Cell Cycle

ISSN: 1538-4101 (Print) 1551-4005 (Online) Journal homepage: https://www.tandfonline.com/loi/kccy20

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To cite this article: Alejandro Vazquez-Martin, Luciano Vellon, Pedro M. Quirós, Sílvia Cufí, Eunate Ruiz de Galarreta, Cristina Oliveras-Ferraros, Angel G. Martin, Begoña Martin-Castillo, Carlos López-Otín & Javier A. Menendez (2012) Activation of AMP-activated protein kinase (AMPK) provides a metabolic barrier to reprogramming somatic cells into stem cells, Cell Cycle, 11:5, 974-989, DOI: 10.4161/cc.11.5.19450

To link to this article: https://doi.org/10.4161/cc.11.5.19450

Published online: 01 Mar 2012.

Cell Cycle 11:5, 974-989; March 1, 2012; © 2012 Landes Bioscience

Activation of AMP-activated protein kinase (AMPK) provides a metabolic barrier to reprogramming somatic cells into stem cells

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Key words: metformin, iPSCs, tumor propagating cells, pluripotency, bioenergetics, AMPK

The ability of somatic cells to reprogram their ATP-generating machinery into a Warburg-like glycolytic metabotype while overexpressing stemness genes facilitates their conversion into either induced pluripotent stem cells (iPSCs) or tumor-propagating cells. AMP-activated protein kinase (AMPK) is a metabolic master switch that senses and decodes intracellular changes in energy status; thus, we have evaluated the impact of AMPK activation in regulating the generation of iPSCs from non-stem cells of somatic origin. The indirect and direct activation of AMPK with the antidiabetic biguanide metformin and the thienopyridone A-769662, respectively, impeded the reprogramming of mouse embryonic and human diploid fibroblasts into iPSCs. The AMPK activators established a metabolic barrier to reprogramming that could not be bypassed, even through *p53* deficiency, a fundamental mechanism to greatly improve the efficiency of stem-cell production. Treatment with metformin or A-769662 before the generation of iPSC colonies was sufficient to drastically decrease iPSC generation, suggesting that AMPK activation impedes early stem cell genetic reprogramming. Monitoring the transcriptional activation status of each individual reprogramming factor (i.e., *Oct4*, *Sox2*, *Klf4* and *c-Myc*) revealed that AMPK activation notably prevented the transcriptional activation of *Oct4*, the master regulator of the pluripotent state. AMPK activation appears to impose a normalized metabolic flow away from the required pro-immortalizing glycolysis that fuels the induction of stemness and pluripotency, endowing somatic cells with an energetic infrastructure that is protected against reprogramming. AMPK-activating anti-reprogramming strategies may provide a roadmap for the generation of novel cancer therapies that metabolically target tumor-propagating cells.

Introduction

The results from studies concerning the reprogramming of adult somatic cells into induced pluripotent stem cells (iPSCs) provide experimental evidence regarding the reliability of tumor-propagating stem cell-like populations as the origin of human tumors.[1-3] There are remarkable similarities between the reprogramming processes and the malignant transformation of adult somatic cells, and similar factors appear to regulate both pluripotency and tumorigenicity (**Fig. 1A**).[4-7] First, both reprogramming and malignant transformation require specific combinations of collaborating oncogenes and tumor suppressor genes that can efficiently produce a less differentiated cell that is able to proliferate and self-renew indefinitely. The four transcription factors originally shown to reprogram somatic cells into iPSCs (i.e., *Oct4*, *Sox2*, *Klf4* and *c-Myc*) are overexpressed in some types of tumors, and *Sox2*, *Klf4* and *c-Myc* are established oncogenes that are linked to iPSC generation and stem cell self-renewal.[7-15] Second, the reprogramming efficiency of somatic cells into iPSCs is negatively regulated by the p53 and $p16^{INK4a}$/retinoblastoma (RB) signaling pathways, which activate cell-intrinsic, cancer-protective programs, such as senescence, which is a major tumor suppressing mechanism that must be overcome during tumorigenesis.[16-22] Reprogramming-induced senescence (RIS) is a crucial reprogramming barrier in response to the expression of stem-cell-specific genes that mimics the senescence response observed during oncogene-induced senescence (OIS) in primary cells, which limits oncogenic transformation.[2,23-27] When senescence is disabled, the cells are more susceptible to either reprogramming or oncogenic transformation.[21-27] Escaping senescence or acquiring immortality, is also a crucial and rate-limiting step toward the establishment of a pluripotent state in somatic cells,[16-20] further

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Submitted: 01/16/12; Accepted: 01/22/12
http://dx.doi.org/10.4161/cc.11.5.19450

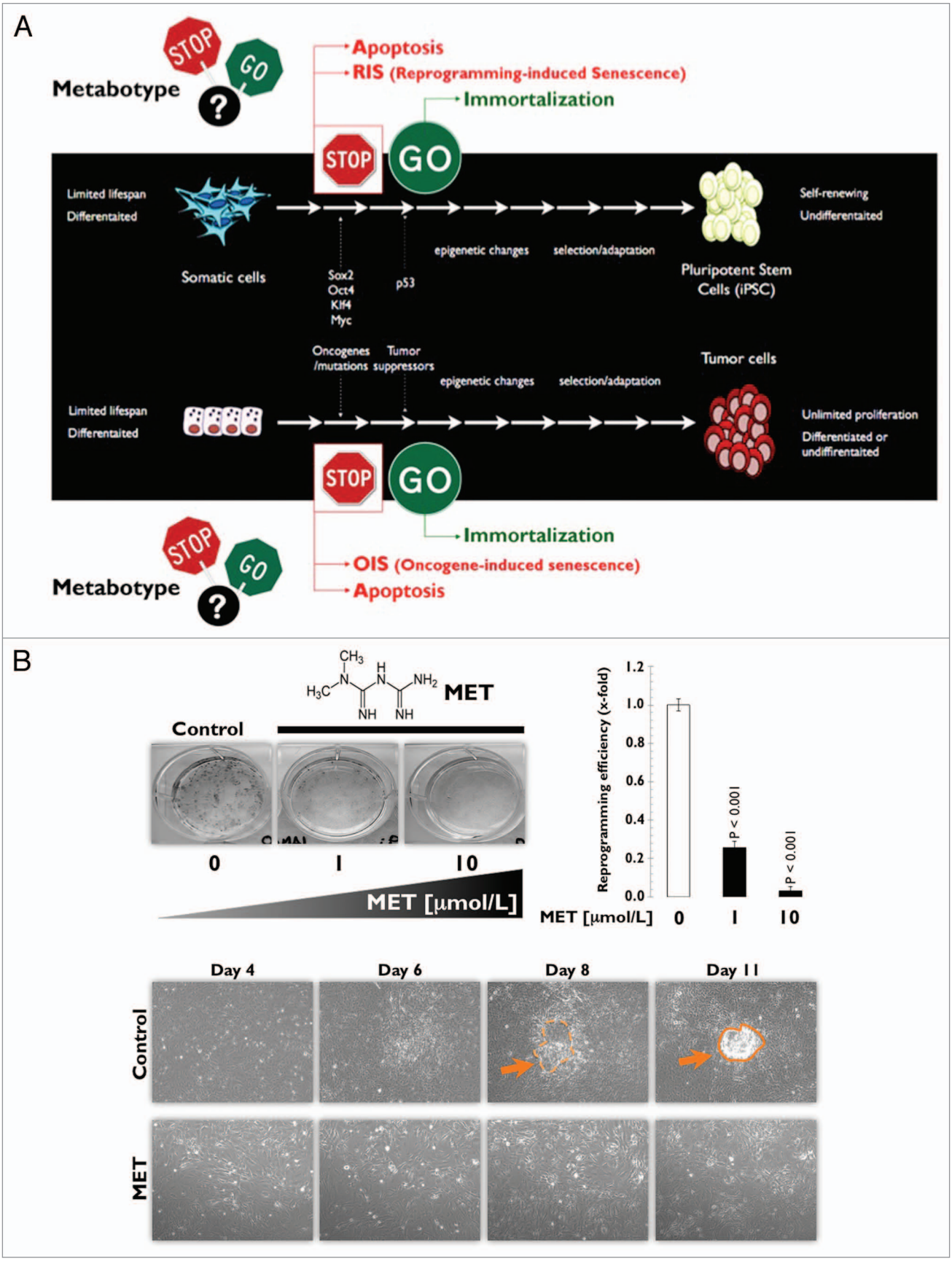

Figure 1. For figure legend, see page 976.

Figure 1 (See previous page). (A) Overlapping mechanisms control iPS-cell and tumor-cell generation. Normal fibroblasts (top) or epithelial cells (bottom), which are mature, differentiated cells with a limited lifespan, can be converted into induced pluripotent stem cells (iPSCs, top) or tumor cells (bottom), respectively, by a combination of defined factors. Both processes require specific combinations of collaborating oncogenes and tumor suppressor genes that can produce a less differentiated, pluripotent cell that is able to proliferate and self-renew indefinitely. The reprogramming stemness factors *Oct4*, *Sox2*, *Klf4* and *c-Myc* promote the reprogramming of fibroblasts into iPSCs in a manner that conceptually parallels their roles in transforming normal epithelial cells into tumor cells. Deletion of the p53 tumor suppressor protein greatly increases the efficiency of oncogene cooperation in transforming normal cells to tumor cells and directly or indirectly limits the reprogramming of fibroblasts into iPSCs or transformed cancer cells by inducing apoptosis or cellular senescence. Somatic cells can reprogram their ATP-generating energy machinery into a Warburg-like glycolytic metabotype, which facilitates conversion of the somatic cells into iPSCs and, perhaps, into tumor-propagating cells. Here, we tested the hypothesis that the predisposition or adaptation of aberrant cell energy metabolism is a key element that fine-tunes the molecular and genetic events associated with immortality and indefinite self-renewal potential. (B) Mouse embryonic fibroblasts (MEFs) fail to reprogram into induced pluripotent stem cells (iPSCs) in the presence of the indirect AMPK activator metformin. Early passage MEFs infected with retroviruses encoding *Oct4*, *Sox2* and *Klf4* (OSK) were cultured in ES medium in the continuous presence or absence of metformin (MET, 1 and 10 mmol/L) or PBS alone as control, as specified. Top: The numbers of AP^+ colonies (microphotographs of representative reprogramming experiments are shown) were counted 14 d after the initial infection and were plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. Bottom: Phase-contrast microphotographs of representative MEFs transduced with OSK at different time-points during the reprogramming process in the absence or presence of continuous metformin (MET, 10 mmol/L). The arrows indicate emerging iPSC-like colonies.

underscoring the similarities between induced pluripotency and tumorigenesis. Third, when oncogenes, transcription factors or onco-microRNAs induce previously differentiated cells to transdifferentiate into iPSCs with stem-like features,[28-30] they spontaneously form teratocarcinomas when transplanted into mice.[1,31] Moreover, one of the primary assays of "stemness" is also a tumor assay, which further illustrates the strong link between reprogramming and stem cell tumorigenicity. Therefore, testing the ability of chemicals specifically targeted against putative survival and self-renewing tumor-initiating mechanisms to negatively regulate the efficiency and kinetics of somatic reprogramming to iPSCs might provide a proof-of-concept validation for novel oncology drug candidates that limit both the number and the aberrant functionality of tumor propagating cells.

Malignant cells have the ability to replace the mitochondrial oxidative phosphorylation that operates in most normal, non-proliferative tissues with a Warburg-like glycolytic metabolism that more efficiently supports the large-scale biosynthetic programs required for continuous cancer cell growth and proliferation.[32-35] Interestingly, recent evidence has also implicated mitochondrial restructuring and bioenergetic plasticity as crucial components that mediate the execution of somatic reprogramming to iPSCs. Thus, a Warburg-like metabolic shift of somatic oxidative energy metabolism to a glycolytic metabotype promotes proficient reprogramming, establishing a novel regulator of acquired stemness.[36-42] Given that (1) Many tumor cells have developed mechanisms to escape the growth-restraining effects imposed by the switching of cellular metabolism from anabolic to catabolic modes that occur upon activation of the energy sensor adenosine monophosphate (AMP)-activated protein kinase (AMPK);[43-47] (2) AMPK is activated by the antidiabetic drug metformin and many natural products, including "nutraceuticals" and compounds used in traditional medicines,[45-49] and (3) AMPK activators potentially have cancer preventative effects, and there is already evidence that metformin usage provides protection against the initiation of several human cancers,[50-54] we hypothesize that AMPK might be an "energy checkpoint" that closely regulates the energetically demanding reprogramming process. Taking advantage of the iPSC-based model, which suggests that inducing a differentiated somatic cell to become more stem-like predisposes that cell to tumorigenicity, we recently tested the effects of two structurally and mechanistically unrelated AMPK-activating drugs (i.e., metformin[55-57] and A-769662[58-60]) in regulating the generation of stem cells from non-stem cells of somatic origin, including mouse embryonic, *p53*-deficient adult mouse and human neonatal foreskin fibroblasts.

We herein provide the first experimental evidence that the cell metabotype imposed upon AMPK activation provides a metabolic barrier to reprogramming somatic cells into pluripotent stem cells. Because the loss of AMPK activation sensitivity in response to metabolic stresses (i.e., the depletion of ATP) is a molecular strategy that might allow somatic cells to de-differentiate and acquire properties of tumor-propagating cells, characterizing the role of AMPK in restraining somatic reprogramming might facilitate the development of clinically applicable AMPK-activating strategies directed against the reprogramming features of tumor-propagating cells.

Results

Metformin impedes the somatic reprogramming of mouse embryonic fibroblasts (MEFs). To address the functional effect of metformin treatment on the generation of iPSCs, we performed comparison experiments using a three-factor (i.e., *Oct4*, *Sox2* and *Klf4*) induction protocol in early passage mouse embryonic fibroblasts (MEFs). MEFs were transduced with individual lentiviruses containing *Oct4*, *Sox2* and *Klf4* genes at a 1:1:1 ratio on day zero. The transduction was repeated every 12 h for two days using the same protocol. On day three after the first transduction, the culture medium was switched to human embryonic stem (hES) cell growth medium supplemented or not with two different concentrations of metformin (i.e., 1 and 10 mmol/L); the ES medium was renewed every two days. From days 10–12, clearly visible, tightly packed colonies similar to hES cells appeared in the metformin-free control cultures. Although previous studies have reported calculating the reprogramming efficiency based solely on the morphologies of the observed iPSC colonies with a success rate of 90–100%, we decided to combine the observations of the ES cell-like morphological changes (e.g., defined boundaries and high nuclear-to-cytoplasm ratio

within individual cells) with the results of staining with the pluripotency marker alkaline phosphatase (AP) to quantify bona fide iPSC colonies on day 14 post-viral transduction (**Fig. 1B**). When the MEFs were transduced with the three stemness factors in the absence of metformin, we consistently obtained approximately 100 colonies from 50,000 starting cells. Using the same rigorous criteria for calculating the reprogramming efficiency, less than 25 AP^+ colonies from 50,000 starting cells (approximately a 75% decrease) were generated in experiments performed in the presence of 1 mmol/L metformin (**Fig. 1B**). Notably, only two to three colonies (more than a 95% decrease) were observed in parallel experiments when the metformin concentration was increased to 10 mmol/L (**Fig. 1B**).

The metformin-induced reduction in reprogramming efficiency we observed was independent of the metformin-induced cell death of the starting somatic population. Indeed, whereas the reprogrammed MEFs displayed an undifferentiated phenotype with distinct ES-like colonies in the absence of MET (**Fig. 1B**), flattened fibroblast-like cells with low nuclear-to-cytoplasm ratios were observed when reprogramming was performed in the presence of metformin (**Fig. 1B**). To further address that the impaired reprogramming efficiency was not due to the metformin-induced inhibition of the established iPSC colonies, we treated the MEFs with metformin either at early stages (days 1 to 7 post-viral transduction) or at late stages of reprogramming (days seven to 14 post-viral transduction) but before colony appearance (day 10). We found that treatment with metformin during the early stages of reprogramming significantly prevented (more than a 95% decrease) the formation of clearly visible iPSC colonies (**Fig. 2A**), indicating that the observed effects of metformin on the reprogramming efficiencies were primarily due to inhibition of the reprogramming process itself and not to a significant impairment of iPSC colony survival or growth. Indeed, when metformin treatment began on day seven, we observed late changes in the cell culture morphologies that were analogous to the "background" or "early" colonies that were observed in the untreated control cultures beginning on day four (**Fig. 2A**). Taken together, these results indicate that reprogramming somatic cells into iPSCs is less efficient and slower in the presence of metformin.

Metformin antagonizes the rescue of reprogramming potential elicited by p53 deficiency in cells that normally fail to reprogram. Molecular changes associated with cellular senescence provide a crucial roadblock for the conversion of somatic cells into iPSCs. Accordingly, MEFs at early passages generate iPSCs more efficiently than MEFs at later passages, which exhibit a concomitant onset of senescence.[18] Moreover, the reprogramming factors themselves trigger senescence (i.e., RIS[2,23]) by generating endogenous DNA damage and activating a p53-regulated DNA damage response (DDR) similar to what is observed during replicative-induced senescence and OIS. Further underscoring the similarities between induced pluripotency and tumorigenesis, the abrogation of *p53* facilitates efficient reprogramming in the face of DNA damage by inactivating the key pathways that control replicative potential and senescence.[16-20] Thus, we decided to test whether the metformin-induced, reprogramming-protected cell metabotype is dominant over the p53 deficiency, which enhances the efficiency of somatic cell reprogramming to a pluripotent state. When adult mouse skin fibroblasts were transduced with the three stemness factors, only four to five colonies were generated from 50,000 starting cells (approximately 20 times less efficiently than early passage cultures of MEFs), confirming that the loss of replicative potential and senescence facilitated reprogramming in old differentiated cells (**Fig. 2B**). We then investigated the regulatory effects of metformin on the efficiency and reprogramming kinetics of adult mouse skin fibroblasts in a *p53*-deficient background. The double-knockout adult fibroblasts lacking *p53* (*$p53^{-/-}$*) were similarly reprogrammed to iPSCs more efficiently than the wild-type (wt) *p53* cells; approximately 30 colonies were obtained from 50,000 starting cells, which represented a 6-fold increase in reprogramming efficiency compared with the wt *p53* adult skin fibroblasts. Remarkably, the *$p53^{-/-}$* adult skin fibroblasts could not be reprogrammed to iPSCs (a greater than 95% decrease) in the presence of metformin despite the fact that the cells have normal proliferation rates (**Fig. 2B**). These observations suggest that while *p53* deficiency expectedly rescues reprogramming potential in cells that normally fail to form iPSCs, metformin treatment activates a metabolic barrier to reprogramming that cannot be bypassed, even in the absence of *p53*.

Activation of AMPK, the mechanism of action of metformin, is sufficient to impede reprogramming of somatic cells to iPSCs. At the cellular level, metformin treatment has been shown to activate AMPK,[61,62] a master energy sensor crucially involved in the regulation of cellular metabolism and activated through increases in the intracellular levels of AMP.[43-49] It is generally accepted that metformin indirectly activates AMPK by disrupting complex I of the mitochondrial respiratory chain,[63,64] which leads to decreased ATP synthesis and an increase in the intracellular AMP:ATP ratio (**Fig. 3A**). Recent studies have demonstrated that the metformin-mediated regulation of glucose metabolism is causally linked to both ATP depletion and a concomitant rise in AMP intracellular levels independent of AMPK activation.[65,66] Therefore, we decided to explore whether the sole activation of AMPK was sufficient to impede the reprogramming of somatic cells to iPSCs. We treated cells with the small-molecule thienopyridone A-769662, which is a direct and potent activator of AMPK that does not cause significant alterations in the AMP/ATP ratio but mimics two of the effects of AMP on the AMPK system[59,60,67] (i.e., the allosteric activation of Thr-172 and the inhibition of Thr-172 dephosphorylation; **Fig. 3A**). A total of 75 AP^+ colonies were obtained from 50,000 starting cells (25% decrease) in the presence of a sub-optimal concentration of A-769662 (i.e., 10 μmol/L; **Fig. 3B**). Notably, approximately 20 AP^+ colonies were generated from 50,000 starting cells (approximately an 80% decrease) in reprogramming experiments that were performed in the presence of an AMPK saturating concentration of A-769662 (i.e., 50 μmol/L; **Fig. 3B**). Moreover, the cellular mimetic of AMP, A-769662, rescued the metformin-mediated inhibition of iPSC formation in adult skin murine fibroblasts lacking the reprogramming barrier of p53 (**Fig. 3C**).

Metformin inhibits the somatic reprogramming of human diploid fibroblasts (HDFs) and represses expression of the

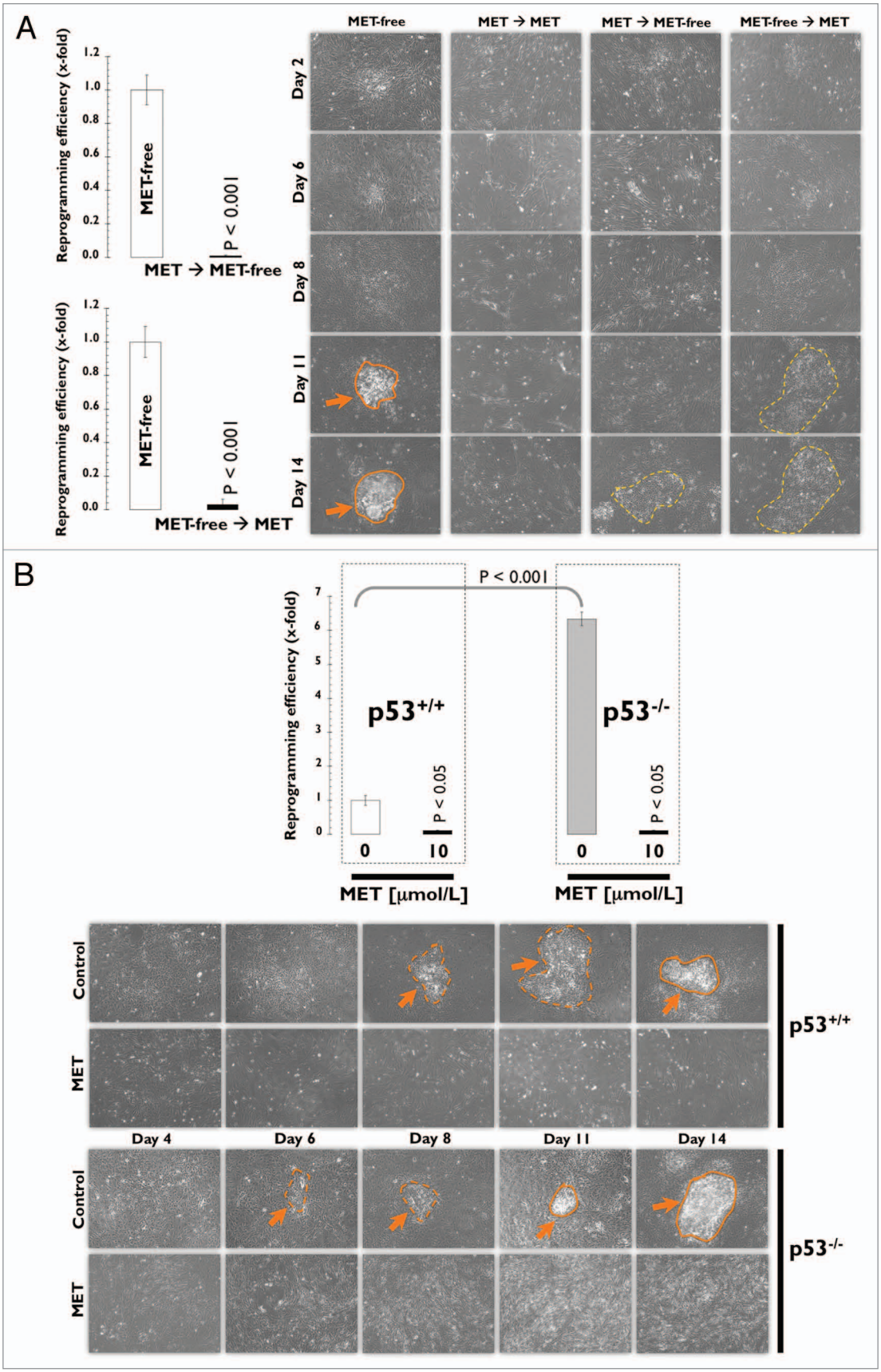

Figure 2. For figure legend, see page 979.

Figure 2 (See opposite page). (A) Indirect AMPK activation impedes early stem cell genetic reprogramming. The early passage MEFs infected with retroviruses encoding the OSK stemness factors were grown in ES medium in the intermittent presence or absence of metformin (MET, 1 and 10 mmol/L) or PBS alone as control, as specified. Left: The numbers of AP^+ colonies were counted 14 d after the initial infection and are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. Right: Phase-contrast microphotographs of representative MEFs transduced with OSK at different time points during the reprogramming process in the absence or presence of intermittent metformin (MET, 10 mmol/L), as specified. Arrows indicate emerging iPSC-like colonies. (B) p53 deficiency cannot bypass metformin-induced inhibition of somatic cell reprogramming. Adult mouse skin fibroblasts (MSFs) from p53-null ($p53^{-/-}$) and wild-type p53 ($p53^{+/+}$) littermates transduced with retroviruses encoding the OSK stemness factors were grown in ES medium in the continuous presence or absence of metformin (MET, 1 and 10 mmol/L) or PBS alone as control, as specified. Top: The numbers of AP^+ colonies were counted 14 to 20 d after the initial infection and are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. Bottom: Phase-contrast microphotographs of representative MSFs transduced with OSK at different time-points during the reprogramming process in the absence or presence of continuous metformin (MET, 10 mmol/L), as specified. The arrows indicate emerging iPSC-like colonies.

master pluripotency gene *Oct4*. To further investigate the metformin-mediated inhibition of the reprogramming of murine fibroblasts to human diploid fibroblasts (HDFs), we first confirmed the metformin-mediated activation of AMPK in HDFs. BJ-1 fibroblasts were treated for 48 h with varying concentrations of metformin, and the cell lysates were analyzed on immunoblots probed with antibodies against phospho-$AMPK\alpha^{Thr172}$ or total AMPK. Similarly, metformin treatment increased AMPK phosphorylation in a dose-dependent manner (**Fig. 1A**); the total amount of AMPK did not change (**Fig. 1A**), and there was no significant change in cell viability (data not shown).

Next, we transduced BJ-1 human foreskin fibroblasts with viruses expressing the four OSKM Yamanaka cocktail factors (i.e., *Oct4*, *Sox2*, *Klf4* and *c-Myc*) and subsequently conducted reprogramming experiments in the absence or presence of two different concentrations of metformin (i.e., 1 and 10 mmol/L). The histone deacetylase inhibitor (HDACi) 2-propylvaleric acid (valproic acid), which greatly increases the efficiency of reprogramming,[68,69] was employed in parallel as a positive control. After 14 d, the colonies were fixed and stained for AP activity (**Fig. 4A**). When BJ-1 fibroblasts were transduced with the four stemness factors in the absence of metformin, approximately 20 colonies were consistently generated from 20,000 starting cells. Eight to nine AP^+ colonies (approximately a 60% decrease) were generated from 20,000 starting cells in reprogramming experiments performed in the presence of 1 mmol/L metformin (**Fig. 4A**). Notably, only three to four AP^+ colonies (more than a 80% decrease) were generated in parallel experiments when the metformin concentration was increased to 10 mmol/L (**Fig. 4A**). Similarly, the exogenous addition of valproic acid significantly increased (>2-fold) the number of AP^+ colonies compared with the untreated controls (**Fig. 4A**). The AP^+ colony quantification also showed that metformin treatment dramatically decreases reprogramming efficiency using OSK (three-factors) transduction (data not shown); the proliferation of fibroblasts transduced using either OSKM or OSK was not significantly affected by metformin treatment.

To assess whether the metformin-mediated attenuation of the number of iPSC colonies formed after OSKM transduction was related to expression of the reprogramming factors, we monitored the expression of each individual reprogramming factor in BJ-1 fibroblasts transduced with lentiviral vectors co-expressing each of the four Yamanaka stemness factors together with a fluorescent protein linked by a 2A peptide.[70] The BJ-1 fibroblasts transduced with each factor were cultured under two concentrations of metformin in DMEM medium for two days prior to performing real-time PCR analyses to assess *Oct4*, *Sox2*, *Klf4* and *c-Myc* mRNA levels. Upon exposure to metformin, we observed that (1) *Klf4* mRNA expression remained largely unaltered; (2) *Sox2* mRNA expression was slightly increased, and (3) *c-Myc* mRNA levels were significantly decreased. Notably, metformin treatment drastically decreased the transcriptional expression of the master pluripotency gene *Oct4* in a dose-dependent manner (>60% reduction in response to 10 mmol/L metformin vs. untreated control cells; **Fig. 4B**).

Discussion

A proper delineation of the parallels between induced pluripotency and tumorigenicity might provide new clues for the discovery of drugs specifically targeting cancer-propagating cells, which are ultimately responsible for tumor formation, treatment failure and metastatic relapse.[71-73] iPSCs offer a unique system to study the cellular and molecular mechanisms underlying the process of transformation, which is initiated when committed cells overcome cellular barriers (i.e., tumor suppressor pathways that, by comparison, also act as roadblocks against reprogramming) to acquire an indefinite self-renewal capacity and a certain degree of pluripotent plasticity (i.e., the two main defining features of iPSCs that are achieved hand-in-hand during successful reprogramming). If the acquisition of stem cell-like properties is closely associated with tumorigenesis, then the mechanisms triggering the expression of reprogramming stemness factors should elicit bona fide tumor suppressor pathways that inherently protect cells against uncontrolled growth. The tumor suppressor gene *p53*, which prevents the initiation of tumor formation by inducing cell cycle arrest, senescence, DNA repair and apoptosis, is activated upon the overexpression of exogenous stemness factors during the reprogramming of somatic cells. Consequently, senescent cells or cells with DNA damage and/or chromosomal abnormalities undergo apoptosis and are excluded from becoming iPSCs. While the inhibition of p53 directly transforms normal stem cells into tumor cells,[74] the teratomas from *p53*-deficient iPSCs generated using the four Yamanaka factors are mostly composed of undifferentiated tissue.[75] Four-factor-generated, p53-deficient iPSCs have the ability to self-renew and aberrantly differentiate, and therefore, these cells resemble malignant tumors.[76] These findings are consistent with the ability of *c-Myc*, in the absence of

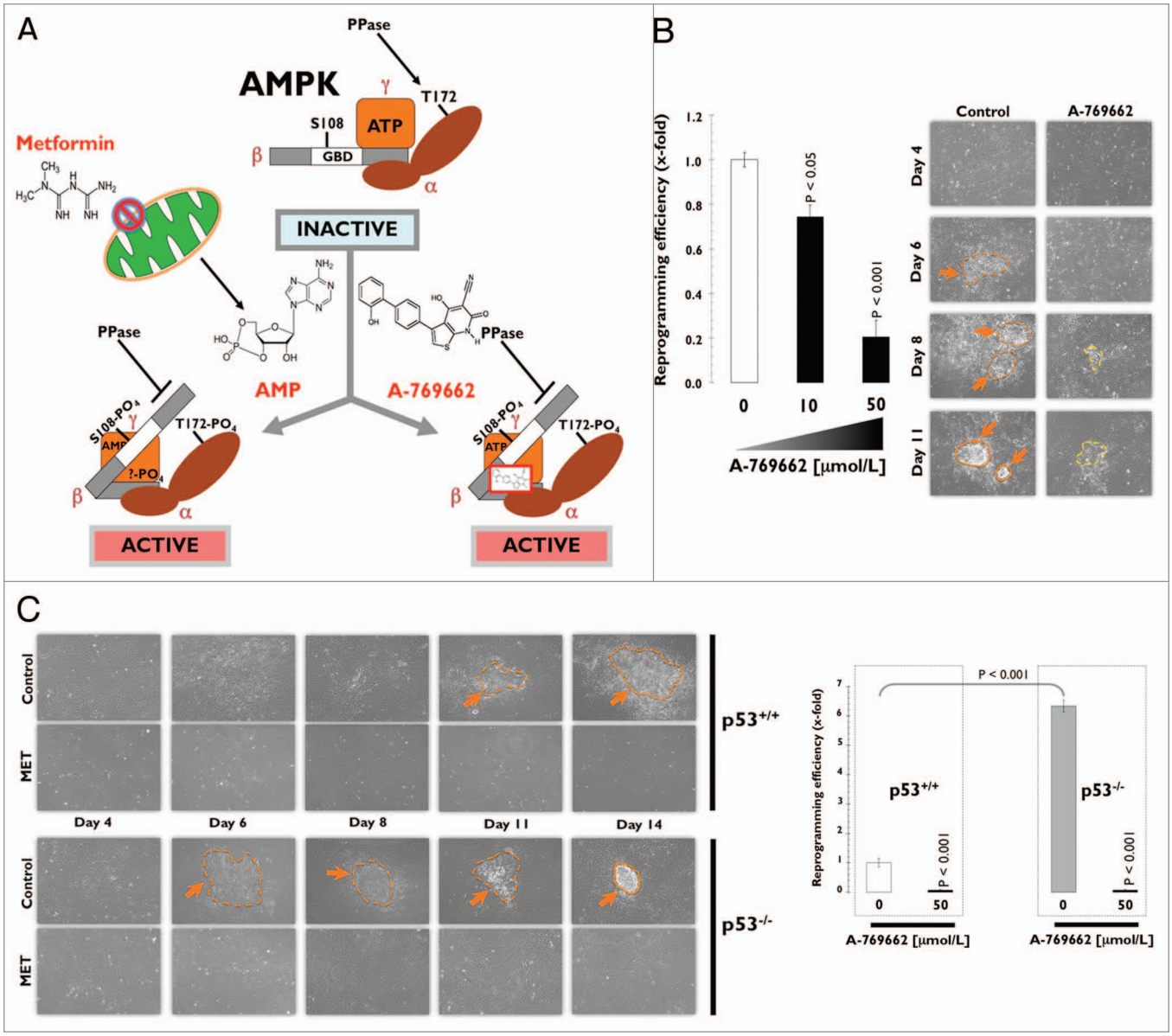

Figure 3. (A) Model for activation of AMPK by metformin or A-769662. In the inactive conformation of AMPK, ATP is bound to the γ-subunit and the Thr-172 (T172)-activating site within the catalytic α-subunit is freely accessible to protein phosphatases (PPase). Therefore, when AMPK is inactive, T172 is maintained predominantly in the unphosphorylated form. The binding of A-769662 stabilizes the AMPK conformation, which efficiently impedes the dephosphorylation of T172. This conformation, which is depicted here as steric hindrance by the β-subunit, also requires the phosphorylation of Ser-108 (S108) within a region of the β-subunit that has been termed the glycogen-binding domain (GBD). The active conformation of AMPK is also promoted when AMP displaces ATP from the γ-subunit. Metformin activates AMPK through this indirect mechanism by inhibiting complex I of the mitochondrial respiratory chain and increasing intracellular AMP and/or ADP, and the phosphorylation of S108 is not sufficient to maintain the active conformation; thus, the addition of phosphorylation sites within either the α- or β-subunits, which are depicted here as ?-PO_4, might be required to maintain the active form of AMPK. (B) Direct activation of AMPK impedes reprogramming of MEFs into iPSCs. Early passage MEFs infected with retroviruses encoding OSK stemness factors were grown in ES medium in the continuous presence or absence of A-769662 (10 and 50 μmol/L) or DMSO alone as a control, as specified. *Left*. The numbers of AP^+ colonies were counted 14 d after the initial infection and are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. Right: Phase-contrast microphotographs of the representative MEFs transduced with OSK at different time points during the reprogramming process in the absence or presence of continuous A-769662 (50 μmol/L). The arrows indicate emerging iPSC-like colonies. (C) Direct activation of AMPK impedes somatic cell reprogramming even in p53-deficient fibroblasts. Adult MSFs from p53-null ($p53^{-/-}$) and wild-type p53 ($p53^{+/+}$) littermates transduced with retroviruses encoding the OSK stemness factors were grown in ES medium in the continuous presence or absence of A-769662 (50 μmol/L) or PBS alone as a control, as specified. Left: Phase-contrast microphotographs of representative MSFs transduced with OSK at different time points during the reprogramming process in the absence or presence of continuous A-769662 (50 μmol/L), as specified. The arrows indicate emerging iPSC-like colonies. Right: The numbers of AP^+ colonies were counted 14 d after the initial infection and are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM.

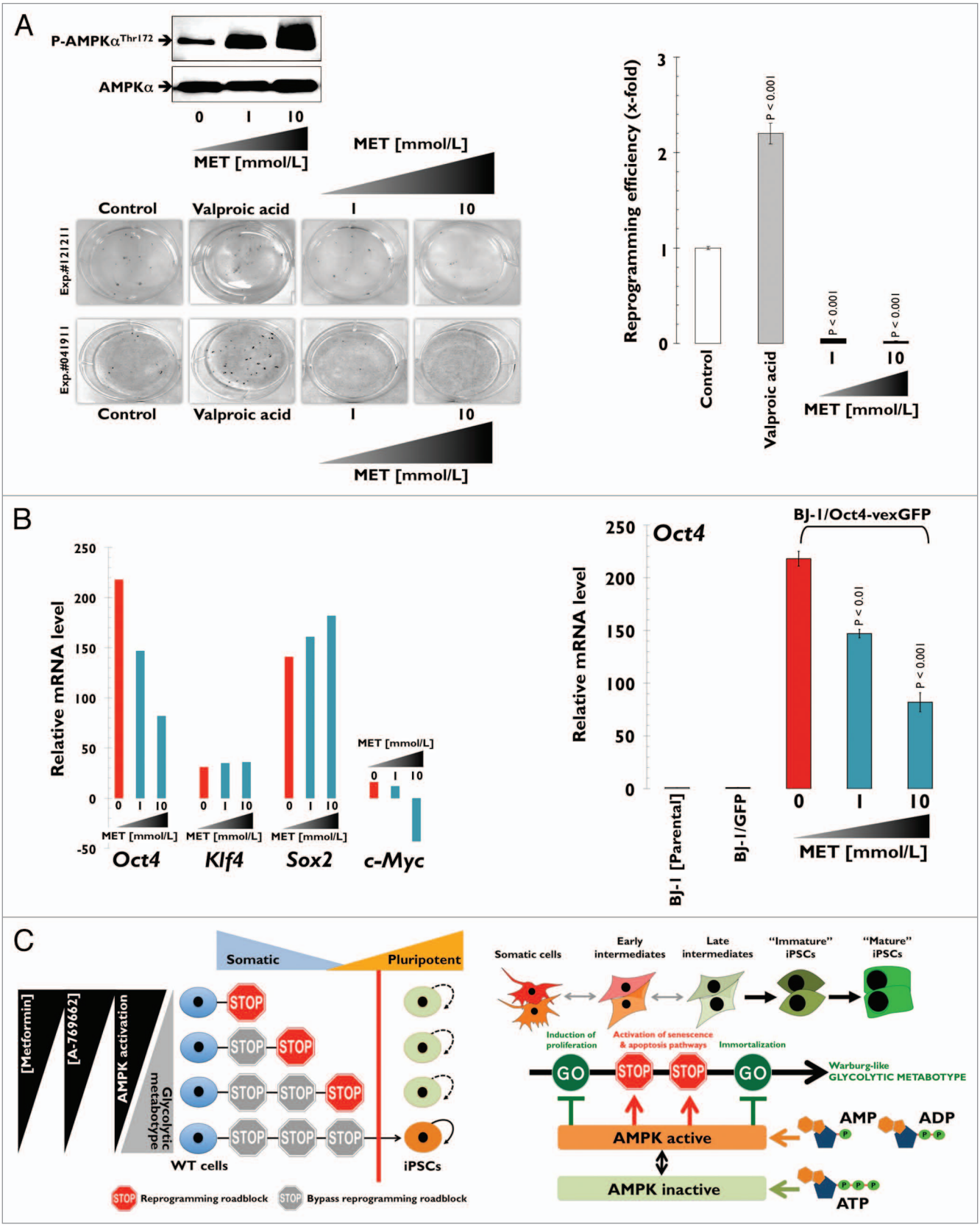

Figure 4. For figure legend, see page 982.

Figure 4 (See previous page). (A) Human BJ-1 neonatal foreskin fibroblasts (MEFs) fail to reprogram into iPSCs in the presence of the indirect AMPK activator metformin. Early passage MEFs infected with retroviruses encoding *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM) were grown in ES medium in the continuous presence or absence of metformin (MET, 1 and 10 mmol/L) or PBS alone as a control, as specified. Top: Representative immunoblot of lysates from BJ-1 fibroblasts treated with metformin (MET, 1 and 10 mmol/L) to detect the phosphorylation response of AMPK at the Thr-172 site. The numbers of AP^+ colonies (microphotographs of representative reprogramming experiments are shown) were counted 14 d after the initial infection and are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. Bottom: Phase-contrast microphotographs of representative MEFs transduced with OSKM at different time points during the reprogramming process in the absence or presence of continuous metformin (MET, 10 mmol/L). The arrows indicate emerging iPSC-like colonies. (B) Oct4 is the primary target of metformin among Yamanaka stemness factors during reprogramming. BJ-1 human fibroblasts were transduced with Yamanaka reprogramming factors as described in Papapetrou et al.[70] Total RNA was isolated following 48 h treatment with varying concentrations of metformin for the real-time RT-PCR analysis of each factor relative to expression in untreated BJ-1 (parental and GFP-transduced) control fibroblasts. The relative mRNA levels are plotted for each condition relative to the controls (x-fold), as specified. The error bars indicate the SEM. (C) AMPK: a metabolic gatekeeper of somatic reprogramming into stem cells. Right: During stemness transcription factor-induced reprogramming, somatic cells encounter different roadblocks (see left part), including the acquisition of immortality, which can rescue almost every cell from any reprogramming roadblock at any time. The a priori bioenergetic signature of somatic cells correlates with their reprogramming efficiencies. Cells that demonstrate oxidative:glycolytic production ratios closer to the glycolytic metabotype of pluripotent cells reprogram more quickly and efficiently than those demonstrating a metabotype closer to the oxidative state of normal, non-proliferative somatic cells. We now reveal that activation of the master energy-sensing protein AMPK imposes a normalized metabolic flow away from the required pro-immortalizing glycolysis that fuels the induction of stemness and pluripotency, endowing somatic cells with an energetic infrastructure protected against reprogramming. The red bar indicates the transition point between the somatic (blue) and the pluripotent (orange) state (modified from Utikal et al.). Left: The early steps during the transition of somatic cells into iPSCs are reversible, as indicated by the reverse arrows. "Immature iPSCs" have already acquired pluripotency but still retain an epigenetic memory of their somatic cell type of origin, while "mature iPSCs" have completely lost this memory. The "stop" and "go" signs below the reprogramming flow indicate assumed reprogramming roadblocks (e.g., induction of proliferation, activation of senescence and apoptosis pathways and immortalization) that the cells encounter at different stages before fully acquiring stemness and pluripotency. Failure to pass any of these roadblocks could result in cell growth that arrests at that stage or, alternatively, senescence or apoptosis. Although somatic oxidative bioenergetic transitions into pluripotency-dependent glycolysis critically underlie reprogramming efficiency and somatic cell fate determination, AMPK activation in response to the AMP or ADP increase (or to drugs that mimic a low energy cellular state) could activate and/or strengthen one or several of the reprogramming roadblocks while concurrently preventing a bioenergetic shift from somatic oxidative mitochondria toward a pro-immortalizing glycolytic metabotype, thus minimizing the efficiency of somatic reprogramming to pluripotency.

p53, to initiate tumor formation in epithelial cells by blocking the differentiation of p53-deficient stem cells.[74,77] Herein, we describe for the first time the function of the metabolic tumor suppressor AMPK[47,78] in terms of somatic reprogramming efficiency to a pluripotent state. AMPK appears to operate upstream of the most common pathways associated with human cancers, suppression of *p53* and activation of *c-Myc*, because AMPK-activating drugs fully impede the reprogramming of somatic cells to iPSCs regardless of the *p53* status and/or the inclusion of *c-Myc* in the reprogramming cocktail. Indeed, while p53 deficiency expectedly rescued the reprogramming potential in cells that normally fail to form iPSCs (i.e., senescent-prone adult mouse fibroblasts), treatment with AMPK-activating drugs imposed a metabolic barrier to reprogramming that cannot be bypassed, even in the absence of p53.

There is an overall correlation between the bioenergetic state of somatic cells and their efficiency to reprogram into stem cells. Folmes et al.[38] have recently shown that the a priori energetic infrastructure of somatic cells is a crucial molecular feature for the optimal successful acquisition of pluripotency. Glycolytic gene potentiation occurs prior to the induction of pluripotent markers, and the stimulation of glycolysis promotes reprogramming efficiency, while the blockade of glycolytic enzymes inhibits this process. Panopoulos et al.[41] have confirmed the idea that a bioenergetic shift from somatic oxidative mitochondria toward an alternative ATP-generating glycolytic phenotype maximizes the efficiency of somatic reprogramming to pluripotency. These authors demonstrated that somatic cells with oxidative:glycolytic energy production ratios closer to those of pluripotent cells were reprogrammed more quickly and efficiently. Therefore, it is reasonable to suggest that the prevention of a glycolytic metabotype may be a crucial mechanism through which AMPK activators, such as metformin and A-769662, efficiently impede nuclear reprogramming. Although data directly linking the activation status of AMPK with glycolysis are currently scarce, studies have suggested that AMPK suppresses glycolysis in tumor cells by inhibiting mammalian target of rapamycin (mTOR).[79] Another paradigm of AMPK regulation of glycolysis is on the activation of p53, a tumor suppressor that regulates the expression of several genes intimately linked to processes of oxidative phosphorylation and glycolysis. First, p53 positively regulates the expression of cytochrome *C* oxidase 2, a crucial regulator of the cytochrome *C* oxidase complex that is essential for mitochondrial respiration.[80-82] Second, p53 positively regulates TP53-induced glycolysis and expression of the apoptosis regulator (TIGAR), which leads to a decrease in the levels of fructose-2,6-phosphate, an activator of glycolysis and inhibitor of gluconeogenesis.[83-85] Third, p53 negatively regulates the expression of phosphoglycerate mutase, which is crucial for establishing cellular immortalization by enhancing the glycolytic flux, allowing indefinite proliferation and rendering the cells resistant to oncogene-induced senescent growth arrest.[86,87] Fourth, by directly preventing the formation of the active glucose-6-phosphate dehydrogenase dimer, p53 negatively regulates the pentose phosphate pathway (PPP), thus suppressing glucose consumption, NADPH production and biosynthesis.[88] Fifth, p53 negatively regulates levels of pyruvate dehydrogenase-2 and formation of the pyruvate dehydrogenase complex, both of which are key regulators of pyruvate metabolism.[89] Sixth, p53 negatively regulates the expression of glucose transporters (e.g., SLC2A12/GLUT12, SLC2A1/GLUT1) and the master transcription factor *c-Myc*, which is another crucial regulator of

cellular bioenergetics.[90-93] Collectively, the net effect of the p53 deficiency is to reduce mitochondrial respiration and strongly activate glycolysis, leading to the Warburg effect. Accordingly, immortal fibroblasts deficient in components of the p53 pathway yield iPSC colonies at a significantly higher efficiency than wild-type cells, endowing almost every somatic cell with the potential to form iPSCs (i.e., approximately 100% reprogramming efficiency). AMPK activation results in the homeostatic regulation of metabolic processes that are crucial for the prevention of cancer and that may promote p53-driven suppression of the glycolytic flux, which is essential both to the reprogramming process and the steps of immortalization and malignant transformation. Perhaps more importantly, it appears that even in the absence of p53, the guardian of the genomic and metabolic checkpoint, AMPK activation imposes a normalized metabolic flow away from pro-immortalizing glycolysis, thus rendering cells metabolically protected against reprogramming (**Fig. 4C**).

Similar to the multistep tumorigenesis process, induced pluripotency is globally considered to be poorly efficient, as the safeguard activity of p53 ensures that only cells with no DNA damage progress to full, well-reprogrammed iPSCs. The function of p53 is critical in both reprogramming and malignant cell transformation to control the spread of damaged cells. The p53-dependent counter selection of DNA-damaged cells during reprogramming has been demonstrated by increased DNA damage foci and increased phosphorylation of the serine/threonine kinase ataxia telangiectasia mutated (ATM),[16-20] a well-known primary regulator of the cellular response to DNA double-strand breaks that has begun to emerge as a central DNA damage checkpoint that connects cancer predisposition with cellular bioenergetics;[94,95] thus, it has been suggested that the DNA damage response (DDR) activated during the induction of pluripotency might be equivalent to the oncogene-induced DDR that occurs during OIS,[2,17] when the cell proliferation and transformation induced during oncogene activation in early tumorigenesis is restrained by cellular senescence. The DDR that is mounted upon expression of the Yamanaka reprogramming factors is not only compatible with OSKM/OSK-driven DNA replication-induced DNA damage, but also with the generation of reactive oxygen species (ROS), which may explain why reprogramming is significantly more efficient under low oxygen conditions or in the presence of antioxidants, such as vitamin C.[96,97] We have recently reported that metformin treatment, through the promotion of enhanced mitochondrial biogenesis and oxidative stress, may lower the threshold for cellular senescence by activating an ATM-dependent pseudo-DDR, which occurs regardless of the p53 status.[98,99] The metformin-driven activation of AMPK appears to sensitize cells against further damage, thus mimicking the precancerous stimulus that induces an intrinsic barrier against carcinogenesis, which, in turn, would accelerate the onset of cellular senescence (OIS/RIS) in response to additional oncogenic-like stresses.[100] Consistent with this notion, we have recently reported that metformin, by activating the ATM/AMPK axis, establishes a stronger DDR-dependent cell cycle arrest that synergistically interacts with hyperoxic culture-induced DNA damage and cellular senescence in cultured human diploid fibroblasts, including the BJ-1 neonatal foreskin fibroblasts used in this study to monitor the effects of metformin on the reprogramming of human somatic cells.[99] Moreover, metformin co-treatment accelerated the DDR-related cellular senescence response imposed by DNA damaging drugs (i.e., doxorubicin), which is somewhat equivalent to the protective responses activated by reprogramming factors.[99,101] Although no direct evidence is provided that AMPK activation reinforces the RIS that occurs during somatic reprogramming experiments, it is reasonable to suggest that AMPK-activating drugs favor a metabolic cellular imbalance to lower the threshold for stress-induced senescence in response to the exogenous overexpression of reprogramming factors, which is a pivotal roadblock when attempting to convert adult somatic cells to pluripotent stem cells. If AMPK-activating drugs strengthen RIS to decrease the rate of conversion of somatic cells into iPSCs, then we can infer that strategies to promote the activation of AMPK should provide a more efficient senescent response in pre-malignant and malignant tissues to prevent stemness in cell populations with tumor-propagating properties.

How is the cell metabotype acquired in response to AMPK-activation associated with the cell phenotype refractory to reprogramming? Prior to the completion of this study, two groups independently reported that metformin treatment negatively regulates *Oct4*, a well-known transcription factor (also named *Pou5f1*) that plays a fundamental role in stem cell self-renewal, pluripotency and somatic cell reprogramming.[102,103] When examining the effect of metformin on the self-renewal capacity of pancreatic cancer stem-like cells, Bao et al. reported that metformin decreases sphere-forming capacity (i.e., three-dimensional pancreatospheres) and increases the disintegration of pancreatospheres. The metformin-induced attenuation of cancer stem cell function was accompanied by significant decreases in the expression of several cancer stem cell markers, including *Oct4*. Using *Oct4* expression as a marker for human breast carcinoma stem cells, Jung et al. demonstrated that metformin reduces the size and number of three-dimensional mammospheres via the inhibition of estrogen receptor-mediated *Oct4* expression. *Oct4* is the master regulator or gatekeeper of self-renewal and pluripotency that cannot be replaced by any other transcription factor in the pluripotency network, as revealed by studies exploring alternative factor-based reprogramming strategies.[106,107] Moreover, the amount of *Oct4* expression is critical in embryonic stem cells (ESCs) and iPSCs, and its up- or downregulation drastically alters the reprogramming efficiency and pluripotent cell phenotype.[70] When monitoring the transcriptional activation status of each individual reprogramming factor in human fibroblasts cultured in the absence or presence of metformin, we confirmed that metformin downregulated the *Oct4* dosage. This result suggests that AMPK activation could negatively impact reprogramming efficiency by preventing the critical regulation of *Oct4* activities that link the genetic and epigenetic regulation of stem cell pluripotency. Moreover, the anti-*Oct4* activity of metformin in reprogramming somatic cells might translate into anticancer effects, if *Oct4*-driven transcriptional genetic modules are specifically reactivated in tumor-propagating cells. Indeed, some

poorly differentiated, biologically aggressive carcinomas appear to hijack self-renewal transcription factor machinery (e.g., *Oct4*) to support aberrant proliferation and tumor initiation. Previous studies have shown that *Oct4* overexpression leads to the generation of dysplastic lesions in epithelial tissues due to aberrant expansion of early progenitor cells in mice.[108] Indeed, *Oct4* is expressed in a subpopulation of breast and ovarian cancer cells that possess self-renewal ability,[109,110] and *Oct4* overexpression is sufficient to enhance tumor-propagating cells in a mouse model of breast carcinoma.[111] Beltran et al. recently confirmed that the sole transduction of *Oct4* in primary human mammary epithelial breast cell preparations from reduction mammoplasty donors was sufficient to generate cell lines possessing tumor-initiating and colonization capacities. Moreover, the *Oct4*-transduced breast cells displayed a gene signature compatible with that of claudin-low breast carcinomas, which is a molecular subtype of breast cancer that is highly enriched in cancer stem cell-like features.[113] Another molecular candidate that might connect AMPK activation with the reprogramming blockade is the Lin28/let-7 axis, which is composed of the RNA binding proteins Lin28a/b, which promote malignancy by inhibiting the biogenesis of let-7,[114] a family of tumor-suppressor miRNAs.[115,116] Lin28 is a gatekeeper of the pluripotent state that binds and represses the processing of let-7, a gatekeeper of the differentiated state, in an intricately designed auto-regulatory loop. While let-7 opposes the actions of cell cycle-regulating miRNAs that maintain self-renewal in embryonic stem cells,[117] Lin28 can functionally replace c-Myc in the original Yamanaka cocktail of reprogramming transcription factors,[118] supporting the notion that Lin28 promotes reprogramming by preventing the production of mature let-7 miRNAs. We have recently shown that short-term exposure to metformin was sufficient to drastically upregulate let-7a and coincidentally reduce the mammosphere-initiating stem cell-like features of breast cancer cells.[119] Bao et al. recently confirmed that metformin treatment causes a significant re-expression of let-7, which is typically inhibited in prostate cancer, especially in pancreatospheres enriched with tumor propagating-like cells. Although we acknowledge that further mechanistic studies are warranted to explore whether metformin might antagonize Lin28a/b expression and/or activity,[120] the impact of metformin on let-7 expression raises the tantalizing possibility that AMPK activation might impede somatic cell reprogramming by altering the Lin28/let-7 axis to maintain differentiation vs. pluripotency.[121] Notably, let-7 overexpression could markedly reduce the expression of key transcriptional inducers of stemness, including *Oct4,* while Lin28 could directly upregulate the expression of *Oct4*,[109,122] suggesting that metformin inhibits expression of the pivotal pluripotency factor *Oct4*, which might reflect a primary consequence of AMPK activation-mediated modulation of the Lin28/let-7 axis. Moreover, three recent studies have revealed that the Lin28/let-7 signaling pathway is a central regulator of mammalian glucose metabolism,[123-125] which suggests the possibility that a layered arrangement of interconnected metabolic controllers (i.e., AMPK and Lin28/let-7) may fine-tune the activation of core reprogramming factors (e.g., *Oct4*) rather than acting as *on/off* reprogramming switches.

Metformin (a derivative of galegine, a potent activator of AMPK naturally occurring in the traditional European herbal medicine *Galega officinalis*), and A-769662 (a direct AMPK activator developed by Abbott) activate AMPK by distinct mechanisms, and it is unlikely that the inhibition of somatic reprogramming to pluripotency could be due to "off-target" (i.e., AMPK-independent) effects.[67,126] Metformin, as with many other AMPK-activating xenobiotics, fails to activate AMPK complexes bearing an R531G mutation, which renders the γ2 isoform insensitive to increases in ADP and AMP; thus, metformin works by increasing cellular AMP and/or ADP to activate AMPK.[126] Although A-769662 has been shown to inhibit Na^+-K^+-ATPase function in skeletal muscle cells,[127] this AMPK-independent effect occurred at concentrations 2- to 4-fold higher than those employed in the present study. Therefore, our current findings reveal for the first time that the activation of AMPK, a master regulator of energy metabolism and oxidative stress that balances oxidative phosphorylation and glycolysis, is sufficient to impose a cell metabotype that is refractory to reprogramming by stemness factors and dominant over *p53* deficiency, a well-recognized mechanism that enhances the efficiency of somatic cell reprogramming to a pluripotent state (**Fig. 4C**).[128] Moreover, our current approach definitively confirms that changes in energy metabolism play a role in initiating the reprogramming process, rather than simply being a consequence of acquiring a pluripotent state. Beyond the importance of AMPK-regulated energy metabolism in inducing pluripotency, our current studies demonstrate that understanding the metabolic changes associated with somatic cell reprogramming might provide crucial insights into the metabolic mechanisms that ultimately regulate the tumor-propagating cell phenotype. Biologically aggressive, poorly differentiated tumors might arise from either stem or progenitor cells that have acquired additional malignant hits or from somatic cells that might dedifferentiate by activating components of the pluripotency machinery, thus demonstrating that the complex genomic and metabolic reprogramming that accompanies induced pluripotency shares many commonalities with the complex, albeit aberrant, genomic and metabolic reprogramming that accompanies malignant transformation. Because not all of the factors required to trigger the reprogramming of somatic cells into iPSCs are necessary for their maintenance,[129,130] and if the so-called tumor-propagating cells indeed initially arise through a reprogramming-like mechanism, many of the oncogenes that promote tumor formation might be poor targets for new anticancer therapies, as they might be dispensable for tumor propagation. Our findings validate the notion that the loss of AMPK activation sensitivity in response to metabolic stresses is a molecular strategy that might allow somatic cells to de-differentiate and acquire properties of tumor-propagating cells and add a new molecular dimension to metformin anticancer stem cell activity.[104,105,119,131-135] Because it is reasonable to expect more AMPK activators with higher specificity and potency to appear in the market in the near future, the fact that AMPK activation reprograms cellular metabolism in a manner that successfully impedes somatic cells from de-differentiating and acquiring stemness properties may provide a roadmap to a new-generation

of anti-reprogramming strategies metabolically targeting tumor propagating cells.

Materials and Methods

Chemicals. Metformin was purchased from the Sigma Chemical Co., and dissolved in phosphate-buffered saline (PBS) immediately before use. A-769662 was purchased from Tocris Biosciences and dissolved in DMSO immediately prior to use.

Generation of mouse iPSCs. The reprogramming of primary (passage 2–4) mouse embryo fibroblasts (MEFs) derived from wild-type embryos (C57BL6 genetic background) was performed as previously described by Marion et al.[17] with modifications from a previous protocol by Blelloch et al.[136] Briefly, retroviral supernatants were produced in HEK-293T cells (ATCC® Number CRL-11268™) transfected with 4 μg of the ecotropic packaging plasmid pCL-Eco (www.addgene.org/12371/) together with 4 μg of one of the following retroviral constructs: pMX-*Oct3/4* (www.addgene.org/13366/), pMX-*Sox2* (www.addgene.org/13367/) or pMX-*Klf4* (www.addgene.org/13370/). Transfections were performed using the Plus™ Reagent (Invitrogen) according to the manufacturer's protocol. Two days later, retroviral supernatants (10 mL) were serially collected during the subsequent 48 h, at 12 h intervals, each time adding fresh medium to the cells (10 mL). The recipient MEFs were seeded the previous day (2 x 10^5 cells per well; 6 well-plates) and received 1 mL of each of the corresponding retroviral supernatants (a total of three). This procedure was repeated every 12 h for 2 d (for a total of four rounds of virus infection). Following transduction, the media was replaced with standard ES media supplemented with the knockout serum replacement (KSR, Invitrogen). The cultures were maintained in the absence or presence of two different concentrations of either metformin (1 and 10 mmol/L) or A-769662 (10 and 50 μmol/L), with medium changes every two days. Reprogramming was assessed on day 14 post-transduction by counting the alkaline phosphatase (AP)-positive colonies. The AP staining was performed according to the manufacturer's instructions (Alkaline Phosphatase Detection kit, Millipore). The reprogramming efficiency was calculated as the number of iPSC colonies obtained at day 14 post-transduction with the three reprogramming factors relative to the total number of cells initially infected (infection efficiency was measured by transducing with the three pMX-*Oct3/4*, pMX-*Sox2* and pMX-*Klf4* retroviruses plus a pMX retrovirus expressing the fluorescent protein tdTomato (RV-tdTomato) in equal proportions and analyzed by immunofluorescence microscopy to detect the proportion of Tomato-positive cells). The colonies were picked on day 20 and expanded on feeder fibroblasts using standard procedures.

Generation of human iPSCs. Constructs encoding the reprogramming factors and fluorescent reporters pLM-*Oct4*-vexGFP (ID 22240), pLM-*Sox2*-mcitrine (ID 23242), pLM-*Klf4*-mcherry (ID 23243) and pLM-*cMyc*-mcerulean (ID 23244) (as described in Papapetrou et al.[70]) were obtained from Addgene. The lentiviral vectors were generated at the Viral Vector Facility of the Fundación INBIOMED. Briefly, constructs encoding each of the Yamanaka factors were co-transfected into HEK293 cells along with the vectors pMDLg/pRRE and pRSV-Rev for third generation viral packaging and with the vector pMD2.G encoding proteins for the VSV-G (Vesicular Stomatitis Virus) coat. The viral supernatants from cultures packaging each of the four viruses were collected at 54 h post-transfection, pooled and concentrated using ultracentrifugation in a Beckman Coulter Optima L-100 XP centrifuge.

The reprogramming of human BJ fibroblasts (ATCC) (75 x 10^3/well in a 6-well plate) was performed using the following protocol: serum-depleted cells were infected during 16–20 h with a 1:1:1:1 ratio of the vectors carrying each of the Yamanaka factors at the MOI required to obtain at least 80–90% transduction efficiency. The transgene expression was examined using fluorescence microscopy and at 5–7 d post-infection, 20 x 10^3 cells/well were seeded on a feeder layer of irradiated human HFF-1 fibroblasts (ATCC) in hES medium [KO-DMEM, KO-Serum Replacement 20%, βFGF 10 nmol/L, Glutamax 2 mmol/L, 2-β-mercaptoetanol 50 μmol/L, non-essential amino acids (Sigma-Aldrich), which were all purchased from Gibco]. Subsequently, the infected BJ cells were incubated in the presence or absence of 1 and 10 mmol/L of metformin or 5 μmol/L of valproic acid as a positive control to increase cellular reprogramming as specified. The metformin was depleted from the medium after two days, whereas valproic acid was added every two days when the cells were fed with fresh medium. The morphological changes were observed as early as 14–20 d post-infection, and the cells were stained for AP activity using an AP detection kit from Millipore according to the manufacturer's instructions.

Immunoblotting. The activation of phosphorylation and total expression of AMPK was assessed using immunoblotting procedures and the AMPK and ACC (#9957) Antibody Kit (Cell Signaling Technology, Inc.). Briefly, metformin-treated and untreated control cells were washed twice with cold PBS and then lysed as described above. Equal amounts of protein (i.e., 50 μg) were resuspended in 5x Laemmli sample buffer (10 min at 70°C), subjected to 10% SDS-PAGE and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked for 1 h at RT with TBS-T buffer [25 mmol/L TRIS-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween 20] containing 5% (*w/v*) nonfat dry milk to minimize non-specific binding. Subsequently, the treated membranes were washed in TBS-T and incubated with phospho-AMPKα^{Thr172} (Clone 40H9) or total AMPK antibodies, as specified, in 1x TBS-T buffer containing 5% *w/v* BSA and 0.1% Tween-20 at 4°C with gentle shaking overnight. The membranes were washed with TBS-T, incubated with horseradish peroxidase-conjugated secondary anti-rabbit IgG in TBS-T for 1 h, and the immunoreactive bands were detected using a chemiluminescence reagent (Pierce). The blots were re-probed with an antibody against β-actin to control for protein loading and transfer. Densitometric values of the proteins bands were quantified using Scion Image software (Scion Corporation).

Quantitative, real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted from the cell cultures

using a Qiagen RNeasy kit and QiaShredder columns according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed to cDNA using a Reaction Ready™ First Strand cDNA Synthesis Kit (SABiosciences) and applied to the Yamanaka reprogramming factors expression array [(IPHS-002), 96-well format] according to the instructions in the SABiosciences RT-PCR manual. The plates were processed in an Applied Biosystems 7500 Fast Real-Time PCR System using an automated baseline and threshold cycle detection. The data were interpreted using the SABiosciences' web-based PCR array analysis tool.

Statistical analysis. The results are represented as the means ± SEM. The statistics were calculated using either unpaired two-tailed Student's t test or one-way ANOVA with a post hoc test if appropriate. p-values < 0.05 were considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

This work was financially supported through funding from the Instituto de Salud Carlos III (Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria (FIS), Spain, Grants CP05-00090 and PI06-0778 and RD06-0020-0028), the Fundación Científica de la Asociación Española Contra el Cáncer (AECC, Spain), and the Ministerio de Ciencia e Innovación (SAF2009-11579, Plan Nacional de I+D+ I, MICINN, Spain). Alejandro Vazquez-Martin received the Sara Borrell post-doctoral contract (CD08/00283, Ministerio de Sanidad y Consumo, Fondo de Investigación Sanitaria-FIS-, Spain). Sílvia Cufí received a Research Fellowship (Formación de Personal Investigador, FPI) from the Ministerio de Ciencia e Innovación (MICINN, Spain).

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