Abstract. There is growing epidemiological evidence indicating an association between diabetes mellitus and the increased incidence of colorectal cancer (CRC). The preferred initial and most widely used pharmacological agent for the treatment of type 2 diabetes is metformin, which in parallel reduces the risk of CRC and improves patient prognosis. AMP-activated protein kinase (AMPK) appears to be tightly associated with the beneficial metabolic effects of metformin, serving as a cellular energy sensor activated in response to a variety of conditions that deplete cellular energy levels. Such conditions include nutrient starvation (particularly glucose), hypoxia and exposure to toxins that inhibit the mitochondrial respiratory chain complex. The aim of the present study was to determine the effect of metformin on CRC cell lines, with different levels of anterior gradient 2 (AGR2) expression, exposed to 5-fluorouracil (5-FU) and oxaliplatin, alone or in combination with metformin. AGR2 has recently emerged as a factor involved in colon carcinogenesis. In AGR2-knockout cells, markedly higher levels of phosphorylated-AMPK were observed in comparison with control cells transfected with GFP-scrambled guide RNA, which indicated that the presence of AGR2 may interfere with the metformin-dependent activation of AMPK. In addition, metformin in combination with 5-FU and oxaliplatin induced ROS production and attenuated autophagy. This effect was enhanced in AGR2-knockout cells.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide; according to GLOBOCAN estimates, there were 1,801,000 new cases of CRC in 2018 worldwide (1). The risk of CRC is increased up to three times by diabetes mellitus, predominately type 2 diabetes mellitus (T2DM) (2-4). T2DM is a chronic metabolic disorder associated with high mortality and morbidity. Patients with diabetes have an increased risk of various malignancies due to numerous putative mechanisms, including hyperinsulinemia, hyperglycemia and inflammation (5). T2DM has been demonstrated to increase CRC aggressiveness and mortality (6,7). The key molecular mechanisms by which T2DM increases the incidence or worsens the prognosis of patients with CRC are associated with nutrient-sensing pathways coupling energy metabolism to signals of cell growth and survival, which are often dysregulated in diabetes, and may be important contributors to cancer development in patients with diabetes (8). The pathophysiology of T2DM involves enhanced oxidative stress induced by high plasma glucose, lipid and cytokine levels, which can trigger endoplasmic reticulum (ER) stress (9). Anterior gradient 2 (AGR2) protein, coded by the AGR2 gene, has recently been described as an important regulator of ER stress. AGR2 is inducible by ER stress, which is associated with the acquisition of a pro-inflammatory phenotype (10). The presence of AGR2 has been detected in a wide range of human malignancies, including CRC (11,12). Functionally, AGR2 belongs to the protein disulfide isomerase family with all the key features of an ER-resident protein responsible for maintaining ER homeostasis (13,14). Metformin, which is a biguanide derivative, is a first-line drug used in T2DM treatment worldwide (15). Considering the epidemiological evidence between T2DM and an increased risk of CRC, the impact of metformin therapy on the incidence and outcome of CRC has been intensively studied (16). The beneficial effects of metformin for patients with CRC and diabetes are already supported by recent clinical trials, which reported prolonged overall survival for metformin users compared with nonusers (17). Studies on CRC cell lines have revealed that metformin inhibits cell proliferation and migration by arresting the cell cycle in the G0/G1 phase, and by transient downregulation of c-Myc and insulin-like growth factor receptor 1 (18).
A recent study on the effects of metformin on ER stress demonstrated that metformin acts as a modulator of ER stress in patients with T2DM by promoting an adaptive unfolded protein response (19). Studies on metformin in combination with 5-fluorouracil (5-FU) and/or oxaliplatin, which are drugs routinely used during standard chemotherapy of patients with CRC, confirmed its synergistic anti-cancer effects (20). The potential association between metformin and AGR2 expression has been demonstrated by transcriptomic analysis identifying AGR2 as one of the most downregulated genes in pancreatic cancer cells exposed to combined treatment with metformin and aspirin (21). Since AGR2 has been identified as a putative marker of chemoresistance (22-24), and AGR2 silencing may sensitize tumor cells to ER stress-induced autophagy (13), the aim of the present study was to elucidate the role of AGR2 in CRC cells exposed to metformin in combination with 5-FU and oxaliplatin.

Materials and methods

Cell lines and culture. Human epithelial colorectal adenocarcinoma cell lines DLD-1 and SW480 (American Type Culture Collection) were maintained in high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% pyruvate and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Unless otherwise stated, cells were grown to 70-80% confluence prior to treatment. In addition, unless otherwise stated, the cells were treated with the following concentrations of drugs: 1 µM baflomycin A1, (Cell Signaling Technology, Inc.), 1% pyruvate and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Transfection. AGR2-knockout (KOAGR2) DLD-1 cells were prepared using CRISPR/Cas9 technology. Briefly, the guide RNA oligonucleotide (5'-AGA GAT ACC ACA TGT TGC GAC TGG -3') was designed using Tools for Guide Design (zlab.bio/guide-design-resources). The guide gene (ENSE00003623642) was designed using Tools for Guide Design (zlab.bio/guide-design-resources). The guide RNA specifically targeted mRNA coding 21-27 aa of the AGR2 N-terminal region, which is important for AGR2 protein-mediated cell adhesion. The GFP-scrambled sequence (5'-AAC AGT CGC GTT TGC GAC TGG-3') served as a control (25). Both sequences were cloned into a LentiCRISPR-v2 vector (cat. no. 52961; Addgene) using Esp3I restriction cloning. DLD-1 cells (1x10⁶ cells) were transfected with LentiCRISPR-v2_AGR2 or LentiCRISPR-v2_scrambled (scr). After 2 days, the cells were exposed to puromycin (2 µg/ml) and the pool of resistant cells was sorted and seeded as single colonies in 96-well plates. KOAGR2 cells were tested for AGR2 expression using western blotting. Two clones with an undetectable expression of AGR2, DLD1 KOAGR2 A9 and F5, were selected for further experiments. SW480 cells (1x10⁶) were transiently transfected using an Amaxa Nucleofector II (Lonza Group Ltd.) with 2 µg pcDNA3-AGR2 or empty pcDNA3 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.), which served as a control, and were subsequently selected with G-418 (400 µg/ml; Sigma-Aldrich; Merck KGaA). A pool of resistant cells was tested for positive AGR2 expression with western blot analysis.

To determine the doubling time of the transfected cancer cell lines with manipulated AGR2 gene expression compared with untransfected cells, equal numbers of cells (5x10⁵) were seeded into the complete media and maintained under the standard conditions for 48 h. The culture medium was removed, and adherent cells were detached by 0.5% trypsin (Gold Biotechnology, Inc.) and counted using CASY Model TT cell counter (Roche Diagnostics).

Western blot analysis. Cells were washed twice with cold phosphate-buffered saline (PBS). The cells were then scraped into NET lysis buffer [150 mM NaCl, 1% NP-40, 50 mM Tris (pH 8.0), 50 mM NaF, 5 mM EDTA (pH 8.0)] supplemented with Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. Protein concentration was determined using the Bradford method and 15 µg proteins were loaded onto 10% acrylamide gels. Following SDS-PAGE, the samples were transferred to nitrocellulose membranes, blocked with 5% milk diluted in PBS supplemented with 1% Tween for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against phosphorylated-AMPKα at Thr172 (p-AMPK; 1:1,000; cat. no. 2535; Cell Signaling Technology, Inc.), β-actin and p62 (both 1:1,000; cat. nos. sc-47778 and sc-28359; Santa Cruz Biotechnology, Inc.), microtubule-associated proteins 1A/1B light chain 3 (LC3)-II (1:1,000; cat. no. NB100-2220; Novus Biologicals, LLC) and AGR2 (in house) (26). The membranes were washed and probed with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (1:1,000; cat. nos. P0217 and P0161; Dako) for 1 h at room temperature. Chemiluminescent signals were developed using a mixture of 1:1 ECL-A and ECL-B solutions [ECL-A: 0.5 M EDTA (pH 8), 90 mM cromatic acid, 1 M luminol, 200 mM Tris (pH 9.4) and ECL-B: 0.5 M EDTA (pH 8), 0.123 g NaBO₃ x 4H₂O, 50 mM CH₃COONa (pH 5)] and visualized with the SYNGENE G:BOX Chem XX6 gel doc system (Syngene; Synoptics Ltd.).

Clonogenic assay. Cells were plated at a density of 250 cells/well in a 6-well plate. Following 24-h incubation at 37°C with 5% CO₂, 500 µM metformin, 5 µM 5-FU and 2 µM oxaliplatin were applied and further incubation for ~10 days was performed. The medium was removed, and the colonies were stained with 1% crystal violet at room temperature for 20 min and counted. Recombinant extracellular AGR2 (eAGR2) was prepared as described previously (27) and was used in the clonogenic assay as an extracellular protein supplied in the medium (1 ng/ml) throughout the experiment.

Determination of mitochondrial depolarization. To analyze mitochondrial depolarization, the JC-1 probe was used. At 24 h post-treatment, ~2x10⁶ cells were harvested and washed with PBS, stained with 5 µg/ml JC-1 dye (Invitrogen; Thermo Fisher Scientific, Inc.) for 30 min at 37°C and analyzed using a BD FACS Aria III flow cytometer (BD Biosciences). Viable population of cells was gated according to forward scatter (FSC) vs. side scatter (SSC). Individual cells were gated by FSC-A vs. FSC-H parameters. The ratio of green (FITC)
to red (phycoerythrin) fluorescence was analyzed by FCS
Express version 4 software (De Novo Software) as a loss of
mitochondrial potential (ΔΨm). Treatment with valinomycin
(25 μM; Thermo Fisher Scientific, Inc.) for 2 h at 37˚C was
used as a positive control for mitochondrial depolarization.

Detection of reactive oxygen species (ROS) production.
CM-H2DCFDA was used as an indicator for ROS in
CRC cells. Briefly, the cells were seeded in 96-well plates
(4x10^3 cells/well) and incubated at 37˚C overnight. The
next day, the cells were treated with respective drugs diluted in
Hank's Balanced Salt Solution (HBSS; Sigma-Aldrich; Merck
KGaA) for 6 h at 37˚C. Hydrogen peroxide (50 μM; Pentra
s.r.o.) served as a positive control, whereas N-acetyl cysteine
(10 mM; Sigma-Aldrich; Merck KGaA) was used to block ROS
production. Subsequently, the drugs were removed, and 5 μM
CM-H2DCFDA (Thermo Fisher Scientific, Inc.) in HBSS was
added for 30 min at 37˚C. The cells were washed twice with
HBSS, and the fluorescence was examined using an Infinite
100 plate reader (Tecan Group, Ltd.).

Statistical analysis. Statistical analyses were performed
using the Online Web Statistical Calculators for Categorical
Data Analysis (https://astatsa.com). One-way ANOVA with
post-hoc Tukey HSD calculator was used to determine statisti-
cally significant differences between the groups. For western
blot analyses, the protein level changes were first normalized
to β-actin and were then statistically analyzed. P<0.05 was
considered to indicate a statistically significant difference.

Results

Effects of metformin in combination with 5-FU and oxali-
platin on CRC cells with manipulated AGR2 expression.
Two clones with AGR2 gene knockout, KOAGR2 A9 and
KOAGR2 F5, were prepared by CRISPR/Cas9 using DLD-1
cells (Fig. 1A). SW480 cells with no detectable AGR2 expres-
sion were used as the second model; an AGR2-knock-in
SW480 cell line was prepared by stable transfection (Fig. 1A).
The effects of AGR2 expression on cell proliferation were
tested in DLD-1 and SW480 cells. Consistently with previous
studies (28,29), the results demonstrated a positive effect of
AGR2 expression on cell proliferation rate in the two models
(Fig. 1B). The cells were then exposed to selected drugs to
determine their effects on the phosphorylation of AMPK. It
has previously been reported that the actions of metformin
are attributable to AMPK (30). Metformin treatment exhib-
ited a negligible effect on AMPK phosphorylation in DLD-1
scr cells. Treatment with metformin in combination with
5-FU or oxaliplatin induced a moderate increase in AMPK
phosphorylation (Fig. 1C). Notably, a significant increase in
the expression of p-AMPK was observed in both KOAGR2
clones treated with 5-FU and oxaliplatin in combination with
metformin (Fig. 1C). To confirm the involvement of AGR2
in the regulation of AMPK activation, the same experi-
ment was performed using SW480 cells. A non-significant
increase in p-AMPK expression was observed in SW480 cells
without AGR2 (SW480-pcDNA3) treated with the combina-
tion of metformin and 5-FU (Fig. 1D). Since both A9 and
F5 KOAGR2 clones showed very similar p-AMPK/AMPK
expression patterns, subsequent experiments were performed
with only KOAGR2 clone A9.

Effects of metformin on colony formation. The effects of
metformin on the ability of cells to sufficiently form colo-
nies, based on AGR2 expression and in response to standard
chemotherapy drugs, were tested using a clonogenic assay.
A significant loss in the ability of cells to develop colonies
was associated with the absence of AGR2 (Fig. 2). Metformin
alone exhibited no effect on the number of colonies developed
by DLD-1 and SW480 cells irrespective of AGR2 expres-
sion. However, metformin sensitized DLD-1 cells producing
AGR2 to 5-FU and oxaliplatin, which was reflected by signifi-
cantly reduced colony formation compared with untreated
ARG2-negative cells (Fig. 2A).

To investigate the impact of eAGR2 on cell chemosen-
sitivity, recombinant eAGR2 protein was added into the
culture medium (Fig. 2B). Similar trends were observed as in
Fig. 2A; however, with eAGR2, twice the number of colonies
was formed compared with cells cultured in medium without
eAGR2. In addition, the effect of eAGR2 was stronger in
AGR2-negative cells treated with chemotherapy drugs, which
exhibited approximately twofold increase in the number of
colonies compared with cells maintained in media without
eAGR2 (Fig. 2A and B).

SW480-AGR2 cells exhibited a significant decrease in
the number of colonies in response to the combination of
metformin and 5-FU (Fig. 2C). The presence of eAGR2 protein
approximately doubled the number of colonies (Fig. 2D). Taken
together, these data support the anti-prolifera-
tive activity of metformin administered in combination with
standard chemotherapy and suggest a significant contribu-
tion of eAGR2 to the resistance of tumor cells to chemotherapy.

Effect of metformin combined with standard chemotherapy
on autophagy and oxidative stress. Since AMPK has been
demonstrated to regulate autophagy, the effects of metformin
on LC3-II cleavage were determined (Fig. 3). In agreement
with the elevated p-AMPK levels in AGR2-negative cells
(Fig. 1), markedly higher signals of LC3-II were detected in
DLD-1 KOAGR2 cells in comparison with DLD-1 scr cells
(Fig. 3), which indicated that AGR2 may attenuate autophagy.
No significant induction of LC3-II was observed in cells
exposed to all drugs compared to untreated cells (Fig. 3A).
5-FU treatment was even associated with a sharp decrease in
LC3-II, indicating that autophagy may serve an important role
in survival of these tumor cells. The role of autophagy may
connect with the high sensitivity of DLD-1 cells to 5-FU,
as demonstrated in Fig. 2. Although the amount of LC3-II
is clearly correlated with the number of autophagosomes,
LC3-II itself is also degraded by autophagy (31). Therefore,
it is important to measure the amount of LC3-II delivered
to lysosomes by comparing LC3-II levels in the presence and
absence of lysosomal protease inhibitors e.g. bafilomycin
A1. However, the addition of bafilomycin A1 was not asso-
ciated with increased LC3-II cleavage in cells treated with
metformin in combination with 5-FU or oxaliplatin (Fig. 3B),
which indicates that autophagy was inhibited. An alternative
method for detecting the autophagic flux is the determination
of p62/sequestosome-1 degradation, since p62 can bind LC3,
serving as a selective substrate of autophagy (32). However, no significant decrease in p62 levels was observed in response to metformin combined with 5-FU or oxaliplatin treatments; by contrast, an increase in p62 levels was observed in response to 5-FU and oxaliplatin administered alone. Western blot analysis of SW480 cells revealed a reduction in LC3-II isoform
expression in response to metformin and its combinations with 5-FU and oxaliplatin (Fig. 3C). These findings support the hypothesis that autophagy-dependent clearance of misfolded proteins in non-metformin-treated patients with T2DM may be suppressed by metformin treatment (19).

Metformin inhibits mitochondrial respiratory complex I at a cellular level, possibly leading to mitochondrial membrane depolarization and the release of ROS (33,34). Therefore, alterations in mitochondrial membrane potential were investigated in cells exposed to metformin in combination with 5-FU or oxaliplatin. JC-1 staining followed by flow cytometric analysis revealed a significant mitochondrial depolarization in response to metformin alone and in combination with oxaliplatin in AGR2-negative DLD-1 cells compared with in untreated cells (Fig. 4A-C).

The impact of metformin on ROS production was monitored using an indicator for reactive oxygen species in cells, CM-H2DCFDA. All tested drugs administered alone resulted in a low or moderate induction of ROS. However, the combined administration of metformin with 5-FU or oxaliplatin significantly increased ROS production in both DLD-1 scr and DLD-1 KOAGR2 cells. The effect was significantly enhanced in AGR2-negative cells exposed to a combination of 5-FU and metformin, whereas combined treatment with oxaliplatin and metformin reached only a marginal effect (Fig. 4D).

Discussion

CRC is a type of cancer, the relative risk of which is increased by diabetes (35); in addition, the outcomes of CRC are significantly worse in patients with diabetes compared with in non-diabetic subjects (36). Metformin is used for treating patients with T2DM, including those with CRC, as demonstrated in the clinical setting (17). Beneficial effects of metformin are not limited to primary CRC. Metformin also significantly increases the therapeutic effectiveness of standard chemotherapeutics, such as 5-FU or oxaliplatin, on recurring CRC by targeting chemoresistant CRC cells enriched in stem or stem-like cells (37,38). The effects of metformin on CRC cells depend predominately on regulation of the AMPK/mammalian target of rapamycin (mTOR) pathway. Under low energy conditions, AMPK phosphorylates specific enzymes and growth control nodes to increase ATP generation and decrease ATP consumption (39). A decreased level of AMPK in T2DM attenuates the inhibition of protein, fatty acid and cholesterol synthesis to favor cancer cell growth. AMPK regulates these processes by interfering with mTOR, which is a regulator of growth. Thus, under nutrient-rich conditions, AMPK is inactive and mTOR is active, whereas under energy deficient conditions, increased AMPK activity leads to a decrease in mTOR activity, resulting in reduced protein synthesis and cell growth (40). The PI3K/AKT/mTOR axis has also been shown to be involved in the positive regulation of AGR2 expression (41-43). Activation of AKT signaling and impaired expression of its negative regulator phosphatase and tensin homolog has been reported in 60-70% of human colon cancer cases (44). Recent meta-analysis has reported that AGR2 overexpression has an unfavorable impact on overall survival and time to tumor progression in patients with solid tumors (45). Although elevated expression of AGR2 is generally perceived as undesirable due to the prediction of poor outcome, in several tumor types, such as lung, ovarian and colorectal cancer, contradictory findings have been reported; therefore, further analyses and clinical trials on certain types of cancer are required (46,47).

Autophagy may exhibit dual functions in tumors, including CRC; it may contribute to cancer development; however, it may also act as a tumor suppressor by inducing...
cell death. The decision to trigger either induction of processes leading to cell death or activation of pro-survival functions depends on the stage of the neoplastic process (48).

Early stages of CRC carcinogenesis are usually associated with the tumor suppressive role of autophagy, whereas late stages are associated with pro-survival functions (49).
Development of chemoresistance represents an event that is frequently observed in later stages of CRC due to activation of autophagy. For instance, Yang et al. (50) demonstrated that autophagy was induced in response to oxaliplatin treatment, along with the enrichment of the CRC stem cell population, which increased cancer cell resistance to chemotherapy and prevented apoptosis. Li et al. (51) reported that the combination of fluorouracil treatment with autophagic inhibitors, such as bafilomycin A1 or 3-methyladenine, enhanced the chemotherapeutic effect of fluorouracil by stimulating cell death.

The results of the present study demonstrated that, compared with in AGR2-positive cells, CRC cells with silenced AGR2 expression exhibited increased levels of autophagy. However, the activation of AMPK in response to chemotherapy combined with metformin did not induce autophagy. A clear decrease in LC3-II was observed in SW480 cells irrespective of AGR2 status. Although the mechanism of metformin as an antineoplastic agent is not yet fully understood (52), its activity is at least partially attributable to AMPK activation through the inhibition of complex I of the mitochondrial respiratory chain, leading to an increased AMP:ATP ratio (30,34), membrane depolarization (33) and the release of mitochondrial ROS (53). Notably, the present study revealed that metformin in combination with 5-FU or oxaliplatin attenuated autophagy in CRC cells, but increased ROS production responsible for decreased cell viability, as demonstrated by the clonogenic assay. This effect was also significantly enhanced by knocking out AGR2, supporting its potential catalytic redox activity in the regulation of redox balance in cells, which is a typical feature of protein disulfide isomerases (54). The results of

Figure 4. Analysis of mitochondrial depolarization and oxidative stress. (A) Flow cytometry plots showing the gating strategy to determine viable and individual cellular population. (B) Representative plots demonstrating mitochondrial depolarization. (C) At 24 h post-treatment with 5-FU (5 µM), OXAL (2 µM), MET (0.5 mM) and their combination, the JC-1 probe was used to measure the FITC/PE ratio to identify alterations in mitochondrial membrane potential compared with in control cells. 25 µM VAL was used as a positive control. (D) ROS production was determined relative to untreated cells. Hydrogen peroxide was used as a positive control, whereas NAC was used to inhibit ROS development. *P<0.05 vs. untreated DLD-1 scr; †P<0.05 vs. untreated DLD-1 KOAGR2; #P<0.05 as indicated. 5-FU, 5-fluorouracil; CTR, control; MET, metformin; NAC, N-acetyl cysteine; OXAL, oxaliplatin; VAL, valinomycin. At least three independent biological experiments were performed to construct the graphs.
the present study are also in agreement with a recent study that demonstrated increased ROS production in CRC cells induced by metformin (18). By contrast, a study on breast cancer MDA-MB-231 cells revealed that low doses of metformin inhibited ROS production and inflammatory signaling (55). This discrepancy suggests that metformin may function through distinct mechanisms at lower versus higher concentrations (56).

Another important aspect is the cellular localization of AGR2. Although AGR2 is overexpressed in various types of human cancer and has been reported to promote aggressive tumor features, including the resistance to anticancer treatment (23,24,26,57), little is known regarding the extracellular functions of AGR2 in tumorigenesis. Secreted eAGR2 has been demonstrated to promote cell migration and metastasis of CRC in vitro and in vivo (12). A comprehensive protein-protein interaction screen identified AGR2 as an interacting partner of the mTOR complex 2 pathway; eAGR2 promoted increased phosphorylation of rapamycin-insensitive companion of mTOR (T1135), whereas intracellular AGR2 antagonized its levels and phosphorylation (58). A subsequent study aiming to distinguish between the roles of intracellular AGR2 and eAGR2 in response to chemotherapy using an in vitro prostate cancer model revealed that eAGR2 promoted significant resistance to docetaxel (58). Recently, the interaction between AGR2 and transmembrane p24 trafficking protein 2 has been described to serve a key role in AGR2 dimerization and following autophagy-dependent release of AGR2 in the extracellular milieu (59). These findings support the present results, which indicated that autophagy may contribute to survival of CRC cells exposed to 5-FU and oxaliplatin, and the presence of eAGR2 may significantly enhance cell survival and colony formation.

Notably, 5-FU and oxaliplatin represent the standard therapy option for patients with CRC, although with a limited therapeutic success rate (60). Therefore, compounds sensitizing CRC cells to these routinely used drugs are urgently required to improve therapeutic outcome. Metformin is a promising candidate, as documented by several clinical trials (61,62). The results of the present study demonstrated that metformin augmented the anticancer activity of 5-FU and oxaliplatin, and that the effect was enhanced in CRC cells with disrupted AGR2 expression. However, the non-canonical mechanisms by which metformin activates AMPK and induces oxidative stress are areas that require further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

AM conducted the majority of western blotting experiments and prepared the manuscript. LS prepared stable AGR2 gene knockout cell lines and prepared the manuscript. KKu was responsible for functional analyses. JP participated in autophagy experiments and reactive oxygen species determination. BV provided materials and tools, designed the preparation of stable AGR2 knockout cell lines and drafted the manuscript. KKa participated in the design of the study and revised the manuscript. RH conceived and approved all experiments, performed functional biological assays and finalized the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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