore, the targeted inhibition of AKT1 and AKT3 prevented glycolysis-related enzyme activation, significantly blocked the production of lactate and diminished the migration and invasion of chemoresistant colon cancer cells [25]. All these data are consistent with our findings in HCC cells, where the knockdown of each of the three AKT isoforms reduced lactate production.

Moreover, we also found that the knockdown of AKT1, AKT2 or AKT3 can decrease the OCR, and the activation of each of the three AKT isoforms can increase OCR in HCC cell lines. Consistently, it has been reported that the inhibition of AKT signaling can decrease the OCR in gastric cancer and esophageal cancer [26,27]. Vice versa, the activation of AKT has been shown to increase the OCR in different types of cancer cell lines such as Rat1a, HEK293, glioblastoma cell line U251 and ovarian cancer cells lines TOV112D and TOV21G [28,29], in line with our results in HCC presented in this study.

Current data suggest an association between upregulated mitochondrial metabolism and carcinogenesis, but the potential molecular mechanisms are still unclear. PDH is a gatekeeper multiprotein complex that catalyzes the conversion from pyruvate to acetyl coenzyme A (acetyl CoA), thereby regulating mitochondria respiration. Additionally, the dephosphorylation of PDH activates its function, but the phosphorylation of PDH at distinct sites blocks its activity [30,31].As the gatekeeper complex for mitochondria respiration, inhibiting PDH activity by the upregulation of its phosphorylation through the downregulation of PDH phosphatase expression was validated and shown to decrease the OCR in glioblastoma [32]. Our study demonstrates that the knockdown of AKT1, AKT2 and AKT3 can increase the inhibitory phosphorylation of PDH at Ser232, while the expression of activated variants of each of the three AKT isoforms can decrease PDH phosphorylation. These data strongly suggest that the AKT-dependent regulation of the oxygen metabolism occurs via AKT-mediated PDH phosphorylation in HCC cells. Because this phosphorylation of PDH occurs in opposition to the AKT kinase activity (knockdown vs. overexpression of activated mutants), we postulate that AKT phosphorylates another protein which then induces the dephosphorylation of PDH at Ser232. A candidate protein might be the PDH phosphatase, which dephosphorylates PDH at this specific residue.

Moreover, phosphorylation at other residues may also add to the post-translational PDH regulation. Cerniglia et al. reported that the genetic knockdown of AKT1 reduced the oxygen consumption rate in human head and neck cancer cell lines in vitro by 30% to 40% and increased the phosphorylation level of the PDH complex at Ser293, which again suppresses PDH activity [33]. In contrast, phosphorylation at Ser264 was unchanged following the inhibition of AKT1 in the prostate cancer cell line PC-3 [24]. Taken together, while our study strongly suggests that all AKT isoforms negatively affect the activity of PDH through phosphorylation at serine 232 in HCC cell lines, PDH is also regulated by

phosphorylation at alternative sites by both AKT and other kinases which might contribute to the metabolic reprogramming towards glycolysis in cancer.

In the 1920s, Otto Warburg demonstrated that cultured tumor tissues show high lactate secretion rates, even in the presence of oxygen (aerobic glycolysis), which is also referred to as the Warburg effect [34]. The Warburg effect was observed in tumor cells and other proliferating cells. This augmentation of aerobic glycolysis in cancer cells is frequently thought to occur together with damage to mitochondrial respiration [35]. These classical notions considered that respiration is impaired in highly glycolytic tumor cells. However, our data demonstrate that the knockdowns of each of the three AKT isoforms can also reduce lactate production in Hep3B and Huh7 cell lines (Figure 9). Recently, several studies have suggested that oxidative respiration is not reduced or even increased in a large number of tumor cells despite high levels of aerobic glycolysis [36,37]. Accordingly, there is a widespread agreement with Warburg's observance that tumor cells have promoted glycolysis; nevertheless, they do still retain oxidative phosphorylation [38]. Moreover, an investigation into hematopoietic and glioblastoma cell lines revealed that the constitutive activation of AKT enables an increased glycolytic rate without changing oxygen consumption [39]. However, as we only analyzed the OCR, ECAR and lactate in HCC cell lines, the other metabolic substrates in the HCC should be confirmed in the future.

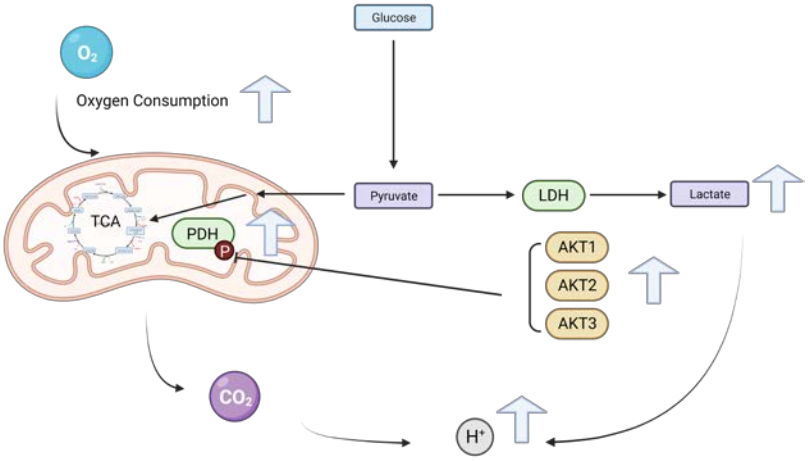

Figure 9. Model of the regulation of oxygen consumption and lactate production by the three AKT isoforms. AKT1, AKT2 and AKT3 increase oxygen consumption in HCC cells by a molecular mechanism leading to dephosphorylation of PDH at the inhibitory regulatory Ser232. The upregulation (blue arrows) of metabolic changes compared with control cells are shown.

Since 2020, for advanced stage HCC, the combination of VEGF inhibitors+ ICI or ICI combination (anti-CTLA + anti-PD-L1) therapy have become the first line therapy. Nonetheless, the usage of novel ICIs and TKIs improve HCC outcomes but the response rates are still low [40].

Drug resistance, which particularly causes the low response rate to HCC treatment, may be attributed to the following reasons. Some TKIs can target several kinase pathways. Consequently, they can also simultaneously or consecutively activate additional regulators and compensatory signaling transduction pathways—for instance, PI3K/AKT and tumor hypoxia—leading to acquired resistance [41]. As for ICIs, they are effective only in a subset of patients due to the many mechanisms that tumors adopt to blunt anti-tumor immunity inflicted by the tumor microenvironment (TME), such as insufficient vascularization, hypoxia, nutrient shortage, lactate accumulation and microenvironment acidification [42]. Sustained sorafenib treatment leads to the reduction in microvessel density, which can promote intratumoral hypoxia [41]. The inhibition of AKT can reduce hypoxia and may thereby reduce the drug resistance of HCC cells. Lactic acid in the tumor microenvironment results in immune cell de-differentiation and the suppression of immune effector cell proliferation [43]. Importantly, the suppression of all three AKT isoforms could reduce

acidification in the HCC microenvironment and thereby may decrease the drug resistance against ICIs like atezolizumab. Thus, targeting the distinctive cross-talk between tumor cells and the tumor microenvironment bears as a promising method to restrain HCC progression and to diminish the immunosuppressive pressure mediated by the hypoxic/acidic metabolism, specifically regarding the potential combination of this strategy with the first-line combination of ICI and TKI anti-cancer therapy [44]. The pan-AKT inhibitor capivasertib, an orally bioavailable small-molecule inhibitor of all three AKT isoforms, has been newly approved by the Food and Drug Administration (FDA) for hormone receptor (HR)-positive, HER2-negative locally advanced or metastatic breast cancer, and might also be considered as a future therapeutic approach for HCC treatment [45].

4. Materials and Methods

4.1. Cell Culture and Culture Conditions

The tumor cell lines Hep3B and Huh7 (human hepatocellular carcinoma) were kindly provided by Prof. Dr. Hans Will from the Leibniz Institute of Virology, Hamburg, Germany. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (#41965-039, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (FCS) (#26140-079, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin mix (#15140-122, Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All cells were tested for mycoplasma contamination regularly. The cells were passaged twice a week, utilizing trypsin/EDTA solution, washed and resuspended in the respective cell culture medium.

4.2. Stable AKT Isoform-Specific Knockdown and Ectopic Expression of Activated AKT Isoforms

pLKO.1-puro vectors encoding either AKT1, AKT2, AKT3 or scrambled shRNA were purchased from Sigma-Aldrich (Taufkirchen, Germany). Activated pseudophosphorylated mutations (AKT-DD) of each AKT isoform (e.g., AKT1-T308D/S473D) were constructed by site-directed mutagenesis and stably expressed in Hep3B and Huh7 cells by lentiviral transduction. The generation of pseudotyped lentiviral particles and cell transductions were performed as previously described [15]. Transduced cells were selected with puromycin (Sigma-Aldrich, Taufkirchen, Germany) in DMEM medium (final concentration: 1.5 µg/mL).

4.3. Western Blot Analysis and Densitometric Quantification

The tumor cell lysates were prepared in NP40 lysis buffer containing Tris 1% NP-40 (#98379, Sigma-Aldrich, St. Louis, MO, USA), 2% Aprotinin (#A162.2, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 2 mM EDTA (#E5134, Sigma-Aldrich, St. Louis, MO, USA), 50 mM NaF (#S7920, Sigma-Aldrich, St. Louis, MO, USA), 10 mM NaPPi (#S6422, Sigma-Aldrich, St. Louis, MO, USA), 10% Glycine (#3908, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 1 mM Sodium orthovanadate (#S6508, Sigma-Aldrich, St. Louis, MO, USA) and 1 mM PMSF (#10837091001, Sigma-Aldrich, St. Louis, MO, USA). Thereafter, the proteins were blotted onto a nitrocellulose membrane and incubated with specific primary antibodies against AKT1 (#2938S, Cell Signaling Technology Inc., Danvers, MA, USA), AKT2 (#5239S, Cell Signaling Technology Inc., Danvers, MA, USA), AKT3 (#8018S, Cell Signaling Technology Inc., Danvers, MA, USA), PDH (#2784S, Cell Signaling Technology Inc., Danvers, MA, USA), p-PDH Ser232 (#15289S, Cell Signaling Technology Inc., Danvers, MA, USA) and GAPDH (#G0622, Santa Cruz Biotechnology Inc., Dallas, TX, USA). Afterwards, the membrane was incubated with the appropriate secondary antibodies against mouse-IgG (#7076, Cell Signaling Technology) or rabbit-IgG (#7074, Cell Signaling Technology). The protein expression was analyzed using the LAS-4000 Imager from Fuji (Raytest, Straubenhardt, Germany). Densitometric quantification was carried out using AIDA Image Analyser Software Version 3 (Elysia-raytest GmbH, Straubenhardt, Germany).

4.4. Seahorse Metabolic Flux Measurement

The XF-96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine the OCR and ECAR. Briefly, 20,000 Hep3B and Huh7 cells per well were plated in XF96-well microplates (Agilent Technologies, Santa Clara, CA, USA) with Dulbecco's Modified Eagle Medium (DMEM) (#41965-039, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplied with 10% fetal calf serum (FCS) (#26140-079, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin mix (#15140-122, Thermo Fisher Scientific Inc., Waltham, MA, USA) as well as 1.5 µg/mL Puromycin. Then, the cells were allowed to settle down at 37 °C, 5% CO_2 overnight. The hydrate cartridge (Agilent Technologies, Santa Clara, CA, USA) added 200 µL ddH_2O per well and then the cartridge was maintained at 37 °C in a CO_2-free incubator overnight. Three hours before the OCR and ECAR determinations, the hydrate cartridge added 200 µL XF Calibrant (#100840 Agilent Technologies, Santa Clara, CA, USA) per well and was then maintained at 37 °C in a CO_2-free incubator. One hour before OCR and ECAR determination, the cell culture medium was carefully removed, wells were washed with 200 µL of assay medium, and, finally, 200 µL of assay medium was added. The assay medium consisted of XF DMEM medium pH 7.4, 500mL (#103575-100 Agilent Technologies, Santa Clara, CA, USA) supplemented with 1 mM sodium pyruvate (#S8636, Sigma Aldrich, St. Louis, MO, USA), 2 mM glutamine (#25030-081, Thermo Fisher Scientific Inc., Waltham, MA, USA), and 10 mM glucose (#A24940-10, Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells in XF96-well microplates were cultured for 60 min at 37 °C in a CO_2-free incubator before the start of the measurement. During OCR and ECAR analysis, cells were treated with 1.0 µM oligomycin (#75351, Sigma Aldrich, St. Louis, MO, USA), 0.5 µM fluorocarbonyl cyanide phenylhydrazone (FCCP) (#15218, Cayman Chemical, Ann Arbor, Michigan, USA), 0.5 µM rotenone (#13955, Cayman Chemical, Ann Arbor, Michigan, USA) and 0.5 µM antimycin A (#A8674, Sigma Aldrich, St. Louis, MO, USA). OCR and ECAR data were calculated using the software (Wave Desktop and Controller 2.6, Agilent Technologies, Santa Clara, CA, USA) provided by the manufacturer.

4.5. Bradford Assay for Normalization of Seahorse Assay Data

The normalization of seahorse assay was conducted by Bradford assay. To prepare the cell samples for the seahorse measurement, all media from the wells of the seahorse plate were removed by aspiration. The wells of the seahorse plate were gently washed with 100 µL PBS, and thereafter, the PBS was completely aspirated. Then, 20 µL of lysis buffer containing 0.1% Triton X-100 (#T8787, Sigma Aldrich, St. Louis, MO, USA) was added to each well. Empty seahorse wells served as controls to evaluate a potential interference of the lysis buffer in the protein assay. Bradford Reagent (#500-0006, BioRad, Hercules, CA, USA) was diluted in a ratio of 3:10 in ddH_2O. Then, 180 µL of diluted Bradford reagent was added to each well of the seahorse plate and mixed well. The total volume in each well was 200 µL. The absorbance was measured at 575 nm by using a microplate reader (Tecan, Männedorf, Switzerland). Thereafter, the zero-protein blank average was subtracted from the absorbance of all wells. The protein concentration of each well in the seahorse assay was calculated based on the absorbance value from the microplate reader.

4.6. Lactate Determination

Hep3B and Huh7 cells were plated at a density of 5000 cells/well into a 96-well flat-bottomed cell culture plate (Greiner Bio-One, Kremsmünster, Austria). For Hep3B and Huh7 cells' cultivation in this assay, Dulbecco's Modified Eagle Medium (DMEM) (#41965-039, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplied with 10% dialyzed fetal calf serum (d-FCS) (#A3382001 Thermo Fisher Scientific Inc., Waltham, MA, USA), 5 mM glucose (#A24940-10, Thermo Fisher Scientific Inc., Waltham, MA, USA), 2 mM glutamine (#25030-081, Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin mix (#15140-122, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used. At the indicated time points, 2.5 µL of the medium from the well was collected,

diluted in 97.5 µL PBS and stored at −20 °C. According to the technical manual from Lactate-Glo Assay (J5021, Promega, Madison, WI, USA), 25 µL of the sample and 25 µL lactate detection reagent was mixed in 384-well plates (Greiner Bio-One, Kremsmünster, Austria) and incubated for 60 min at room temperature. The luminescence was measured using a microplate reader (Tecan, Männedorf, Switzerland).

4.7. Statistics

All statistical analyses were performed with implemented functions in GraphPad Prism 8.4.1 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was tested using unpaired two-tailed Student's t-test when comparing two groups and using one-way ANOVA with Dunnett's post hoc test when comparing more than two groups. Results were considered significant if $p < 0.05$. p values were encoded into asterisks, as follows: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

5. Conclusions

In summary, our data demonstrate that the targeting of a single AKT isoform was sufficient to inhibit the oxidative respiration and lactate production in HCC cell lines, underlining the essential role of all three isoforms in the metabolic regulation in HCC tumor cells. By reducing oxygen consumption and simultaneously reducing lactate production, AKT inhibition could help to reduce tumor hypoxia and the acidification of the tumor microenvironment. Therefore, AKT could be a valuable target to reduce or even reverse pathological changes in the metabolism of HCC patients, which may be useful in combination with other therapeutic approaches.

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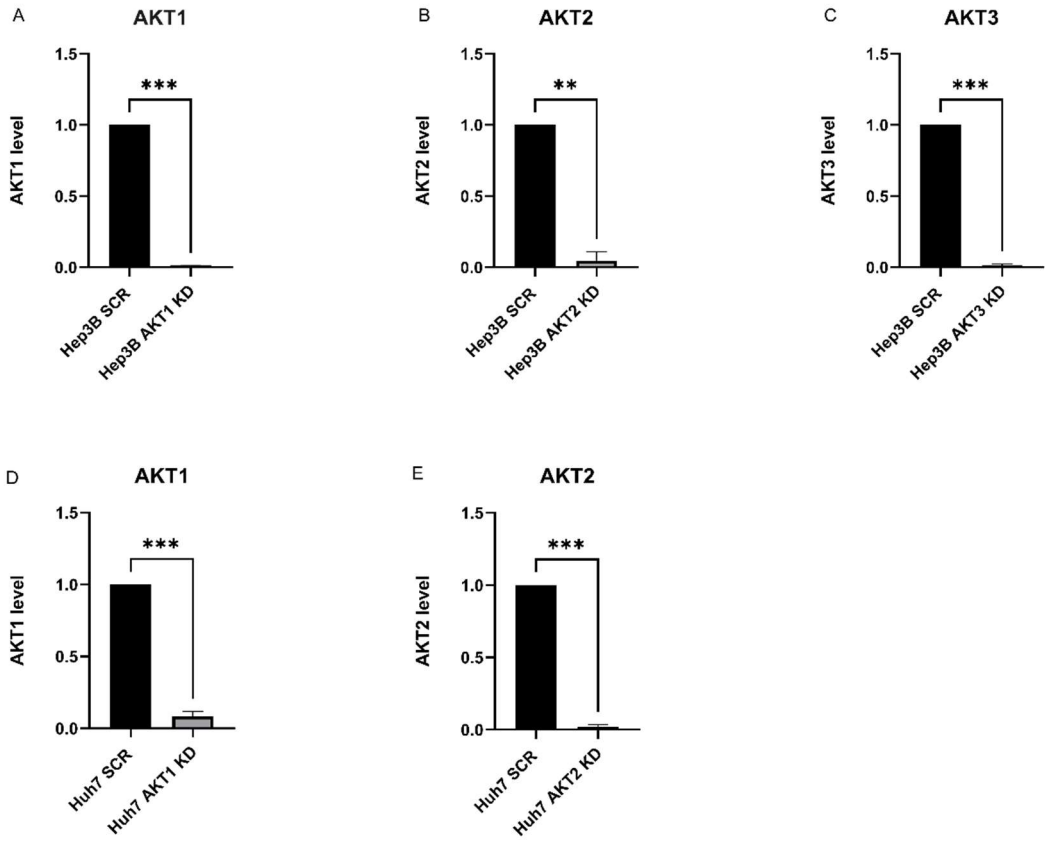
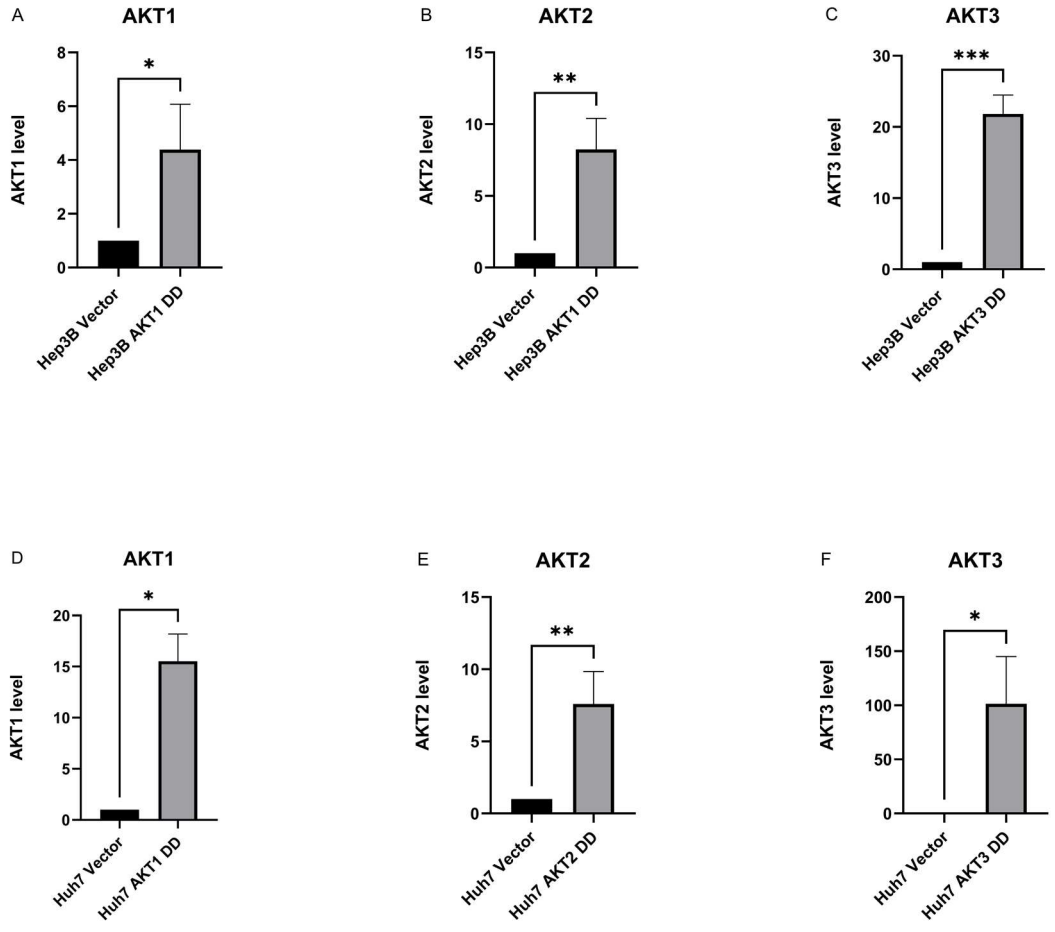
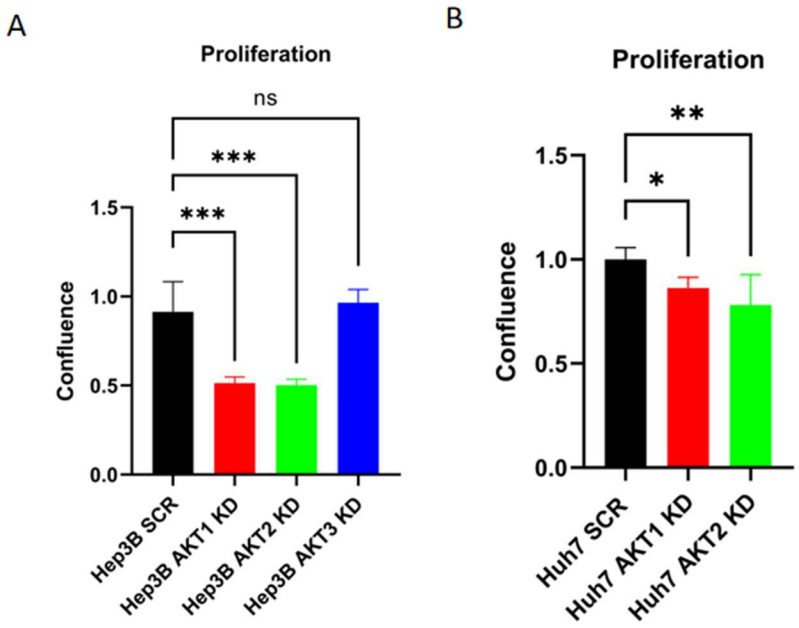


Supplementary Figure S1 Relative levels of knockdown of AKT1, AKT2 and AKT3 in HCC cell lines. Lentiviral mediated knockdowns of AKT isoforms in Hep3B and Huh7 cell lines were compared to the parental cells by Western blot analysis. **(A-C)** Quantification of AKT isoform performed from Western blot analysis of triplicates and normalized to intensity of Hep3B parental cells signals. **(D-E)** Quantification of AKT isoforms performed from Western blot analysis of triplicates and normalized to intensity of Huh7 parental cells signals. Bars indicate mean with SD; ** = $p < 0.01$; *** = $p < 0.001$..



Supplementary Figure S2 Relative levels of overexpression of AKT1, AKT2 and AKT3 in HCC cell lines. Hep3B and Huh7 cells were transduced with lentiviral vectors with the constitutively activated AKT isoforms (AKT-DD) and overexpression was compared to HCC parental cells. Expression levels were quantified by Western blot analysis of triplicates. **(A-C)** Quantification of AKT isoform overexpression in Hep3B cells **(D-F)** Quantification of AKT isoform overexpression in Huh7 cells. Bars indicate mean with SD; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$..



Supplementary Figure S3 Effects of knockdowns of AKT isoforms on the proliferation of HCC cells. (A) Hep3B cells harboring AKT isoform knockdowns were seeded in a 96-well plate and confluence was monitored with the IncuCyte live cell imaging system. (B) Huh7 cells with AKT isoform knockdowns were seeded in a 96-well plate and confluence was monitored with the IncuCyte live cell imaging system. Bars indicate mean with SD; * = $p < 0.05$, ** = $p < 0.01$.*** = $p < 0.001$

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Cc:Smit, Daniel <d.smit@uke.de>; Nadezhda V. Popova <npopova@gmail.com>; Horn, Stefan <s.horn@uke.de>; Velasquez, Lis Noelia <l.velasquez@uke.de>; Huber, Samuel <s.huber@uke.de>; Jücker, Manfred <juecker@uke.de>; IJMS Editorial Office <ijms@mdpi.com>; Krzysztof Lustofin <krzysztof.lustofin@mdpi.com>;

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Authors: Ling-Yu Tian, Daniel J. Smit, Nadezhda V. Popova, Stefan Horn, Lis Noelia Velasquez, Samuel Huber, And Manfred Jücker *
Received: 15 Jan 2024
E-mails: l.tian.ext@uke.de, d.smit@uke.de, npopova@gmail.com, s.horn@uke.de, l.velasquez@uke.de, s.huber@uke.de, juecker@uke.de
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3. Presentation of the Publication

3.1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world and represents a major challenge to global healthcare. HCC is the sixth leading cause of cancer death in Western countries. The similarity between incidence and mortality highlights the poor prognosis of HCC[1]. The main risk factors for HCC include hepatitis, viral infections, autoimmune diseases, drug and non-drug toxicity, and non-alcoholic fatty liver disease (NAFLD), and the development of HCC is closely related to the presence of chronic liver disease. The global incidence varies due to regional differences in risk factors[2].

NAFLD is emerging as an important cause of HCC in developed regions. Prospective studies have elucidated the NAFLD as a risk factor for HCC; retrospective studies have shown that metabolic syndrome, including diabetes and obesity, is strongly associated with HCC in patients with NAFLD[3, 4]. Diabetes is an independent risk factor for HCC, for patients with a high baseline body mass index (BMI) having a HCC mortality rate five times that of patients with a normal BMI[3-5]. Smoking is associated with increased risk of HCC, whereas coffee intake is associated with decreased risk[6, 7]. Based on these findings, there is growing evidence that metabolic factors are strongly associated with HCC incidence[2]. Recently, the impact of metabolic dysregulation on HCC carcinogenesis has attracted increasing research attention[8].

Oncogenic signaling pathways like phosphoinositide 3-kinase(PI3K)/AKT/mammalian target of rapamycin (mTOR) transduction pathway, play pivotal roles in the progression of HCC[9]. The signaling cascade of PI3K/AKT/mTOR governs critical cellular functions such as cell survival, metastasis, and metabolism. Dysregulation of this pathway can occur through various mechanisms, including genomic changes and mutations affecting PIK3CA, AKT, and mTOR, as well as the loss of tumor suppressor phosphatase and tensin homolog (PTEN)[10]. Tumors displaying activated PI3K/AKT signaling often exhibit an aggressive phenotype. The activation

of PI3K/AKT signaling also considered as a significant risk factor for early recurrence and unfavorable prognosis among patients diagnosed with HCC[11, 12].

The PI3K/AKT signaling network exerts various downstream effects on cellular metabolism by directly regulating nutrient transporters and metabolic enzymes, as well as by controlling the expression of key proteins in metabolic pathways through transcription factors[13]. In HCC patients with increased uptake of 2[18F] fluoro-2-deoxy-D-glucose (FDG), some downstream molecules of the PI3K/AKT/mTOR signaling pathway are particularly increased, indicating that activation of this pathway may regulate the metabolism of HCC[14].

The AKT family consists of three members (AKT1/PKBα, AKT2/PKBβ, and AKT3/PKBγ), which exhibit high structural homology. However, studies involving AKT isoform-specific gene knockout mice suggest that the diversity of AKT signaling transduction may be partially attributed to the distinct functions of the three members within the AKT family[15]. AKT1 gene knockout (KO) mice exhibit growth restriction but do not show metabolic abnormalities. AKT2 gene KO mice display glucose intolerance and manifest systemic insulin resistance. However, AKT3 gene KO mice have reduced brain volume but maintain glucose homeostasis[16]. In tumor metabolism research, the activation of AKT1 is associated with the accumulation of aerobic glycolysis metabolites in prostate cancer[17]. Furthermore, AKT2 plays a significant role in the occurrence and progression of colorectal cancer. Studies have found that AKT2 can interact with and phosphorylates the glycolytic rate-limiting enzyme hexokinase 2 (HK2). Additionally, serine phosphorylation of HK2 significantly enhances its catalytic activity and augments glycolysis. Mechanistically, AKT2 phosphorylation of HK2 at the T473 site increases hexokinase activity and lactate production, demonstrating that AKT2 phosphorylation of hexokinase 2 enhances its activity and lactate production in colorectal cancer[18]. AKT plays a crucial role in cell proliferation and survival. However, the isoform-specific roles of AKT in mitochondrial function have not been thoroughly investigated to date. Studies have found that stable knockdown of AKT isoforms in human bladder cancer cells leads to a significant increase in mitochondrial mass in AKT1 and AKT3 knockdown cells, while AKT2 knockdown does not produce similar effects. Interestingly, AKT3 knockdown

also results in severe mitochondrial structural defects, increased doxorubicin-induced senescence, and impaired cell proliferation in galactose medium, accompanied by a significant decrease in mitochondrial oxygen consumption rate in AKT3 knockdown cells. Reduced mitochondrial respiration was also observed in A549 lung cancer cells with AKT3 deficiency[19]. These findings suggest that AKT isoforms play distinct roles in mitochondrial function, with AKT3 being crucial for normal mitochondrial respiration in human cancer cells[19]. The liver plays a crucial role in maintaining metabolic balance in the human body and is involved in regulating various metabolic processes. However, the functional roles of the three AKT isoforms in metabolic regulation in HCC have never been simultaneously studied[20].

In summary, abundant evidence indicates that abnormal expression of the three AKT isoforms is present in various common human malignancies, and these dysregulated isoforms play a crucial role in tumor metabolic regulation. As HCC is a tumor closely associated with metabolic factors, the three AKT isoforms may play important roles for the metabolism in HCC. Therefore, my study primarily investigates the impact of each of the three AKT isoforms i.e. AKT1, AKT2, and AKT3, on the metabolic behavior of HCC cells. Furthermore, employing the mass spectrometry approach to identify downstream regulatory proteins of AKT1, AKT2, and AKT3 aims to reveal their potential mechanisms of action. This research aims to provide a new theoretical basis for elucidating the pathogenesis of HCC and the role of AKT isoforms in HCC, ultimately offering insights into biological targets for HCC treatment.

3.2 Results

In order to investigate the effect of the three AKT isoforms on the metabolic phenotype of HCC cell lines, it was first necessary to examine the basal expression levels of the three AKT isoforms, AKT1, AKT2 and AKT3, in the HCC cell lines. For this purpose, two common HCC cell lines (human hepatocellular carcinoma cell lines Hep3B and Huh7, kindly provided by Prof. Hans Will, Leibniz Institute of Virology, Hamburg, Germany) were selected for Western Blot (WB) experiments. WB experiments showed that Hep3B cells expressed all three AKT isoforms, AKT1, AKT2, and AKT3 whereas Huh7 cells expressed AKT1 and AKT2, but very low levels

of AKT3. Based on these results, knockdowns of all three AKT isoforms were performed in the Hep3B cell line and AKT1 and AKT2 knockdowns in the Huh7 cell line. In addition, overexpression of all the three AKT isoforms were performed in Hep3B and Huh7 cells in order to investigate the effects of the three AKT isoforms on the metabolic phenotypes of HCC cells. In Hep3B cells, the knockdown efficiencies of AKT1, AKT2 and AKT3 were 98.9%, 95.3% and 98.9%, respectively. In Huh7 cells, the knockdown efficiencies of AKT1 and AKT2 were 91.4% and 98.2%, respectively.

Increased aerobic glycolysis is an important hallmark of cancer metabolism, and we used the Seahorse XF Cell Mitochondrial Stress Test assay to determine the oxygen consumption rate of the stable transduced cell lines of Hep3B and Huh7 cells in order to analyze the effect of AKT isoforms on aerobic respiration in HCC cells. We observed that the oxygen consumption rate (OCR) was reduced after knockdown of AKT1, AKT2 and AKT3 in Hep3B cells in comparison to the Hep3B control cells. Detailed data analysis further showed that AKT1, AKT2 and AKT3 knockout cells had significantly lower basal OCR, maximum respiration and ATP production compared to control cells. In addition, AKT1, AKT2 and AKT3 knockout cells had reduced spare respiratory capacity, which refers to the percentage of the total unutilized oxygen-consuming capacity that can be used to respond to increased metabolic demands. The same results were also observed in the Huh7 cell line.

To further investigate the impact of the three AKT isoforms on the metabolic roles of Hep3B cells, stable overexpression of constitutively activated forms of each of the three AKT isoforms was performed in Hep3B and Huh7 cells. We observed that Hep3B cells with activated variants of AKT1, AKT2, and AKT3 exhibited higher OCR compared to vector transduced control cells. Additionally, baseline OCR, maximal respiratory capacity, ATP production, and spare respiratory capacity in cells overexpressing AKT1, AKT2, and AKT3 were all significantly higher than their respective control cells. Similarly, we found that basal respiration, maximal respiration, ATP production, and spare respiratory capacity in Huh7 cells overexpressing constitutively active AKT1, AKT2, and AKT3 were significantly higher than their control cells. These data indicate that each AKT isoform can upregulate oxygen metabolism in HCC cells.

Pyruvate dehydrogenase (PDH) complex is the key regulator of the mitochondrial tricarboxylic acid (TCA) cycle, generating precursors for oxidative phosphorylation (i.e., NADH and $FADH_2$). We found that overexpression of activated AKT1, AKT2, and AKT3 in Huh7 cells results in reduced inhibitory phosphorylation at Ser232. Conversely, knockdown of AKT1, AKT2, and AKT3 in Hep3B cells leads to increased phosphorylation of PDH at Ser232.

To further elucidate the differences in cellular metabolism induced by AKT isoforms, extracellular acidification rates (ECAR) were assessed using a Seahorse Analyzer. In order to depict the maximal metabolic potential of the cells under stress conditions, ECAR was estimated before (basal) and after (stimulated) injection of ATP synthase inhibitor oligomycin and mitochondrial uncoupler FCCP. In Hep3B cells, basal and stimulated ECAR were significantly reduced in AKT1, AKT2, and AKT3 knockdown cells in comparison to control cells. Consistent with our findings in Hep3B cells, knockdown of AKT1 and AKT2 also decreased basal and stimulated ECAR in the Huh7 cell line. In summary, these results demonstrate that knockdown of AKT isoforms reduces both ECAR and OCR in HCC cell lines.

As lactate production contributes to changes in ECAR, we further analyzed whether the observed AKT-mediated effects on ECAR could potentially be due to AKT-dependent regulation of lactate. Therefore, after culturing Hep3B and Huh7 cells with AKT isoform knockdowns in lactate-free fresh media for 8, 24, 48, and 72 hours, we measured lactate secretion levels. Following knockdown of AKT1, AKT2, and AKT3, there was a significant reduction in lactate secretion in Hep3B and Huh7 cells.

3.3 Discussion

In this study, the metabolic roles of each of the three AKT isoforms were analyzed in human HCC cell lines[21]. The experimental data indicate that all three AKT isoforms can upregulate the oxygen metabolism in human HCC cells. Overexpression of AKT1, AKT2, and AKT3 isoforms leads to increased OCR in both tested HCC cell lines. This is consistent with previous findings showing that AKT activation can enhance OCR in various cancer cell lines, such as HEK293, glioblastoma cell line U251, and ovarian cancer cell lines TOV112D, as observed in this study with HCC[22, 23]. We also

found that all three AKT isoforms can upregulate the ECAR and lactate production. Knockout of AKT1 and AKT2 reduces lactate production in the prostate cancer cell line PC3[24]. Targeted inhibition of AKT1 and AKT3 prevents activation of glycolysis-related enzymes, significantly inhibits lactate production, and reduces migration and invasion of chemotherapy-resistant colon cancer cells[25]. Recently, some studies have indicated that despite high levels of aerobic glycolysis, oxidative respiration in many tumor cells is not reduced and may even be increased[26, 27]. Additionally, studies have shown that the use of the AKT inhibitor MK2206 can inhibit glycolysis and oxygen consumption in the circulating breast cancer cell line MDA-MB-468 ASC[28].

PDH is a gatekeeper multiprotein complex that regulates mitochondrial respiration[29]. Our results indicate that knockdown of AKT1, AKT2, and AKT3 can increase inhibitory phosphorylation of PDH at Ser232, while overexpression of activated variants of these three AKT isoforms can decrease PDH phosphorylation. Similar results have been observed by inhibiting PDH activity in glioblastoma, which has been demonstrated to reduce OCR[30].

Of the three AKT isoforms, AKT1 is aberrantly expressed in HCC cells and is closely associated with cell proliferation, survival, metabolism, and carcinogenesis[31]. A study using immunohistochemical methods examined the expression of AKT2 in tumor and non-tumor tissues from 56 patients with HCC: high expression of AKT2 was detected in 21 cases (38%) of HCC tissues. Additionally, low expression of AKT2 was associated with histological differentiation, portal vein invasion, and tumor nodules. A multivariate analysis revealed that AKT2 is an independent prognostic marker for cancer relapse, suggesting that AKT2 is a novel independent predictor for the occurrence and progression of HCC[32]. In a study involving 130 specimens of HCC and matched adjacent non-tumorous liver tissues, upregulation of AKT1, AKT2, and AKT3 proteins was significantly associated with tumor invasiveness and poor prognosis in HCC patients[33]. Based on the collective findings, all three isoforms of AKT emerge as potential targets for the prevention and treatment of HCC[34].

Interfering with tumor metabolism is an emerging therapeutic strategy for cancers resistant to standard treatments[35]. The liver, being a vital metabolic organ, and

HCC with its rapid proliferation and exacerbated glycolysis, create a highly hypoxic environment, leading to excessive lactate secretion and acidification. These metabolic conditions promote the invasiveness of HCC and immune suppression in the tumor microenvironment[36]. Therefore, targeting the tumor metabolism is a promising approach to inhibit HCC progression and to alleviate hypoxia/acidosis-mediated immune suppression. This is particularly promising considering the potential of combining this strategy with first-line anticancer therapies such as immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs)[36].

The pan-AKT inhibitor Capivasertib is an orally bioavailable small molecule inhibitor that targets all three isoforms of AKT. It received approval from the United States Food and Drug Administration (FDA) in November 2023 for the treatment of hormone receptor (HR)-positive, HER2-negative locally advanced or metastatic breast cancer. It may also be considered as a potential approach for the treatment of hepatocellular carcinoma (HCC) in the future[37].

3.4 Material and Methods

The tumor cell lines Hep3B and Huh7 (human hepatocellular carcinoma) were generously provided by Prof. Dr. Hans Will from the Leibniz Institute of Virology, Hamburg, Germany. AKT isoform-specific knockdowns were generated by lentiviral transduction of shRNA against AKT1, AKT2 or AKT3. Western blot analysis using antibodies against AKT1, AKT2 and AKT3 were performed as described previously [38]. The XF-96 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA, USA) was used to determine the OCR and ECAR. 20,000 Hep3B or Huh7 cells per well were plated in XF96-well microplates (Agilent Technologies, Santa Clara, CA, USA) and cells were treated with 1.0 µM oligomycin (#75351, Sigma Aldrich, St. Louis, MO, USA), 0.5 µM fluor carbonyl cyanide phenylhydrazone (FCCP) (#15218, Cayman Chemical, Ann Arbor, Michigan, USA), 0.5 µM rotenone (#13955, Cayman Chemical, Ann Arbor, Michigan, USA) and 0.5 µM antimycin A (#A8674, Sigma Aldrich, St. Louis, MO, USA). OCR and ECAR data were analyzed by using the software (Wave Desktop and Controller 2.6, Agilent Technologies, Santa Clara, CA, USA). Then, activated pseudophosphorylated mutations (AKT-DD) of each AKT

isoform (e.g., AKT1-T308D/S473D) were constructed in HCC cells by lentiviral transduction. Subsequently, the OCR, ECAR and lactate production were measured as previously described[21].

Proliferation was evaluated by measuring confluence using the IncuCyte® Zoom Live Cell Analysis System (Essen BioSciences, Ann Arbor, USA). The lactate production was measured by Lactate-Glo Assay (J5021, Promega, Madison, WI, USA). The luminescence which represents the lactate secretion of the cells was measured using a microplate reader (Tecan, Männedorf, Switzerland). The statistical analysis of the data was performed using GraphPad Prism Version 8.4.1 software (unpaired two-tailed Student's t-test when comparing two groups and using one-way ANOVA with Dunnett's post hoc test when comparing more than two groups.) (GraphPad Software Inc., San Diego, USA).

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4. Zusammenfassung / Summary

Der PI3K/AKT/mTOR-Signalweg spielt eine wichtige Rolle bei der Entwicklung des hepatozellulären Karzinoms (HCC) und ist mit einem aggressiven Phänotyp, einem frühen Wiederauftreten und einer ungünstigen Prognose für HCC-Patienten assoziiert. Veränderungen im Zellstoffwechsel gelten als ein Kennzeichen von Krebs, und es hat sich gezeigt, dass der PI3K/AKT/mTOR-Signalweg an der Regulierung von Stoffwechselprozessen beteiligt ist. Die Ergebnisse dieser Studie zeigen, dass die gezielte Reduktion jeder der drei AKT-Isoformen, d. h. AKT1, AKT2 und AKT3, ausreicht, um die Sauerstoffverbrauchsrate (OCR), die extrazelluläre Ansäuerungsrate (ECAR) und die Laktatproduktion in den HCC-Zelllinien Hep3B und Huh7 zu verringern. Umgekehrt kann die Überexpression jeder der drei AKT-Isoformen die OCR, die ATP-Produktion, die maximale Atmung und die freie Atmungskapazität sowohl in Hep3B als auch in Huh7 erhöhen. Dies unterstreicht die bedeutende Rolle aller drei AKT-Isoformen bei der Regulierung des Zellstoffwechsels in HCC. Mechanistisch gesehen war die beobachtete Verringerung der OCR nach Knockdown der AKT-Isoformen mit einer erhöhten Phosphorylierung der Pyruvatdehydrogenase an Ser232 verbunden, die die Aktivität dieses Gatekeepers der mitochondrialen Atmung negativ reguliert. Zusammenfassend zeigen diese Daten, dass alle drei AKT-Isoformen die OCR-, ECAR- und Laktatproduktion in menschlichen HCC-Zellen hochregulieren können.

Zielgerichtete Therapien und Immuntherapien werden bei der Behandlung von HCC eingesetzt, jedoch ist die Arzneimittelresistenz einer der Gründe für die niedrige Ansprechrate der HCC-Behandlung. AKT-Inhibitoren könnten dazu beitragen, die Tumorhypoxie und die Übersäuerung der Tumormikroumgebung zu reduzieren, indem sie den Sauerstoffverbrauch und gleichzeitig die Laktatproduktion verringern. AKT-Inhibitoren könnten in Kombination mit anderen zielgerichteten Therapien und Immuntherapien bei HCC-Patienten einen therapeutischen Nutzen bringen, insbesondere für Patienten mit fortgeschrittenem HCC, bei denen eine chirurgische Resektion nicht mehr in Frage kommt. Der pan-AKT-Inhibitor Capivasertib, ein oral bioverfügbarer niedermolekularer Inhibitor, der alle drei AKT-Isoformen hemmt, wurde vor kurzem von der US-Arzneimittelbehörde (FDA) für die Behandlung von fortgeschrittenem oder metastasiertem Brustkrebs zugelassen. Somit könnte AKT ein wertvolles metabolisches therapeutisches Ziel sein, um die Stoffwechselpathologie bei HCC-Patienten zu reduzieren oder sogar umzukehren, und könnte in Kombination mit gezielten und immuntherapeutischen Ansätzen für die Behandlung von HCC-Patienten nützlich sein.

The PI3K/AKT/mTOR signaling pathway plays an important role in the development of hepatocellular carcinoma (HCC) and is associated with an aggressive phenotype, early recurrence and an unfavorable prognosis for HCC patients. Changes in cell metabolism are considered as a hall mark of cancer and PI3K/AKT/mTOR signaling has been implicated in the regulation of metabolic processes. The results of this study indicate that targeting of each AKT isoforms i.e. AKT1, AKT2 and AKT3 is sufficient to reduce the oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and lactate production in the HCC cell lines Hep3B and Huh7. Vice versa, overexpression of each of the three AKT isoforms can increase the OCR, ATP production, maximal respiration and spare respiratory capacity in both Hep3B and Huh7. This underscores the significant role of all three AKT isoforms in regulating tumor cell metabolism in HCC. Mechanistically, the observed reduction of the OCR after knockdown of AKT isoforms was associated with an increased phosphorylation of pyruvate dehydrogenase at Ser232, which negatively regulates the activity of this

gatekeeper of mitochondrial respiration. In summary, our data indicate that all three AKT isoforms can upregulate OCR, ECAR, and lactate production in human HCC cells.

Targeted therapy and immunotherapy have been used in the treatment of HCC, however, drug resistance is one of the reasons for the low response rate of HCC treatment. AKT inhibitors may help to reduce tumor hypoxia and tumor microenvironment acidification by decreasing oxygen consumption and simultaneously reducing lactate production. AKT inhibitors in combination with targeted and immunotherapies may provide more significant therapeutic benefits for patients with hepatocellular carcinoma, and have potential therapeutic significance for patients with advanced HCC that is not amenable to surgical resection. The pan-AKT inhibitor Capivasertib, an orally bioavailable small molecule inhibitor that inhibits all three AKT isoforms was recently approved by the U.S. Food and Drug Administration (FDA) for the treatment of advanced or metastatic breast cancer. Thus, AKT may be a valuable metabolic therapeutic target for reducing or even reversing metabolic pathology in HCC patients, and may be useful in combination with targeted and immunotherapeutic approaches for the treatment of HCC patients.

5.Declaration of contribution to the publication

The study was planned by myself and Professor Dr. rer. nat. Manfred Jücker. The experiments were all carried out independently by me.
As part of the seahorse assay, I received technical support from Dr. Lis Noelia Velasquez and Prof. Dr. Samuel Huber for the measurement and evaluation from I. Department of Medicine and Hamburg Center for Translational Immunology at the University Medical Center Hamburg-Eppendorf.
The lenti-virus used in the publication, Dr. Daniel Smit and Dr. Stefan Horn kindly made it available to me for these studies.
The interpretation of the collected data was carried out together with Professor Dr. rer. nat. Manfred Jücker. The first version of the manuscript was written by me and

revised by Professor Dr. rer. nat. Manfred Jücker and Dr. Daniel Smit. The other co-authors (Dr. Nadezhda Popova, Dr. Stefan Horn, and Dr. Lis Noelia Velasquez) were involved in the critical revision of the manuscript.
No further assistance was sought.

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7.CURRICULUM VITAE

Lebenslauf aus datenschutzrechtlichen Gründen nicht enthalten.

8. Complete list of publications

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Tian LY, Smit DJ, Popova NV, Horn S, Velasquez LN, Huber S, Jücker M. All Three AKT Isoforms Can Upregulate Oxygen Metabolism and Lactate Production in Human Hepatocellular Carcinoma Cell Lines. (2024) International Journal of Molecular Sciences. doi.org/10.3390/ijms25042168

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* Authors contributed equally as first author to this work

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9. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....