CD147 a direct target of miR-146a supports energy metabolism and promotes tumor growth in ALK+ ALCL

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We recently reported that miR-146a is differentially expressed in ALK+ and ALK− anaplastic large cell lymphoma (ALCL). In this study, the downstream targets of miR-146a in ALK+ ALCL were investigated by transcriptome analysis, identifying CD147 as a potential target gene. Because CD147 is differentially expressed in ALK+ ALCL versus ALK− ALCL and normal T cells, this gene emerged as a strong candidate for the pathogenesis of this tumor. Here we demonstrate that CD147 is a direct target of miR-146 and contributes to the survival and proliferation of ALK+ ALCL cells in vitro and to the engraftment and tumor growth in vivo in an ALK+ ALCL-xenotransplant mouse model. CD147 knockdown in ALK+ ALCL cells resulted in loss of monocarboxylate transporter 1 (MCT1) expression, reduced glucose consumption and tumor growth retardation, as demonstrated by [18F]FDG-PET/CT analysis. Investigation of metabolism in vitro and in vivo supported these findings, revealing reduced aerobic glycolysis and increased basal respiration in CD147 knockdown. In conclusion, our findings indicate that CD147 is of vital importance for ALK+ ALCL to maintain the high energy demand of rapid cell proliferation, promoting lactate export, and tumor growth. Furthermore, CD147 has the potential to serve as a novel therapeutic target in ALK+ ALCL, and warrants further investigation.

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INTRODUCTION

Anaplastic large cell lymphoma (ALCL) is a T-cell non-Hodgkin lymphoma with anaplastic morphology and strong CD30 expression [1]. Two distinct disease entities are recognized based on the expression or lack of the anaplastic lymphoma kinase (ALK). ALK+ ALCL frequently carries the chromosomal translocation t(2;5)(p23;q35) involving the anaplastic lymphoma kinase (ALK) and the nucleophosmin (NPM) gene, leading to the expression and constitutive activation of chimeric ALK fusion protein. ALK fusion proteins activate several key signaling pathways involved in transformation, cell proliferation and survival, including STAT3, AKT/mTOR, RAS/MAPK, PLCγ and PI3K [2]. A central target gene of the JAK/STAT signaling pathway is the transcription factor C/EBPβ, which is over-expressed in ALK+ ALCL [3–5]. C/EBPβ is also able to control tumorigenesis through regulation of gene expression by miRNA up- and downregulation [6]. Several deregulated miRNA have been identified in different studies, including the downregulated miR-16, miR-21, miR-26a, miR-29, miR-96, miR-101, miR-146a and miR-155; besides the upregulated miR-135b and miR-17-92 cluster, suggesting that these miRNAs might contribute to ALK-mediated oncogenesis and/or tumor biology [7–11]. So far only few target genes of deregulated miRNAs have been revealed in ALK+ ALCL. ZNF652, BACH1, RB1, E2F2 and TP53INP1 were detected as target genes of miR-155 [12], whereas MCL-1, INOS and ALK are target genes of miR-29a, miR-29a and miR-96, respectively [7, 10, 13]. FOXO1, STAT6 and GATA3 were identified as target genes of the highly expressed miR-135b [9].

The intrinsic low expression of miR-146a previously reported in ALK- ALCL in comparison to ALK+ ALCL, normal T cells and lymph nodes [6, 8], suggested that this miRNA might have an impact in the tumorigenesis of ALK+ ALCL. Importantly, miR-146a...
has been found to function as a potent tumor suppressor gene, is associated with the T-helper 1-(Th1)-phenotype and is involved in the development of lymphoid neoplasms [14]. Furthermore, in hepatocellular carcinoma (HCC) and non-small cell lung carcinoma (NSCLC), the hypermethylation of the promoter of miR-146a is associated with increased cell proliferation, cell survival, invasion, migration and metastasis. Hence, the use of miR-146a appears to be effective in the treatment of NSCLC patients [15, 16].
Fig. 1 miR-146a target genes. A RT-qPCR results of miR-146a expression in primary ALK+ ALCL cases (ALK+ five cases, ALK− seven cases and four reactive lymph nodes) and cell lines. For cell line analysis ALK+ ALCL cell lines (SUDHL-1, KUJ, Karpas 299) were compared to the ALK− ALCL cell line (Mac-1) and CD3+ T cells from 3 healthy donors. Every point represents the average of three independent measurements. For RT qPCR quantifications values were normalized to miR-106b and analyzed using the 2−ΔΔCT method [6]. B Comparison of miR146a expression levels in ALK+ ALCL cells (SUDHL-1, Karpas 299, KUK, SR786, SUP-M2), ALK− ALCL cells (FE-PD, Mac-1 and Mac2a), CD3+ T cells from 3 healthy donors, leukemia cells (Jurkat and HL-60), B-cell lymphoma cells (SUDHL-4, Jeko-1 Rec-1 and and KMS-12) and carcinoma cell lines (HeLa, HEK293-T). Every point represents the average from three independent measurements. For RT qPCR quantifications values were normalized to miR-106b and analyzed using the 2−ΔΔCT method. C RT-qPCR results of relative miR-146a expression levels in untransfected, and miR-146a mimic-transfected SUDHL-1 (blue) and Karpas 299 (orange) cells. For RT-qPCR quantification, values were normalized to miR-106b, and data were analyzed according to the 2−ΔΔCT method. Results are depicted as mRNA levels relative to mean value levels of HiperFect treated cells. D Overview of the reads generated in the transcriptome analysis by NGS, and number of deregulated genes comparing control/ miR-146a overexpression. E Volcano plot of RNA-seq transcriptome data displaying the differentially expressed genes (FDR-corrected p ≤ 0.05). Significantly regulated genes after miR-146a overexpression (>0.5 Log2 Fold change) are depicted with orange dots (four downregulated genes left side). Coordinates of CD147 is marked with an orange star. F Bar graphs represent Gene Ontology pathways enriched in the Gene Set Enrichment Analysis (GSEA) of the differentially expressed genes ranking list. Signaling pathway with FDR q-value <0.05 are highlighted in gray. G RT-qPCR analysis of miR-146a regulated target genes. Relative expression levels of the four selected candidate genes (ZNF275, SRPRB, PNPO and CD147) regulated by miR-146a were determined by RT-qPCR analysis in miR-146a transfected or untransfected SUDHL-1 (blue) and Karpas 299 (orange) cells. Relative mRNA downregulation of the candidate genes was identified using RT-qPCR quantification to ACTB and data were analyzed according to the 2−ΔΔCT method. Each plot represents the average from biological triplicates. For statistical analysis unpaired t-test (target vs control) was performed. Results are depicted as mRNA levels relative to mean value levels of HiperFect treated SUDHL-1 and Karpas 299 cells (control).

Therefore, the aim of this study was to identify miR-146a target genes by transcriptome analysis using next generation sequencing (NGS) in ALK+ ALCL and further analyze relevant candidates.

MATERIALS AND METHODS

Cell culture and patient samples

Five ALK+ ALCL cell lines (SUDHL-1, KUK, Karpas 299, SUP-M2 and SR-786), three ALK− ALCL cell lines (Mac-1, Mac2a and FE-PD), HEK293T and HeLa cells were cultured as previously described (Supplementary Methods) [3, 17]. Formalin-fixed paraffin embedded (FFPE) primary tumor samples of 81 ALK+ and 14 ALK− ALCLs patients were collected from the archives of the Institutes of Pathology from the University of Tübingen and the University of Kiel, Germany. Ethics approval for the study (620/2011BO2) was obtained from the University of Tübingen.

RNA isolation

Total RNA and miRNA were isolated from cell lines using the RNeasy Mini Kit and miRNeasy Mini Kit, respectively (Qiagen, Hilden, Germany) (Supplementary Methods).

Overexpression of miR-146a

Overexpression of miRDIAN miRNA Mimic 146a, Mimic Housekeeping Positive Control #2 (GAPD) and miRDIAN microRNA Mimic Negative Control #1 (GE Healthcare, Buckinghamshire, United Kingdom) was performed in SUDHL-1 or Karpas 299 (Supplementary Methods).

Transcriptome analysis

Transcriptome analysis using NGS was performed by CeGaT (Tübingen, Germany) (Supplementary Methods).

Real-time quantitative RT-PCR

cDNA synthesis from RNA and real-time quantitative RT-PCR analysis (RT-qPCR) to quantify the mRNA level of CD147 was performed using Universal Probe Library (UPL) assays (Roche Applied Science, Penzberg, Germany). Mature miRNA quantification, cDNA synthesis and RT-qPCR analysis were performed as previously described (Supplementary Methods) [18].

Western blot analysis

Lysis of cells and Western blotting were performed as described elsewhere [4, 19]. The antibodies used are listed in the Supplementary Information.

Cloning of CD147-shRNA constructs, Virus Production and Viral Infections

Oligonucleotides containing CD147-shRNA sequences were cloned into pSUPER and pFUGW (pF-CD147), as previously described (Supplementary Methods) [3]. Production of virus containing lentiviral vector pFUGW was performed as recently described [20]. Transduction efficiency was determined as previously specified [3, 17, 19].

CD147 CRISPR/Cas9 system knockout

Individual lentiviral CRISPR plasmids targeting a single genomic locus - CD147 - were designed and constructed according the lentCRISPRv2 #52961, (Addgene Watertown, Massachusetts, USA), as previously described [21]. Pathogenic mutations leading to CD147 protein damage were investigated with targeted NGS as previously reported (Supplementary Methods).

Luciferase reporter assay

The regions of the CD147 and SRPRB 3′-UTRs, including the miR-146a binding site predicted by the miRanda tool were amplified from human genomic DNA using PCR [22]. For luciferase reporter assays, HEK293T and HeLa cells were transfected (Supplementary Methods).

Cell proliferation and viability assay (MTS assay)

Cell viability and growth retardation was determined by the MTS cell proliferation assay (AQueous CellTiter 96, Promega) [3, 23]. Apoptosis analysis was done by annexin V (AnnexinV-APC, Invitrogen) and propidium iodide (PI) (Sigma-Aldrich) stainings according to manufacturer protocols, followed by flow cytometry (FACS Calibur, BD, Franklin Lakes, NJ, USA).

Crizotinib treatment

ALK+ ALCL cells were treated with increasing concentrations of Crizotinib (25, 50 and 100 nM). Protein and miRNA were isolated after 72 h (Supplementary Methods).

Experimental mice

All animal experiments were approved by the Regierungspräsidium Tübingen and performed according to animal use and care protocols of the German Animal Protection Law (Supplementary Methods).

Sequential PET/MRI

For the PET-measurements, the tracer [19F]FDG was used, which was synthesized in a FDG MicroLab module (GE Healthcare, Münster, Germany) as described previously [24]. Sequential [19F]FDG-PET/MRI scans were performed 3 and 4 weeks after tumor cell implantation (Supplementary Information).

Immunohistochemistry

Explanted xenograft and primary ALCL tumors were stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Inc.)
according to the company’s protocols for open procedures (Supplementary Information).

**Transmission electron microscopy (TEM)**

FFPE materials were used for the analysis (Supplementary Information).

**Non-targeted metabolomics**

Metabolic profiling was performed by LC-QTOF-MS analysis as described [25] (Supplementary Information). Data were preprocessed by targeted feature extraction of annotated metabolite species [25]. Peak areas were log$_2$ transformed and normalized by median normalization.
prior the assessment of fold changes between CD147 knockdown (KD) tumors and controls.

Targeted metabolomics
Pyruvate, aconitase, fumarate, α-ketoglutarate, malate and citrate were quantified by GC-MS analysis and lactate by LC-MS-MS as described previously (Supplementary Information) [26–29].

XF cell mito stress test using seahorse XFe96 analyzer
SUDHL-1 and KUK cells (control and CD147-KD) were grown in suspension after viral infection and incubated for at least 72 h. Oxygen consumption rates (OCR) were measured using Agilent Seahorse XFe96 Analyzer (Seahorse Bioscience; Agilent Technologies, Inc., Santa Clara, CA, USA) and the Seahorse XF Cito Mito Stress Test Kit (Agilent) [30], according to the manufacturer’s protocol (Supplementary Information).

Measurement of mitochondrial membrane potential (ΔΨm) using FACS analysis
SUDHL-1 cells, SUDHL-1 cells with CD147-KO and SUDHL-1 cells under stress conditions (6 days starvation) were stained with Mito Tracker Green (MG) and Mito Tracker Deep Red (MDR) (ThermoFisher Scientific). FACS analysis was performed and data were analyzed using FlowJo V9.9.6 (Supplementary Information) [31].

Statistical analysis
Specific details concerning statistical tests for individual experiments are noted in the figure legends and in Supplementary Information. P values <0.05 were considered significant.

RESULTS

Identification of miR-146a target genes by transcriptome analysis using NGS
In a previous study, we demonstrated that ALK+ ALCIL cell lines and ALK+ ALCIL primary cases show very low miR-146a expression in comparison to the moderate expression (4 folds higher) in ALK− ALCIL cases and the high expression of reactive lymph nodes (7 folds higher) (Fig. 1A) [6]. miR-146a expression levels are high in T cells (≥10 folds higher) in comparison to T- and B-cell lymphomas, leukemia and carcinoma cell lines (Fig. 1B, Supplementary Fig. 1). In order to investigate the downstream targets of the tumor suppressor miR-146a, ALK+ ALCIL cell lines were transfected with miR-146a mimic and analyzed using RNA-seq analysis. ALK+ ALCIL cell lines SUDHL-1 and Karpas 299 showed strong overexpression of miR-146a (Fig. 1C). Efficiency of the transfection was confirmed by parallel GAPDH downregulation by the miR-positive control (Supplementary Fig. 2). SUDHL-1 cells transfected with miR-146a or untransfected were analyzed by RNA-seq analysis using NGS. For both samples more than 100 million reads were generated, which were mapped to the human genome. RNA seq data showed 113 genes with statistically differential expression (-log10 q-value > 0.5, log2Fold Change > 0.5) (Fig. 1D, Supplementary Table 1). GSEA of a ranked list of differentially expressed genes revealed higher enrichment score (NES) of gene ontology pathways related to vascular endothelial growth factor and phospholipase C activating protein (FDR q-value < 0.5), supporting the angiogenic role of miR-146a (Supplementary Fig. 3, Fig. 1F) [32]. Eight candidate genes were selected for further validation including the four strongest downregulated genes (PSENEN, SRPRB, ZNF275, PNP0), and four genes with high expression and known oncogenic functions related to ALK+ ALCIL (BSG/C14D7, CASP2, ADAM17, PIK3AP) (Supplementary Table 1, Supplementary Fig. 4). Validation of these eight genes by RT-qPCR confirmed a strong downregulation of four genes; ZNF275, SRPRB, PNP0 and C14D7 (BSG/Basigin, EMMPRIN) to 44–54% in SUDHL-1 and 31–67% in Karpas 299 cells after miR-146a overexpression compared to untransfected control cells (Fig. 1G). A potential direct regulation was investigated for two genes: CD147 and SRPRB. Relative luciferase activity was strongly reduced for CD147 and SRPRB in both cell lines after transfection of miR-146a indicating that both genes are direct targets of miR-146a (Fig. 2A, Supplementary Fig. 5). Because we recently reported that CD147 is differentially expressed in ALK+ ALCIL [33], we concentrating further on the analysis of this target gene. Western blot analysis showed downregulation of CD147 and p-STAT3 protein expression. Each lane contained 25 µg protein extract. α-Tubulin was used as loading control. Western blot analysis of ALK, pSTAT3 and CD147 in ALK+ ALCIL cell lines (SUDHL-1 and Karpas 299) after 48 h treatment with different doses of Crizotinib, as indicated. Untreated cells were loaded as control. Western blot analysis demonstrates a complete absence of ALK activity after Crizotinib treatment and a reduction of CD147 and pSTAT3 protein expression. Each lane contained 25 µg protein extract. α-Tubulin was used as loading control. H Quantification of p-STAT3 or CD147 obtained from independent biological triplicate samples, normalized to α-Tubulin and represented in the bar graph. For statistical analysis unpaired t-test was used, "p < 0.05, **p < 0.001, ***p < 0.0001."
and five ALK− ALCL primary patient samples. ALK+ cases showed higher mRNA levels than ALK− ALCL cases, (p = 0.07) (Fig. 2C). To corroborate the differential expression of CD147 protein in primary cases, 95 ALCL cases were investigated (ALK+, 81 cases and ALK− 14 cases). ALK+ ALCL cases revealed a moderate to strong positive membranous staining in the majority of tumor cells, as opposed to the weak or absent staining observed in ALK− ALCL cases (histoscore 264.64+/− 40 vs 127.14+/− 105, p = 0.0001, Fig. 2D, E). To investigate whether the expression of CD147 and miR-146a were ALK-dependent, two ALK+ ALCL cell lines
where treated with increasing concentrations of Crizotinib, which led to inhibition of P-ALK and P-STAT3 with subsequent increase in miR146a (Fig. 2F), and decrease in CD147 (Fig. 2G, H). These results indicate that ALK regulates the expression of miR-146a and CD147.

**CD147 contributes to the survival and proliferation of ALK+ ALCL cells in vitro**

To evaluate the effect of CD147 in ALK+ ALCL cells, a double approach was pursued using CD147 shRNA KD and CD147 knockout (KO) by CRISPR/Cas9 system (Fig. 3A). CD147 shRNA was selected by testing 5 different shRNA constructs (Supplementary Fig. 6A). CD147 shRNA “A” and “B” were selected for further analyses and transduced in SUDHL-1 and KJK (Fig. 3, Supplementary Fig. 6B–E). Flow cytometric analysis showed that CD147-shRNA (pF-CD147) was effectively transduced into the cell lines with infection rates of 97.8% to 99.45% with both shRNAs (Supplementary Fig. 6C). Three days after the second transduction, CD147 was successfully downregulated at mRNA and protein level, as demonstrated by Western blot and RT-qPCR analyses (Fig. 3B, C, Supplementary Fig. 6D, E). RT-qPCR confirmed the CD147 mRNA downregulation to 8% or 4% in KiJK and SUDHL-1 cells compared to control cells, respectively. Growth curves were generated after seven or eight days of the secondary infection demonstrating growth retardation of 70% in SUDHL-1 using CD147 shRNA B or of 59% in SUDHL-1 and 47% in KJK using CD147 shRNA A (Fig. 3D, Supplementary Fig. 6E), despite the normal expression of ALK, P-STAT3, P-STAT5 and P-STAT1 (Fig. 3C). Cell cycle analysis demonstrated a decrease in S phase of 11–19.2% in CD147-KD cells in comparison to controls, and an increase in G0/G1 of 8.9–15.4% after four days of infection (Fig. 3E), corroborating a G0/G1 cell cycle arrest. Flow cytometric analysis with annexin V/propidium iodide revealed increased apoptosis when compared to the control cells (11.6–24.6%) (Fig. 3E).

CD147-KO after CRISPR/Cas9 editing was confirmed at the protein level for three cell clones harboring different CD147 damaging mutations. Western blot analysis demonstrated complete lack of CD147 protein that correlated with reduced MCT1 expression but normal CD30, ALK and pSTAT3 expression (Fig. 3F). Growth curves of the different SUDHL-1 clones show a growth retardation of 48–68% in comparison to the untreated control after 10 days (Fig. 3G).

**CD147 contributes to tumor growth of ALK+ ALCL cells in vivo**

To further investigate the influence of CD147 in survival and tumor growth of ALK+ ALCL cells in vivo, KJK and SUDHL-1 cells with (n = 10; 5 KJK, 5 SUDHL-1) and without (n = 10; 5 KJK, 5 SUDHL-1) CD147-KD were subcutaneously implanted in 6–8 weeks old female NOD scid gamma immune deficient mice and analyzed by PET/MRI (Fig. 4A–C). After 3 and 4 weeks, KJK + CD147-KD tumors (CD147-KD: 3.42 ± 4.37 mm3 vs untreated: 256.4 ± 153.7 mm3), were 75-fold smaller compared to untreated KJK-tumors (CD147-KD: 3.42 ± 4.37 mm3 vs untreated: 256.4 ± 153.7 mm3), (Fig. 4B, F). After 4 weeks most of untreated KJK-lymphomas reached already the final tumor stage and the animals were sacrificed before the last PET/MRI measurements. Similar results were obtained after implantation of SUDHL-1 + CD147-KD tumor cells, where apparent engraftment was visible after 4 weeks in only 2/5 animals (40%), (Fig. 4A–C). After 3 and 4 weeks, KJK + CD147-KD tumors (CD147-KD: 9.4 ± 12.06 mm3), untreated SUDHL-1 cells (CD147-KD: 3.42 ± 4.37 mm3), showed a significantly increased uptake of 2.3 fold (Fig. 4B). These results clearly demonstrate that CD147 is necessary for engraftment and growth of ALK+ ALCL cells.

To corroborate the lack of CD147 expression in CD147-KD cells, immunohistochemical analysis was performed in the murine tumors (Fig. 4H). Tumors with CD147-KD showed complete lack of CD147 expression, as compared to the untreated cells. Lack of CD147 expression correlated with lack of MCT1 expression in vivo (Fig. 4H) indicating destabilization of the CD147-MCT1 complexes. Additional functions of CD147 include induction of matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF). To investigate whether these functions were impaired, MMP7, VEGFR2 and CD31 stains were performed. The lack of MMP7 expression and the decrease in angiogenesis was evident in the CD147-KD cells when compared to controls, confirmed by reduced CD31 and VEGFR2 histoscopes in tumors with CD147-KD (Fig. 4G). There were no differences in proliferation and apoptosis, as shown by MI81 and caspase 3 activated, respectively (data not shown). These findings further establish CD147 as a functionally relevant protein in ALK+ ALCL cells necessary for survival and tumor growth.

**ALK+ ALCL cells are dependent on CD147-MCT1 transmembrane complexes for glucose metabolism and tumor growth**

CD147-MCT1 transmembrane complex has been shown to have a central role in cellular metabolism – particularly glycolysis – a major source of energy production in cancer cells [34]. Accordingly, in vivo PET analysis revealed a remarkably reduced uptake.
of the glucose analog [18F]FDG in CD147-KD KUJ or SUDHL-1 tumors when compared to untreated tumor (Fig. 4A, E). These results indicate that ALK+ ALCL have high-energy consumption and therefore addiction to lactate transport through the CD147-MCT1 complex and point towards increased glycolysis of neoplastic ALK+ ALCL, a phenomenon described as the Warburg effect. To investigate further the impact of CD147 blockade in relation to tumor energy metabolism, the mitochondrial fitness of...
tumor cells was investigated. Measurement of ΔΨm revealed a loss of mitochondrial depolarization in SUDHL-1 cells with CD147-KD compared to control (SUDHL-1 CD147 WT cells). CD147-KD cells showed decreased mitochondrial activity per mitochondrial mass demonstrated by higher percentage of cells with low staining for MDR compared to MG, referred as MDR/MG low population. Conversely, the control cells show higher MDR staining compared to MG referred as MDR/MG ratios (Fig. 5A, B). A similar scenario occurs in SUDHL-1 under hypoxic conditions (Fig. 5A, B). The TEM assay performed in SUDHL-1 and KJK supported these findings, revealing swollen mitochondria with disrupted cristae structure and reduced mitochondrial cristae number and length (Fig. 5C, D).

For a more detailed investigation, metabolomic analyses both in vivo and in vitro were performed. Initially, exploratory analysis was carried out in vivo by liquid-chromatography mass spectrometry-based non-targeted metabolomics (i.e., metabolomic profiling), a hypothesis-free approach that aims to capture as many metabolites as possible in a single analysis. Metabolomic profiling of SUDHL-1 tumors of mice showed changes of metabolites involved in amino acid metabolism, nucleotide biosynthesis and lipid metabolism (Fig. 6A). Despite reduced glucose uptake, an increased intratumoral level of glucose was observed in KJK + CD147-KD-tumors compared to controls. Higher levels of TAG species are in line with the accumulation of lipid vesicles in vitro observed in TEM (Fig. 5C). Further changes related to lipid metabolism indicated an increase of lyso-phospholipids (e.g., Lyso PC 16:0) and free fatty acids in KJK + CD147-KD-tumors (Fig. 6A). To assess a potential increased entry of glucose in the TCA cycle, TCA intermediates were quantified with a targeted approach by mass spectrometric methods. These analyses are complementary to the profiling experiment as they capture pre-defined analytes with high accuracy and precision. As a result, concentrations of citrate, aconitase and α-ketoglutarate (AKG) were increased, while TCA intermediates downstream of AKG, such as succinate and fumarate, were found reduced in tumors depleted of CD147 (Fig. 6B). This metabolite pattern hinted to an impaired electron transport chain (ETC) and maintenance of TCA cycle by increased entry of glutamine into the TCA cycle with subsequent reductive carboxylation of AKG to citrate. Indeed, the metabolic ratio AKG/citrate as an indicator of reductive glutamine carboxylation [35] increased considerably upon CD147-KD (Fig. 6C).

Cellular experiments confirmed these changes albeit to a smaller extent. CD147-KD cells showed higher intracellular concentrations of lactate, pyruvate and TCA cycle intermediates (Supplementary Fig. 8A) and an increase of the AKG to citrate ratio (Fig. 6C). Next, in order to assess a more specific contribution of CD147 to mitochondrial function, we analyzed the OCR as an indicator of mitochondrial respiration by real time metabolic flux measurements in cell culture. Analyses of the OCR in SUDHL-1 and KJK cells; however, indicated an increased basal and maximal respiration of CD147-KD cells compared to control cells (Supplementary Fig. 8B).

**DISCUSSION**

In this study, we aimed to identify the downstream targets of miR-146a, a tumor suppressor miRNA in ALK+ ALCL. We focused on CD147, because we recently reported that this protein is induced by C/EBPβ and it is differentially expressed in ALK+ versus ALK− ALCL cases, indicating a specific role of CD147 in ALK+ ALCL tumor development [33]. This finding was corroborated in a large cohort of ALK+ and ALK− ALCL primary cases (p = 0.0001).

We now confirmed CD147 as miR-146a direct target gene and demonstrate that functional CD147-MCT1 transmembrane complexes are necessary for cellular metabolism supporting tumor growth, angiogenesis, and invasion in vitro and in vivo in ALK+ ALCL cells (Fig. 7) [36]. Accordingly, CD147 as a direct target of miR-146a has been demonstrated in solid cancers such as NSCLC, HCC and renal cancer [37, 38].

CD147 or extracellular matrix metalloproteinase inducer (EMM-PRIN) is upregulated in T cells upon activation; however, its expression both at protein and mRNA levels is rather strong in ALK+ ALCL cases when compared with ALK− ALCL cases or normal reactive T cells [33]. CD147 is a transmembrane glycoprotein of the immunoglobulin superfamily expressed in a wide variety of cell types and tumors with pleiotropic functions including development, activation, proliferation, migration, adhesion and invasion, which are important for the pathogenesis of various diseases [33, 39, 40]. Induction of matrix metalloproteinases (MMP) is considered an important oncogenic aspect of CD147 function [40, 41]. Accordingly, the downregulation of CD147 resulted in lack of expression of its downstream target MMP7 and correlated with lack of engraftment of ALK+ ALCL cells or striking tumor growth retardation. These results show that CD147 expression is needed for engraftment and growth of ALK+ ALCL tumors.

A widespread hallmark of cancer cells is an altered energy metabolism, where the cells activate glycolysis to meet their energy demand for rapid proliferation, known as “Warburg effect” [39]. CD147 is an important modulator of the “Warburg effect” by sustaining glycolysis and inhibiting mitochondrial biogenesis and oxidative phosphorylation in tumor cells [42, 43]. A side effect of glycolysis is the accumulation of lactic acid by products. As CD147 also acts as a chaperone for MCT1, it seems to regulate the altered energy metabolism in cells by shuttling MCT1 to the plasma membrane, thereby mitigating the toxic buildup of lactic acid [44, 45]. We now demonstrate that CD147 is essential for ALK+ ALCL tumor viability. Accordingly, PET analysis showed a reduced
Our metabolic analyses suggest that suppression of CD147 might target aberrant glycolysis, thus impairing the major energy source of tumor cells with consequent strong tumor growth restriction. Accordingly, repressing CD147 has been proposed as a novel therapeutic strategy for HCC and malignant melanoma. Interestingly, a recent study showed that CD147 is also known to stimulate the expression of VEGF and MMP in tumor and stromal compartment leading to angiogenesis. CD147 is also known to stimulate the expression of VEGF and MMP in tumor and stromal compartment leading to angiogenesis. CD147 is also known to stimulate the expression of VEGF and MMP in tumor and stromal compartment leading to angiogenesis. CD147 is also known to stimulate the expression of VEGF and MMP in tumor and stromal compartment leading to angiogenesis. CD147 is also known to stimulate the expression of VEGF and MMP in tumor and stromal compartment leading to angiogenesis.
Fig. 6  Investigation of metabolic alterations upon CD147-KD in vivo. A Non-targeted profiling of metabolites in a mouse xenotransplant model that differ between CD147-KUJ-1 tumors and controls with an absolute log2 fold change >0.5 (mean ± SEM, n = 2 for controls, n = 3 for CD147-KD). B Concentrations of glycolytic metabolites and TCA cycle intermediates in CD147-KUJ tumors and controls (mean ± SEM, n = 2 for controls, n = 3 for CD147-KD). C Changed metabolite ratio α-ketoglutarate (AKG) to citrate in CD147- KUJ tumors and controls (mean ± SEM, n = 2 for controls, n = 3 for CD147-KD) and in SUDHL-1 and KUJ CD147-KD cells compared to controls (n = 1 biological replicate per cell line) indicates shift of metabolism to reductive glutaminolysis due to compromised mitochondrial quality. Unpaired t-test, *p < 0.05.
lenalidomide and pomalidomide act by disrupting the cereblon-CD147-MCT1 axis to exert their antitumor activity, highlighting the importance of the CD147-MCT1 complexes for survival of tumor cells [60].

In conclusion, our data show that low miR-146a expression in ALK+ ALCL results in high CD147 expression. Due to CD147 involvement in multiple tumor-promoting mechanisms, CD147 has the potential to serve as a novel therapeutic target in ALK+ ALCL and warrants further investigation.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CMG, IB, LQ-M. Performed the animal model and PET analysis: CMG, UK, IB. Performed transcriptome and KD experiments: JS, IB, AR, A-KG, H-CL, I-AM-M. Performed the metabolic experiments: MH, ES and MS. Performed the EM analysis: PF-B. Analyzed and interpreted the data: JS, I-AM-M, CMG, IB, UK, MH, ES, MS, FF, LQ-M. Supervised the experimental work IB, LQ-M. Contributed reagents/materials/analysis tools: BJP, MS, FF, LQ-M. Wrote the paper: I-AM-M, LQ-M. Helped writing the paper: JS, FF, MS, IB.

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The authors declare no competing interests.

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