Regulation of hypoxia-induced autophagy in glioblastoma involves ATG9A

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Background: Hypoxia is negatively associated with glioblastoma (GBM) patient survival and contributes to tumour resistance. Anti-angiogenic therapy in GBM further increases hypoxia and activates survival pathways. The aim of this study was to determine the role of hypoxia-induced autophagy in GBM.

Methods: Pharmacological inhibition of autophagy was applied in combination with bevacizumab in GBM patient-derived xenografts (PDXs). Sensitivity towards inhibitors was further tested in vitro under normoxia and hypoxia, followed by transcriptomic analysis. Genetic interference was done using ATG9A-depleted cells.

Results: We find that GBM cells activate autophagy as a survival mechanism to hypoxia, although basic autophagy appears active under normoxic conditions. Although single agent chloroquine treatment in vivo significantly increased survival of PDXs, the combination with bevacizumab resulted in a synergistic effect at low non-effective chloroquine dose. ATG9A was consistently induced by hypoxia, and silencing of ATG9A led to decreased proliferation in vitro and delayed tumour growth in vivo. Hypoxia-induced activation of autophagy was compromised upon ATG9A depletion.

Conclusions: This work shows that inhibition of autophagy is a promising strategy against GBM and identifies ATG9 as a novel target in hypoxia-induced autophagy. Combination with hypoxia-inducing agents may provide benefit by allowing to decrease the effective dose of autophagy inhibitors.

Despite considerable advancement in the molecular characterisation of glioblastoma (GBM), survival of patients under current treatment regimen remains disappointing. Treatment failure is partially due to the capacity of tumour cells to activate pro-survival pathways in an unfavourable microenvironment. The GBM vasculature is poorly functional, leading to insufficient oxygen supply and necrotic areas (Evans et al., 2004). Hypoxia and angiogenic factors are correlated with tumour grade and poor patient prognosis in brain tumours (Yang et al., 2012) and are linked to radiation- and chemotherapy resistance (Vaupel and Mayer, 2007). Although targeting angiogenesis has long been regarded as an attractive therapeutic approach, anti-angiogenic agents are incapable to halt tumour progression and improve patient survival (Gilbert, 2016). We have previously shown that administration of bevacizumab, an antibody against vascular endothelial growth factor (VEGF), resulted in an adaptive metabolic switch leading to an increased hypoxia and induction of glycolysis (Keunen et al., 2011; Fack et al., 2015). However, the
exact mechanism of GBM cell survival and adaptation under hypoxia are still incompletely understood.

Solid tumours use autophagy as one of the survival mechanisms upon various stressors including metabolic stress and starvation (Yang et al., 2011), hypoxia (Rabinowitz and White, 2010; Roushchef et al., 2010), chemotherapy (Kanzawa et al., 2004; Ciechomska et al., 2013) and radiotherapy (Firat et al. 2012). In physiological situations, autophagy has an important role in organelle turnover, degradation of proteins, cellular differentiation and aging (Glick et al., 2010). During stress, autophagy protects cells by eliminating damaged organelles and proteins via autophagosomes. Autophagosomes fuse with lysosomes to form the autolysosome responsible for enzymatic self-digestion of cellular waste. Recycled cellular components may serve as an energy source during periods of starvation, hypoxia or high-energy demand. Under physiological hypoxia (0.1–3%O₂), the autophagic response is HIF1α-dependent (Mazure and Pouyssegur, 2010) and relies on the induction of the pro-autophagic genes BNIP3 (BCL2/adenovirus EIB (BCL2/adenovirus EIB interacting protein 3) and BNIP3L (BNIP3-like) (Pouyssegur et al., 2006; Bellot et al., 2009). Furthermore, autophagy is strongly dependent on the synchronised action of autophagy-related (ATG) genes. Although many ATG genes are modulated upon induction of autophagy (Gasch et al., 2000), their specific roles are not always fully elucidated. ATG9A is the only transmembrane autophagy-related protein and has been associated with the regulation of autophagosome formation (Jin and Klionsky, 2014). ATG9A cycles between the Golgi network, endosomes and the so-called ‘ATG9A reservoir’, and ATG9A-containing vesicles in cytoplasm, creating a ready source to support autophagosome formation (Reggiori and Tooze, 2012). Although the detailed mechanism is poorly understood, it is thought to support the growth and maturation of autophagic membranes by recruiting membrane structures to the LC3-positive autophagosomes (Orsi et al., 2012; Yamamoto et al., 2012; Corcelle-Termeau et al., 2012; Lamb et al., 2016).

Following up on our earlier studies (Fack et al., 2015; Sanzey et al., 2015), we addressed the role of autophagy in enabling cell survival in severe hypoxia and during anti-angiogenic treatment. We show that GBM cells activate autophagy in hypoxia and that ATG9A has an essential role in the autophagic response of GBM.

**MATERIALS AND METHODS**

**GBM patient material.** Human GBMs were obtained from the Neurosurgery Department of the Centre Hospitalier in Luxembourg (CHL) (T16) or the Department of Neurosurgery, Haukeland University Hospital in Bergen (P3, P8), Norway. All patients had provided informed consent, tumour collection was approved by the National Research Ethics Committee for Luxembourg (CNER) or by the Regional Ethical Board at the Haukeland University Hospital in Bergen. All biopsies were primary GBM based on neuropathological diagnosis and genomic analysis (Supplementary Table S1). The original organotypic GBM spheroids from patient samples were prepared as previously described (Keunen et al., 2011; Golebiewska et al., 2013; Bougnaut et al., 2016) and maintained in spheroid medium (DMEM medium, 10% FBS, 2 mM l-Glutamine, 0.4 mM NEAA and 100 μM Pen-Strep; Lonza, Basel, Switzerland) in agar pre-coated flasks for 7–10 days.

**Orthotopic patient-derived GBM xenografts.** Serial transplantation of PDx in eGFP-expressing NOD/SCID mice were used to expand the tumour material and prepare spheroids for in vitro assays, as previously described (Niclou et al., 2008; Bougnaut et al., 2016). For treatment experiments, P3 and T16 GBM spheroids expressing RFP were orthotopically implanted into the right frontot lobe of Swiss nude mice (6 per mice). Tumour growth was monitored by in vivo fluorescence imaging (IVIS Lumina Fluorescence system; PerkinElmer, Waltham, MA, USA). Three weeks post implantation mice were randomly allocated into treatment groups (6–7 mice per group). Bevacizumab, chloroquine and normal saline were delivered by intraperitoneal injections. The treatment schedule is summarised in Supplementary Table S2. NCH421k and NCH644 harbouring Scramble or ATG9A shRNA were stereotactically implanted in NOD/SCID mice (13 7500 NCH421k cells or 50 000 NCH644 cells per animal; 6–7 per group). Animals were monitored daily and the following criteria were evaluated: (1) loss of >10% of body weight, (2) exhibition of strong neurological signs (3) increased lordosis or (4) swollen belly. The criteria were scored as: 0 = none, 1 = early, 2 = established, 3 = severe signs and animals were killed when three criteria with grade 2 or 1 criteria with grade 3 were reached. All procedures were approved by the national authorities responsible for animal experiments in Luxembourg.

**Immunohistochemistry.** For mouse-specific CD31 staining cryostat sections (10 μm) of flash-frozen brains were fixed in ice-cold acetone and acetone:chloroform (1:1) for 5 min each. Sections were blocked for 1 h in TBS/2% FCS, followed by a 1 h incubation in rat anti-mouse CD31 antibody (Merck Millipore, Nottingham, UK, 1:200). Alexa Fluor 488-conjugated secondary antibodies were applied for 1 h. Sections were analysed by fluorescence microscopy. Quantification of vessel staining was done using ImageJ (NHS, Bethesda, MA, USA) from 3–4 images per group (9–34 images per mouse).

**Western blotting.** GBM cells were cultured in normoxia or 0.1% O₂ hypoxia for 48 h. When indicated, 20 μM chloroquine was added 16 h before cell collection. Cultured cells or spheroids were lysed in RIPA buffer (Merck Millipore) with 0.1% SDS. Overall, 30 μg of proteins were loaded and separated in a NuPAGE Novex 4–12% Bis-Tris Gels (Life Technologies, Merelbeke, Belgium) followed by electroblot transfer to a PVDF membrane (Novex, InvitroCon PVDF, Life Technologies). Membranes were blocked with 2% non-fat milk in Tris-buffered saline containing 0.1% Triton-X before incubation with primary antibodies (LC3B: Cell Signaling Technology, Danvers, MA, USA, 1:2000; p62: BD Bioscience, Erembodegem, Belgium, 1:1000; Actin: Millipore, 1:10 000; Tubulin: Millipore, 1:5000). Secondary coupled to horseradish peroxidase were detected by enhanced chemiluminescence (ECL) (Lumigen TMA6, GE Healthcare) with luminescent image analyser (Image Quant LAS4000, GE Healthcare, Diegem, Belgium). Quantification was performed with the ImageQuant TL. Owing to the substantial normalisation problems linked to disturbed actin and tubulin signal in hypoxic cells upon induction of autophagy (Klionsky et al., 2016), WB signals were normalised to total protein content.

**Cell viability in GBM spheroids.** Cell viability after 72 h of treatment with inhibitors was assessed by double labelling with 2 μM Calcein AM and 4 μM Ethidium homodimer-1 (LIVE/DEAD Viability/Cytotoxicity assay kit, Molecular Probes, Eugene, OR, USA) for 6 h. Measurements of viable (‘green’) and dead (‘red’) cells were performed using fluorescence confocal microscopy (Zeiss LSM STO META, Zeiss, Zaventem, Belgium) by obtaining 20–25 stacks of two-dimensional images from successive focal planes (5 μm). Quantification was performed using IMARIS software (Bitplane, Belfast, UK). The volume of viable and dead cells within a spheroid was calculated by multiplying the surface area of each component per stack by the total height of the image stacks. The percentage of dead cell volume was calculated as: % dead cell in spheroids (volume) = Dead cell volume (‘red’)×100/Total spheroid volume (‘green’ + ‘red’). Experiments were carried out three times with at least five spheroids each.

**Cell culture.** The primary adherent P3 cells (P3A) was derived from patient xenograft-derived P3 3D spheroids grown in uncoated flasks until a confluent adherent culture was obtained.
P3A, U87, U251 and T98G cells were cultured as monolayers in DMEM containing 10% FBS, 2 mM l-glutamine and 100 U ml⁻¹ Pen-Strep (Lonza). The normal human astrocytes (NHA) (kindly provided by Dr Uros Rajcevic, Ljubljana, Slovenia) grew in DMEM, 10% FBS, 2 mM l-glutamine and 100 U ml⁻¹ Pen-Strep (Lonza). GBM stem-like cultures (NCH421k, NCH660h, NCH465, NCH601 and NCH644) were kindly provided by Christel Herold-Mende (University of Heidelberg, Germany) and were cultured as previously described (Sanze et al., 2015). Normoxic cultures were performed at 37°C under 5% CO₂ atmospheric oxygen. Hypoxic conditions at 0.1–0.5% O₂ were maintained in the hypoxic incubator chamber (Galaxy 48R incubator, New Brunswick, Eppendorf, RotSELL, Belgium).

**Cytotoxicity assay.** Cells were plated at semi-confluency in 96 well plates. NCH644 were attached on ECM Cell-Tak (VWR, Leuven, Belgium) precoated plates. Increasing concentrations of tested compounds (chloroquine diphosphate (Sigma, Overijse, Belgium; C6628) and melfloquine hydrochloride (Sigma, M23191)) were applied for 72 h. Induction of cell death was measured after 72 h with the Sulforhodamine (SRB) assay (*In Vitro Toxicology Assay Kit*, Sigma). The optical density was measured at 540 nm. The percentage inhibition of cell mass was determined as: % cell mass reduction = (Mean OD_control – Mean OD_sample) × 100/Mean OD_control. IC₅₀ was determined with the GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

**Gene expression analysis.** The gene expression profiles were analysed as described previously (Sanze et al., 2015). Lists of differentially expressed genes (DEGs) were obtained with ANOVA (false discovery rate (FDR) < 0.01, any FC). The Ingenuity Pathway Analysis (IPA) (Ingenuity Systems) was used for data mining. Right-tailed Fisher’s exact test was used to calculate a value for functional enrichment analysis (threshold: -log(P value) > 1.3). Upstream regulator analysis was used to detect potential transcriptional regulators (an overlap of P value < 0.05 and activation z-score > 2). Venn diagram analysis was performed with the SUMO software (http://angiogenesis.dkfz.de/oncoexpress/software/). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3085.

**Real-time quantitative PCR.** Overall, 1 μg of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad, Temse, Belgium). Quantitative PCR (qPCR) was carried out using Fast SYBR Green Master Mix and the Viia 7 Real Time PCR System (Life Technologies) with ATG9A (F: GCCAGACGGCTTTTGGCTGC; R: TAGGGATGCGCAAGCGGTGC) and EZRN (F: TGGCCCACTGCTGAACT; R: CCGGCATATAAACCCTATGG) primers. Fold-change (FC) was calculated using the ΔΔCt method (QBase).

**shRNA-mediated knockdown of ATG9A.** A control shRNA (shScramble, Open Biosystems, RHS4346) or a shRNA targeting ATG9A (Open Biosystems, RHS4430-99150604) were introduced using lentiviral particles. Individual pGIPZ shRNAmir constructs were obtained as E. coli cultures in LB-chenix medium with 8% glycerol, 100 μg ml⁻¹ carbenicillin and 25 μg ml⁻¹ zeocin. Lenti-viral particles were produced in HEK cells by co-transfection of the pGIPZ vector with the viral core packaging construct pCMVdel-tarR8.74 and the VSV-G envelope protein vector pMD.G.2. Supernatant containing viral particles was used to transduce 100 000 cells and puromycine selection permitted to obtain 100% of stably transduced GFP-positive cells (0.5 μg ml⁻¹ for NCH421k and U87; 1 μg ml⁻¹ for NCH644 for at least 2 weeks). Cells were regularly verified for GFP expression via flow cytometry and puromycine selection was repeated, if required.

**Transient transfection with LC3B.** U87 and U251 were seeded in ibidi iTreat μ-Dish transfected using lipofectamine (Thermo Fisher, Illkirch, France) with 2 μg of LC3B-GFP or LC3-Tomato plasmid for 3 h. Transfected cells were incubated for 16 h in either normoxia or 0.1–0.5% O₂ hypoxia in the presence of 20 μM chloroquine. Nuclei were visualised with Hoechst33342. Images were taken using fluorescence confocal microscopy (Zeiss LSM STO META) by obtaining 20–25 stacks of two-dimensional images from successive focal planes (10–15 μm total). Quantification of autophagosomes was performed with ImageJ. Experiments were performed twice, 35 individual cells were acquired in total for analysis.

**Cell proliferation assay.** shScramble and shATG9A transfected NCH421k, NCH644 (10 000 cells) and U87 (5000 cells) were plated in 6 well plates. Cells were cultured for 4, 7 and 11 days. At each time point, total number of viable cells was measured with a Countess cell counter (Thermo Fisher). Experiments were performed three times with three replicates each.

**Statistical analysis.** The data was analysed with unpaired independent-samples t-test (Excel software, Microsoft, Redmond, Seattle, WA, USA). Kaplan–Meier survival curves, log-rank test for survival analysis and IC₅₀ were calculated with the GraphPad Prism5. Data were considered statistically significant with a P value < 0.05.
**Figure 1. Hypoxia sensitises GBM cells to autophagy inhibitors.** Chloroquine and bevacizumab were administered as single agents or simultaneously in P3 (A: 20 mg kg\(^{-1}\)) and T16 (B: 20 and 50 mg kg\(^{-1}\)) PDXs. Kaplan–Meier graphs show the survival of mice upon treatment. See Supplementary Table S1 for summary. Abbreviations: Bev = Bevacizumab; CQ = chloroquine; log-rank test, * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\).

(C) Blood vessels from control and treated P3 PDXs were visualised by mouse-specific anti-CD31 (scale bars 100 \(\mu\)m).

(D) Quantification of vessel number per mm\(^2\) upon treatment (mean ± s.e.m., * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\)).

(E) The cytotoxic effect of inhibitors (chloroquine 20 \(\mu\)M, mefloquine 10 \(\mu\)M) was analysed for PDX-derived spheroids and NHA after 72 h treatment in normoxia and hypoxia. Representative images of treated spheroids are presented (‘green’ = viable, ‘red’ = dead).

(F) Quantification of cell death upon treatment displayed as % of dead cells/volume (\(n \geq 5\), * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\)).

(G) Sensitivity of GBM cultures to chloroquine and mefloquine 72 h after treatment. Concentration gradients were used to determine the median inhibitory concentration (IC\(_{50}\)). IC\(_{50}\) are expressed as mean ± s.e.m. (\(n \geq 3\), * \(P<0.05\), ** \(P<0.01\), *** \(P<0.001\)).
GBM cells exhibit increased sensitivity to chloroquine in hypoxia. To further confirm a role of hypoxia in the outcome of anti-autophagy treatment, we assessed the efficacy of two autophagy inhibitors, chloroquine and mefloquine, at different oxygen levels. We have first assessed the cytotoxic effects in primary PDX-derived 3D spheroids standardised for drug testing (Supplementary Figure S1), known to recapitulate well the genetic makeup of patient tumours (De Witt Hamer et al, 2008; Bougnau et al, 2016) (Supplementary Table S1) and drug responses (Hirschhaeuser et al, 2010). Non-transformed human astrocytes (NHA) cultured under identical conditions were used as a control. Spheroids treated for 72 h with chloroquine (20 μM) or mefloquine (10 μM) in normoxia or severe hypoxia (0.1% O₂; Figure 1E and F) displayed a heterogeneous response to autophagy inhibitors. Little cell death was observed within P3 and T16 spheroids treated with chloroquine in normoxia, whereas cell death was markedly increased in hypoxia (Figure 1E and F). P8 spheroids were already sensitive to chloroquine in normoxia and exhibited no further increase in sensitivity under hypoxia. Mefloquine, a more potent lysosomalotropic agent, was generally more toxic already in normoxia. In P3 spheroids, sensitivity, however strongly increased in hypoxia, which appeared relatively resistant to mefloquine in normoxia. At the indicated concentration, chloroquine and mefloquine did not induce cell death in astrocytes (Figure 1E and F), suggesting that astrocytes are less dependent on autophagy compared to GBM cells.

We further determined the half maximal inhibitory concentration (IC₅₀) for chloroquine and mefloquine in a panel of GBM cultures. Out of six cultures tested NCH644, U87 and T98G exhibited increased sensitivity to chloroquine in hypoxia (Figure 1G). U251 and P3A were already very sensitive under normoxia and no additive effect was observed in hypoxia (Figure 1G). Again, mefloquine was generally more potent in normoxia, and increased sensitivity in hypoxia was observed only for NCH644, which displayed highest IC₅₀ at normal oxygen levels (Figure 1G).

Taken together, we show that hypoxia potentiates the cytotoxic effect of autophagy inhibitors in GBM spheroids and in GBM cultures. Similar to the in vivo situation, the GBM response is heterogeneous and the additive effect is observed in hypoxic cells only when the treatment reaches mild/moderate effect in normoxia.

**Induction of autophagy at the transcript and protein level.** We have recently shown that GBM cells can survive under long-term severe hypoxia, undergoing transcriptional changes and increasing dependency on glycolysis (Sanze et al, 2015). Although autophagy is known to be regulated mainly at the post-transcriptional level, transcriptional regulation has an important role in the induction of the process (Moussay et al, 2011). We therefore investigated transcriptional regulation of autophagy-associated genes. Gene expression patterns were obtained from a panel of GBM cultures including glioma stem-like cells (NCH421k, NCH644) and classical GBM lines (U87, U251), cultured under short (12 h) and long-term (7 days) hypoxia. Differentially expressed genes (FDR<0.01; any FC) were further subjected for functional enrichment analysis by IPA. As genes associated with the autophagy pathway, genes regulating autophagy are poorly annotated in ontology databases we applied an in-house gene list (244 genes referred as 'Autophagy pathway') (Moussay et al, 2011), revealing strong modulation of the autophagy pathway upon both short- and long-term hypoxia (Figure 2A). As expected, the upstream regulator analysis by IPA predicted the hypoxia inducible factor 1-alpha (HIF1A) transcription network to be strongly activated upon hypoxia (P value<0.05; z-score > 2; Figure 2B), as was FOXO3A – one of the transcription factors responsible for induction of autophagy (Figure 2B).

Activation of autophagy was further visualised via increased conversion of LC3-I to LC3-II isoform under hypoxia (Figure 2C). To appropriately detect changes in the autophagic flux, experiments were performed in the absence and in the presence of the lysosomotropic agent chloroquine, which inhibits both the fusion of autophagosomes with lysosome and lysosomal protein degradation. Contrary to the previous experiments where chloroquine was used as a treatment agent (Figure 1), the inhibition of the autophagic flux was detectable upon short chloroquine treatment (3–16 h) according to well-established protocols (Shintani and Klionsky, 2004; Klionsky et al, 2016). High levels of the LC3-II isoform were detected in all GBM cells treated with chloroquine upon hypoxia as reflected in the LC3-II/LC3-I ratios. Interestingly, NCH421k and U251 cells displayed high levels of LC3-II already in normoxia, suggesting their strong dependency on autophagy in normal conditions (Figure 2C). This is in accordance with the high sensitivity of U251 to chloroquine in both conditions (Figure1G). Induction of autophagy by hypoxia was further confirmed by a decrease in p62 (Figure 2D) and an increase in the number of autophagosomes visualised via transient LC3-GFP transfection (Figure 2E). In conclusion, these data indicate that autophagy is induced under severe hypoxia in GBM cells. The heterogeneous sensitivity to autophagy inhibition corroborates with the differential basal level of autophagy in normoxia and further activation of autophagy in hypoxic GBM cells.

**ATG9A is involved in the hypoxia-dependent autphagic response.** To further explore the GBM-specific response to hypoxia we focused on 98 specific regulators of autophagy (71-positive and 27-negative regulators, Supplementary Table S3). Although the number of deregulated genes and the extent of deregulation was variable, we found four commonly deregulated genes shared between short-term and long-term hypoxia (ATG9A, BNIP3, BNIP3L and PIK3C3) (Figure 3A; Supplementary Table S3), showing increased levels upon hypoxia. BNIP3 and BNIP3L were previously associated with the autophagic response in hypoxic conditions (Mazure and Pouyssegur, 2010), whereas PIK3C3 is a well-known partner in the autophagy onset mechanism (Munson and Ganley, 2015). Of note, MTOR, a negative regulator of autophagy and of PIK3C3, was significantly downregulated in 3 out of 4 GBM cultures (Supplementary Table S3).

Interestingly, within the ATG family, only ATG9A was upregulated in all GBM cell lines (Figure 3B; Supplementary Table S3), ATG2A was high only in 5 out of 8 conditions (Supplementary Table S3). The upregulation of ATG9A was confirmed by qPCR in GBM stem-like cells (NCH644, NCH421k, NCH660h, NCH601, NCH465) and adherent cultures (U87, U251) (Figure 3C).

Interestingly, analysis of the ATG9A gene promoter revealed the presence of five hypoxia response elements (HREs) in close proximity to the canonical transcription start site, confirmed to be functional according to the TRANSFAC database (Matys et al, 2006; Mole et al, 2009) (Supplementary Table S4). This was true also for BNIP3, BNIP3L and PIK3C3 promoters, and is in line with the HIF-dependent regulation reported for the BNIP3 and BNIP3L (Kothari et al, 2003; Mole et al, 2009; Slemc and Kunej, 2016). In summary, we show for the first time that ATG9A expression is strongly induced in hypoxic conditions, implicating ATG9A as a new player of hypoxia-dependent autophagic response in GBM.

**Silencing of ATG9A affects GBM cell proliferation and tumour growth in vivo.** To study the involvement of ATG9 in autophagy, we generated an efficient long-term ATG9A knockdown (75–98%; Figure 4A) in three GBM cultures, resulting in reduced proliferation both in normoxia and hypoxia (Figure 4B). Contrary to the control, ATG9A-depleted U87 cells did not increase the number of LC3-positive vesicles upon hypoxia (Figure 4C), suggesting inefficient activation of autophagy. To examine the effect of ATG9A silencing on tumour growth in vivo, we implanted shATG9A NCH421k and NCH644 cells into the brain of immunodeficient mice. ATG9A knockdown led to a significant increase in mouse survival (+12–18%; Figure 4D). Of note, two of the autophagy-associated genes, ATG9A and BNIP3L, were included.
in our previously reported targeted shRNA screen, where we examined the essentiality of 55 genes for survival of GBM cells in vitro and in vivo (Sanzey et al., 2015). Interestingly, ATG9A but not BNIP3L was also depleted both in vitro and in vivo (Figure 4E), indicating that ATG9A is essential for general GBM cell survival. Taken together, our data show that ATG9A is important for GBM growth both in normoxic and hypoxic conditions, and regulates activation of autophagy upon hypoxia. Interfering with ATG9A expression efficiently blocks tumour growth in vivo.

**DISCUSSION**

Hypoxia is a characteristic feature of malignant gliomas and drives tumour progression by adaptive cellular responses including angiogenesis, changes in tumour metabolism, motility and survival (Bertout et al., 2008). Increased hypoxia is also one of the escape mechanisms driving resistance to anti-angiogenic treatment in GBM. Here we find that the autophagy pathway is strongly induced in GBM under hypoxia, and we identify ATG9A as a novel regulator of autophagy induction. Inhibiting autophagy was shown to potentiate various anti-cancer therapies in vitro, including gliomas (Kanzawa et al., 2004; Shingu et al., 2009), where cells were subjected to external stress. Although, there are currently over 20 clinical studies involving the use of chloroquine and hydroxychloroquine in cancer treatment, in GBM both agents showed limited effect in non-toxic doses (Sotelo et al., 2006; Rosenfeld et al., 2014). Here we show a significant increase in survival of GBM PDXs when chloroquine was administered as a single agent, although with different effective dose. This is in accordance with the recent clinical trial showing dose-dependent inhibition of autophagy by hydroxychloroquine in GBM patients (Rosenfeld et al., 2014) and suggests that dosing needs to be adapted to the specific patient tumour. Of note, we show that certain tumour cells were sensitive to autophagy inhibitors also at normal oxygen, indicating a strong dependence on autophagy without additional environmental stress in a subgroup of GBM. This heterogeneous response suggests that the genetic background, for example, PTEN deletion, p53 mutation or EGFR amplification, may differentially
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Figure 3. ATG9A is specifically activated upon autophagic response to hypoxia. (A) Genes directly related to autophagy [knowledge-driven selection] were extracted from DEG lists between hypoxia 12 h vs normoxia and hypoxia 7 days vs normoxia (FDR < 0.01; any FC) for each culture (n = 3–6). Venn diagrams reveal commonly deregulated genes. (B) Heatmap shows expression levels for selected genes in NCH421k in normoxia, 12 h and 7 days hypoxia. See Supplementary Table S3 for more autophagy-related genes. (C) QPCR confirmed increased ATG9A expression in hypoxia. EZRIN was used as a reference (mean ± s.e.m.; n = 3; *P < 0.05, **P < 0.01, ***P < 0.001). NCH421k cells were used as an internal calibration (value = 1).

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...chloroquine cannot be excluded (Maycotte et al., 2012; Maes et al., 2014), we did not observe vessel normalisation upon chloroquine treatment. In line with a previous study (Chen et al., 2008), normal astrocytes remained unaffected at the lowest chloroquine concentration affecting GBM cells, confirming more substantial dependence of tumour cells on autophagy. Although more potent inhibitors are warranted, our data suggest the existence of a ‘therapeutic window’ for autophagy inhibitors in GBM, and that co-treatment with anti-angiogenic agents allows to significantly lower effective doses.

We found that activation of autophagy in hypoxia was linked to transcriptional changes of numerous genes associated with autophagy, among which BNIP3, BNIP3L, ATG9A and PIK3C3 were upregulated in all GBM cells. BNIP3 and BNIP3L, while activated by HIF1α, mediate autophagy by releasing Beclin1 from complexes with Bcl-2 and Bcl-Xl (Zhang et al., 2008; Bellot et al., 2009). Interestingly, within the ATG family, only ATG9A was transcriptionally activated in all GBM cells. Contrary to other ATG family members such as ATG5 and ATG7, but similarly to BNIP3 and BNIP3L, we identify ATG9A as potentially HIF1α responsive gene. These transcriptional changes were observed also in GBM cells that exhibit high basal autophagy at normal oxygen levels, suggesting that specific upstream molecules such as FOXO3A are involved in the regulation autophagy pathway at...
different oxygen levels. Pro-autophagic genes, such as Beclin1, ATG5, ATG7, BNIP3 and BNIP3L were previously found to be essential for autophagy in cancer cells (Zhang et al, 2008; Mazure and Pouyssegur, 2009). Here we show that ATG9A also represents an important pro-survival molecule, with ATG9A depletion leading to a strong reduction of tumour growth, thus confirming the relevance of autophagy as a promising target for GBM treatment. Of note, ATG7 knockdown displayed a therapeutic outcome only during anti-angiogenic treatment (Hu et al, 2012).

ATG9A was shown to be essential for autophagosome biogenesis and membrane maturation; however, its mode of action remains enigmatic. Recent data suggest that the Pho–Rpd3 complex regulates expression of ATG9A and other ATG genes upon induction of autophagy (Jin and Klionsky, 2014) and that ATG9A-containing vesicles are generated de novo upon starvation (Yamamoto et al, 2012). Here we show that upon ATG9A depletion, GBM cells were not able to activate autophagy upon hypoxia. We propose that the lack of autophagic activation upon hypoxia may be due to inhibition of de novo autophagosome synthesis. This is in accordance with a recent report, where ATG9A was shown to have a key role in autophagosome formation during hypoxic stress (Weerasekara et al, 2014). Thus, ATG9A may become essential upon autophagy induction and an increased demand for new autophagosome membranes (Orsi et al, 2012).
In conclusion, our data support the notion that inhibiting autophagy represents an effective therapy in primary GBM, although it may be concentration and patient dependent. Anti-autophagy treatment using genetic and pharmacological intervention was effective as a single treatment. However, currently available drugs, including chloroquine and hydroxychloroquine are non-curative in non-toxic doses and novel more potent agents will be necessary for GBM patients. Drugs directly targeting essential proteins such as ATG9A may be of particular interest and a combination with anti-angiogenic therapy may be beneficial. Finally, the hypoxic microenvironment also contributes to immunoresistance and hypoxia-induced autophagy impairs cytotoxic T-lymphocyte-mediated cell lysis of tumour cells (Noman et al, 2011, 2012) and NK-mediated target cell apoptosis (Baginska et al, 2013; Viry et al, 2014). Therefore, targeting autophagy in tumour cells may not only lead to increased tumour cell death but also sensitize tumours to immunotherapies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Baginska J, Viry E, Berchem G, Poli A, Noman MZ, van Moer K, Medves S, Zimmer J, Oudin A, Niclou SP, Bleakley RC, Goping IS, Chouaib S, Janji B (2013) Granzyme B degradation by autophagy decreases tumour cell susceptibility to natural killer-mediated lysis under hypoxia. Proc Natl Acad Sci USA 110: 17450–17455.

Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazeau NM (2009) Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 29: 2570–2581.

Bertout JA, Patlal SA, Simon MC (2008) The impact of O2 availability on human cancer. Nat Rev Cancer 8: 967–975.

Bozgnaud S, Golubiewska A, Oudin A, Keunen O, Harter PN, Madre L, Azzueja F, Fritha S, Stieber D, Koama T, Vallar L, Brons NH, Daubon T, Miletic H, Sundström M, Herold-Mende C, Mittelbronn M, Bjerkvig R, Niclou SP (2016) Molecular crosstalk between tumour and brain parenchyma instructs histopathological features in glioblastoma. Oncotarget.

Chen Y, Henson ES, Xiao W, Huang D, McMillan-Ward EM, Israels SJ, Gibson SB (2016) Tyrosine kinase receptor EGFR regulates the switch in cancer cells between cell survival and cell death induced by autophagy in hypoxia. Autophagy 12: 1029–1046.

Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB (2008) Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 15: 171–182.

Cichomska IA, Gabrusewicz K, Szczepankiewicz AA, Kaminska B (2013) Endoplasmic reticulum stress triggers autophagy in malignant glioma cells undergoing cyclosporine-a-induced cell death. Oncogene 32: 1518–1529.

Corcelle-Terreau E, Vindelov SD, Hamalisto S, Mograbi B, Keldsø A, Bransen JH, Favaro E, Adam D, Sznajerowski P, Hofman P, Krautwald S, Farkas T, Petersen NH, Rohde M, Linkermann A, Jøttelø M (2016) Excess sphingomyelin disturbs ATG9A trafficking and autophagosome closure. Autophagy 12: 833–849.

De Witt Hamer PC, Van Tilborg AA, Eijk PP, Sminia P, Troost D, Van Noorden CJ, Ylstra B, Leenstra S (2008) The genomic profile of human malignant glioma is altered early in primary cell culture and preserved in spheres. Oncogene 27: 2091–2096.

Demeure K, Fack F, Duriez E, Tiemann K, Bernard A, Golubiewska A, Bougnaut S, Bjerkvig R, Domon E, Niclou SP (2015) Targeted proteomics to assess the response to anti-angiogenic treatment in human glioblastoma. Mol Cell Proteomics 15: 481–492.

Evans SM, Judy KD, Dunphy I, Jenkins WT, Nelson PT, Collins R, Wileyto EP, Jenkins K, Hahn SM, Stevens CW, Judkins AR, Phillips P, Georger B, Koch CJ (2004) Comparative measurements of hypoxia in human brain tumours using needle electrodes and EF5 binding. Cancer Res 64: 1886–1892.

Fack F, Espedal H, Keunen O, Golubiewska A, Obad N, Harter PN, Mittelbronn M, Bahr O, Weyerbrock A, Stuhr L, Miletic H, Sakariassen PA, Stieber D, Rygh CB, Lund-Johansen M, Zheng L, Gottlieb E, Niclou SP, Bjerkvig R (2015) Bevacizumab treatment induces metabolic adaptation toward anaerobic metabolism in glioblastomas. Acta Neuropathol 129: 115–131.

Firat E, Weyerbrock A, Gaedicke S, Grosu AL, Niedermann G (2012) Chloroquine or chloroquine-Pi3K/Akt pathway inhibitor combinations strongly promote gamma-irradiation-induced cell death in primary stem-like glioma cells. PLoS ONE 7: e47357.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO (2000) Genomic expression programs in the response of yeast cells to environmental changes. Mol Biol Cell 11: 4241–4257.

Gilbert MR (2016) Antiangiogenic therapy for glioblastoma-complex biology and complicated results. J Clin Oncol 34: 1567–1569.

Glick D, Barth S, Macleod FK (2010) Autophagy: cellular and molecular mechanisms. J Pathol 221: 3–12.

Golubiewska A, Bougnaut S, Stieber D, Brons NH, Vallar L, Hertel F, Klink B, Schrock E, Bjerkvig R, Niclou SP (2013) Side population in human glioblastoma is non-tumorigenic and characterizes brain endothelial cells. Brain 136: 1462–1475.

Hirschhaeuser F, Menne H, Dittfeld C, West J, Mueller-Klieser W, Kunz-Schughart LA (2010) Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol 148: 3–15.

Hu TL, DeLay M, Jahangiri A, Molinaro AM, DeLay M, Jahangiri A, Molinaro AM, Rose SD, Carbonell WS, Aghi MK (2012) Hypoxia-induced autophagy promotes tumor cell survival and adaptation to antiangiogenic treatment in glioblastoma. Cancer Res 72: 1773–1785.

Jin M, Klionsky DJ (2014) Transcriptional regulation of ATG9 by the Pho23-Rpd3 complex modulates the frequency of autophagosome formation. Autophagy 10: 1681–1682.

Kanzawa T, Germaino IM, Komata T, Ito H, Kondo Y, Kondo S (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. Cell Death Differ 11: 448–457.

Keunen O, Johansson M, Oudin A, Sanzey M, Abdul Rahim SA, Fack F, Thorsen F, Taxt T, Bartos M, Irik R, Miletic H, Wang J, Stieber D, Stuhr L, Moin E, Rygh CB, Bjerkvig R, Niclou SP (2011) Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. Proc Natl Acad Sci USA 108: 3749–3754.

Klionsky DJ, Abdelmohsen K, Abe A, Abin JD, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K, Adhidipty PN, Adler SG, Agam G, Agarwal R, Aghi MK, Aignell M, Agustini P, Aguilar PV, Aguirre-Ghiso J, Airoldi EM, Ali-Sti A-S, Akematsu T, Alkopriaite A, Al-Rubeai M, Albicata GM, Albanese C, Albani D, Albert ML, Albidio J, Algil H, Aliereazi M, Alloza I, Almasan A, Almonte-Beceril M, Alnemri ES, Alonso C, Altan-Bonnet N, Altieri DC, Alvarez S, Alvarez-Erviti L, Alves S, Amadoro G, Amano A, Amanzini C, Ambrosio S, Ambrosio I, Amer AO, Amessou M, Amon A, An Z, Ananina FA, Andersen SU, Andrey UP, Andreadi CK, Aurelien L, Aurelian L, Auroux J, Araya J, Arcaro A, Arias E, Arimoto H, Ariosa AR, Armstrong JL, Apostolova N, Aquila S, Aquilano K, Araki K, Arama E, Aranda A, Araki K, Arama E, Aranda A, Araya J, Arcaro A, Arias E, Arimoto H, Ariosa AR, Armstrong JL, Arnould T, Arsov I, Ascania K, Askanas V, Asselin E, Atarashi R, Atkinson SS, Atkin JD, Attardi LD, Auburger P, Auburger G, Aurelian L, Autelli R, Avagliano L, Avantaggiati ML, Avrahami L, Awale S, Azad N, Bacheti T, Backer JM, Bae DH, Bae JS, Bae ON, Bae SH, Baechere EH, Baek SH, Baghdigiousian S, Bagiewska-Zadworna A, Bai H, Bai J, Bai Y, Bai XY, 821
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