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Fulvio Chiacchiera & Cristiano Simone

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Article Addendum

Inhibition of p38α unveils an AMPK-FoxO3A axis linking autophagy to cancer-specific metabolism

Fulvio Chiacchiera and Cristiano Simone*

Laboratory of Signal-dependent Transcription; Department of Translational Pharmacology (DTP); Consorzio Mario Negri Sud; Santa Maria Imbaro (Ch), Italy

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Autophagy is an essential process for the maintenance of cellular and metabolic homeostasis. Indeed, it is required for the recovery of ATP-generating substrates in cells subjected to different types of stress insults. Thus, the activity of the autophagic machinery strongly depends on the metabolic status of the cell.[1] It has been proposed that this principle applies not only to normal, but also to cancer cells,[2] despite the profound differences in their metabolism. Cancer cells predominantly produce ATP through the constitutive activation of aerobic glycolysis, a process that generally relies on the stabilization and activation of the transcription factor HIF1α, which regulates the expression of glycolytic genes.[3] We recently showed that p38α is required to sustain the expression of HIF1α target genes, and that its inhibition causes a rapid drop in ATP levels in colorectal cancer cells (CRCs). This acute energy need triggers AMPK-dependent nuclear accumulation of FoxO3A and subsequent activation of its transcriptional program, leading to sequential induction of autophagy, cell cycle arrest and cell death. In vivo, pharmacological blockade of p38α has both a cytostatic and cytotoxic effect on colorectal neoplasms, associated with nuclear enrichment of FoxO3A and expression of its target genes p21 and PTEN.[4] Our data suggest that CRCs impaired in their glycolytic metabolism trigger autophagy as a reversible recovery mechanism and undergo cell cycle arrest; however, the persistence of the stress insults inevitably leads to cell death.

Autophagy is an evolutionarily conserved process consisting in the sequestration of cytoplasmic material and organelles into autophagosomes and their subsequent degradation within autolysosomes. This self-digestion provides nutrients that preserve vital cellular functions under metabolic stress. Indeed, autophagy is activated as an adaptive catabolic process in response to starvation, growth factor deprivation and hypoxia, to generate amino acids and fatty acids to sustain cellular ATP production.[5]

In the past decade, tumorigenesis has emerged as one of the first pathological processes genetically linked to the imbalance of the autophagic machinery. Indeed, deletions of autophagy-related genes are commonly found in human malignancies. Inactivation of these genes, such as *ATG6/beclin 1*, results in increased tumor formation in mice, and their enforced expression inhibits breast tumor growth in mice models. Accordingly, several anticancer agents are potent inducers of autophagy.[6] Moreover, there is accumulating evidence that regulation of the autophagic signaling is linked by an inverse relationship to that of the oncogenic signaling. Several commonly activated oncogenes (class I PtdIns3K, Akt, TOR, Bcl-2) inhibit autophagy, whereas commonly mutated or epigenetically silenced tumor suppressor genes (p53, PTEN, TSC1/TSC2) promote autophagy.[7]

Intriguingly, these factors also modulate cancer-specific metabolism by functional interaction with the transcription factor HIF1α, which directly links aerobic glycolysis to carcinogenesis.[3] These pathways regulate important steps in HIF1α stabilization and the activation of its transcriptional program resulting in increased glycolysis even in the presence of high oxygen tension and leading to decreased mitochondrial oxidative phosphorylation (the so-called 'Warburg effect').[8]

Our studies indicate that inhibition of the p38α signaling pathway causes a significant decrease in the intracellular levels of ATP in CRCs that correlates with impaired expression of rate-limiting enzymes involved in aerobic glycolysis, the main source of energy for these cells.[4] Indeed, we detected a time-dependent reduction of HIF1α protein stability and the consequent down-regulation of its target genes *GLUT1*, *HK2*, *PKM2* and *LDHA*.[4] The resulting acute energy need triggers the AMPK-dependent nuclear accumulation of the transcription factor FoxO3A, its binding to DNA cognate sequences and the activation of the FoxO3A transcriptional program. This program consists of the sequential expression of target genes whose protein products are

*Correspondence to: Cristiano Simone; Laboratory of Signal-dependent Transcription; Department of Translational Pharmacology (DTP); Consorzio Mario Negri Sud; Santa Maria Imbaro (Ch) 66030 Italy; Tel.: +39.0872570344; Fax: +39.0872570299; Email: simone@negrisud.it

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involved in autophagy, cell metabolism, cell cycle arrest and cell death.[4]

The first induced genes are reported to play a role in autophagosome assembly. Indeed, in the first 24 hours of SB202190 (a p38α inhibitor) treatment, the expression of members of the *ATG8* family—*MAP1LC3*, *GABARAP* and *GABARAPL1*—was rapidly upregulated (Fig. 1), in accordance with the rate of autophagic vesicle formation in CRCs[9] (Fig. 2A). Moreover, increased transcription of these genes was consistently observed at all time points analyzed.

The expression of other autophagy-related (*ATG*) FoxO3A-target genes, including *ATG5*, *ATG6/BECN1*, *ATG12* and *ATG7*, was also upregulated throughout the treatment (Fig. 1), albeit with different kinetics. *ATG* genes were significantly induced 24 hours after the addition of SB202190 and reached a plateau at 48 hours of treatment. Collectively, these data are in agreement with several studies demonstrating that induction of autophagy is often sustained by the upregulation of autophagy-related genes from yeasts to humans.[10-12]

The time-dependent upregulation of *ATG* genes correlates well with the accumulation of the lipidated form of MAP1LC3 (LC3-II) (Fig. 2C) and with the induction of cytoplasmic MAP1LC3 dots (Fig. 2B). Moreover, the large SB202190-dependent vacuoles were positive for LAMP-2, a lysosomal membrane marker, and associated with MAP1LC3 dots, thus suggesting their autolysosomal origin (Fig. 2B). Indeed, the employment of 3-methyladenine (3MA), an inhibitor of the initial nucleation step of the autophagic process, or bafilomycin A_1, an inhibitor of the maturation step that prevents formation of autolysosomes,[9,10] completely abolished SB202190-dependent vacuolation (Fig. 2D) and significantly affected lipidation of MAP1LC3 or the subsequent degradation of LC3-II, respectively (Fig. 2E). Importantly, the protein product of *GABARAPL1*, the gene that showed the highest transcriptional induction upon SB202190 treatment in CRCs,[4] accumulated in a time-dependent manner (Fig. 2F) forming cytoplasmic dots, like MAP1LC3, and localized to large vacuoles (Fig. 2G).

In parallel with the upregulation of the *ATG8* family genes, FoxO3A induced the expression of target genes involved in cell metabolism (Fig. 1), suggesting that, in response to p38α inhibition, CRCs activate a transcriptional program leading to the expression of enzymes capable of transforming the final autophagic products, amino acids and fatty acids, into energy fuel to survive. Indeed, autophagy induction in CRCs correlated with the upregulation of FoxO target genes coding for PGC1α, a transcriptional coactivator involved in glucose uptake, gluconeogenesis and mitochondrial oxidation, PEPCK, the most important enzyme in gluconeogenesis, and UCP2, which protects cells against the formation of reactive oxygen species (ROS). Moreover, SB202190-treated CRCs also upregulated CPT-1, the carrier of fatty acids across the outer mitochondrial membrane, and MCAD, an enzyme involved in fatty acid oxidation.[4]

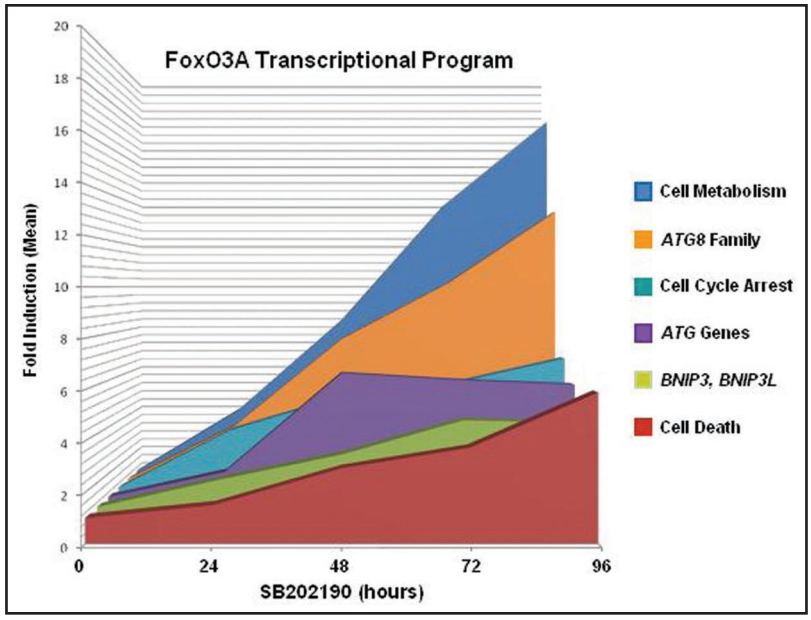

Figure 1. Transcriptional profile of FoxO3A target genes. FoxO3A target genes induced by SB202190 (10 μM) treatment were grouped into six different categories depending on the functional role of their protein products: *Cell Metabolism* (PGC1α, PEPCK, UCP2); *ATG8 Family* (MAP1LC3, GABARAP, GABARAPL1); *Cell Cycle Arrest* (p21, p27, p57, Bcl6, CycG, RBL2); *ATG genes* (ATG5, ATG6/BECN1, ATG12, ATG7); BNIP3 and BNIP3L; *Cell Death* (PUMA, Bim, PTEN). The indicated values are representative of the means of relative expression for each group.

SB202190-dependent autophagy was followed by reduced growth and viability (Fig. 2H), due to sequential induction of cell cycle arrest (24 h–48 h) and cell death (48 h and beyond).[4,13] Hence, we extended our analysis to FoxO3A target genes involved in the control of proliferation and survival (Fig. 1). In the first 24 hours of SB202190 treatment, transcription of FoxO3A target genes coding for cell cycle regulators (p21, p27, p57, Bcl6, CycG, RBL2) was rapidly induced and remained elevated throughout the entire treatment (Fig. 1). Persistent inactivation of p38α reduced the survival rate of CRCs (Fig. 2H) by inducing nonapoptotic cell death.[4,13] Gene expression profiles of the cell death group suggested an important role in SB202190-dependent cell death for the BH3-only proteins PUMA and Bim, and for the class I PtdIns3K-specific phosphatase PTEN (Fig. 1). The other two FoxO3A target genes coding for the BH3-only proteins BNIP3L and BNIP3 are described to be involved in autophagy and/or cell death depending on the experimental model and conditions. In our studies their expression profiles were similar to those of the cell death group (Fig. 1), but for the moment we cannot exclude their contribution to SB202190-dependent autophagy. Our data are not yet conclusive about the type of cell death triggered by p38α blockade in CRCs and to the relative contribution of the autophagic machinery to this process. According to the recommendations of the Nomenclature Committee on Cell Death 2009,[14] we can exclude the possibility that SB202190-treated CRCs die by apoptosis, but further characterization of the death program is required.

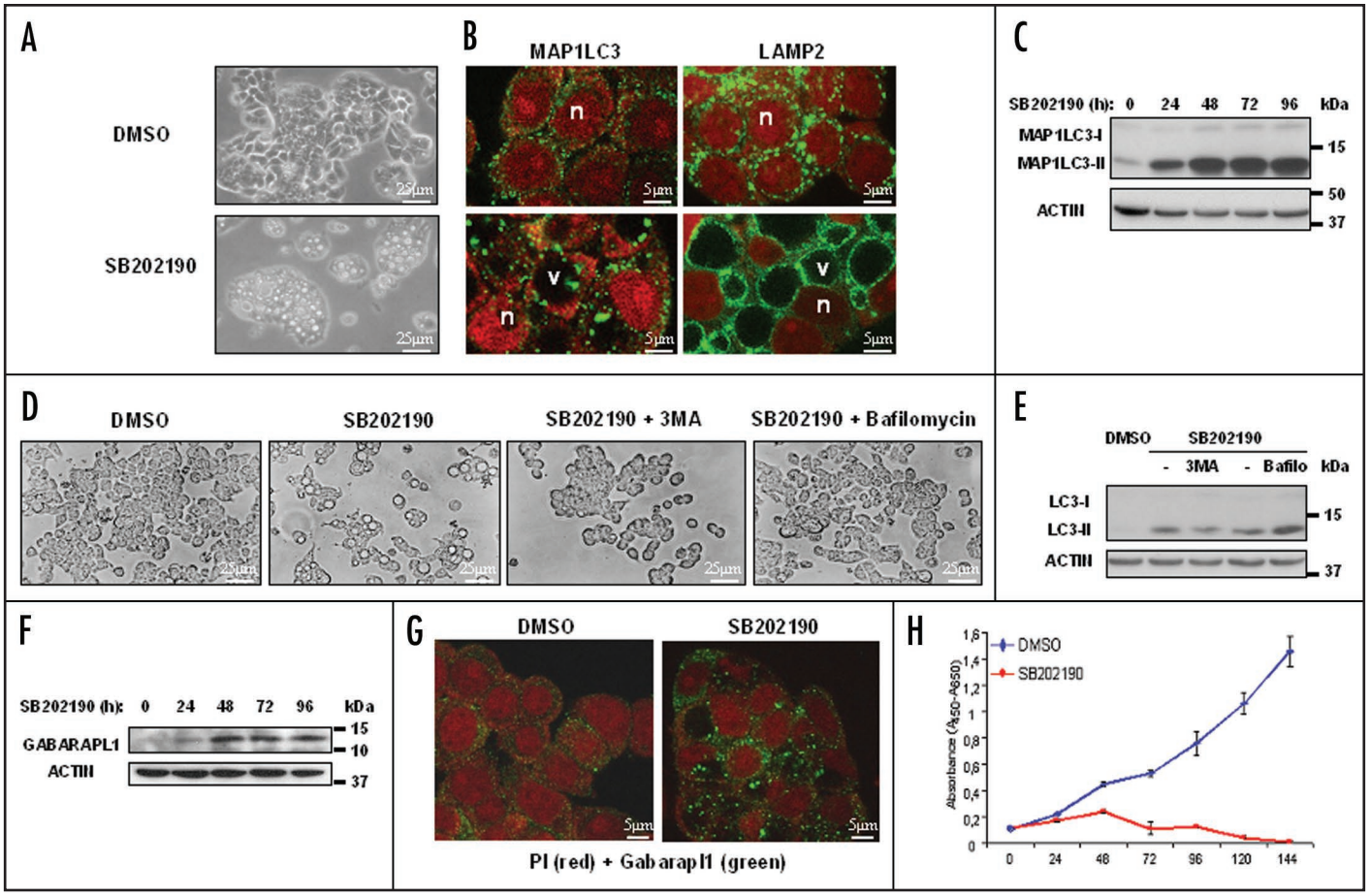

Figure 2. Bright field (A) and confocal images (B) of HT29 CRCs treated for 24 h with SB202190 (10 μM). Confocal analysis demonstrates an increase in punctate MAP1LC3 staining (green) and reveals the localization of both MAP1LC3 and LAMP-2 (green) to large vacuoles (v) in SB202190-treated HT29 cells. Nuclei (n) were stained with propidium iodide (PI). (C) Immunoblots showing the expression of the cytosolic (LC3-I) and PE-conjugated (LC3-II) forms of MAP1LC3 in HT29 cells cultured for up to 96 hours in the presence of SB202190 (10 μM). (D and E) SB202190 treatment increases the autophagic flux. HT29 cells were cultured for 12 hours in the presence of SB202190 (10 μM) or a combination of SB202190 (10 μM) and 3-methyladenine (3MA; 10 mM) or SB202190 (10 μM) and bafilomycin (Bafilo; 0.1 nM). Bright field images (D) show that both 3MA and bafilomycin reduce or abrogate the formation of large vacuoles. Immunoblot analysis of the same cells reveals the accumulation of LC3-II in cells treated with SB202190 plus bafilomycin compared to cells treated with SB202190 alone. (F and G) Immunoblot analysis and confocal microscopy images demonstrating the SB202190-dependent accumulation of GABARAP-L1. Nuclei were stained with propidium iodide. (H) WST1 viability assay. 2 x 10^3 HT29 cells were plated and cultured in either the absence or the presence of SB202190 (10 μM) for the indicated periods of time. The assay shows that SB202190 treatment induces growth arrest in the first 48 h and cell death after 72 h.

Conclusions

Cancer-specific glycolytic metabolism is emerging as one of the more promising characteristics of tumor biology that marks a profound difference with normal tissues. We propose the AMPK-FoxO3A axis as a metabolic switch capable of sensing variations in the AMP/ATP ratio and which is still responsive in cancer cells, at least in CRCs. Manipulation of this pathway could represent a valid strategy, in combination with drugs targeted to the molecular machinery responsible for the 'Warburg effect' and/or to autophagy, for future therapeutic approaches aimed to selectively kill cancer cells.

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