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The AMPK-FoxO3A axis as a target for cancer treatment

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FoxO proteins are an evolutionarily conserved subfamily of transcription factors involved in tumor suppression, regulation of energy metabolism and development in several tissues, and are mainly regulated by phosphorylation-dependent nuclear/cytoplasmic shuttling. The transcriptional activity of FoxO3A, one of the four members of the family, is further modulated by AMPK, one of the key regulators of cellular metabolism, which basically shifts cell machinery from energy-consuming to energy-producing pathways.

We recently demonstrated that the AMPK/FoxO3A energy sensor pathway is still inducible in human cancer cells in response to metabolic stress, as it becomes activated in colorectal and ovarian cancer cells in response to the inhibition of p38α. Activation of the FoxO3A transcriptional program initially induces autophagy as an attempt to retain energy to survive, whereas under persistent stress conditions it triggers autophagic cell death.

In this review, we focus on the connections between AMPK and FoxO3A, describing their central role as modulators of fundamental processes such as stress resistance, cell metabolism, autophagy and cell death, and highlighting the therapeutic potential of pharmacological modulation of the AMPK-FoxO3A axis.

FoxO3A: The "Silent Guardian" against Age-Related Diseases

The *forkhead-box* (Fox) gene family of transcription factors includes more than 100 members, with evolutionarily conserved roles in stress resistance and metabolism, development, differentiation, proliferation and survival. In humans, at least 43 members have been identified and classified in subfamilies recognized by letters (e.g., FoxA).[1]

The FoxO subfamily is composed of FoxO1, FoxO3A, FoxO4, that are ubiquitously expressed, and FoxO6, whose expression is restricted to neural cells. These transcription factors recognize and bind the conserved consensus core recognition motif FHRE (5'TTGTTTAC3') on target gene promoters.[2] As the consensus sequence is common to the various members of the FoxO family, additional mechanisms of regulation are needed to ensure the specificity of their transcriptional activity (i.e., multiple interactions with co-regulators, differential expression levels and cell-type and tissue specificity). FoxO proteins are evolutionarily conserved from *C. elegans* to mammals. In invertebrates, the homologous DAF-16 promotes longevity; in mammals, the members of the FoxO family play a role in proliferation/arrest, survival/death, metabolism and autophagy, and have been involved in tumor suppression, regulation of energy metabolism and development in a number of tissues. All these functions are mediated by the specific activation of a coordinated transcriptional program.[3]

The functions of the FoxO proteins have been deeply characterized by employing various animal models.[4] As mentioned above, the first studies were conducted in *C. elegans*, where DAF-16 has been proven to increase lifespan and regulate nutrient sensing. These functions are conserved in Drosophila, where dFOXO is also involved in insulin signaling. Since in mammals at least three FoxOs are ubiquitously expressed and share several target genes, experiments of individual gene disruption have been necessary to evaluate their physiological roles in development and adult life. FoxO1-null mice display an embryonic lethal phenotype as a consequence of incomplete vascular development.[5] Conversely, FoxO3A- and FoxO4-null mice are viable and grossly indistinguishable from their littermate controls, indicating their dispensability for normal vascular development. However, FoxO3A-null mice show age-dependent infertility due to abnormal ovarian follicular development leading to degeneration,[5,6] and presented severe muscle regeneration impairment.[7] Taken together, these experiments indicate that some degree of functional diversification exists between FoxO proteins both in developing and adult animals, without excluding, however, the possibility of functional redundancy. To clarify this issue, all three genes were simultaneously disrupted.[8,9] The conditional triple knock-out developed thymic lymphomas and haemangiomas, suggesting that FoxO proteins may play redundant functions at least in suppressing tumorigenesis, but they are characterized by cell-type and tissue specificity.

In multicellular organisms, all the above mentioned functions carried out by FoxO proteins result in the regulation of the response to oxidative stress, starvation and calorie restriction with the ultimate effect of increasing lifespan and counteracting age-related diseases, such as diabetes and cancer.[10] It is important to highlight that under pathological conditions FoxO activation often results in a phenotypically different outcome compared to what happens in normal conditions. The role of FoxO1 in

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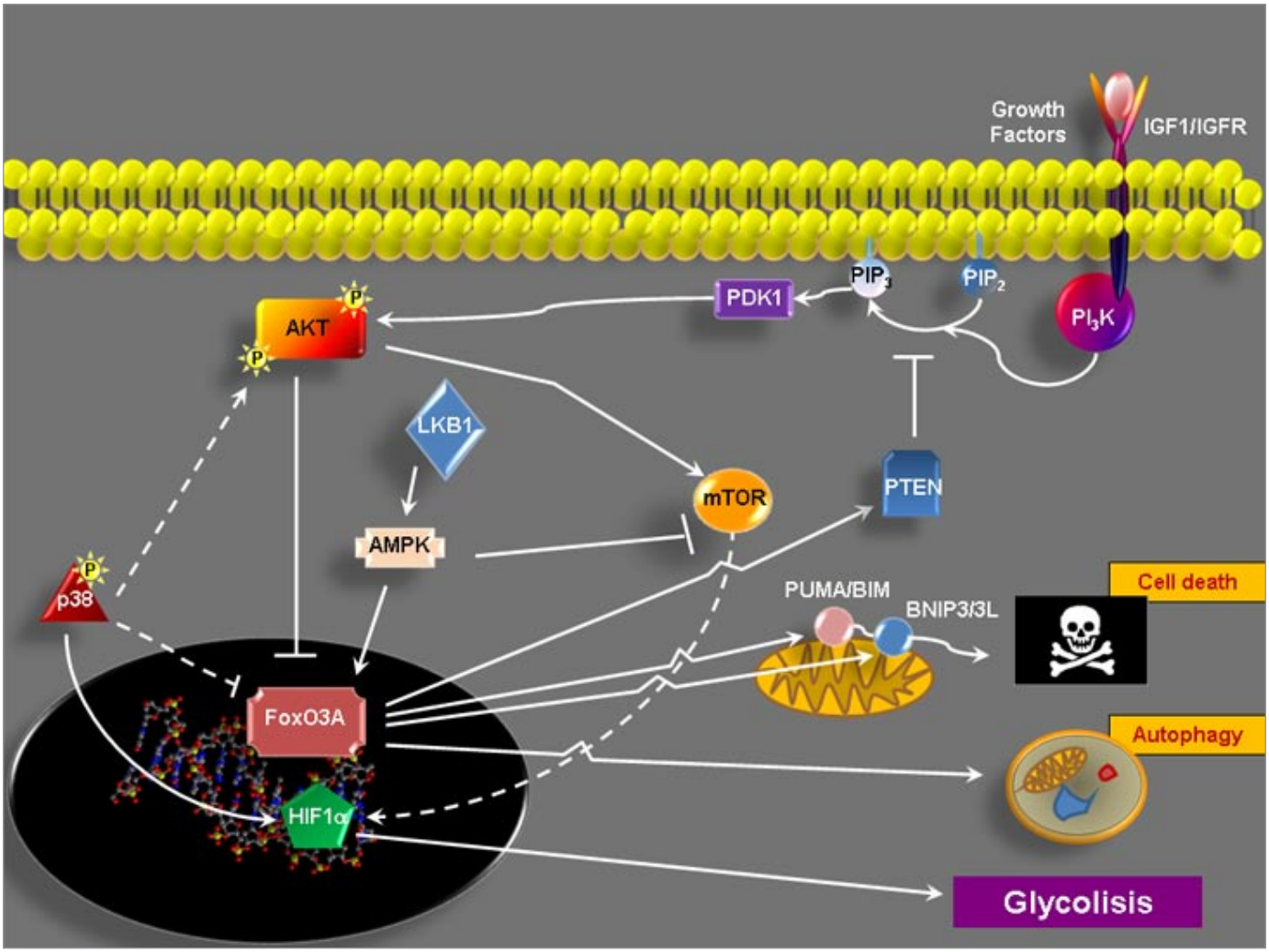

Figure 1. Signaling cascades regulating the balance between energy-consuming and energy-producing pathways.

diabetes,[11] and that of FoxO3A in cancer[12-15] are just two examples. Indeed, albeit gene disruption experiments in mice did not highlight a role for FoxO3A in tumor development, and despite the fact that triple FoxO knockout mice developed only thymic lymphomas and haemangiomas, but no other tumors, FoxO3A is required for colorectal cancer cell death induced by cisplatinum[15] and p38α inhibitors.[12]

FoxO3A is able to bind to promoters and induce transcription of target genes involved in cell cycle arrest (p21, p27, *CYCLIN G2*, *RBL2*, *BCL6*), cell death (*GADD45*, *BIM, FasL, PUMA, PTEN, BNIP3, BNIP3L*), cell metabolism (*PGC1α*, *PEPCK*, *UCP2*) and stress resistance (*SOD2*, *Catalase*).[12,13,15-17] Besides, some other genes, like those coding for cyclin D, E and A, are reported to be negatively regulated by FoxOs in a direct or indirect manner.[16,18,19] Moreover, FoxO3A regulates several autophagy-related genes—such as *MAP1LC3*, *Gabarapl1*, *ATG12*, *BNIP3* and *BNIP3L*—in muscle cells in response to atrophic signals and in cancer cells in response to the downregulation of the glycolytic cascade promoted by inhibition of the p38α-HIF1α pathway.[12,13,15,20,21] The involvement of FoxO in the regulation of autophagy seems to be evolutionarily conserved, as indicated by the role of dFOXO in Drosophila.[22]

The above mentioned functions of FoxO3A indicate that it could have an important role in the control of tumor growth. Indeed, FoxO3A overexpression has been shown to inhibit tumor growth in vitro and tumor size in vivo in breast cancer cells.[23,24] Furthermore, cytoplasmic localization of FoxO3A seems to correlate with poor survival in patients with breast cancer.[23] The significance of FoxO3A antitumoral activity is also underscored by studies conducted in leukemia,[25] prostate cancer[26-28] and glioblastoma.[29]

The emerging picture suggests that FoxO3A does not act as a crucial gatekeeper for the well being of the cell, rather it seems to operate as a 'silent guardian', which is called into action when things get worse. In fact, it is not devoted to the monitoring and preservation of the health state of the cells in non-stressed conditions. Conversely, it seems to be required following exogenous stress stimuli (i.e., oxidative or metabolic stress conditions) to help restoring the metabolic homeostasis of the cell. To our knowledge, functional or structural modifications favoring

or contributing to the onset of diseases have not been reported to date in FoxO3A locus, except for a chromosomal translocation, t(6; 11), found in a few cases of acute myeloid leukemia, which involves a fusion between MLL and FoxO3A.[25] On the other hand, FoxO3A appears to be preferentially inactivated by deregulated upstream signaling pathways, suggesting that its inactivation might be beneficial to the development of pathological conditions. As a consequence, FoxO3A is still functional and might be a promising target to be activated in order to counteract age-related diseases such as cancer.

Kinase-Dependent Regulation of FoxO3A: The Role of AMPK

FoxO proteins are regulated by phosphorylation-dependent nuclear/cytoplasmic shuttling as a result of the activity of the Akt and JNK kinases, which is evolutionarily conserved from invertebrates to humans.[3] Akt directly phosphorylates FoxOs (at the T32, S253 and S315 residues in the case of FoxO3A) and targets them to bind to the 14-3-3 nuclear export protein. This binding excludes FoxO factors from the nucleus, leading to their cytoplasmic accumulation and subsequent degradation. In addition, other kinases involved in oncogenic signals, such as SGK, CK1, DYRK1A and IKKβ, can inactivate FoxOs with a similar mechanism. On the other hand, JNK1 phosphorylates the 14-3-3 protein inhibiting its binding to FoxOs and promoting their nuclear localization. The observation that stress stimuli trigger the nuclear localization of the FoxO proteins even in the presence of growth factors illustrates the hierarchy of the regulation of these transcription factors, with phosphorylation by JNK (at Thr447 and Thr451 in the case of FoxO4) overriding the effect of phosphorylation by Akt.

As a further mechanism of regulation, it has been recently shown that the activation of the energy sensor pathway, which is triggered by an increased AMP/ATP ratio, leads to the AMPK-mediated phosphorylation of FoxO3A.[30] AMPK, the AMP-responsive kinase, is a central metabolic switch found in all eukaryotes that governs glucose and lipid metabolism in response to alterations in nutrients and intracellular energy levels. Importantly, AMPK has been identified as a direct downstream effector of the LKB1 tumor suppressor kinase in a mechanism representing a direct connection between energy metabolism and cell growth control.[31] Indeed, the LKB1/AMPK pathway is clearly involved in the regulation of cell growth of gastrointestinal hamartomas and many other malignancies including those arising in colon, breast, ovarian, pancreatic and lung tissues (the so called Peutz-Jeghers Syndrome, PJS), as well as in melanoma, breast and prostate cancer.[31-35]

AMPK is able to associate with and phosphorylate FoxO both in nematodes and mammalian cells. In nematodes, AMPK phosphorylates FoxO on six residues: T166, S202, S314, S321, T463 and S466. Also mammalian FoxO3A can be phosphorylated in vitro by AMPK on six sites (T179, S399, S413, S555, S588, S626), five of which are located to the transactivation domain.[30] Mutation of the six residues strongly reduces (84%), but not completely abrogates FoxO3A phosphorylation, suggesting the existence of other putative target sites. FoxO3A is phosphorylated by 2-deoxyglucose-activated AMPK in mammalian cells. The FoxO3A mutant in which all six residues found to be phosphorylated by AMPK were replaced by alanine was severely impaired in transcription, but maintained the ability to bind its cognate sequences, at least in vitro, and to perform nucleo-cytoplasmic shuttling depending on external cues. Even if these data strongly suggest that AMPK plays a role in FoxO3A dependent transcription, they do not exclude the existence of other signaling pathways converging on the same residues to modulate FoxO3A transcriptional activity, nor do they prove a direct role of AMPK-mediated FoxO3A phosphorylation in transcriptional modulation. It was recently nicely demonstrated that AMPK is able to modulate NAD^+ metabolism and SIRT1 deacetilase activity, a well known FoxO coactivator.[36-38] In particular, AMPK activation results in increased NAD^+ levels that lead to SIRT1 activation and deacetylation-induced activation of its downstream targets, including PGC1α, FoxO1 and FoxO3A. Taken together, these studies depict an intricate scenario, in which AMPK is able to indirectly modulate FoxO3A transcriptional activity by activating SIRT1 under metabolic stress. Besides, AMPK is also required for FoxO3A-dependent transcription of thioredoxin during oxidative stress response.[39] Moreover, recent evidences indicate the existence of an alternative pathway of AMPK activation, triggered by mitochondrial ROS and involving FoxO3A and its target genes (SOD, catalase, PGC1α).[40]

Yet, several questions remain unanswered. Although it is largely accepted that activated AMPK is able to directly phosphorylate FoxO3A, the physiological or patho-physiological stimuli triggering AMPK-dependent FoxO3A phosphorylation still have to be identified and, most importantly, it is not clear yet which FoxO3A functions are actually regulated by these multiple phosphorylations.

The AMPK-FoxO Axis: Balancing Nutrient Sensing and Healthspan/Lifespan

The effect of food restriction on lifespan was first described in rats in 1935 by McCay and colleagues.[41] Several studies have extended their results to other species from nematodes to mice.[42] Dietary restriction, without malnutrition, extends lifespan and reduces age-dependent diseases including, but not restricted to, cancer and atherosclerosis.[43-45] It has to be noted that different dietary regimens seem to activate different molecular pathways, probably depending on specific limiting nutrients, and that various tissues may be involved as signaling emitting centers or signaling responding centres.[46-50] Several pathways, including insulin/IGF1 and AMPK signaling, were shown to have a role in dietary restriction-dependent lifespan extension. Reduced activity of the insulin/IGF1 pathway was sufficient but not strictly required to extend lifespan in a variety of organisms including worms, flies and mice.[51] Animal experiments indicated that the inverse relationship existing between these two pathways (inhibition of PI3K/Akt and activation of AMPK) represents an essential intracellular switch for transducing the inhibitory effect of dietary energy restriction on mammary carcinogenesis.[52] Among the several

molecular pathways analyzed, activation of the AMPK-FoxO axis was shown to be sufficient and required for the dietary restriction effects to occur on both healthspan and lifespan.[53] In particular, Greer and colleagues have shown that dietary restriction-dependent lifespan extension was suppressed in AMPK defective nematodes and significantly reduced in FoxO mutant strains. On the contrary, the long lifespan of insulin receptor mutant nematodes was further enhanced by dietary restriction. The AMPK-FoxO axis is also required for resistance to oxidative stress, a response that is tightly linked to lifespan extension. Of note, in nematodes AMPK-mediated FoxO-dependent lifespan extension under dietary restriction is not reliant on a transcriptional role of FoxO, as this protein fails to accumulate into the nuclei and maintains a cytoplasmic localization. In contrast, the resistance to oxidative stress mediated by a constitutively active AMPK subunit requires the FoxO-dependent transcription of sod-3. These observations suggest a role for FoxO in lifespan extension in at least two different pathways, which are both dependent on AMPK activity.

The AMPK-FoxO3A Axis in Cancer

We recently reported that the conserved AMPK/FoxO3A energy sensor pathway is still inducible in human cancer cells in response to metabolic stress. Indeed, colorectal and ovarian cancer cells can activate a FoxO3A-dependent transcriptional program in response to decreased glycolysis caused by inhibition of the p38α/HIF1α pathway, first leading to autophagy and cell cycle arrest as an attempt to retain energy and increase ATP levels, but then triggering autophagic cell death in conditions of persistent stress.[12,13,15] In these cells, FoxO3A induced the transcription of genes whose protein products promote the overall autophagic flux (ATG6, ATG7, ATG12, GABARAP, GABARAPL2), with MAP1LC3 and Gabarapl1 localizing to large vacuolar structures characterized as autophagolysosomes.[12,13,15] The upregulation of FoxO3A target genes involved in metabolism, such as *PGC1α*, *PEPCK* and *UCP2*, suggests that, in response to energy depletion triggered by p38α blockade, cancer cells undergo a gene expression reprogramming leading to the expression of enzymes responsible for the conversion of metabolites produced by the autophagic machinery—mainly fatty acids and aminoacids—into energy fuel to survive. Indeed, colorectal cancer cells also expressed CPT-1 and MCAD, two enzymes involved in fatty acid catabolism.[12] Following p38α inhibition and in response to the acute energy demand that triggered autophagy, cells exited the cell cycle and accumulated in the G_1 phase, probably as an attempt to retain energy for survival. This was achieved by the FoxO3A-mediated upregulation of the cyclin D transcriptional repressor Bcl-6, of the cdk inhibitors p21 and p27, and of the retinoblastoma family protein p130, finally leading to inhibition of the G_1/S transition. Prolonged inactivation of p38α by its specific inhibitor SB202190 promoted an increase in the expression levels of FoxO target genes encoding for the BH3-only proteins PUMA, Bim, BNIP3 and BNIP3L, which correlated to the induction of autophagic cell death.[12,13,15] Interestingly, also PTEN, a phosphatase that inhibits the progression of the PI_3K/Akt signaling, was found to be upregulated, with FoxO3A being localized to its promoter together with activated PolII, thus suggesting the existence of a negative feedback loop that possibly accounts for the time-dependent reduction of Akt phospho-activation and kinase activity observed in SB202190-treated colorectal and ovarian cancer cells.[12,15] The analysis of other pathways involved in the regulation of FoxO function revealed that JNK—a MAPK that is often turned on as an alternative cascade to p38—was activated together with AMPK in cancer cells.[12,15] The AMPK pathway, which becomes activated when the levels of AMP overcome those of ATP, was required for FoxO3A nuclear accumulation and for the subsequent transcriptional activation of target genes involved in autophagy, metabolism, cell cycle arrest and cell death. At the cellular level, AMPK was necessary for the induction of the autophagic phenotypes observed in SB202190-treated colorectal cancer cells.[12] These data were obtained by using pharmacological and genetic approaches to knock-down AMPK in cancer cells and suggest that AMPK plays an important role—whether direct or indirect—in the regulation of FoxO3A intracellular localization. Moreover, our preliminary results showed a significant decrease in the overall amount of FoxO3A in cells in which AMPK was inhibited or depleted. Our results also evidenced that the nuclear pool of endogenous FoxO3A was both enriched and hyperphosphorylated at serine and threonine residues in response to p38α inhibition (Chiacchiera and Simone, unpublished results). At present, however, due to the simultaneous perturbation of other pathways affecting the phospho-status of FoxO3A, a direct mechanism involving the phosphorylation of FoxO3A by AMPK can only be hypothesized. As mentioned above, Greer and coworkers[30] described the direct phosphorylation of FoxO3A by AMPK. Moreover, data gathered in experiments performed on 293 cells expressing exogenous wild type FoxO3A or FoxO3A phosphorylation mutants suggested that AMPK-dependent post-translational modifications are responsible for the induction of FoxO3A transcriptional activity, without affecting its cellular localization. Although our observations led to slightly different conclusions, they might not be in contrast, as different experimental procedures, cell type and stimuli were used in the two studies.

In a variety of cells and tissues analyzed, activated AMPK inhibited ACC, which regulates the formation of fatty acids, and induced CPT-1 activity and beta-oxidation with a consequent increase in the ATP/AMP ratio. These findings are in accordance with the activation of the transcriptional program observed following p38α blockade in colorectal cancer cells. Collectively, our results showed that p38α blockade causes tumor growth inhibition both in vitro and in vivo by inducing a FoxO3A-dependent transcriptional program. Indeed, SB202190-treated colorectal tumors showed a striking reduction in cell growth, accompanied by nuclear accumulation of FoxO3A and its target genes p21 and PTEN.[12]

These data confirm the potential of antitumoral strategies designed to interfere with the Warburg effect, which accounts for the constitutive activation of aerobic glycolysis commonly occurring in cancer cells. In this broader frame, modulation of the AMPK pathway might prove an effective approach. In fact,

several evidences suggest that deregulation of the AMPK pathway could significantly contribute to the Warburg Effect. For example, mTOR has been identified as an indirect link between AMPK and HIF1α. Indeed, AMPK suppresses mTOR-dependent expression and transcriptional activation of HIF1α both in vitro and in vivo.[54] According to studies performed in fibroblasts with overactivated mTOR signaling, fibroblasts deficient for LKB1 or AMPK, tumors from PJS patients and LKB1$^{+/-}$ mice showed increased levels of HIF1α and its target genes.[55]

Taken together, these data suggest that AMPK mediators and effectors, when still functional, might be successfully exploited in cancer therapy to counteract tumor-specific metabolism.

Perspectives

Understanding the connections between AMPK and FoxO3A is of utmost importance as these proteins play a central role as modulators of fundamental processes such as stress resistance, cell metabolism, autophagy and cell death. As deregulation of these processes is involved in age-related diseases and tumor formation and progression, the ability to modulate the activity of these proteins may represent a promising therapeutic strategy.

The compelling evidences presented in this review highlight that pharmacological manipulation of the AMPK-FoxO3A axis might represent a beneficial approach for cancer treatment. Indeed, FoxO3A is emerging as a key downstream effector of various anticancer therapeutics, such as p38 inhibitors,[12,13,15] cisplatin (a DNA cross-linker),[14] paclitaxel (a microtubule stabilizer), doxorubicin (a DNA intercalating agent), imatinib (a multikinase inhibitor), OSU-03012 (a novel PI3K-Akt inhibitor), trastuzumab and cetuximab (EGFR/HER2 inhibitors), ionizing radiation, and even gene therapy (adenoviral delivery of FoxO3A in melanoma cells).[56] AMPK agonists (i.e., metformin) have a great deal of pharmaceutical interest since they have been in clinics for many years now (for type 2 diabetes, insulin resistance, metabolic syndrome) and are well tolerated by patients. Other agonists include phenformin (currently withdrawn from clinical use because of side effects causing fatal lactic acidosis), AICAR and A769662 (a small molecule directly activating AMPK), which have been shown to inhibit the growth of a plethora of tumor cells in vitro, in tumor xenografts and in animal cancer models in vivo.[31,57,58] Given the great number of patients taking metformin every day, epidemiologic studies have evaluated the effects of this drug on cancer incidence reporting statistical effectiveness in cancer prevention and pathological complete response rate, to such an extent that a large phase III clinical trial in breast cancer is in progress.[31,59-61]

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