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Dynamics of oxygen level-driven regulators in modulating autophagy in colorectal cancer cells

Shing Yau Tam, Vincent W.C. Wu**, Helen K.W. Law*

Department of Health Technology and Informatics, Faculty of Health and Social Science, The Hong Kong Polytechnic University, Hung Hom, Hong Kong

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ABSTRACT

Colorectal cancer is a common cancer with metachronous distant metastases still threatening overall survival. Tumor oxygen level influences tumor radiosensitivity in relation to autophagy and apoptosis. The objective of this study is to evaluate the expression and interaction between multiple key regulators in different oxygen levels. Human colorectal adenocarcinoma HT-29 cells were cultured in 1% or 10% oxygen level and irradiated by 2 Gy with different incubation time. Autophagy key regulators, AMPK, HIFs and JNK were evaluated by Western blot. Sequential autophagy key regulator activation was observed in the order of AMPK, HIF-1α, HIF-2α and JNK. 10% oxygen level could promote autophagy with similar degree of autophagy activation as 1% oxygen level in 48-h while irradiation could slightly inhibit autophagy. The results of this study supported prior evaluation of oxygen level and autophagy regulators for improving treatment efficacy and indicated the possible directions in developing individualized radiotherapy by selective targeting of hypoxic regions.

1. Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide which ranks third in men and second in women [1]. About two-third occurred in sigmoid colon or rectum and required chemoradiotherapy apart from the primary surgical treatment [2]. Although local disease control is effective due to development of preoperative radiotherapy and total mesorectal excision, overall survival is still threatened by metachronous distant metastases.

Hypoxia has been identified as an important factor for radiotherapy failure as hypoxia reduces radiosensitivity, and increases invasiveness and metastatic power [3]. The proposed mechanisms for radiosensitivity reduction included the promotion of pro-survival autophagy and the suppression of pro-death apoptosis [4,5]. The process is complex and multiple key regulators including AMP-activated protein kinase (AMPK), hypoxia-inducible factors (HIFs), mechanistic target of rapamycin (mTOR), c-Jun N-terminal kinase (JNK) and unfolded protein response (UPR), have been identified [5–8].

While there are multiple pathways proposed in autophagy and apoptosis modulation under hypoxic condition, the link between hypoxia-stimulated autophagy and radiotherapy efficacy remains unclear. Rouschop et al. [5] indicated that chloroquine (autophagy inhibitor) could increase radiosensitivity and improve radiotherapy efficacy among hypoxic tumor cells in vitro and in vivo. Radiation is usually regraded as an autophagy stimulator through mTOR or endoplasmic reticulum stress pathways [9], but research in this area remains scarce.

Although tumor cells may experience a wide range of oxygen level in the body from close to anoxia to about 10% during metastasis in arterial bloodstream, most of the previous studies studied oxygen level ≤2% (pathological or radiobiological hypoxia) and in short time intervals (<24 h) with single signaling pathway [10,11]. Moreover, single oxygen concentration was studied with normoxia (21% O_2) as control, however this level would never be experienced by tumor cells in body [10]. The interplay between different autophagy signaling pathways, the influence of higher oxygen level and

Abbreviations: 4E-BP1, eIF4E-binding protein 1; AMPK, AMP-activated protein kinase; BAK, Bcl-2 homologous antagonist killer; BAX, Bcl-2-like protein 4; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra large; BNIP3, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3; BNIP3L, Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like; CRC, Colorectal cancer; HIF, Hypoxia-inducible factor; JNK, c-Jun N-terminal kinase; LC3B, Microtubule-associated proteins 1A/1B light chain 3B; MAPK, Mitogen-activated protein kinase; mTOR, Mechanistic target of rapamycin; ULK1, Unc-51 like autophagy activating kinase 1; UPR, Unfolded protein response.

* Corresponding author.
** Corresponding author.
E-mail addresses: shing-yau.tam@connect.polyu.hk (S.Y. Tam), htvinwu@polyu.edu.hk (V.W.C. Wu), hthelen@polyu.edu.hk (H.K.W. Law).

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chronic hypoxia on autophagy have received little attention. As blood oxygen level may provide clues of tumor cell functions during metastasis and chronic hypoxia may affect tumor cell functions different from acute hypoxia, we hypothesized that different oxygen level and different time points may induce multiple autophagy pathways in CRC cells. We studied the impact of oxygen level in both pathological hypoxia (1%) and blood oxygen concentration (10%) in longer incubation time on various target molecules aiming to reveal the complex kinetics in autophagy.

2. Materials and methods

Human colorectal adenocarcinoma cell line HT-29-Red-FLuc (PerkinElmer, Inc., Waltham, MA) was employed and maintained as instructed by American Type Culture Collection (ATCC). The following antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA): β-Actin (D6A8) Rabbit, AMPKα (D63G4) Rabbit, phosphor-AMPKα (Thr172) (40H9) Rabbit, 4E-BP1 (53H11) Rabbit, phosphor-4E-BP1 (Thr37/46) (236B4) Rabbit, Bcl-2 (Human Specific) Rabbit, Bcl-xL (54H6) Rabbit, Beclin-1 Rabbit, BNIP3 (D7U1T) Rabbit, BNIP3L/Nix (D4R4B) Rabbit, HIF-2α (D9E3) Rabbit, LC3B Rabbit, mTOR Rabbit, phosphor-mTOR (Ser2448) (D9C2) XP Rabbit, SQSTM1/p62 Rabbit, SAPK/JNK Rabbit and phosphor-SAPK/JNK (Thr183/Tyr185) Rabbit. Purified mouse anti−HIF−1α antibody was obtained from BD Transduction Laboratories. Secondary antibody used were anti-rabbit IgG, HRP-linked and anti-mouse IgG, HRP-linked from Cell Signaling Technology, Inc.

Two Million HT-29 cells were seeded in T25 flask with complete McCoy's 5A modified medium supplemented with 10% FBS. The cells were then incubated in different oxygen level for 24 h. SCI-tive Dual chamber hypoxia workstation (Baker Ruskinn, Sanford, ME), was employed to maintain different oxygen level conditions (1% O_2, 5% CO_2, 94% N_2 or 10% O_2, 5% CO_2, 85% N_2) within the incubation period while 21% O_2 and 5% CO_2 (normoxia) experiments were conducted in conventional cell incubator (Thermo Fisher Scientific, Waltham, MA).

Some cells were lysed for western blotting after 24 h. Other cells were air-sealed for keeping the constant oxygen level and taken out of the chamber to receive 0 Gy or 2 Gy of irradiation at 225 kV, 2.5 Gy/min by MultiRad 225 (Faxitron Bioptics, LLC, Tucson, AZ). After that the cells were returned to their respective chamber for another 24 h of incubation before harvesting.

In western blotting, after removing the culture medium and gently washed by PBS twice, the cells were scraped in Laemmli buffer on ice by a cell scraper. The harvested protein was then centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant denatured at 95 °C for 5 min. The protein samples were resolved in 8−12% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA). Blots were blocked in 5% BSA in TBST for 1 h. For protein detection, blots were incubated overnight at 4 °C with a primary antibody in recommended dilution in TBST containing 5% BSA. Bound antibodies were visualized by 2-h incubation of secondary anti-rabbit or anti-mouse IgG antibodies conjugated with HRP diluted at 1:3000 in TBST containing 5% nonfat dry milk and Western Lightning Plus-ECL (PerkinElmer, Inc.) according to the supplier's instruction.

Data was presented as means ± SEM of more than 4 independent experiments and the differences between groups were analyzed by student's t-test (2 groups) or Kruskal-Wallis H test (3 or more groups) by SPSS version 22 (IBM Corp). $p < 0.05$ was considered as statistically significant.

3. Results

3.1. The dynamics of autophagic flux

Increased microtubule-associated proteins 1A/1B light chain 3B (LC3B)-II expression and LC3B-II/LC3B−I ratio were the key markers of autophagy activation [12]. We first assessed the level of autophagy in HT-29 cells in the two time points of different oxygen level incubation (24 and 48 h) among unirradiated and irradiated samples (48 h only) by western blotting. LC3B-II expression and LC3B-II/LC3B−I ratio increased substantially among 1% and 10% O_2 after 24 h (Fig. 1A). While in 48 h, LC3B-II in 1% O_2 showed slight decrease when compared with normoxia control whereas LC3B-II in 10% O_2 showed a greater increase when compared with control and 24-h. Moreover, LC3B-II/LC3B−I ratios increased in both 1% and 10% O_2 (Fig. 1B).

Irradiation gave diverse impact on LC3B-II level among different oxygen levels, LC3B-II levels slightly increased among 1% and 21% O_2 while LC3B-II level slightly decreased in 10% O_2. Irradiation slightly decreased LC3B-II/LC3B−I ratios among all oxygen levels (Fig. 1B).

The expression of p62, another autophagy indicator, was decreased in 1% O_2 sample but no change in expression in 10% O_2 sample in 24-h (Fig. 1C). Whereas p62 slightly increased in 48-h 10% O_2 (Fig. 1D). Irradiation slightly increased p62 among all oxygen levels (Fig. 1D).

3.2. Expression of AMPK, HIFs and JNK in the regulation of autophagic flux

After demonstrating autophagic flux change by different oxygen level and irradiation, we investigated the expressions of AMPK, HIFs and JNK. AMPK activation was determined by phosphorylated-AMPKα/total-AMPKα (*p*-AMPKα/AMPKα) ratio. Both 1% and 10% O_2 indicated considerable increases in *p*-AMPKα/AMPKα ratio in 24-h with 10% O_2 showing greater increase (Fig. 2A). The situation experienced changes in 48-h with *p*-AMPKα/AMPKα ratio in 1% O_2 was significantly smaller than control. The *p*-AMPKα/AMPKα ratio in 10% O_2 had no significant change while irradiation could only pose minor increase in *p*-AMPKα/AMPKα ratio among 10% and 21% O_2 (Fig. 2B).

Both HIF-1α and HIF-2α levels were investigated. As shown in Fig. 2, HIF-1α was strongly expressed in 1% O_2 of both time points with stronger promotion at 24-h. For HIF-1α expression in 10% O_2, milder increases were recorded when compared with 1% O_2 with stronger promotion in 48-h. Irradiation further increased HIF-1α expression in 1% O2, but it did not greatly affect HIF-1α expression in 10% O_2. Also, slight increase in HIF-1α expression in 21% O_2 was found (Fig. 2C−D).

HIF-2α experienced milder increase than HIF-1α. HIF-2α was increased among 1% and 10% O_2 in both time points with stronger expression in 1% O_2. Also, differences in expression between 1% and 10% O_2 were slightly smaller in 48-h. Irradiation did not influence the HIF-2α levels except an increase in 21% O_2 (Fig. 2C−D).

Since AMPK and HIFs may influence the expression of mTOR and its downstream targets such as eIF4E-binding protein 1 (4E-BP1), ratios of *p*-mTOR/mTOR and *p*-4E-BP1/4E-BP1 were used for assessing their activations. Among all samples, *p*-mTOR/mTOR ratio were similar (Fig. 3A−B). While for *p*-4E-BP1/4E-BP1 ratio, there were no significant changes in 24-h, but significant decreases among 1% O_2 were found in 48-h while no significant changes were found in 10% O_2 or irradiation samples (Fig. 3C−D).

Apart from influencing mTOR and its downstream target, HIF-1α may also affect the expressions of B-cell lymphoma 2 (Bcl-2)/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and

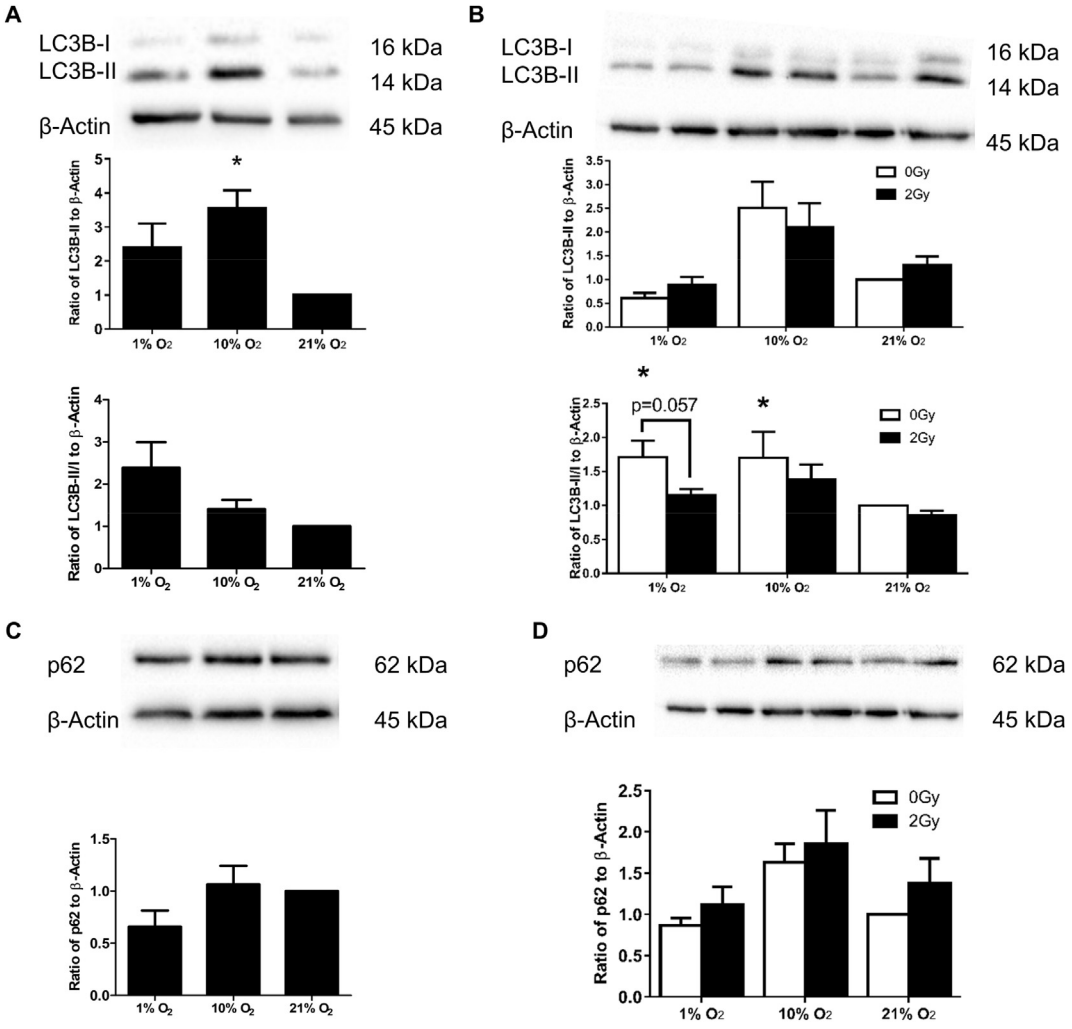

Fig. 1. Hypoxia or blood oxygen level induced autophagy and radiation partially reversed the effect in HT-29 cells. Conversion of LC3B—I to LC3B-II in HT-29 cells subjected to 1% O_2 or 10% O_2 for 24 h (A) or 48 h with or without irradiation (0 Gy or 2 Gy) (B) measured by Western blotting. The expression of LC3B-II and the ratio of LC3B-II/I increased with the decrease in oxygen level and irradiation slightly reversed the effect. p62 level as secondary autophagy evaluation target at 24 h (C) or 48 h (D). Increase trend of p62 expression in 10% O_2 with or without irradiation at 48 h can be observed. The data (means ± SEM) was expressed as the relative changes compared with unirradiated 21% O_2 group.*$p < 0.05$ versus unirradiated 21% O2, N = 4 (24 h) or 5 (48 h).

Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L). BNIP3 was generally weakly expressed (Fig. 3E—F). While BNIP3L was significantly induced in 1% O_2 with 24-h and irradiated samples attaining higher expression. For 10% O_2 samples, only slight increase has been found among 24-h and irradiated samples. Moreover, irradiation could significantly decrease BNIP3 expression in 21% O_2 samples (Fig. 3E—F).

mTOR-independent JNK pathway was also evaluated by *p*-JNK/JNK ratio, and its potential downstream targets including Bcl-2 and B-cell lymphoma extra large (Bcl-xL). For the ratio of *p*-JNK/JNK, slight down-regulation of JNK was found in 24-h 1% O_2 (Fig. 4A) while up-regulation of JNK were found in 48-h 1% and 10% O_2 (Fig. 4B). Irradiation reduced JNK activation except a slight increase in 21% O_2 (Fig. 4B).

Weak Bcl-2 and strong Bcl-xL expressions were generally recorded. There were moderate increases of the two proteins in 1% and 10% O_2 except slight down-regulation in 48-h 1% O_2 (Fig. 4C—D). Irradiation increased both protein expressions in 1% O_2 while only Bcl-2 level was increased in 10% O_2. Irradiation did not influence expressions of both proteins in 21% O_2 (Fig. 4D). There were also no significant changes in Beclin-1 levels among all samples (Fig. 4E—F).

4. Discussion

4.1. Autophagy in different oxygen level and irradiation

Autophagy is a highly conserved process to enable tumor cells to survive under stressful conditions such as hypoxia and irradiation [9]. Various autophagy modulation agents have been suggested to reduce treatment resistance due to hypoxia and/or radiation-driven autophagy [5,9]. However, conclusions of efficacy of these drugs were based on comparison with normoxia (21% O_2). Our results showed that both hypoxia (1% O_2) and arterial blood oxygen concentration (10% O_2) could induce autophagy when compared with normoxia. This novel finding drove us to rethink the use of autophagy modulation agents in improving treatment efficacy based on single oxygen level comparison with normoxia while the seemingly 'non-hypoxic' 10% O_2 could also stimulate autophagy.

While in the context of irradiation, we found that irradiation

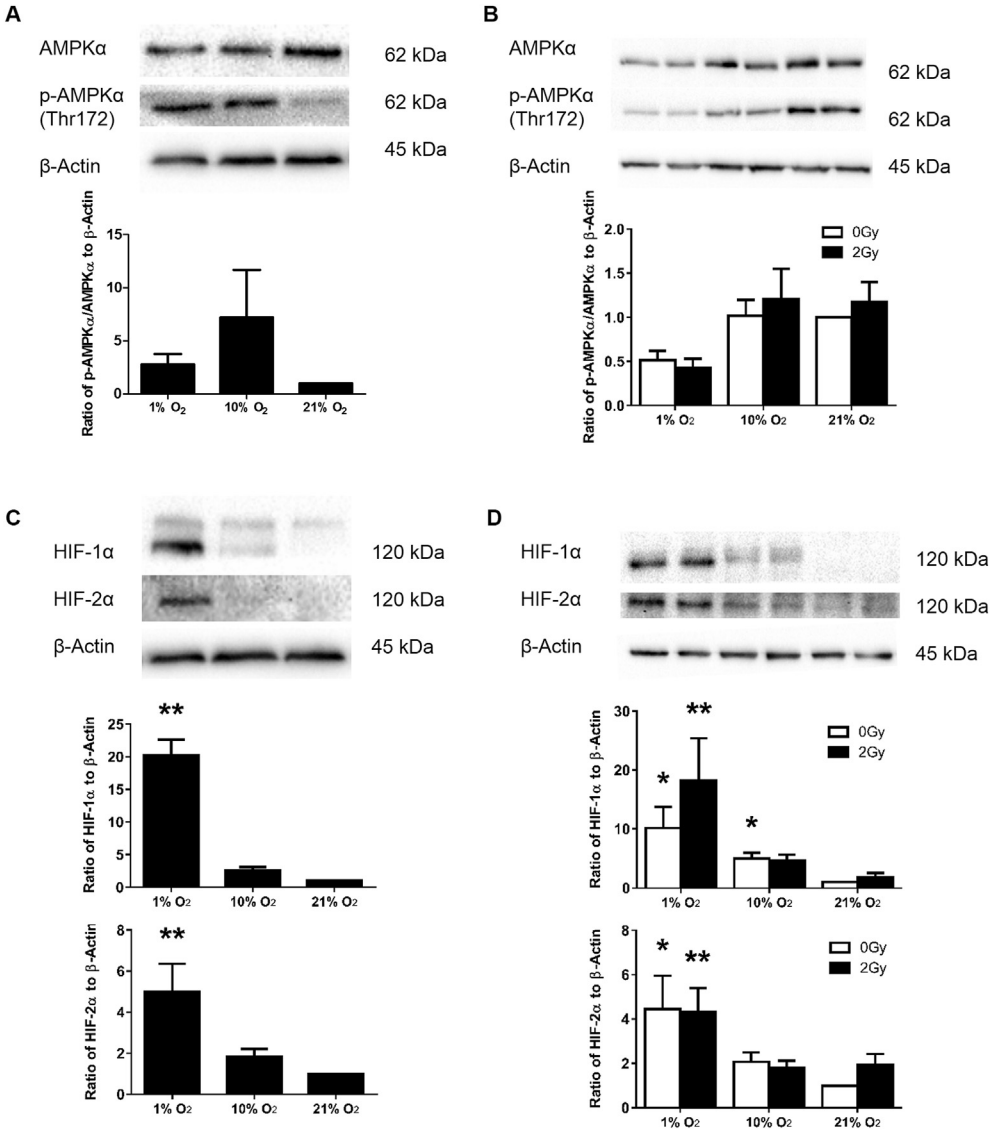

Fig. 2. The early onset and rapid suppression of AMPKα and the dynamics of HIFs. The ratio of *p*-AMPKα/AMPKα and the expression levels of HIF-1α and HIF-2α were used to evaluate the activity of AMPKα and HIFs by Western blotting in HT-29 cells subjected to different O_2 level (1% and 10%) for 24 h (A/C) and 48 h of with or without irradiation (0 Gy or 2 Gy) (B/D). Increase trend of AMPKα expression in 1% O_2 and 10% O_2 demonstrated the early activation of AMPKα (A) and significant decrease in AMPKα expression in 1% O_2 at 48 h (B) showed the rapid suppression of AMPKα. Significant up-regulation of HIF-1α and HIF-2α was found in 1% O_2 in both time points (C/D) whereas 10% O_2 also showed significant increase in HIF-1α expression at 48 h (D). The data (means ± SEM) was expressed as the relative changes compared with unirradiated 21% O_2 group. $^{*}p < 0.05$, $^{**}p < 0.01$ versus unirradiated 21% O_2, N = 4 (24 h) or 5 (48 h).

had less influence on autophagy than the difference in oxygen level. This also led us to rethink the influence of radiation on autophagy, which may be affected by other cellular process such as apoptosis. For p62 level, although lowered p62 expression has been well-regarded as an indicator of autophagy [13], p62 level could be up-regulated under stressful conditions through mitogen-activated protein kinase (MAPK) signaling such as JNK [14], which was also stimulated by 1% or 10% O_2 in 48-h. This made the complexity of establishing p62 as a marker of autophagic flux in stressful conditions.

4.2. The expression and relationship of AMPK and HIFs in autophagy regulation

AMPK, HIFs and JNK have been established as key autophagy regulators during oxidative stress. However, their interactions and dynamics in different oxygen level and time point are not well studied. In this study, AMPK was stimulated in 24-h among 1% and 10% O_2, showing that AMPK aimed to restore energy homeostasis by reducing ATP production and the 'non-hypoxic' 10% O_2 was also considered as a stressful condition [15]. AMPK also promoted autophagy through mTOR inhibition and Unc-51 like autophagy activating kinase 1 (ULK1)/Beclin-1 stimulation. Since AMPK is also a tumor suppressor by inhibiting Warburg effect and tumor growth, and AMPK is antagonized by HIF-1α activity [16], 10% O_2 could attain a higher AMPK activation level than 1% O_2. Moreover, this study showed that AMPK activation has limited duration as it returned to normal level in 48-h with the continued HIF-1α activity inhibited AMPK in 1% O_2. This demonstrated the removal of tumor suppressing function conferred by AMPK and allowed further tumor growth in chronic hypoxia.

Besides interfering AMPK activation, HIFs also impact autophagic flux in various aspects. First, HIF-1α promotes autophagy through inhibiting mTOR while HIF-2α inhibits autophagy through

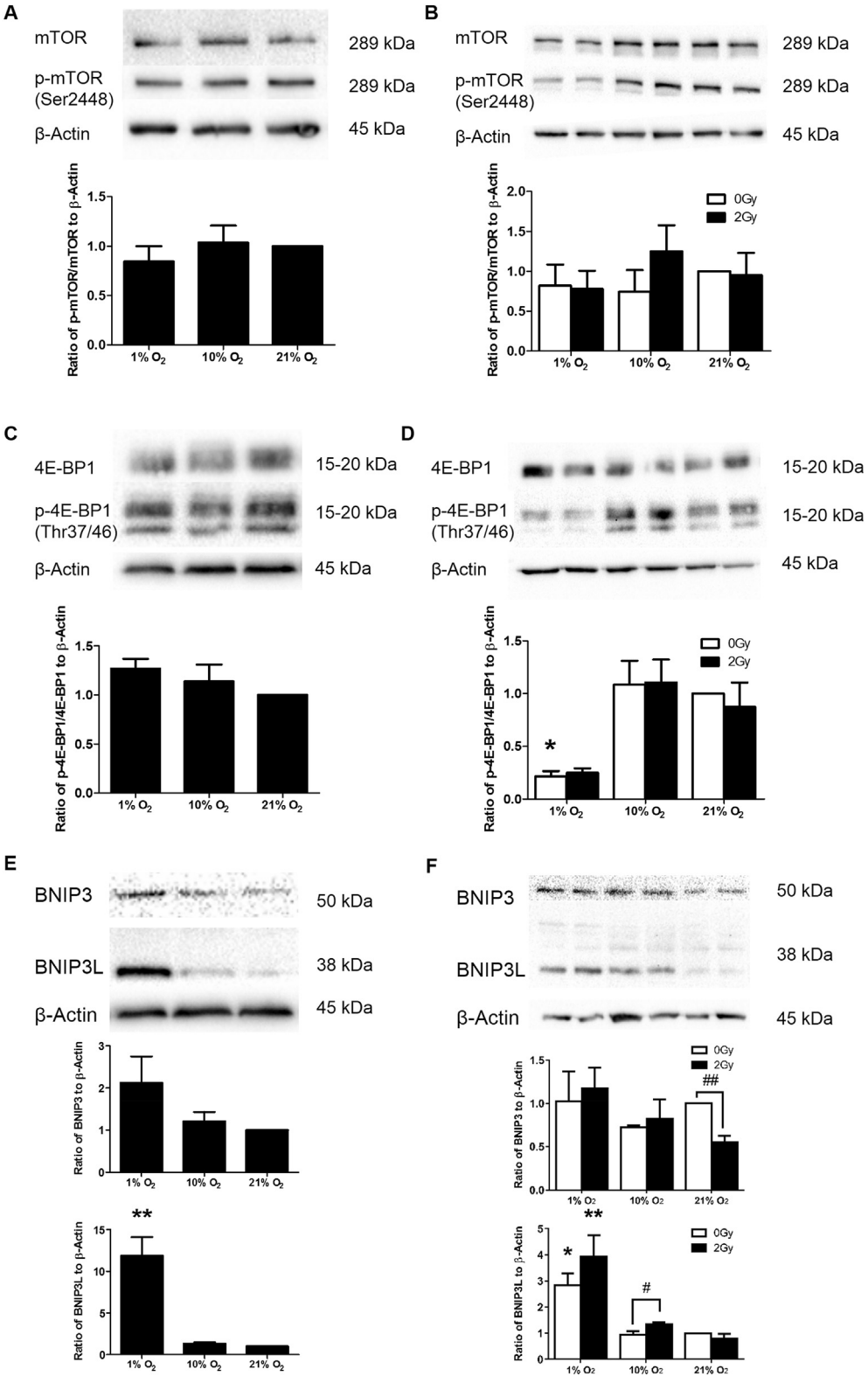

Fig. 3. Modulation of mTOR activity in different oxygen level and the HIF-1α mediated BNIP3L stimulation as a mTOR-independent autophagy pathway. The change of mTOR activity in 1% O_2 or 10% O_2 on HT-29 cells was not apparent in 24 h (A) or 48 h with or without irradiation (0 Gy or 2 Gy) (B) from the phosphorylation level of mTOR evaluated by Western blotting. However, the involvement of mTOR regulation was observable through the hypophosphorylation of 4E-BP1 in 1% O_2 at 48 h (D) but not at 24 h (C). The downstream targets of HIF-1α includes BNIP3 and BNIP3L. BNIP3 achieved much weaker stimulation due to histone deacetylation and methylation while BNIP3L was significantly up-regulated by HIF-1α in 1% O_2 at both time points (E/F). The data (means ± SEM) was expressed as the relative changes compared with unirradiated 21% O_2 group. $^{*}p < 0.05$, $^{**}p < 0.01$ versus unirradiated 21% O_2 (Kruskal-Wallis test), $\#p < 0.05$, $\#\#p < 0.01$ unirradiated versus irradiated (Student's t-test). N = 4 (24 h) or 5 (48 h).

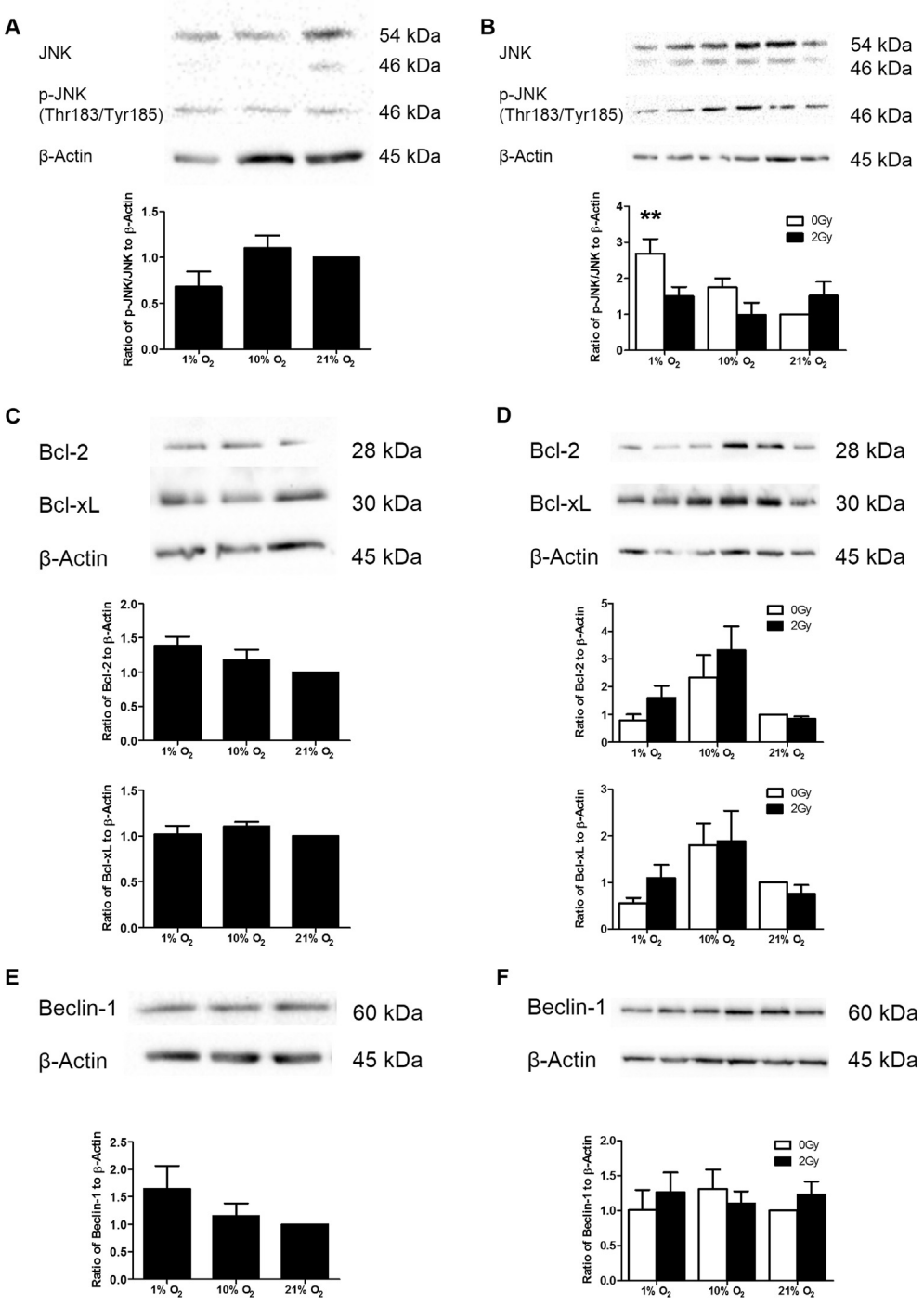

Fig. 4. The delayed JNK pathway and the strong expression of anti-apoptotic Bcl-xL. The ratio of *p*-JNK/JNK was used to investigate JNK activity of HT-29 cells at different oxygen levels in 24 h (A) or 48 h (B) by Western blotting. JNK was generally up-regulated by 1% O_2 and 10% O_2 levels at 48-h while radiation partially reversed the effect except in normoxia. Expression levels of Bcl-2 and Bcl-xL showed that HT-29 generally had large amount of Bcl-xL, which greatly reduced the JNK mediated apoptosis stress in both 24 h (C) and 48 h (D). There were no significant changes in the expression level of Beclin-1 in both 24 h (E) and 48 h (F). The data (means ± SEM) was expressed as the relative changes compared with unirradiated 21% O_2 group. $^{**}p < 0.01$ versus unirradiated 21% O_2, N = 4 (24 h) or 4–5 (48 h).

stimulating mTOR. Although mTOR status had no significant changes observed in this study, hypophosphorylation of 4E-BP1 was found in 48-h 1% O_2. 4E-BP1 was controlled by mTOR as inactive hyperphosphorylated state while mTOR inhibition by HIF-1α could hypophosphorylate and activate 4E-BP1. Thus, hypophosphorylation of 4E-BP1 provided a clue that HIF-1α promoted autophagy through inhibiting mTOR. Also, the activated 4E-BP1 bound with eIF4E and suppresses cap-dependent mRNA translation. Thus, translation of IRES-containing mRNAs was promoted and this signaled a switch from cap-dependent to cap-independent translation. Elevated 4E-BP1 expression has been associated with poor prognosis in CRC as this leads to selective translation of IRES-containing mRNAs such as HIF-1α, VEGF and Bcl-2 [17,18]. While HIF-1α experienced a decreased expression from 24-h to 48-h in 1%

O_2 and delayed up-regulation in 10% O_2, HIF-2α has sustained up-regulation with comparatively higher expression level in 10% O_2. This result agrees with previous literature and leads to the phenomenon of autophagy inhibition by mTOR promotion and partially influence the reduced autophagic flux in 1% O_2 [19]. Although HIF-1α and HIF-2α have antagonistic roles in mTOR regulation, HIF-2α could be another promoter of tumorigenesis [20]. Therefore, both HIF-1α and HIF-2α are worthy to be studied in greater depth as they could influence cellular functions in wide range of oxygen level.

The second direction for HIF-1α acting as autophagy promoter is from up-regulating BNIP3/BNIP3L. BNIP3/BNIP3L stimulates autophagy and the binding of BNIP3/BNIP3L with Bcl-2/Bcl-xL also prevents cell death through apoptosis [21]. Due to histone deacetylation and methylation, BNIP3 expression is silenced among majority of colorectal and gastric cancer cell lines including HT-29, which is thought to prevent hypoxic cell death [22]. Our results showed that BNIP3 expressions were weak as described in previous literatures. However, strong inductions of BNIP3L by hypoxia were observed in both time points. The induced BNIP3L could promote mitophagy while liberated Beclin-1 could promote autophagy [23]. Also, the triggering of Bcl-2-like protein 4 (BAX)- Bcl-2 homologous antagonist killer (BAK)-dependent apoptosis by BNIP3L regulation was prevented by Bcl-2/Bcl-xL binding. Thus, induced BNIP3L could be seen as a survival response under hypoxia while the trend of BNIP3L followed the decrease of HIF-1α in 48-h and thus contributing the decrease in autophagic flux in 48-h 1% O_2.

4.3. The delayed activation of JNK

JNK pathway also affects Beclin-1 status and influences both autophagy and apoptosis under stressful conditions. We found that JNK was up-regulated in both 1% and 10% O_2 in 48-h but not 24-h, demonstrating JNK activation as a delayed response when compared with AMPK or HIF-1α. Although only up-regulation in 48-h 1% O_2 achieved statistical significance, the up-regulation trend of p62 in 48-h 10% O_2 could serve as a secondary evidence of JNK activation [14]. Moreover, we discovered irradiation could suppress JNK rather than promote JNK among 1% and 10% O_2. As JNK activation also links to apoptosis promotion apart from autophagy regulation, we proposed that the reduction of JNK activation by irradiation as a mechanism to reduce apoptosis activation and thus preventing cell death with the expense of reducing autophagy stimulation, which was seen in the decreased LC3-II/LC3-I ratio among irradiated samples. This finding demonstrates the complex regulation of autophagy and apoptosis in maintaining cellular homeostasis.

JNK activates autophagy in a similar way as HIF-1α-BNIP3L by disrupting Bcl-2/Bcl-xL-Beclin-1 complex, activated JNK phosphorylates Bcl-2/Bcl-xL and liberates Beclin-1 in the process. This autophagy activation mechanism is also pro-apoptotic as it involves the phosphorylation of anti-apoptotic Bcl-2/Bcl-xL [24]. However, we propose that the phosphorylation of Bcl-2/Bcl-xL should have minimal effect on apoptosis activation due to high Bcl-xL level among samples. Bcl-xL is approximately ten times more effective than Bcl-2 in anti-apoptosis role [25]. Therefore, targeting Bcl-xL may prove to be useful in inducing apoptotic cell death especially under chronic stressful conditions.

4.4. Autophagy modulations on future studies

Our results concluded the sequential autophagy key regulator activation, i.e. AMPK for early autophagy activation, HIF-1α and HIF-2α for longer term autophagy modulation, and finally JNK for delayed autophagy activation as a novel key finding. Also, we found that 10% O_2 could promote autophagy with similar degree of autophagy activation as hypoxia in long term as another novel key finding. We suggest that the decision of using these autophagy modulation agents should base on autophagy status at multiple oxygen levels including the 'non-hypoxic' region such as blood oxygen level.

We suggest that cells incubated in blood oxygen level remain as important targets for further investigations since tumor cells metastasize by bloodstream. While we found that CRC cells had high autophagy flux at blood oxygen level as a novel finding, modulation of autophagy level may provide rewarding results in treatment against metastasis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.07.043.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.07.043.

References

[1] J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray, Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012, Int. J. Cancer 136 (2015) E359–E386.
[2] J. Simpson, J.H. Scholefield, Treatment of colorectal cancer: surgery, chemotherapy and radiotherapy, Surgery 26 (2008) 329–333.
[3] R.P. Hill, R.G. Bristow, A. Fyles, M. Koritzinsky, M. Milosevic, B.G. Wouters, Hypoxia and predicting radiation response, Semin. Radiat. Oncol. 25 (2015) 260–272.
[4] J.T. Erler, C.J. Cawthorne, K.J. Williams, M. Koritzinsky, B.G. Wouters, C. Wilson, C. Miller, C. Demonacos, I.J. Stratford, C. Dive, Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance, Mol. Cell. Biol. 24 (2004) 2875–2889.
[5] K.M. Rouschop, T.v.d. Beucken, L. Dubois, H. Niessen, J. Bussink, K. Savelkouls, T. Keulers, H. Mujcic, W. Landuyt, J.W. Voncken, P. Lambin, A.J.v.d. Kogel, M. Koritzinsky, B.G. Wouters, The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5, J. Clin. Investig. 120 (2010) 127–141.
[6] B. Keith, R.S. Johnson, M.C. Simon, HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression, Nat. Rev. Cancer 12 (2011) 9–22.
[7] K.R. Laderoute, K. Amin, J.M. Calaoagan, M. Knapp, T. Le, J. Orduna, M. Foretz, B. Viollet, 5′-AMP-Activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments, Mol. Cell. Biol. 26 (2006) 5336–5347.
[8] K.R. Laderoute, J.M. Calaoagan, C. Gustafson-Brown, A.M. Knapp, G.-C. Li, H.L. Mendonca, H.E. Ryan, Z. Wang, R.S. Johnson, The response of c-jun/AP-1 to chronic hypoxia is hypoxia-inducible factor 1α dependent, Mol. Cell. Biol. 22 (2002) 2515–2523.
[9] S.Y. Tam, V.W.C. Wu, H.K.W. Law, Influence of autophagy on the efficacy of radiotherapy, Radiat. Oncol. 12 (2017) 57.
[10] S.R. McKeown, Defining normoxia, physoxia and hypoxia in tumours—implications for treatment response, Br. J. Radiol. 87 (2014), 20130676.
[11] A. Wouters, B. Pauwels, F. Lardon, J.B. Vermorken, Review: implications of in vitro research on the effect of radiotherapy and chemotherapy under

hypoxic conditions, The Oncologist 12 (2007) 690–712.
[12] I. Tanida, T. Ueno, E. Kominami, LC3 conjugation system in mammalian autophagy, Int. J. Biochem. Cell Biol. 36 (2004) 2503–2518.
[13] M. Komatsu, Y. Ichimura, Physiological significance of selective degradation of p62 by autophagy, FEBS Lett. 584 (2010) 1374–1378.
[14] A. Puissant, N. Fenouille, P. Auberger, When autophagy meets cancer through p62/SQSTM1, Am J Cancer Res 2 (2012) 397–413.
[15] G. Zadra, J.L. Batista, M. Loda, Dissecting the dual role of AMPK in cancer: from experimental to human studies, Mol. Cancer Res. : MCR 13 (2015) 1059–1072.
[16] B. Faubert, G. Boily, S. Izreig, T. Griss, B. Samborska, Z. Dong, F. Dupuy, C. Chambers, B.J. Fuerth, B. Viollet, O.A. Mamer, D. Avizonis, R.J. DeBerardinis, P.M. Siegel, R.G. Jones, AMPK is a negative regulator of the Warburg Effect and suppresses tumor growth in vivo, Cell Metabol. 17 (2013) 113–124.
[17] S. Braunstein, K. Karpisheva, C. Pola, J. Goldberg, T. Hochman, H. Yee, J. Cangiarella, R. Arju, S.C. Formenti, R.J. Schneider, A hypoxia-controlled cap-dependent to cap-independent translation switch in breast cancer, Mol. Cell 28 (2007) 501–512.
[18] M.W. Chao, L.T. Wang, C.Y. Lai, X.M. Yang, Y.W. Cheng, K.H. Lee, S.L. Pan, C.M. Teng, eIF4E binding protein 1 expression is associated with clinical survival outcomes in colorectal cancer, Oncotarget 6 (2015) 24092–24104.
[19] C.P. Bracken, A.O. Fedele, S. Linke, W. Balrak, K. Lisy, M.L. Whitelaw, D.J. Peet, Cell-specific regulation of hypoxia-inducible factor HIF-1α and HIF-2α stabilization and transactivation in a graded oxygen environment, J. Biol. Chem. 281 (2006) 22575–22585.
[20] J. Zhao, F. Du, G. Shen, F. Zheng, B. Xu, The role of hypoxia-inducible factor-2 in digestive system cancers, Cell Death Dis. 6 (2015) e1600.
[21] G. Bellot, R. Garcia-Medina, P. Gounon, J. Chiche, D. Roux, J. Pouyssegur, N.M. Mazure, Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains, Mol. Cell. Biol. 29 (2009) 2570–2581.
[22] M. Murai, M. Toyota, H. Suzuki, A. Satoh, Y. Sasaki, K. Akino, M. Ueno, F. Takahashi, M. Kusano, H. Mita, K. Yanagihara, T. Endo, Y. Hinoda, T. Tokino, K. Imai, Aberrant methylation and silencing of the BNIP3 gene in colorectal and gastric cancer, Clin. Cancer Res. 11 (2005) 1021–1027.
[23] P.A. Ney, Mitochondrial autophagy: origins, significance, and role of BNIP3 and NIX, Biochim. Biophys. Acta 1853 (2015) 2775–2783.
[24] Y.-Y. Zhou, Y. Li, W.-Q. Jiang, L.-F. Zhou, MAPK/JNK signalling: a potential autophagy regulation pathway, Biosci. Rep. 35 (2015), e00199.
[25] A.A. Fiebig, W. Zhu, C. Hollerbach, B. Leber, D.W. Andrews, Bcl-XL is qualitatively different from and ten times more effective than Bcl-2 when expressed in a breast cancer cell line, BMC Canc. 6 (2006) 213.