Modulation of Pancreatic Neuroendocrine Neoplastic Cell Fate by Autophagy-Mediated Death

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Keywords

Autophagy · Pancreatic neuroendocrine cancer · Activated protein kinase · Cell death · Insulinoma · Spheroids

Abstract

Introduction: Autophagic cell death in cancer cells can be mediated by inhibition of deacetylases. Although extensive studies have focused on the autophagic process in cancer, little is known about the role of autophagy in degrading cytosolic and nuclear components of pancreatic neuroendocrine neoplastic (pNEN) cells leading to cell death, thus improving the therapy of patients affected by pNEN. **Methods:** 2D and 3D human pNEN and pancreatic stellate cells were treated with panobinostat and bafilomycin. Autophagy markers were detected by RT-qPCR, immunofluorescence, and Western blot. Autophagosomes were detected by electron microscopy and their maturation by real-time fluores-

cence of LC3B stable transfected cells. ChIP was performed at the cAMP responsive element. Immunofluorescence was performed in murine pancreatic tissue. **Results:** We observed that pan-deacetylase inhibitor panobinostat treatment causes autophagic cell death in pNEN cells. We also found that although AMPK- α phosphorylation is counterbalanced by phosphorylated AKT, it is not capable to inhibiting autophagic cell death. However, the binding activity of the cAMP responsive element is prompted by panobinostat. Although autophagy inhibition prevented autophagosome synthesis, maturation, and cell death, panobinostat treatment induced the accumulation of mature autophagosomes in the cytosol and the nucleus, leading to disruption of the organelles, cellular digestion, and decay. Observation of autophagosome membrane proteins Beclin1 and LC3B aggregation in murine pancreatic islets indicates that autophagy restoration may also lead to autophagosome aggregation in murine insulinoma cells. A basal low expression of autophag-

gy markers was detectable in patients affected by pNEN, and, interestingly, the expression of these markers was significantly lower in metastatic pNEN. **Discussion/Conclusion:** Our study highlights that the autophagy functional restoration and prolongation of this catabolic process, mediated by inhibition of deacetylase, is responsible for the reduction of pNEN cells. Prompting of autophagy cell death could be a promising strategy for the therapy of pNEN.

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Introduction

Pancreatic neuroendocrine neoplasms (pNEN) are a heterogeneous group of rare neoplasms derived from endocrine pancreatic cells with an incidence of approximately 0.8/100,000 per year [1]. They include hormone active functioning tumors and the more frequent non-functioning tumors [2]. Pan-histone deacetylase inhibitors promote histone acetylation and other nuclear and cytosolic nonhistone proteins, thus facilitating the transcription of autophagy-related genes leading to cell cycle arrest and death [3], as shown in hepatocellular carcinoma and osteosarcoma cells [4–7]. Interestingly, it serves as a degradation mechanism in the nucleus as well [8] and can also initiate cellular senescence by degrading the nuclear lamina in response to oncogenic stress, thereby protecting against tumorigenesis [9]. There is a balance between the energy sensor AMP-activated protein kinase (AMPK), which stimulates autophagy in response to cellular metabolic distress, and its counterpart AKT, which suppresses autophagy activity via activation of mammalian target of rapamycin (mTOR) or direct interactions with AMPK [10]. In addition to AMPK, the transcription factor cAMP response element-binding protein (CREB) induces autophagy in response to fasting and increased AMP levels [11]. Similarly, the transcription factor EB (TFEB) promotes the expression of various autophagic and lysosomal genes involved in autophagosome initiation and autolysosome activity [12]. UVRAG (UV radiation resistance-associated gene protein) interacts and, thereby activates, the Beclin1 complex [13], which is capable of inhibiting tumorigenesis in breast cancer cells [14]. LC3B protein, encoded by the *MAP1LC3B* (microtubule-associated proteins 1A/1B light chain 3B) gene, interacts with SQSTM1 (sequestosome1) in order to concert the aggregation of the ubiquitinated proteins during autophagosome formation [15]. We show that panobinostat can cause autophagic cell death in pNEN. Functional

inhibition of AMPK α does not impede autophagy, which is further prompted by an increase of CREB binding at its promoter region. The autophagic process takes place not only in the cytosolic compartment, but also in the nuclear compartment, leading to cellular disruption and dismantling of spheroid organization. However, the impairment of autophagy in pNEN cannot be further clarified due to the lack of an in vivo functional model which could be only tested here for the expression of Beclin1 and LC3B. Instead, the 3D model can be a useful model for future autophagy studies in pNEN. Additionally, identification of autophagy marker expression in patients affected by pNEN could unmask the role exerted by this catabolic process in the tumorigenesis of pancreatic neuroendocrine neoplasia.

Materials and Methods

Cell Lines

Human pancreatic neuroendocrine tumor cell line BON1, the mouse insulinoma cell line HMEG2725, derived from the Rip1Tag2 transgenic mouse model of pancreatic beta-cell carcinogenesis, and the human pancreatic stellate cell line HPSC2.2, a kind gift from Malte Buchholz and Heidi Griesmann (Philipps University Marburg and University Hospital Halle), were grown in DMEM (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL) at 37°C in a humidified atmosphere containing 5% CO₂. The human somatostatinoma cell line QGP1 (Malte Buchholz) was grown in RPMI 1640 medium (Gibco® by Life Technologies™, Carlsbad, CA, USA), with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μ g/mL).

Isolation and Culture of the Murine HMEG Insulinoma Cell Line

The murine insulinoma cell line was generated from 15-week-old RIP1Tag2 mice as previously described [16]. The pancreatic insulinoma was dissected, disrupted, and transferred to cell culture dish containing DMEM media with 2.5% FCS and 15% horse serum. The established insulinoma cell line (HMEG2725) was further cultured in DMEM supplemented with 10% FCS.

Mouse Strain

The transgenic Rip1Tag2 mouse model (B6.D2-Tg(RIP1Tag2)2Dh) has been previously described [17, 18] and was purchased from the NCI Mouse Repository. Mice were kept in a C57BL/6N background and maintained in a climate-controlled specific pathogen-free facility. Genotyping was done by PCR using the following primers (Biomers GmbH, Ulm): FW: 5'-GGACAAACCA-CAACTAGAATGCA-3'; RV: 5'-CAGAGCAGAATTGTG-GAGTG-3'. Rip1Tag2 is characterized by the transgenic expression of the simian virus 40 large T antigen (Tag) oncogene and the rat insulin promoter gene 1 (Rip1) that causes malignant transformation of β -cells [17]. Normal and hyperplastic islets of Rip1Tag2 mice have been identified with a diameter <400 μ m, whereas insu-

linomas must have a diameter >400 µm and show a well-marked angiogenesis [19].

Substances

100 pM bafilomycin A1 (Sigma-Aldrich, St. Louis, MO, USA), 1–100 nM panobinostat (Novartis AG, Basel, Switzerland), and 200 nM wortmannin (tlrl-wtm; InvivoGen, San Diego, CA, USA) were dissolved in sterile DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany).

Spheroid Formation

BON1 and HPSC2.2 spheroids were formed on 50 µL 1.5% peq-GOLD Universal Agarose (PEQLAB Biotechnology GmbH, Erlangen, Deutschland) in a flat-bottom 96-well plate (SARSTEDT AG and Co., KG, Nümbrecht, Germany) for 6 days (HPSC2.2)/7 days (BON1) without medium change [20]. Spheroids from HMEG2725 cells were formed in a 96-well, round-bottom low-attachment plate (4515; Corning, Corning, NY, USA) for 4 days. 1,000 BON1 cells, 2,000 HPSC2.2 cells, and 5,000 HMEG2725 cells were plated in 200 µL medium in a humidified atmosphere containing 5% CO₂ at 37°C, whereby BON1 and HPSC2.2 were placed on an orbital shaker with a shaking speed of 40 rpm overnight. Before treatment, 100 µL of medium was removed from each well containing a single spheroid. 100 µL medium with bafilomycin or panobinostat was added to the remaining 100 µL medium. The working concentration was 100 pM bafilomycin and 10 nM panobinostat.

Real-Time Cell Viability Analysis

BON1, QGP1, and HPSC2.2 cells were cultured on E-plates (05232368001; OLS, Bremen, Germany), and real-time cell viability was measured after treatment with 100 pM bafilomycin and 1–100 nM panobinostat by the xCELLigence RTCA system (Roche). xCELLigence continuously measured (120 h) the impedance to quantify the adherence of the cells on the plate's electrodes.

Quantitative RT-PCR

Total RNA was isolated with the RNeasy Mini Kit (74106; Qiagen, Hilden Germany) according to the manufacturer's protocol. Reverse transcription of mRNA was performed with the iScriptTM cDNA synthesis kit (170-8891; Bio-Rad, Hercules, CA, USA) on FlexCycler (Analytik Jena AG, Jena, Deutschland). Qiagen primers for human *BECN1* (QT00004221), *UVRAG* (QT00034328), *MAP1LC3B* (QT00055069), *SQSTM1* (QT00095676), *TFEB* (QT00069951), *PRKAA1_1* (QT00009436), *PRKAA2_1* (QT00042077), and *GAPDH* (QT01192646) and primers for mouse *Becn1* (QT00139006), *Uvrage* (QT00171563), *Map1lc3b* (QT01750322), *Sqstm1* (QT00127855), *Prkaa1* (QT01551970), *Prkaa2* (QT01062383), and *Gapdh* (QT01658692) were used with GoTaq[®] qPCR Master Mix (Promega, Madison, WI, USA) on an RT-qPCR thermocycler CFX96TM real-time system (Bio-Rad Laboratories, Hercules, CA, USA). Results were analyzed with the Bio-Rad CFX-Manager (Bio-Rad Laboratories) and normalized with GAPDH mRNA content for each sample. Raw data were further analyzed with Rest2009 (relative expression software tool V.2.0.13; Qiagen).

Western Blot Analysis

Whole cell lysates were prepared with Jie's buffer (10 mM NaCl, 0.5% Nonidet P40, 20 mM Tris-HCL pH 7.4, 5 mM MgCl₂, 1 mM PMSF, complete protease inhibitor, and phosphatase inhibitor

[Roche, Basel, Switzerland]). The proteins were separated through SDS-PAGE (NP0342; Life Technologies, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (10600009; GE Healthcare Life Science, Chicago, IL, USA) by semidry-blotting with Trans-Blot[®]TurboTM Transfer System (Bio-Rad Laboratories). The membranes were further sliced according to the required molecular weight of the proteins of interest, blocked in 4% BSA (23208; Thermo Fisher Scientific, Waltham, MA, USA) in TBS-Tween 20 (0.5%), and incubated with primary antibodies against Beclin1 (ab92389; Abcam, Cambridge, UK), UVRAG (U7508; Sigma-Aldrich, St. Louis, MO, USA), LC3B (ab51520; Abcam), SQSTM1 (ab96706; Abcam), AMPK-α (2532S; Cell Signaling Technology, Danvers, MA, USA), Phospho-AMPK-α (T172) (2525S; Cell Signaling Technology), AKT1 (ab54752; Abcam), Phospho-AKT1 (Ser473) (9271S; Cell Signaling Technology) [21], Phospho-AKT1 (T 308) (9275S; Cell Signaling Technology), β-actin (A5441; Sigma-Aldrich, St. Louis, MO, USA), and GAPDH (ab9485; Abcam). Bound primary antibodies were detected by secondary horseradish-labeled goat anti-rabbit (A0545; Sigma-Aldrich) and goat anti-mouse (A9917; Sigma-Aldrich) antibodies and SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The immunodetection was quantified using the Fusion image capture (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany) and Bio-1D analysis system (Vilber Lourmat Deutschland GmbH).

Fixation and Paraffin Embedding of Spheroids

Spheroids were collected, washed twice with PBS, immobilized in plasma-Thromborel S (Siemens Healthineers, Erlangen, Germany) 1:1 mixture clot, and fixed in 4% formaldehyde before paraffin embedding.

Immunofluorescence on Paraffin-Embedded Spheroids/Tissue

5-µm thin sections of 4% formaldehyde-fixed paraffin-embedded spheroids and Rip1Tag2 pancreas were cut, rehydrated, and deparaffinized. Antigen retrieval was performed in citrate buffer (pH = 6) in a microwave with 480 Watt for 10 min. The endogenous peroxidase was blocked with 3% H₂O₂ for 10 min. The sections were permeabilized by 0.5% Triton X-100 (Carl Roth GmbH & Co., KG) in PBS buffer (Life Technologies) for 10 min. Unspecific bindings were blocked through 30-min incubation in 10% immunized serum. The slides were then incubated with 1 µg/mL primary antibodies to TFEB (ab220695; Abcam), Beclin1 (ab114071; Abcam), and/or LC3B (51520; Abcam) in 1% BSA-PBS-0.5% Tween 20 overnight at 4°C. The bound primary antibodies were labeled with 2 µg/mL Alexa Fluor[®] 568 F (ab')₂ fragment of goat anti-rabbit IgG (H + L) (A21069; Invitrogen)/Alexa Fluor[®] 488 goat anti-mouse IgG (H + L) secondary antibodies. Nuclei were stained with 1 µg/mL Hoechst 33342 (Sigma-Aldrich) in 1% BSA-PBST. After 90-min incubation with secondary antibodies and Hoechst, the spheroid slides were washed and mounted with Fluoromount (Sigma), and the tissue slides of Rip1Tag2 were processed with Vector[®] TrueVIEWTM Autofluorescence Quenching Kit (Vector Laboratories, Burlingame, CA, USA) and mounted with VECTASHIELD[®] VibranceTM Antifade Mounting Medium (Vector Laboratories). LAS AF and LAS X software (Leica Microsystems, Wetzlar, Germany) was used for the analysis of fluorescence images acquired with the wide-field fluorescence microscope LEICA DM 5500 and the confocal microscope Leica TCS SP8.

Stable Transfection

BON1 and HPSC2.2 were stably transfected with an *E. coli* plasmid encoding for RFP-GFP-MAP1LC3B (ptfLC3 was a gift from Tamotsu Yoshimori) (Addgene plasmid # 21074; <http://n2t.net/addgene:21074>; RRID: Addgene_21074) [22] by incubation with 20 µg/mL plasmid in serum-free medium and FUGENE® HD Transfection Reagent (Promega). The selective agent G-418 (Roche Diagnostics GmbH, Risch-Rotkreuz, Switzerland) was added to fresh medium 3 days later. In the following weeks, transfected cells were separated from negative cells by scratching with a pipette under the fluorescence microscope and plating positive ones on a new dish with fresh medium containing G-418. Spheroids obtained with the transfected cells were treated with 100 pM bafilomycin and 10 nM panobinostat, and their fluorescence status was monitored by IncuCyte® S3 Live-Cell Analysis System (Sartorius, Göttingen, Germany).

Chromatin Immunoprecipitation

BON1 spheroids were treated with 100 pM bafilomycin and 10 nM panobinostat for 72 h. The phospho CREB (Ser133)-chromatin immunoprecipitation was performed with EZ-Magna ChIP™ Hi-Sense Chromatin Immunoprecipitation Kit (Merck Millipore, Burlington, MA, USA) and ChIPAb+™ Phospho-CREB (Ser133) – ChIP Validated Antibody and Primer Set (Merck Millipore).

Cross-linking of proteins to DNA and cell lysis were followed by manufacturer's instruction. DNA was sheared by sonication for 5 cycles of 30 s ON and 30 s OFF on high mode with the water bath sonicator Bioruptor™ Twin (Diagenode, Liege, Belgium). For immunoprecipitation of cross-linked protein/DNA, 3-µg anti-phospho CREB (Ser133) (17-10131; Merck Millipore) per reaction, 2-µg normal anti-rabbit IgG as negative control, and 2-µL anti-trimethyl-histone H3 (Lys4) (17-614; Merck Millipore) as positive control were used according to the manufacturer's instructions.

The immunoprecipitated DNA was processed by qPCR. C-Fos CRE promoter DNA sequence was amplified by the use of the following oligos: forward: GGCCACGAGACCTCTGAGACA and reverse: GCCTTGGCGGTGCTTAATCT. The data were normalized to fold enrichment.

Electron Microscopy

Spheroids of BON1 and HPSC2.2 cells were collected after 24, 48, and 72 h of treatment with 100 pM bafilomycin and 10 nM panobinostat. The spheroids were washed twice with Ca²⁺/Mg²⁺-free phosphate-buffered saline. Spheroids were then suspended in 2.5% glutaraldehyde 100 mM phosphate buffer (Sörensen's buffer), incubated for 90 min at 4°C, and then centrifuged at 1,000 rpm for 10 min at 4°C. The spheroid pellet was washed twice with Sörensen's buffer and then suspended in the same buffer and kept at room temperature for 7 days. Spheroid pellet was postfixed in Sörensen's buffer for 6 h at 4°C, rinsed in Sörensen's buffer overnight, and postfixed in 1% osmium tetroxide combined with 1.5% potassium ferricyanide [23] for 4 h (4°C). Following dehydration in graded series of ethanol and propylene oxide, cell pellets were placed in capsules and polymerized at 60 and 80°C for 24 h each. Single or serial silver ultrathin sections (approximately 70 nm) were cut, mounted on slot grids (Oval hole; Plano, Wetzlar, Germany), and stained with lead citrate and uranyl acetate. Section thickness was determined using the interference color of sections floating on water and the minimal folds method [24]. Ultrathin sections were examined with the Zeiss 906 electron microscope

Table 1. Forty-nine patients affected by pNEN included in the study for the expression of the autophagy markers

	Female	Male	Total
Gender	23	26	49
Age <50 years	15	13	28
Age >50 years	8	13	21
Nonmetastatic pNEN	19	16	35
Metastatic pNEN	4	10	14
Sporadic insulinomas	13	7	20
Hereditary insulinomas	2	3	5
Sporadic gastrinomas	0	4	4
Hereditary gastrinomas	3	3	6
Sporadic NF-pNET	1	2	3
Hereditary NF-pNET	2	1	3
NEC	2	6	8
Sporadic pNEN	16	19	35
Hereditary pNEN	7	7	14

Hereditary indicates patients affected by MEN1 syndrome. pNEN, pancreatic neuroendocrine neoplasia; NF-pNET, non-functional pancreatic neuroendocrine tumor; NEC, neuroendocrine carcinoid.

(LEO, Oberkochen, Germany) from 2,156 to 35,570 magnifications. Images created with electron microscopy were digitalized with the Agfa SnapScan e50 scanner (Agfa-Gevaert, Mortsel, Belgium) and adapted for brightness and contrast by Adobe Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA, USA) software. Black/white plates were created by using CorelDraw software (CorelDraw version 11; Corel Corporation, Ottawa, ON, Canada) [25]. Characteristic double membrane autophagosomes were counted as initial/early autophagic vacuoles, whereas autophagosomes that had fused with vesicles originated from the endo/lysosomal compartment were counted as degradative/late autophagic vacuoles. Data represent mean vesicle number per cell ± SD.

Collection, Processing, and Analysis of Patient Material

Total RNA was isolated from snap-frozen tumor tissue collected from 49 patients (Table 1) who undergone surgical resection at the University Hospital of Marburg between 1989 and 2016 by following the manufacturer's protocol of the RNA RNeasy Mini Kit (74106; Qiagen). Reverse transcription of mRNA was performed with iScript™ cDNA Synthesis Kit (170-8891; Bio-Rad) on Flex-Cycler (Analytik Jena AG). Qiagen primers for human *BECN1* (QT00004221), *UVRAG* (QT00034328), *MAP1LC3B* (QT00055069), *SQSTM1* (QT00095676), *TFEB* (QT00069951), *PRKAA1_1* (QT00009436), *PRKAA2_1* (QT00042077), and *GAPDH* (QT01192646) were used with GoTaq® qPCR Master Mix (Promega) on the RT-qPCR thermocycler CFX96™ Real-Time System (Bio-Rad Laboratories). Results were analyzed with the Bio-Rad CFX-Manager (Bio-Rad Laboratories) and normalized with *GAPDH* mRNA content for each sample. Raw data were further analyzed with Rest2009 (relative expression software tool V.2.0.13; Qiagen). The data were further statistically/graphically

processed and shown as box and whisker plots. Commercially available pancreas islets (2,000) collected from a healthy donor (00202998; Lonza, Basel, Switzerland) were processed for RNA isolation and qPCR and used as control to normalize the data of pNEN patients. All patients signed the informed consent, and the study was performed by following the approval of the Philipps University Marburg Ethics Committee (206/10).

Statistical Analysis

Statistical analysis was performed using SPSS 15.0.1 for Windows (SPSS Inc., Chicago, IL, USA). Significance was calculated using the *t* test for paired samples, ANOVA-Bonferroni post hoc test, and Kolmogorov-Smirnov test for normal distribution. ***p* < 0.01 and **p* < 0.05 were regarded as significant.

Results

Real-Time Cell Viability Measurement of pNEN Cells and Stellate Cells

pNEN cells BON1 and QGP1 and the human pancreatic stellate cells HPSC2.2, which were included as to control cell physiology and to monitor cytotoxic effects on pancreatic parenchymal cells, were incubated for 120 h with 100 pM of bafilomycin and 1–100 nM of panobinostat. The vacuolar H⁺ ATPase inhibitor bafilomycin, which acts as an autophagy inhibitor by preventing autophagosome fusion lysosome, increased BON1 cell proliferation after 50 h of treatment. Interestingly, 1 nM of panobinostat caused a significant decrease in cell growth after 40 h. Alternatively, 10 and 100 nM of panobinostat induced a decrease in cell viability after 12 h. Cell viability was significantly reduced at 120 h in comparison with untreated and bafilomycin-treated cells (*p* < 0.05) (Fig. 1a).

HPSC2.2 pancreatic stellate cell viability was not impaired by treatment with 100 pM of bafilomycin. Interestingly, 1 nM panobinostat slightly improved cell growth, whereas 10 nM panobinostat showed no effects compared to untreated cells. Exclusively, 100 nM panobinostat caused a significant reduction of cell viability (Fig. 1b). Forty hours of treatment with 100 pM of bafilomycin induced a strong increase of QGP1 cell proliferation. Instead, 1–100 nM of panobinostat caused a significant reduction in QGP1 cell viability (Fig. 1c). Phase contrast images verified that treatment with 100 pM of bafilomycin did not affect BON1 (Fig. 1a), HPSC2.2 (Fig. 1b), and QGP1 (Fig. 1c) cell growth and cell morphology after 24 h. Otherwise, 10 nM panobinostat induced a decrease in BON1 and QGP1 cell density after 24 h of treatment (Fig. 1a, c), while HPSC2.2 cells displayed no improvement (Fig. 1b).

Expression of Autophagic Markers in pNEN and Pancreatic Stellate Monolayer Cell Culture

Genes with regulatory functions in the transcription (*TFEB*), initiation (*MAP1LC3B*), nucleation (*MAP1LC3B*, *BECN1*, and *UVRAG*), and maturation (*MAP1LC3B*, *SQSTM1*) of autophagosomes were analyzed to demonstrate the ability of panobinostat to trigger autophagy in a 2D cell model of pNEN and pancreatic stellate cells. Inhibition of autophagy mediated by treatment with 100 pM of bafilomycin showed no significant changes in the level of autophagy transcripts in BON1, QGP1, and HPSC2.2 cells (Fig. 1a–c). Notably, 10 nM of panobinostat caused a significant increase in *MAP1LC3B* and *SQSTM1* in BON1 and QGP1 after 24 h of treatment, while *BECN1*, *UVRAG*, and *TFEB* remained stable (Fig. 1a, c). In HPSC2.2, 10 nM of panobinostat induced only a significant increase of *SQSTM1* and a significant decrease of *TFEB* transcript expression, while the expression of *BECN1*, *MAP1LC3B*, and *UVRAG* transcripts was not significantly affected compared to the untreated 2D cell model (Fig. 1b).

The densitometry analysis of autophagy marker protein level showed that 100 pM of bafilomycin caused a significant increase in the LC3B-I protein level of BON1 cells. Alternatively, 10 nM of panobinostat provided a reduction of Beclin1 and *UVRAG*, while p62 protein levels were increased. LC3B-I was stable. The lower band (LC3B-II) reflecting the lipidated active form of LC3B was not observable (Fig. 1a lowest panel). In the QGP1 cells, 100 pM of bafilomycin and 10 nM of panobinostat resulted in significant increase in Beclin1 and p62, while the LC3B-I and *UVRAG* protein levels remained stable. LC3B-II was not detectable (Fig. 1c lowest panel). In HPSC2.2, 100 pM of bafilomycin lowered the protein level of Beclin1 and LC3B-I. Stable expressions of LC3B-II, p62, and *UVRAG* have been observed. Furthermore, a significant decrease in Beclin1 and increase in LC3B-II and p62 proteins after treatment with 10 nM of panobinostat (Fig. 1b lower panel) could be shown.

Autophagic Marker Expression in Spheroids of pNEN Cells and Pancreatic Stellate Cells

The involvement of autophagy was analyzed in pNEN cells and HPSC2.2 spheroids that, compared to the traditional two-dimensional cell culture model, represent a three-dimensional model very close to the reality of the tissue structure. Treatment with 100 pM of bafilomycin did not cause any improvement in BON1 spheroid morphology after 72 h. Interestingly, 10 nM of panobinostat showed a cytotoxic effect in pNEN spheroids character-

Fig. 1. Monolayer cell viability and autophagy markers. BON1 (a), HPSC2.2 (b), and QGP1 (c) cells were cultured in 96-well E-plates and were treated with 100 pM of bafilomycin or 1–100 nM of panobinostat after 24 h. Cell impedance was measured continuously for 120 h. Shown are means of normalized cell index $\pm$ SD of 3 independent experiments performed in triplicates. BON1 (a), HPSC2.2 (b), and QGP1 (c) cells (middle panels) were treated with 100 pM of bafilomycin or 10 nM of panobinostat for 24 h. Inverted light microscopy pictures' magnification is $\times 100$. Expression of autophagy marker transcripts *BECN1*, *MAP1LC3B*, *SQSTM1*, *UVRAG*, and *TFEB* was determined in BON1 (a), HPSC2.2 (b), and QGP1 (c) monolayer cells treated for 24 h with 100 pM of bafilomycin or 10 nM of panobinostat (lower middle panel). Shown are means $\pm$ SEM of 3 independent experiments performed with biological duplicates. Level of autophagy proteins Beclin1, LC3B-I, UVRAG, and p62 was detected in BON1 (a), HPSC2.2 (b), and QGP1 (c) monolayer (lower panels) after 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat. Densitometry results were normalized to β -actin content. * $p < 0.05$: untreated versus bafilomycin- or panobinostat-treated cells.

UVRAG, and *TFEB* was determined in BON1 (a), HPSC2.2 (b), and QGP1 (c) monolayer cells treated for 24 h with 100 pM of bafilomycin or 10 nM of panobinostat (lower middle panel). Shown are means $\pm$ SEM of 3 independent experiments performed with biological duplicates. Level of autophagy proteins Beclin1, LC3B-I, UVRAG, and p62 was detected in BON1 (a), HPSC2.2 (b), and QGP1 (c) monolayer (lower panels) after 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat. Densitometry results were normalized to β -actin content. * $p < 0.05$: untreated versus bafilomycin- or panobinostat-treated cells.

Fig. 2. Spheroids and autophagy markers. Light microscopy (magnification $\times 100$) of BON1 (**a**) and HPSC2.2 (**b**) spheroids after 72 h of treatment with 100 pM of bafilomycin and 10 nM of panobinostat (upper panels). Expression of autophagy markers *BECN1*, *MAP1LC3B*, *SQSTM1*, *UVRAG*, and *TFEB* was detected in BON1 (**a**) and HPSC2.2 (**b**) spheroids after 72 h of treatment with 100 pM of bafilomycin and 10 nM of panobinostat. Shown are means $\pm$ SEM of 240 spheroids performed in duplicates (middle panels). Immunofluorescence-labeled TFEB and MAP1LC3B of BON1 (**a**) spheroids after 72 h and HPSC2.2 (**b**) spheroids after 24 h of treat-

ment with 100 pM of bafilomycin and 10 nM of panobinostat (magnification $\times 400$) (lower middle panels). Scale bar represents 20 μ m. Expression of autophagy marker proteins and densitometry of Beclin1, LC3B-I, LC3B-II, p62/SQSTM1, and UVRAG in BON1 spheroids (72-h treatment) (**a**) and HPSC2.2 spheroids (24-h treatment) (**b**) treated with 100 pM of bafilomycin and 10 nM of panobinostat. Densitometry results were normalized to β -actin content (mean of 240 spheroids). * $p < 0.05$: untreated versus bafilomycin- or panobinostat-treated cells (lower panels).

ized by an interruption of spheroid structure within 72 h (Fig. 2a). HPSC2.2 were morphologically unaltered after 72 h of treatment with 100 pM of bafilomycin. Instead, 10 nM of panobinostat released the spheroid intercellular adhesion and caused a loss of the 3D structure within 72 h (Fig. 2b). However, 100 pM of bafilomycin induced only a significant overexpression of the *MAP1LC3B* transcript, and 10 nM of panobinostat demonstrated its fine mechanism of action by inducing a significant overexpression of all autophagic marker transcripts in BON1 spheroids (Fig. 2a). In particular, *BECN1* (2.6-fold), *MAP1LC3B* (31-fold), and *UVRAG* (10-fold) were strongly upregulated. Also, the autophagy gene key regulator *TFEB* was 5-fold increased as well as *SQSTM1* (2.8-fold increased). In HPSC2.2 spheroids (Fig. 2b), a major overexpression of the *TFEB* transcription stage was triggered by 100 pM of bafilomycin. Nonetheless, 10 nM of panobinostat was able to induce a substantial overexpression of *MAP1LC3B* and *UVRAG* and, surprisingly, a significant reduction of *BECN1* and *TFEB*.

TFEB and LC3B proteins were identified by immunofluorescence in BON1 spheroids after 72 h of treatment (Fig. 2a). Treatment with 100 pM of bafilomycin did not modulate TFEB fluorescence in comparison with untreated spheroids, whereas LC3B immunofluorescence was slightly increased. 10 nM of panobinostat, instead, enhanced the TFEB fluorescent signal, which indicates a contribution of TFEB to panobinostat-mediated autophagy. In addition, LC3B immunofluorescence was strongly enhanced after 72 h of treatment (Fig. 2a).

HPSC2.2 spheroids were collected after 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat to prevent the massive shrinkage due to the long exposure to panobinostat that would affect the quality of protein isolation and formalin-fixed paraffin embedding. 100 pM of bafilomycin induced a slight reduction of the TFEB and LC3B immunofluorescence signals in these cells after 24 h. The treatment with 10 nM of panobinostat resulted in an increase of TFEB and LC3B expression in HPSC2.2 spheroids (Fig. 2b). BON1 spheroids showed a significant increase of LC3B-II and UVRAG protein levels after 72 h of treatment with 100 pM of bafilomycin, indicating accumulation of autophagosome proteins through the inhibiting effect of bafilomycin. Beclin1 has remained unchanged, and p62 slightly reduced compared to untreated spheroids. In contrast, 72 h of treatment with 10 nM of panobinostat caused lower regulation of the LC3B-I, LC3B-II, and UVRAG proteins. The protein level of Beclin1 was upregulated together with p62 (Fig. 2a lower left panel). Interestingly, both the unconjugated

form of LC3B-I (upper LC3B) and its phosphatidylethanolamine conjugated LC3B-II form (lower LC3B on Western blot gel shown in Fig. 2 lower left panel) bound to the autophagosome membrane were detected in BON1 spheroids treated for 72 h with bafilomycin or panobinostat (Fig. 2a lower left panel). In particular, the decrease in both LC3B forms, following treatment with 10 nM of panobinostat, suggests that the active autophagy mechanism promotes lipidation of LC3B-I for the formation of the autophagosome inner membrane and its further degradation by autophagosome lysosome fusion. While p62 is highly degraded during autophagy, p62 protein tends to recover after prolonged autophagy activation [26] due to the high p62 transcription compensation. Therefore, a detection of higher protein level may be consistent with the active autophagy mediated by treatment with 10 nM of panobinostat (Fig. 2a).

After 24 h of treatment with 100 pM of bafilomycin, HPSC2.2 spheroids showed a significant downregulation of Beclin1 and UVRAG protein levels. The accumulated LC3B-II and p62 were stable. On the other side, 10 nM panobinostat induced the overexpression of the LC3B-I and p62 protein levels. LC3B-II and UVRAG protein levels were stable while Beclin1 was downregulated (Fig. 2b lower right panel). These data suggest that the 3D model of pNEN and HPSC2.2 cells is more sensitive to treatment with compounds. While BON1 monolayer cells treated with panobinostat showed the overexpression of some autophagy transcripts, BON1-derived spheroids treated with panobinostat showed a significant overexpression of all autophagy transcripts and the appearance of active LC3B-II. Thus, suggesting that the use of 3D spheroids, instead of monolayer, is a functional model to monitor autophagy process. In addition, the spheroids could better clarify the involvement of the analyzed autophagy markers in the process of autophagosome synthesis and maturation. Treatment with panobinostat caused a change of the transcript and the protein level of *BECN1*, *MAP1LC3B*, and *UVRAG*, key players of initiation and nucleation of the autophagosome vesicles, and of p62

Fig. 3. Autophagosome maturation monitoring. Dynamic of autophagosome maturation in BON1 (a) and HPSC2.2 (b) spheroids stably transfected with RFP-GFP-MAP1LC3B. BON1 (a) and HPSC2.2 (b) spheroids were treated with 100 pM of bafilomycin and 10 nM of panobinostat for 24–72 h. RFP lightening appeared stronger than green fluorescence at late treatment time. Control cells are shown at time zero of treatment.

(For figure see next page.)

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(SQSTM1) which is the key player of the elongation of the autophagosome vesicle membranes.

Monitoring of Autophagic Process in BON1 and HPSC2.2 Spheroids

The autophagic process was monitored in BON1 and HPSC2.2 via stable plasmid transfection with GFP-RFP-MAP1LC3B. Double-labeled LC3B allows autophagic maturation and terminal degradation activity after fusion with the lysosome, where LC3B and the acid-sensitive GFP are degraded by the acidic milieu while the acid-stable RFP retains its fluorescence. Fluorescence status and spheroid morphology were continuously tracked with Incucyte for 72 h. Untreated and 100 pM bafilomycin-treated BON1 spheroids showed stable green (GFP) and red (RFP) fluorescence confirmed by green/yellow fluorescent detection. The spheroid size increased, and the morphology resulted unchanged. It should be noted that 10 nM panobinostat induced a decrease in green fluorescence and a corresponding increase in red fluorescence, suggesting a continuous autophagy process with significantly increased autolysosomal activity. In addition, spheroid size decreased after treatment with 10 nM of panobinostat due to active cell death (Fig. 3a; online suppl. Video; for all online suppl. material, see www.karger.com/doi/10.1159/000512567). After treatment with 100 pM of bafilomycin, HPSC2.2 spheroids showed a stable double fluorescence and a size comparable to untreated spheroids. Notably, 10 nM of panobinostat caused a strong reduction in green fluorescence and a change in fluorescence from yellow to red, indicating an active autophagic process in human pancreatic stellate cells (Fig. 3b; online suppl. Video).

Modulation of AMPK in 2D and 3D Cell Models

AMPK is an energy sensor that activates autophagy in response to cellular metabolic stress [27]. Expression of the *PRKAA1* and *PRKAA2* genes encoding for the catalytic subunits of AMPK- α was stable after 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat in both pNEN and HPSC2.2 2D cell models (Fig. 4a). BON1 monolayer showed a stable protein level of AMPK- α and a significantly higher level of the phosphorylated P-AMPK- α (T172) active form after 24 h of treatment with 100 pM bafilomycin. In parallel, 10 nM panobinostat caused a significant reduction in the overall AMPK- α and a significant increase in P-AMPK- α (T172). In the HPSC2.2 monolayer (2D), 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat resulted in a reduction of AMPK- α protein and a significant

downregulation of active P-AMPK- α (T172) protein. After 24 h of treatment with 100 pM of bafilomycin or 10 nM of panobinostat (Fig. 4b), QGP1 monolayer (2D) showed a stable protein level of both AMPK- α and P-AMPK- α (T172).

BON1 (72 h) and HPSC2.2 (24 h) spheroids treated with 100 pM of bafilomycin showed stable *PRKAA1* transcript expression and significantly increased expression of *PRKAA2*. On the other hand, BON1 spheroids showed significant overexpression of *PRKAA1* and *PRKAA2* transcripts after treatment with 10 nM of panobinostat (Fig. 4c). HPSC2.2 spheroids treated with 10 nM of panobinostat showed only *PRKAA2* transcript upregulation (Fig. 4c).

Detection of the total AMPK- α protein in BON1 spheroids showed significant overexpression after 72 h of treatment with 100 pM of bafilomycin; the P-AMPK- α protein level was stable. Curiously, 72 h of treatment with 10 nM panobinostat resulted in a significant downregulation of AMPK- α and its active phosphorylated form. After 24 h of treatment with 100 pM of bafilomycin, HPSC2.2 spheroids showed significant downregulation of the AMPK- α and P-AMPK- α (T172) protein levels, while treatment with 10 nM of panobinostat did not cause significant changes in the AMPK- α and P-AMPK- α proteins (Thr-172) (Fig. 4d). Interestingly, panobinostat induced a downregulation of AMPK- α in HPSC2.2 monolayers and spheroids and in BON1 spheroids. Given AMPK- α suppression, the autophagy mechanism was active as previously confirmed.

Fig. 4. AMPK- α expression in 2D and 3D models. **a** Expression of the genes *PRKAA1* and *PRKAA2*, encoding for the catalytic subunit of the autophagy regulator AMPK α , detected in BON1, HPSC2.2, and QGP1 monolayer after 24-h treatment with 100 pM of bafilomycin and 10 nM of panobinostat. Shown are means $\pm$ SEM of 3 independent experiments performed in biological duplicates. **b** Protein level of AMPK α and its activated form P-AMPK- α detected in BON1, HPSC2.2, and QGP1 monolayer cell culture after 24-h treatment with 100 pM of bafilomycin and 10 nM of panobinostat. Densitometry results were normalized to β -actin content. * $p < 0.05$ was considered significant: untreated versus bafilomycin- or panobinostat-treated cells. **c** *PRKAA1* and *PRKAA2* gene expression in BON1 spheroids (72 h) and HPSC2.2 spheroids (24 h) treated with 100 pM of bafilomycin and 10 nM of panobinostat. **d** Level of AMPK- α and P-AMPK- α proteins detected in BON1 spheroids (72 h) and HPSC2.2 spheroids (24 h) after treatment with 100 pM of bafilomycin and 10 nM of panobinostat. Proteins were isolated from 240 spheroids each experiment. Densitometry results were normalized to β -actin content. * $p < 0.05$ was considered significant: untreated versus bafilomycin- or panobinostat-treated cells.

(For figure see next page.)

Fig. 5. AKT expression in 2D and 3D models. **a** Protein level of AKT1 and P-AKT was determined in BON1 monolayer cell culture (24-h treatment) and BON1 spheroids (72-h treatment) after treatment with 100 pM of bafilomycin and 10 nM of panobinostat. Densitometry results were normalized to β -actin content. $*p < 0.05$ was considered significant: untreated versus panobinostat-treated cells or combined treatment of cells with panobinostat and wortmannin. **b** RT-qPCR immunoprecipitation of p-CREB (Ser133) was performed in BON1 spheroids after 72-h treatment with 100 pM of bafilomycin and 10 nM of panobinostat. ChIP-qPCR data were normalized to the background signal (fold enrichment).

c Light microscopy of BON1 monolayer cell culture (24-h treatment) and BON1 spheroids (72-h treatment) treated with 10 nM of panobinostat and 200 nM of wortmannin (magnification $\times 100$). **d** Protein level of AKT, P-AKT, AMPK- α , and P-AMPK- α was determined in BON1 monolayer cell culture after 24-h treatment and BON1 spheroids after 72-h treatment with 10 nM of panobinostat and 200 nM of wortmannin. Densitometry results were normalized to β -actin. $*p < 0.05$ was considered significant: untreated versus panobinostat-treated cells or combined treatment of cells with panobinostat and wortmannin.

Regulation of AMPK- α Phosphorylation Is Dependent of AKT

In order to clarify the importance of AMPK- α suppression, we decided to investigate AKT1 expression in the proposed model. AKT1 interacts with AMPK- α via mutual phosphorylation. AKT1 is responsible for the phosphorylation of AMPK- α at Ser-487/491, which, in turn, suppresses the phosphorylation of AMPK- α at Thr-172 and thus inhibits its activation [10]. The status of AKT1 phosphorylation was analyzed in BON1 in order to determine any influence of its activity in panobinostat-mediated autophagy.

Both the total AKT1 protein level and its Ser-473-phosphorylated form were downregulated by treatment with 100 μ M of bafilomycin in BON1 monolayer (2D) after 24 h and in spheroid (3D) models after 72 h. Treatment with 10 nM of panobinostat also determined a major downregulation of both proteins in the monolayer model. Instead, the BON1 spheroids showed a significant upregulation of both the total AKT1 protein level and its Ser-473-phosphorylated form (Fig. 5a). The AKT1 phosphorylation site at Thr-308 was not observed in untreated and treated BON1 cells.

In order to check the inhibitory effect of P-AKT1 (Ser-473) on AMPK- α , BON1 monolayer and spheroids were simultaneously treated for 24 h (monolayer)/72 h (spheroids) with 10 nM panobinostat and 200 nM wortmannin, a well-known PI3K/AKT pathway inhibitor. Inhibition of PI3K/AKT through wortmannin did not cause any additional morphological cytotoxic effects in panobinostat-treated BON1 (Fig. 5c). Indeed, the addition of 200 nM wortmannin was able to restore the protein levels of AMPK- α and P-AMPK- α by counteracting the total protein levels of AKT1 and its Ser-473-phosphorylated form in BON1 cells, compared to 10 nM panobinostat alone (Fig. 5d).

Metabolic Stress Caused by Panobinostat Induces p-CREB Transcriptional Activity

In order to prove the activation of the cyclic AMP cascade and to exclude any possible involvement of the AMPK- α suppression associated with this pathway, the binding activity of the cAMP responsive element was analyzed in the BON1 spheroids. Seventy-two hours of treatment with 10 nM of panobinostat induced a significant increase (6.03-fold enrichment) of the amplified DNA sequence corresponding to the promoter region of the *c-FOS* gene relative to untreated spheroids (3.95-fold enrichment). Interestingly, 100 μ M of bafilomycin caused a significant downregulation of the *c-FOS* promoter re-

gion (1.36-fold enrichment) (Fig. 5b). In conclusion, panobinostat caused a significant cAMP-dependent increase in P-CREB activity, thus promoting the activation of the cAMP cascade, the genetic response, and the activation of AMPK- α , which is distinctly controlled by AKT1 in the autophagy process of BON1 spheroids.

Electron Microscopy Imaging Autophagosome Accumulation in BON1 and HPSC2.2 Spheroids

Autophagosome vesicle status and cellular localization are crucial to highlight the autophagy process. Challenging the localization of autophagosomes into the cellular compartment of a 3D body mass will explain the maturation status of these vesicles and their distribution in a tissue-like structure. BON1 and HPSC2.2 spheroids were treated with 100 μ M of bafilomycin and 10 nM of panobinostat for 72 h. As shown in Figure 6a, untreated BON1 spheroids cells present, predominantly in their cytosolic compartment, early and later stage autophagosomes lower in number than in treated spheroids. Treatment with 100 μ M of bafilomycin caused significant accumulation ($p < 0.05$) of early autophagosomes after 24 h. Late autophagosomes number did not increase significantly. The alteration/disassembly of cellular compartments and the ultrastructure of spheroids were not detectable. Interestingly, autophagosomes were evident in the nuclei of cells treated with bafilomycin.

Panobinostat-mediated autophagic cell death was further confirmed by electron micrographs. In particular, the treatment with 10 nM of panobinostat (Fig. 6a) resulted in a massive accumulation of early and late autophagosomes in the cytosolic cell compartments after 24 h, accompanied by a strong cellular digestion and disaggregation of the spheroid structure (48 and 72 h), sustained by a disappearance of the perinuclear endoplasmic reticulum (ER) acting as a major donor of membranes for the synthesis of autophagosomal vesicles. The number of nuclear autophagosomes was significantly higher after 72 h. Additionally, the micrographs proved absent apoptotic bodies and apoptotic blebbing, thus excluding an apoptotic process in BON1 treated with panobinostat. TUNEL assay proved absent DNA fragmentation in BON1 treated with 10 nM of panobinostat, further confirming the absence of apoptotic process (see online suppl. Fig. 2).

HPSC2.2 spheroids were also distinguished by basal autophagosome aggregation in untreated spheroids in the cytosolic and nuclear cellular compartments (Fig. 6b). Treatment with 100 μ M of bafilomycin showed an accumulation of early autophagosomes in the cytosolic compartment without affecting the integrity of the spheroid.

Fig. 6. Electron microscopy of spheroids. Transmission electron microscopy of BON1 spheroids (a) and HPSC2.2 spheroids (b) treated with 100 pM of bafilomycin or 10 nM of panobinostat for up to 72 h. Magnification is $\times 2,156$ and $\times 35,570$, and scale bars represent 5 and 10 μm . Arrows indicate cytosolic and nuclear autophagosomes. Statistical analysis of electron microscopy detected

early, late, and nuclear autophagosomes in untreated BON1 (upper right graph) and HPSC2.2 (lower right graph) spheroids and after treatment with 100 pM of bafilomycin or 10 nM of panobinostat. $*p < 0.05$ was considered significant: untreated versus bafilomycin- or panobinostat-treated cells. $\#p < 0.05$ was considered significant: bafilomycin versus panobinostat-treated cells.

The number of nuclear autophagosomes has been stable. The alteration/disassembly of cellular compartments and the ultrastructure of spheroids were not detectable. As with BON1 spheroids, 10 nM panobinostat caused massive dismantling of the spheroid architecture in HPSC2.2 spheroids and progressive cell digestion characterized by large accumulation ($p < 0.05$) of early and late cytosolic autophagosomes at all time points and ER dismantling

(Fig. 6b). Nuclear autophagosomes have increased significantly ($*p < 0.05$). The treatment with panobinostat did not cause neither an accumulation of apoptotic bodies nor an apoptotic blebbing, thus excluding the induction of apoptotic process. TUNEL assay proved absent DNA fragmentation in HPSC2.2 treated with 10 nM of panobinostat, further confirming the absence of apoptotic process (online suppl. Fig. 2).

Fig. 7. Monitoring of autophagy in the insulinoma mouse model. HMEG2725 monolayer (magnification $\times 100$) treated for 24 h (a) and HMEG2725 spheroids (magnification $\times 100$) treated for 72 h (c) with 100 pM of bafilomycin and 10 nM of panobinostat. Scale bars represent 50 and 100 μ m. Expression of autophagy marker transcripts *Becn1*, *Map1lc3b*, *Sqstm1*, *Uvrags*, *Prkaa1*, and *Prkaa2* detected in HMEG2725 monolayer (b) and HMEG2725 spheroids (d) after 24-h treatment with 100 pM of bafilomycin and 10 nM of panobinostat. For monolayer are shown means $\pm$ SEM of biological

cal duplicates. For 3D are shown means $\pm$ SEM of 240 spheroids in duplicates. e Immunofluorescence-labeled TFEB and LC3B in HMEG2725 spheroids after 24-h treatment with 100 pM of bafilomycin and 10 nM of panobinostat (magnification $\times 630$). Scale bar represents 20 μ m. f Confocal imaging of double immunofluorescence-labeled Beclin1 (green) and LC3B (red) in insulinomas of the transgenic Rip1Tag2 mouse model and pancreatic islets of wild-type mice. Magnification is $\times 630$. Scale bar represents 25 and 8 μ m.

Monitoring Autophagy in Rip1Tag2-Derived Insulinoma Cells

Autophagy process was monitored in HMEG2725 mouse insulinoma cells. While 100 pM bafilomycin did not affect the monolayer cell morphology after 24 h of treatment, 10 nM of panobinostat induced a reduction in cell density (Fig. 7a). In addition, substantial overexpression of *Map1lc3b* (2.7-fold) and *Prkaa2* (2.7-fold) transcripts was observed after treatment with 10 nM of

panobinostat, whereas 100 pM bafilomycin resulted in no significant change of autophagy gene expression (Fig. 7b).

In addition, 100 pM of bafilomycin did not cause any alteration of the spheroid morphology or the expression of autophagy marker transcripts in HMEG2725 spheroids. The cytotoxic effects of 10 nM panobinostat, already observed in BON1 spheroids, were visible under light microscopy in HMEG2725 too (Fig. 7c). A significant in-

Fig. 8. Autophagy marker expression in HMEG2725. **a** Protein level of Beclin1, Lc3b, Uvrage, and p62 in HMEG2725 monolayer (left panel) and spheroids (right panel) after 24-h treatment with 100 μ M of bafilomycin and 10 nM of panobinostat. Densitometry results were normalized to Gapdh protein content (mean of 240 spheroids). * $p < 0.05$ was considered significant: untreated versus bafilomycin- or panobinostat-treated cells. **b** Protein level of autophagy regulators Ampk- α , P-Ampk- α (thr172), Akt, and P-Akt (Ser473) in HMEG2725 monolayer cell culture and spheroids after 24-h treatment with 100 μ M of bafilomycin and 10 nM of panobinostat. Protein extracted from 240 spheroids. Densitometry results were normalized to GAPDH content. * $p < 0.05$ was considered significant: untreated versus bafilomycin- or panobinostat-treated cells.

crease in all autophagic marker transcripts *Becn1*, *Map1lc3b*, *Sqstm1*, *Uvrag*, *Prkaa1*, and *Prkaa2* was observed after 72 h of treatment (Fig. 7d). Treatment with 100 pM of bafilomycin resulted in a decrease in the fluorescent signal of TFEB and an increase in the fluorescent signal of LC3B in HMEG2725 spheroids. Alternatively, both TFEB and LC3B fluorescence increased significantly after treatment with 10 nM of panobinostat (Fig. 7e).

Localization of the autophagic markers Beclin1 and LC3B was observed by confocal microscopy immunofluorescence in pancreatic islets of wild-type mice and murine insulinomas Rip1Tag2 (Fig. 7f). Pancreatic islets, particularly the outer cells, showed a colocalization of Beclin1 (green) and LC3B (red) confirmed by the yellow color generated by the double staining overlap. Rip1Tag2 insulinomas showed a diffuse distribution of the protein Beclin1 and LC3B. No colocalization was found, suggesting a faulty autophagosome maturation process (Fig. 7f).

Expression of Autophagy Proteins and AKT1 in HMEG2725 Mouse Insulinoma Cells

HMEG2725 monolayer treated with 100 pM of bafilomycin for 24 h showed a downregulation of Beclin1, Uvrag, and p62. Lc3b-I and Lc3b-II protein level was, instead, upregulated. Interestingly, 24 h of treatment with 10 nM of panobinostat resulted in a significant upregulation of Lc3b-I, Lc3b-II, and Uvrag protein levels, where Beclin1 and p62 were downregulated (Fig. 8a left panels).

The protein expression of HMEG2725 spheroids was examined after 24 h of treatment because the long-time exposure to panobinostat affected the consistency and amount of isolated HMEG2725 proteins. The treatment with 100 pM of bafilomycin resulted in an increase of Lc3b-I. In addition, a significant reduction of Beclin1 and Uvrag was observed, while p62 remained stable. Treatment with 10 nM of panobinostat then resulted in increased levels of Beclin1, Lc3b-I, Lc3b-II, and Uvrag proteins. p62 was downregulated (Fig. 8a right panels).

Furthermore, bafilomycin increased the protein level of P-Ampk- α (threonine 172) in monolayer and spheroids, while the protein level of Ampk- α was stable. Additionally, bafilomycin caused a significant increase of Akt1 in 2D and 3D models. The P-Akt1 protein level was significantly lower in monolayer, whereas it increased in spheroids (3D). Treatment with 10 nM of panobinostat caused a significant decrease in the amount of P-Ampk- α protein in monolayers and a significant increase in total Akt1. In spheroids, both Ampk- α and P-Ampk- α were downregulated. Akt1 protein level was stable, and P-Akt1 was significantly increased (Fig. 8b left and right panels).

Interestingly, monolayer and spheroids were characterized by a panobinostat-mediated downregulation of Ampk- α and P-Ampk- α (T172). The total Akt1 level was stable, and its phosphorylated form at Ser-473 was upregulated showing a similar pattern observed in BON1 spheroids.

Expression of Autophagy Markers in pNEN Patients

The expression of *BECN1*, *MAP1LC3B*, *SQSTM1*, *UVRAG*, *TFEB*, *PRKAA1_1*, and *PRKAA2_1* transcripts was further detected in primary tumors of 49 patients affected by pNEN including those affected by metastasis (14) (Table 1). As shown in Figure 9, the results highlighted that the autophagy markers were detectable in all patients. The expression of the *BECN1* transcript was stable both in nonmetastatic and metastatic patients. Interestingly, *MAP1LC3B*, *SQSTM1*, *UVRAG*, *TFEB*, *PRKAA1_1*, and *PRKAA2_1* transcripts were significantly downregulated ($p < 0.05$) in metastatic patients. The downregulation of the autophagy markers could trigger an impairment of autophagy process in metastatic patients, which would then evidence an intrinsic difference between patients affected by pNEN.

Discussion/Conclusion

Knowing cellular processes such as autophagy is necessary to develop new strategies for effective treatment of neuroendocrine cancer and human pancreatic diseases. Panobinostat, a pan-deacetylase inhibitor currently approved for the treatment of multiple myeloma [3], increased the expression of *MAP1LC3B* and *SQSTM1* transcripts, which coincidentally decreased cell viability in the 2D pNEN cell lines. Furthermore, the protein levels of the Beclin1, UVRAG, and p62 autophagic players have been altered by panobinostat. Alternatively, inhibition of autophagy process mediated by treatment with bafilomycin did not alter the expression of autophagy transcripts, but resulted in an upregulation of Beclin1 and LC3B-I proteins in both pNEN cells. However, the activity of panobinostat was weak in terms of autophagy modulation.

Alternatively, the application of panobinostat to spheroids demonstrated a greater efficiency in the overexpression of all autophagic transcripts. Also, an increase in TFEB and aggregation/accumulation of LC3B proteins have been observed. The presence of the double-band LC3B [28], reflecting its cytosolic form LC3B-I and its membrane-bound form LC3B-II, is observed in BON1 spheroids, given that the active vesicle-bound LC3B

Fig. 9. Expression of autophagy markers in patients affected by pNEN. Box and whisker plots showing the expression of the autophagy transcripts *BECN1*, *LC3B*, *SQSTM1*, *UVRAG*, *TFEB*, *PRKAA1_1*, and *PRKAA2_1* in patients affected by pNEN. The box

plots indicate nonmetastatic and metastatic pNEN. The expression was normalized to pancreatic islets collected from healthy donor and commercially available. GAPDH was detected as the house-keeping gene. pNEN, pancreatic neuroendocrine neoplasms.

spheroids are a functional model for the study of autophagy.

SQSTM1 may be stable or even increased during active autophagy [26], in order to maintain the first autophagy phase. As a result, its protein level increased in BON1 spheroids treated with panobinostat due to the significant upregulatory transcription of its corresponding gene. In addition, both LC3B and UVRAG proteins, the fusion proteins of the autophagosomal membrane, due to an improved turnover of autophagy process induced by panobinostat, were downregulated. Bafilomycin-mediated inhibition of autophagy caused, instead, an overexpression of *MAP1LC3B* transcripts and protein levels, as well as an accumulation of Beclin1, UVRAG, and p62 proteins. The prompted course of autophagy process, necessary to sustain the increased catabolism of autophagy markers [29], was proven by the fluorescent switch of ectopic LC3B detected in BON1 and HPSC2.2 spheroids treated with panobinostat. ER is actively involved in the formation of autophagosomes, and due to its main role of a membrane donor, reduces dramatically its volume, as observed after electron microscopy imaging, leading to the total disappearance of its perinuclear distribution in favor of a massive cytosolic autophagosomes synthesis. However, autophagosomes have also been detected in the nuclear compartment of pNEN cells, the function of which could be attributed to their own peculiar process of degradation of subnuclear organelles and genomic material.

Likewise, the inhibitory interaction existing between AKT1 and AMPK- α was neutralized by the PI3K/AKT pathway inhibitor wortmannin, which restored AMPK- α phosphorylation. However, autophagy was neither hindered by the inactive AMPK- α nor prompted by a phosphorylated active form of it. Increased binding of c-Fos to the panobinostat-induced P-CREB promoter could further support the activation of the cAMP signaling pathway and the activity of cAMP independent of the AMPK- α status. Instead, bafilomycin-mediated inhibition of autophagy impeded cAMP-regulated transcriptional activity. HPSC2.2 spheroids treated with panobinostat did not show any resemblance to the pNEN cells in the pattern of expression of autophagy transcripts and proteins, showing significant downregulation of *Beclin1* and *TFEB* transcripts and significant overexpression of *MAP1LC3B* and *UVRAG* transcripts. Analyzing the protein levels of autophagic markers, it was possible to detect increases in TFEB, p62, LC3B-I, and LC3B-II protein levels, while Beclin1 and UVRAG were downregulated. Additionally, the major disparity between the cells of the pNEN was demonstrated by stellate cells carrying active AMPK- α . More-

over, panobinostat again showed weak efficacy in murine insulinoma cells HMEG2725 monolayer by inducing only significant overexpression of *Map1lc3b* and *Prkaa2* transcripts. Instead, a substantial overexpression of all autophagic transcripts and proteins was found in spheroids, with the only exception for *Sqstm1*, which was downregulated. Once again, as it has been observed for pNEN cells, increased expression of autophagic markers has been associated with suppression of AMPK- α and increased or stable AKT1 protein levels.

Islet cells of wild-type mice were distinguished by the colocalization of the autophagosome markers Beclin1 and Lc3b. Despite the basal expression of these markers, Rip1Tag2 insulinomas lacked colocalization, indicative of an aberrant/deficient autophagy process that would contradict previous findings [30], thus illustrating the possibility of restoring autophagy-dependent cell death by excessive autophagy induction. Unfortunately, due to its heterogeneity [31, 32], the Rip1Tag2 mouse model would not be appropriate for an in vivo panobinostat functional assay. Instead, further functional studies could be easily established and rapidly monitored in newly established spheroid models derived from human and murine pNEN cells.

Recent advances proposed that sunitinib, a multityrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors, can trigger cell death by inhibiting autophagy in pNEN cells by combining its administration with chloroquine [30]. Instead, panobinostat is able to prompt autophagy leading to death of pNEN cells. Patients affected by pNEN showed a basal expression of autophagy markers, which are significantly downregulated in metastatic pNEN. Recent advances reported that pNEN are characterized by mutations occurring at several genes implicated in PI3K/mTOR, chromatin remodeling, and DNA damage repair [33]. In particular, somatic mutations of such genes could affect the expression of contiguous genes. Mutation occurring at *BRCA1* (Breast Cancer type 1 susceptibility protein), responsible for its suppression, causes the loss of expression of the neighbor gene *BECN1* [34]. Stressing autophagy by treatment with panobinostat can further lead to cell death in pancreatic neuroendocrine tumor cells and human pancreatic stellate cells independently of AMPK- α activation. This process is acting not only in the cytosol but also in the nuclear compartment of pNEN and stellate cells. Patients affected by pNEN, where expression of autophagy markers is indicative of a sensitivity to autophagy process, would benefit by the restoration of cell death processes driven by au-

tophagy. Furthermore, the possibility to target autophagy bypassing the activation of AMPK- α could offer a new perspective for the therapy of human diseases characterized by insulin resistance [35].

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Statement of Ethics

The patients included in the study signed the inform consent, and the study was conducted by following the approval of the local ethics committee (206/10). No animals were involved in this study. The tissue slides of Rip1Tag2 mice were gently provided by Heidi Griesmann (University Hospital Halle).

Conflict of Interest Statement

The authors declare no potential conflicts of interest.

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Author Contributions

S.M., S.J., D.W., D.N., H.G., I.M., and P.D. performed the experiments and analyzed the data. H.G. isolated the HMEG2725 cells. M.W. and T.S. supervised Incucyte experiments. S.M., H.G., and P.D. wrote the manuscript. T.T.W., M.B., E.K., and D.K.B. revised the manuscript. N.d.P. and V.A.G. critically revised and formatted the manuscript and contributed to the interpretation of the data. All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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