

# Role of AMP-activated protein kinase in regulating hypoxic survival and proliferation of mesenchymal stem cells

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## Aims

Mesenchymal stem cells (MSCs) are widely used for cell therapy, particularly for the treatment of ischaemic heart disease. Mechanisms underlying control of their metabolism and proliferation capacity, critical elements for their survival and differentiation, have not been fully characterized. AMP-activated protein kinase (AMPK) is a key regulator known to metabolically protect cardiomyocytes against ischaemic injuries and, more generally, to inhibit cell proliferation. We hypothesized that AMPK plays a role in control of MSC metabolism and proliferation.

## Methods and results

MSCs isolated from murine bone marrow exclusively expressed the AMPK $\alpha$ 1 catalytic subunit. In contrast to cardiomyocytes, a chronic exposure of MSCs to hypoxia failed to induce cell death despite the absence of AMPK activation. This hypoxic tolerance was the consequence of a preference of MSC towards glycolytic metabolism independently of oxygen availability and AMPK signalling. On the other hand, A-769662, a well-characterized AMPK activator, was able to induce a robust and sustained AMPK activation. We showed that A-769662-induced AMPK activation inhibited MSC proliferation. Proliferation was not arrested in MSCs derived from AMPK $\alpha$ 1-knockout mice, providing genetic evidence that AMPK is essential for this process. Among AMPK downstream targets proposed to regulate cell proliferation, we showed that neither the p70 ribosomal S6 protein kinase/eukaryotic elongation factor 2-dependent protein synthesis pathway nor p21 was involved, whereas p27 expression was increased by A-769662. Silencing p27 expression partially prevented the A-769662-dependent inhibition of MSC proliferation.

## Conclusion

MSCs resist hypoxia independently of AMPK whereas chronic AMPK activation inhibits MSC proliferation, p27 being involved in this regulation.

## Keywords

AMPK • Mesenchymal stem cells • Hypoxia • Glycolysis • Proliferation

## 1. Introduction

Trials of mesenchymal stem cell (MSC) transplantation into infarcted myocardium have demonstrated positive effects on recovery of cardiac function.<sup>1,2</sup> The therapeutic efficacy of MSCs is closely related to their survival capacity in hypoxic/infarcted regions where they

are transplanted and to their ability to trans-differentiate into cardiomyocyte-like cells. Therapeutic benefit has been demonstrated when stem cells are cultured under hypoxia prior to transplantation.<sup>3</sup> We hypothesized that targeting AMP-activated protein kinase (AMPK), an enzyme involved in hypoxic tolerance, cell proliferation, and differentiation, could increase the efficacy of stem cell therapy.

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AMPK is a heterotrimeric protein kinase composed of one catalytic ( $\alpha$ ) and two regulatory ( $\beta/\gamma$ ) subunits existing as several isoforms.<sup>4,5</sup> Cellular energy imbalance, observed during myocardial ischaemia, and the resulting increase in an intracellular AMP/ATP ratio promote AMPK activation.<sup>5–7</sup> Ischaemia-mediated AMPK activation mainly arises from its phosphorylation on Thr172 by LKB1.<sup>8</sup> Once activated, AMPK functions to maintain cellular energy balance by switching on catabolic pathways while switching off anabolism.<sup>5,6</sup> This re-establishes energy balance and enhances cell survival in the absence of oxygen. Genetic inactivation of the cardiac AMPK $\alpha$ 2 isoform elicits energetic imbalance accompanied by rapid and severe ischaemic contracture.<sup>9</sup> Similar results have been obtained using mouse expressing a kinase-dead form of AMPK.<sup>10</sup> Independently of changes in energy charge, AMPK can be phosphorylated on Thr172 by Ca<sup>2+</sup>/calmodulin-dependent kinase kinase- $\beta$  (CaMKK $\beta$ ) in response to elevation of cytosolic [Ca<sup>2+</sup>],<sup>11</sup> which is involved in the regulation of non-metabolic processes, such as smooth muscle contraction and cytoskeleton organization.<sup>12,13</sup> These non-metabolic functions include the stimulation of mitochondrial biogenesis and the inhibition of cell proliferation. AMPK is known to induce expression of the proliferator-activated receptor gamma coactivator-1 $\alpha$  (PGC1 $\alpha$ ), a regulator of mitochondrial biogenesis.<sup>14</sup> The importance of mitochondrial biogenesis is obvious for cardiac differentiation of MSCs, because these cells are characterized by immature mitochondria.<sup>15</sup> Concerning cell proliferation, AMPK activation in mouse embryonic fibroblasts results in p53 phosphorylation, which induces the expression of p21, an inhibitor of cell cycle progression.<sup>16</sup> Moreover, AMPK activation in cancer cells suppresses their proliferation by elevating levels of the cell cycle inhibitory protein p27.<sup>17</sup> AMPK is also known to inhibit the mammalian target of rapamycin/p70 ribosomal S6 protein kinase (p70S6K) and eukaryotic elongation factor 2 (eEF2) signalling pathways, involved in the down-regulation of protein synthesis and cell growth.<sup>6,7</sup> All these aspects are crucial because suppressed cell proliferation has been shown to favour differentiation and vice versa. Indeed, the overexpression of Cyclin D1, a positive cell cycle regulator, inhibited myogenic differentiation.<sup>18</sup> Conversely, p27 overexpression in intestinal epithelial cells leads to an increase in differentiation markers.<sup>19</sup> Finally, an interactomic study has demonstrated that expression of the proliferation-linked protein network is negatively correlated with that of differentiation.<sup>20</sup>

Given that AMPK: (i) controls cardiomyocyte survival under hypoxia, (ii) inhibits cell proliferation, and (iii) increases mitochondrial biogenesis, we hypothesized that AMPK is a key target for improving MSC deployment in cardiac stem cell therapy. In the present study, we initially characterized AMPK isoform expression and pharmacological activation in MSCs in comparison with adult cardiomyocytes. We then investigated AMPK's role in the control of MSC survival under hypoxia, in MSC proliferation, and in mitochondrial biogenesis.

## 2. Methods

For a detailed description, see the expanded materials and methods section in Supplementary material online.

### 2.1 Ethics statement

All animal studies were approved by the Animal Research Committee of Université catholique de Louvain (Approval ID: UCL/MD/2007/049) and performed in agreement with guidelines on animal experimentation at our institution (UCL). This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### 2.2 Isolation and culture of adult cardiomyocytes and MSCs

Animals were anaesthetized with ketamine/xylazine (i.p., 80/10 mg/kg) and sacrificed by cervical dislocation before removing tissues. Anaesthesia depth was monitored by limb withdrawal using toe pinching. Cultured cardiomyocytes were processed as described previously.<sup>21</sup> Cultured mouse MSCs were prepared according to a protocol designed for rat MSCs.<sup>22</sup> Quality of the MSC preparations was evaluated by measuring the expression of height-different surface antigens (see Supplementary material online for details). They were positive for CD29 and CD44 and negative for CD11b/CD45/CD34/CD31/CD117. The differentiation capacity of MSCs was also verified (see Supplementary material online, Figure S1). Silencing experiment protocol is described in Supplementary material online.

### 2.3 Cell treatment and lysis

Cells were treated as described in figure legends. After treatment, the medium was removed and the cells were lysed in ice-cold lysis buffer containing 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (pH 7.5), 50 mM potassium fluoride, 1 mM potassium phosphate, 5 mM ethylene diamine tetraacetic acid, 5 mM ethylene glycol tetraacetic acid (EGTA), 15 mM  $\beta$ -mercaptoethanol, complete protease inhibitor (Roche), 1 mM vanadate, and 1% (vol/vol) Triton X-100. The lysates were then centrifuged (10 000 g, 15 min, 4°C), and the supernatants stored at –80°C. Cell fractionation is detailed in Supplementary material online.

### 2.4 Detection and quantification of total and phosphorylated proteins

The antibodies deployed to evaluate the protein levels of AMPK isoforms, acetyl-CoA carboxylase (ACC), Histone H3, p27 and eEF2, and the phosphorylation state of Thr172 AMPK $\alpha$ , Ser79 ACC, Thr389 p70S6K, Thr56 eEF2, and Ser10 H3 are described in Supplementary material online. Immunoblots were quantified after normalization (see Supplementary material online).

### 2.5 Metabolite concentrations and oxygen consumption rate

Lactate production, adenine nucleotides, and oxygen consumption rate (OCR) were measured as described in Supplementary material online.

### 2.6 Cell proliferation, cell cycle distribution, cell death, and apoptosis

MSC proliferation capacity and cell cycle distribution were tested by direct DNA synthesis measurement using 5-ethynyl-2'-deoxyuridine and propidium iodide, respectively. Viable, necrotic, and apoptotic MSCs were identified by the fluorescent dye Annexin V-fluorescein isothiocyanate apoptosis detection kit (Sigma). Adult necrotic cardiomyocytes were detected by incubation with 50  $\mu$ M propidium iodide.<sup>23</sup>

### 2.7 Protein synthesis

Cells were incubated for 48 h with [<sup>14</sup>C]-phenylalanine (0.75  $\mu$ Ci/mL) in the presence and absence of A-769662. They were then lysed and incubated with 10% trichloroacetic acid for 20 min at 4°C to precipitate proteins. Precipitated material was neutralized with 100 mM NaOH. The samples were precipitated a second time with 10% trichloroacetic

acid for 20 min at 4°C. After re-suspension of protein pellets in formic acid, incorporated radioactivity was measured by liquid scintillation.

## 2.8 Quantitative PCR

Quantitative PCR (qPCR) was performed as described in Supplementary material online. mRNA levels were expressed relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative changes in the target gene-to-GAPDH mRNA ratio were measured according to the formula  $2^{-\Delta\Delta C_t}$ .

## 2.9 Protein measurement and statistics

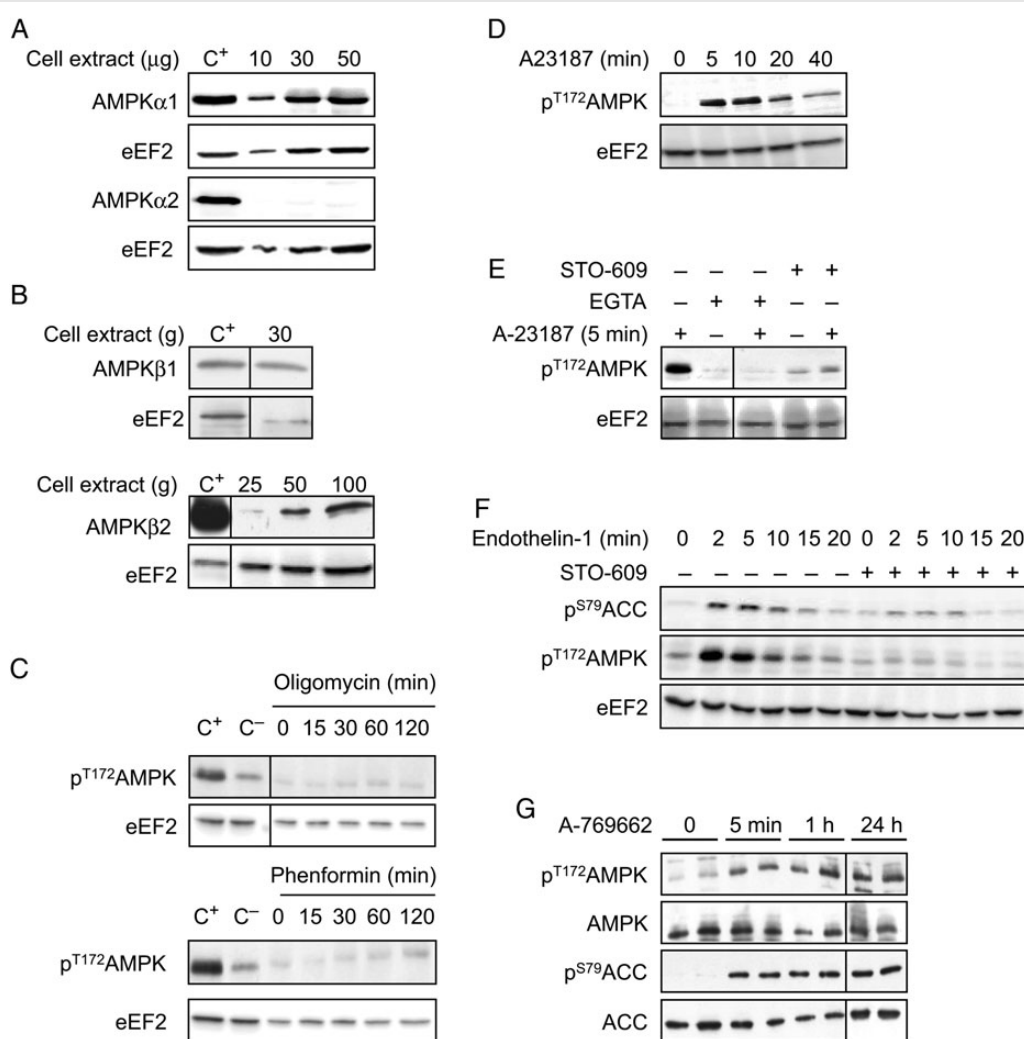
Protein content was estimated by the Bradford method with bovine serum albumin as standard. The results are expressed as means  $\pm$  SE. Statistical significance was calculated by two-tailed unpaired Student's

t-test and one-way analysis of variance with the Bonferroni post hoc test for simple and multiple comparisons, respectively.

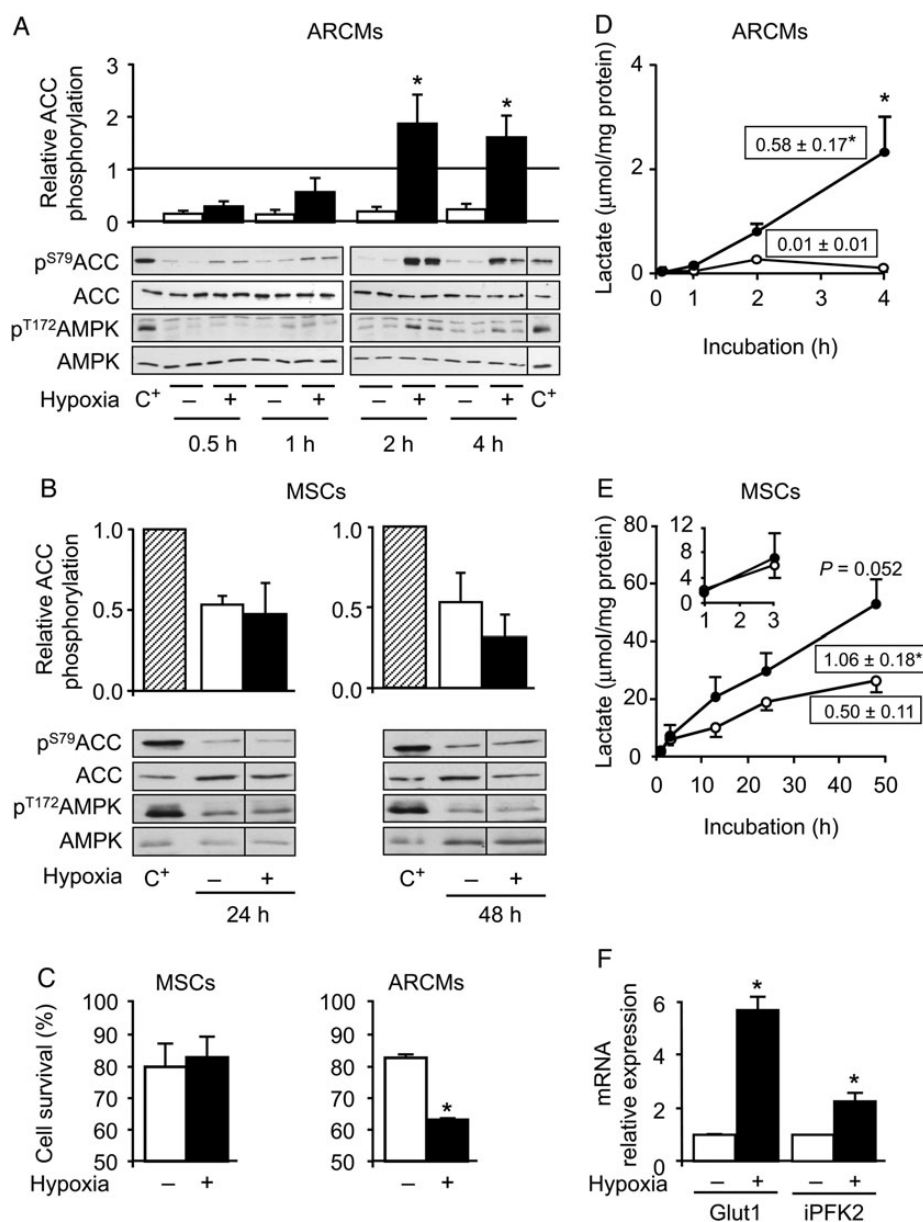
## 3. Results

### 3.1 AMPK expression and activation in MSCs

We defined the expression profiles of AMPK catalytic ( $\alpha$ ) and regulatory subunits ( $\beta$  and  $\gamma$ ) in MSCs. While adult cardiomyocytes express both AMPK $\alpha$ 1 and AMPK $\alpha$ 2 isoforms, we found that MSCs exclusively express AMPK $\alpha$ 1 (Figure 1A). Both AMPK $\beta$ 1 and AMPK $\beta$ 2 isoforms were detected in MSCs and cardiomyocytes (Figure 1B). Finally, MSCs expressed AMPK $\gamma$ 1 and slightly AMPK $\gamma$ 2 (see Supplementary material online, Figure S2). Collectively, we observed that MSCs mainly express



**Figure 1** Differential expression of AMPK subunits (A and B) and effect of AMP-dependent (C) or calcium-dependent (D–F) AMPK activators and A-769662 (G) on AMPK and/or ACC phosphorylation state in MSCs. (A and B) MSC extracts were blotted to identify the AMPK subunits expressed. C<sup>+</sup> (control) represents extracts of the mouse heart (A) and adult rat cardiomyocytes (B). (C) MSCs were incubated with oligomycin (1  $\mu$ M) or phenformin (0.5 mM) for 15–120 min. Controls were cardiomyocytes incubated with (C<sup>+</sup>) or without (C<sup>-</sup>) oligomycin (1  $\mu$ M, 60 min). (D and E) MSCs were incubated with A23187 (10  $\mu$ M) for 5–40 min alone or in combination with ethylene diamine tetraacetic acid (EGTA) (5 mM) or STO-609 (10  $\mu$ M). (F) MSCs were incubated with endothelin-1 (20 nM) for 2–20 min alone or in combination with STO-609 (10  $\mu$ M). (G) MSCs were incubated with A-769662 for 5 min to 24 h. Supernatants of MSC lysates were immunoblotted. eEF2 served as a loading control and was evaluated on the same blot after stripping. The blots are representative of at least three different experiments.



**Figure 2** Effect of hypoxia on AMPK and ACC phosphorylation state (A and B), on cell survival (C), lactate production in adult cardiomyocytes (D) and MSCs (E), and Glut1 and iPFK2 expression in MSCs (F). Adult rat cardiomyocytes (ARCMs) and MSCs were submitted to different periods of normoxia or hypoxia. (A–B) Immunoblots evaluating AMPK and ACC phosphorylation representative of at least three experiments. Positive controls were oligomycin-treated cardiomyocytes and A23187-treated MSCs. ACC phosphorylation was quantified and presented relative to positive controls (C<sup>+</sup>). For cardiomyocytes, two blots performed in parallel were required to compare the elevated number of samples, but aliquots of the same positive control were used in each blot to allow comparisons. Values are the means ± SE. (C) Cell survival was evaluated after 24 and 17 h of incubation for MSCs and ARCMs, respectively. (D and E) ARCMs and MSCs were incubated for the indicated periods of normoxia (open circles) or hypoxia (solid circles). Lactate production rates are indicated in boxes. (F) Glut1 and iPFK2 mRNA expression in MSCs were quantified by qPCR after 24 h of treatment. Values are relative to normoxia. Values are the means ± SE of at least three experiments. \**P* ≤ 0.05 vs. corresponding normoxia-treated cells.

AMPKα1β1γ1 and AMPKα1β2γ1 complexes. Two upstream kinases of AMPK, LKB1 and CaMKKβ, were detected in cell lysates derived from MSCs (data not shown).

Oligomycin and phenformin, which cause ATP depletion by poisoning mitochondria, are known to promote AMPK activation in cardiomyocytes.<sup>21</sup> However, neither oligomycin nor phenformin induced AMPK phosphorylation in MSCs (Figure 1C), correlating with the absence of energy depletion as assessed by measuring the AMP/ATP ratio (see

Supplementary material online, Figure S3). In contrast, the calcium ionophore A23187 and endothelin-1, both known to increase cytosolic Ca<sup>2+</sup> concentration, promoted transient AMPK phosphorylation (Figure 1D–F). It also correlated with increased phosphorylation of ACC, a bona fide substrate of AMPK (Figure 1F). AMPK and ACC phosphorylation was blunted by the calcium chelator EGTA and/or by STO-609, a selective CaMKK inhibitor (Figure 1E and F). We finally tested the A-769662 compound, known to directly stimulate AMPK

through its binding to AMPK $\beta$ 1.<sup>24–26</sup> A-769662 induced sustained AMPK and ACC phosphorylation at the frequently administered concentration of 100  $\mu$ M (Figure 1G).

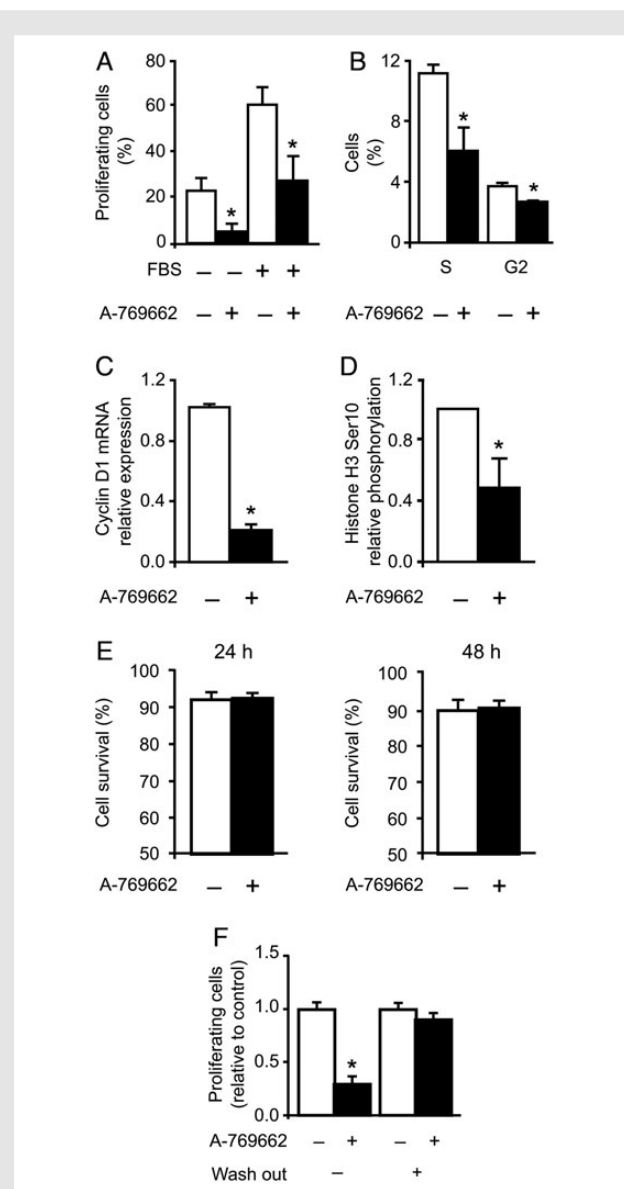
### 3.2 Differential effect of hypoxia on MSCs and cardiomyocytes

MSCs were subjected to hypoxia to mimic injection in hypoxic/ischaemic tissue during cardiac cell therapy. The impact of hypoxia on AMPK activity, cell survival, and metabolism was assessed. MSCs were compared with cardiomyocytes where both AMPK and ACC started to be phosphorylated after 2 h of hypoxia (Figure 2A). In MSCs, neither AMPK nor ACC phosphorylation was altered in response to hypoxia, even after 48 h (Figure 2B). Cardiac AMPK activation by hypoxia is known to result from both ATP decline and AMP increase.<sup>27</sup> The absence of AMPK activation in MSCs correlated with the maintenance of ATP concentration (normoxia:  $19.9 \pm 1.5$ ; 24 h hypoxia:  $19.4 \pm 2.9$  nmol ATP/mg protein, not significant—NS). Similarly, AMP remained unchanged throughout hypoxia (normoxia:  $4.7 \pm 0.7$ ; 24 h hypoxia:  $6.2 \pm 1.8$  nmol AMP/mg protein, NS). Conservation of energetic balance during hypoxia correlated with the maintenance of MSC survival (Figure 2C). Cardiomyocytes were more sensitive to the absence of oxygen. Indeed, while AMPK activation contributed to the protection of cardiomyocytes during the first period of hypoxia (normoxia:  $82.5 \pm 2.2$  vs. hypoxia 2 h:  $80.5 \pm 1.0\%$  of living cells, NS), cell death increased two-fold after 17 h of hypoxia (Figure 2C). To explain the high resistance of MSCs to hypoxia, their metabolism was evaluated by measuring lactate production, the end product of anaerobic glycolysis, and the main ATP provider during hypoxia. No lactate accumulation was seen in normoxic cardiomyocytes, indicating their exclusive oxidative metabolism (Figure 2D). Lactate production occurred under hypoxia, revealing a shift towards anaerobic metabolism (Figure 2D). In contrast, normoxic MSCs produced lactate at a rate similar to that in hypoxic cardiomyocytes (Figure 2E), demonstrating that MSCs are mainly glycolytic in basal condition. Lactate production by MSCs was further enhanced under hypoxia (Figure 2E). Anaerobic stimulation correlated with the increased expression of two critical glycolytic regulators, namely glucose transporter Glut1 and inducible 6-phosphofructo-2-kinase (iPFK2; Figure 2F), as described in cancer cells.<sup>28</sup> The glycolytic dependence of MSCs was confirmed by performing glucose deprivation (see Supplementary material online, Figure S4). The absence of glucose induced energetic imbalance (increase in the AMP/ATP ratio) and reduced MSC survival. The increase in the AMP/ATP ratio being accompanied by the activation of the AMPK pathway demonstrates the ability of AMPK to be activated in an AMP/LKB1-dependent manner.

### 3.3 Effect of AMPK activation on MSC proliferation

We investigated the effect of AMPK activation on MSC proliferation. We tested A-769662 as it causes sustained AMPK activation (Figure 1G). A-769662 significantly decreased the MSC proliferation rate both in the presence or in the absence of serum (Figure 3A). This correlated with a decreased cell number in the S and G2 phases (Figure 3B). The five-fold decline of Cyclin D1 expression and the two-fold reduction of Histone H3 phosphorylation, both being proliferation markers, confirmed the inhibition of MSC proliferation (Figure 3C and D). Interestingly, cell cycle inhibition was not associated with increased cell death (Figure 3E) and was reversed by A-769662 washout (Figure 3F).

AMPK $\alpha$ 1 is the sole AMPK catalytic isoform detectable in MSCs. We isolated MSCs from AMPK $\alpha$ 1-knockout (KO) mice to confirm the role of AMPK in A-769662-induced arrest of proliferation. AMPK $\alpha$ 1 was absent, and there was no detectable compensation by AMPK $\alpha$ 2 (Figure 4A). A-769662-dependent ACC phosphorylation was severely blunted in AMPK $\alpha$ 1KO-derived MSCs, confirming the loss of AMPK



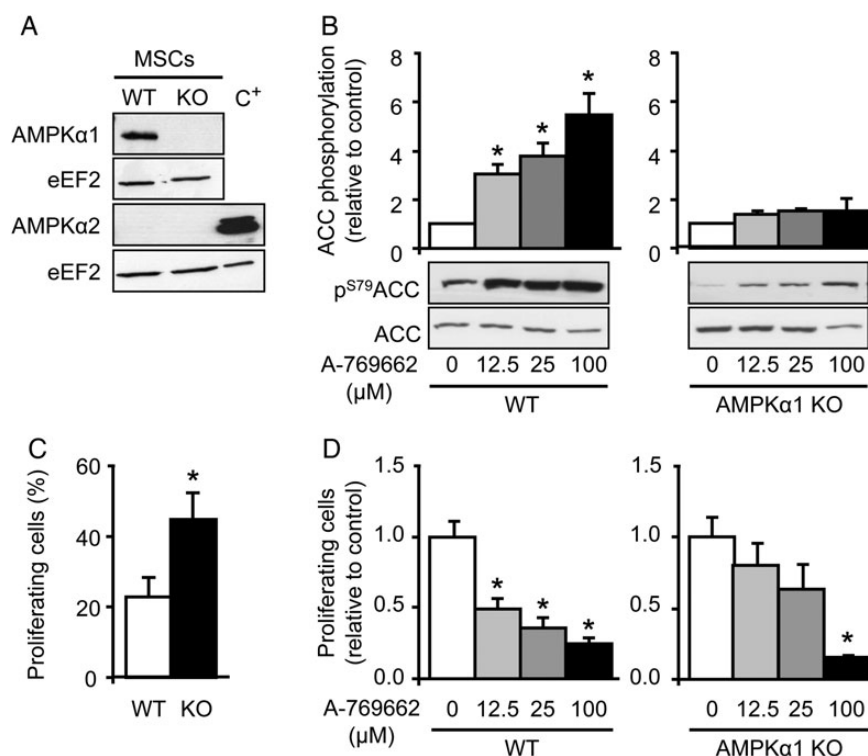
**Figure 3** Effect of A-769662 on MSC proliferation (A), cell cycle (B), Cyclin D1 expression (C), Histone H3 phosphorylation state (D), cell survival (E), and effect of A-769662 washout (F). MSCs were incubated with (solid bar) or without (open bar) A-769662 (100  $\mu$ M). (A) MSCs were incubated in the presence or absence of 1% FBS, and proliferation state was measured after 48 h of treatment. (B) Cell cycle S and G2 phases were evaluated after 48 h of treatment. (C and D) Cyclin D1 mRNA expression and Histone H3 phosphorylation state were quantified after 24 h of incubation. Values are relative to untreated cells. (E) Cell survival was evaluated after 24 or 48 h of treatment. (F) MSCs were incubated with A-769662 for 48 h, and then A-769662 was removed or not. Values are the means  $\pm$  SE of at least three different experiments. \* $P \leq 0.05$ .

activity (Figure 4B). In line with the anti-proliferative action of AMPK, AMPK $\alpha$ 1KO-derived MSCs manifested two-fold greater proliferation capacity than wild-type (WT) MSCs under basal condition (Figure 4C). We incubated both MSC populations with escalating concentrations of A-769662 (Figure 4D). At lower concentrations (12.5 and 25  $\mu$ M), A-769662 inhibited proliferation of WT MSCs, whereas it had no effect in AMPK $\alpha$ 1KO-derived MSCs, confirming the essential involvement of AMPK in A-769662 action. The inhibitory effect of A-769662 at lower concentration was further confirmed by evaluating the distribution of MSCs under the different cycle phases (see Supplementary material online, Figure S5), but was independent of Cyclin D1 whose expression was not modulated (data not shown).

Unexpectedly, A-769662 was still able to block AMPK $\alpha$ 1KO-derived MSC proliferation at higher concentration. This could derived from cell death that occurred in the absence of AMPK (see Supplementary material online, Figure S6) and revealed an AMPK-independent action of the compound at this concentration.

We identified the molecular mechanism by which A-769662-mediated AMPK activation inhibits cell proliferation, by examining known AMPK downstream targets: p70S6K, eEF2, p21, and p27 (Figure 5). p70S6K phosphorylation was suppressed by the highest concentration of A-769662 in WT MSCs, and this inhibition almost completely disappeared in AMPK $\alpha$ 1KO-derived MSCs (WT:  $73 \pm 15$  vs. KO:  $22 \pm 7\%$  of inhibition,  $P \leq 0.05$ ). However, lower A-769662

concentrations did not affect p70S6K phosphorylation in WT MSCs, ruling out p70S6K involvement in the AMPK-specific action of A-769662 on cell proliferation. Similarly, eEF2 phosphorylation (corresponding to its inhibition) occurred in A-769662-treated WT cells and not in AMPK $\alpha$ 1KO-derived MSCs. This AMPK-mediated inhibition was statistically significant at 25 and 100  $\mu$ M, but not at 12.5  $\mu$ M, where arrest of proliferation had already occurred. To definitively exclude a role of p70S6K or eEF2 in A-769662 action, we evaluated the protein synthesis rate. Protein synthesis, although reduced at 100  $\mu$ M, was not impacted by lower A-769662 concentrations (see Supplementary material online, Figure S7). Concerning cell cycle, p21 and p27 expression were enhanced by A-769662 in WT MSCs, and this increase was abolished by AMPK $\alpha$ 1 deletion (Figure 5C and D). However, only p27 expression was increased by low concentrations of A-769662, stimulation of p21 expression being only observed at 100  $\mu$ M. We further confirm the role of p27 by silencing its expression (Figure 5E–G). A reduction of 50% in p27 expression increased the basal proliferation rate of MSCs, demonstrating the role of p27 in this process (Figure 5E and F). Furthermore, the silencing of p27 expression induced a 50% reduction in the inhibitory action of A-769662 on MSC proliferation (Figure 5G and Supplementary material online, Figure S8A). From these results, we can conclude that p27 is, at least partially, involved in the AMPK-specific action of A-769662 on MSC proliferation. It must be noted that the increase in p27 expression induced by AMPK



**Figure 4** Effect of A-769662 treatment on ACC phosphorylation state and on the proliferation of WT and AMPK $\alpha$ 1KO-derived MSCs. (A) Expression of different AMPK $\alpha$  isoforms. Positive controls (C<sup>+</sup>) represented extracts of adult rat cardiomyocytes. (B) WT and AMPK $\alpha$ 1KO-derived MSCs were exposed to different concentrations of A-769662 for 1 h. Cell lysates were then analysed by immunoblotting phosphorylated ACC. Values are relative to respective untreated MSCs. (C) Basal proliferation rate of WT and AMPK $\alpha$ 1KO-derived MSCs. (D) Effect of A-769662 on proliferation capacity of WT and AMPK $\alpha$ 1KO-derived MSCs. Values are relative to respective untreated cells. Values are the means  $\pm$  SE of at least three different experiments. \* $P < 0.05$  vs. respective controls.

resulted in its accumulation in the cytoplasm rather than in the nucleus (see Supplementary material online, Figure S8B).

### 3.4 Effect of AMPK activation on markers of mitochondrial biogenesis

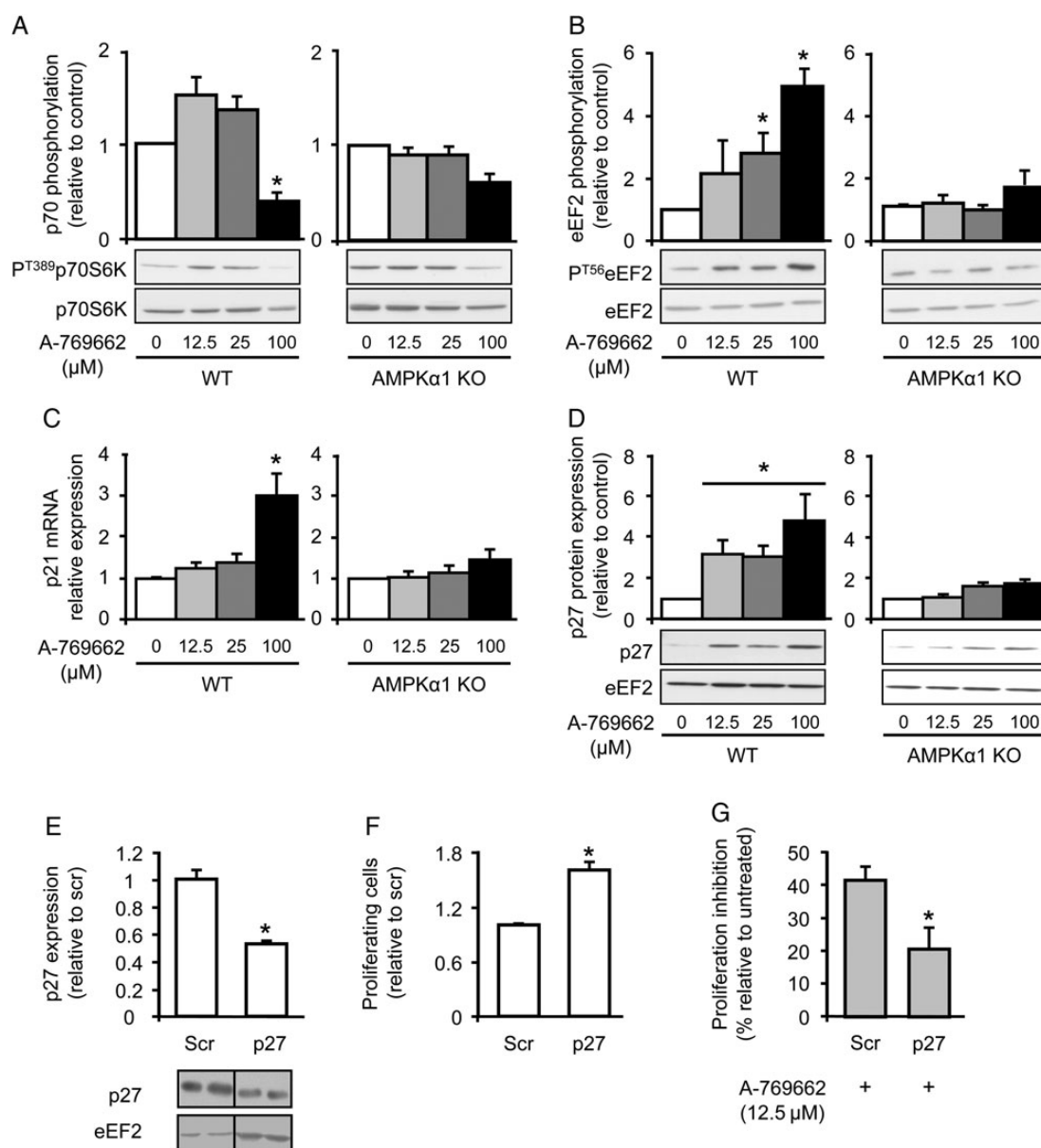
Next, we tested if AMPK activation was sufficient to initiate mitochondrial biogenesis in MSCs (Figure 6). Both low and high concentrations of A-769662 increased PGC1 $\alpha$  expression in WT, but not in AMPK $\alpha$ 1WT-derived MSCs. A similar expression profile was assigned to mitochondrial transcription factor A (mtTFA), a PGC1 $\alpha$  downstream target involved in mitochondrial DNA replication. However, a 5-day incubation with A-769662 is not sufficient to increase the mitochondrial OCR of MSCs (Figure 6C and Supplementary material online, Figure S9).

## 4. Discussion

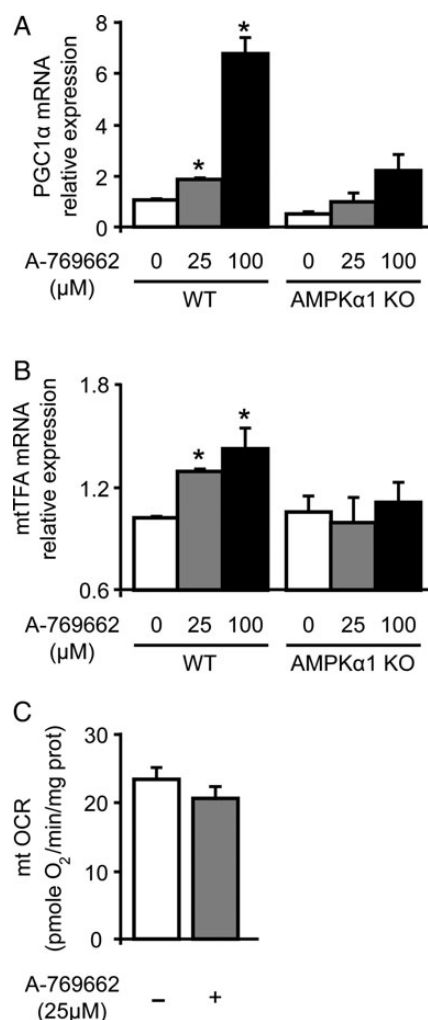
We investigated: (i) the role of AMPK in the regulation of MSC metabolism under hypoxic conditions and (ii) the impact of AMPK activation on MSC proliferation capacity and mitochondrial biogenesis.

### 4.1 Comparison between MSCs and cardiomyocytes in terms of AMPK isoform expression and pharmacological activation

Whereas cardiomyocytes express the catalytic isoforms AMPK $\alpha$ 1/ $\alpha$ 2, MSCs exclusively express AMPK $\alpha$ 1. Knowing that AMPK $\alpha$ 2 is important for the control of cardiac metabolism under ischaemia,<sup>9</sup> the shift from



**Figure 5** Effect of A-769662 treatment on p70S6K (A) and eEF2 (B) phosphorylation state, p21 mRNA expression (C) and p27 expression (D) in WT and AMPK $\alpha$ 1KO-derived MSCs, and effect of siRNA-mediated p27 silencing on its expression (E) and on MSC proliferation under basal (F) or A-769662-treated conditions (G). Values are relative to respective untreated cells (A–D, G) or cells transfected by control siRNA (scramble, Scr) (E and F) and are the means  $\pm$  SE of at least three different experiments. \* $P$  < 0.05 vs. respective controls.



**Figure 6** Effect of A-769662 treatment on PGC1α (A) and mtTFA (B) expression and on mitochondrial (mt) OCR (C). Cells were treated with A-769662 during 24 h (A and B) or 5 days (C). For A and B, values are the means  $\pm$  SE of at least three different experiments. \* $P < 0.05$ . For C, values are shown as means  $\pm$  SE of eight samples and are representative of three independent experiments.

AMPKα1 to AMPKα2 could be a critical step in the differentiation process of MSCs to (pre)cardiomyocytes.

In MSCs, AMPK can be easily activated through a  $\text{Ca}^{2+}$ -dependent pathway. This was expected knowing that AMPKα1 is particularly sensitive to this pathway.<sup>29</sup> In contrast, mitochondrial inhibitors, oligomycin and phenformin, that activate AMPK via an increased AMP/ATP ratio did not induce AMPK activation despite the presence of LKB1. This correlated with the absence of changes in the AMP/ATP ratio, which could be linked to the low activity of immature MSC mitochondria.<sup>30</sup> However, the AMP-mediated/LKB1-dependent pathway is functional, because glucose deprivation was able to activate AMPK. Finally, we showed that A-769662 efficiently activated AMPK in MSCs. A-769662 is of particular interest, because: (i) it directly binds to AMPK in contrast to other agents that act indirectly via AMP or  $\text{Ca}^{2+}$  and (ii) its action on AMPK persists for at least 2 days.

## 4.2 MSCs are resistant to hypoxia independently of AMPK

AMPK controls cardiac metabolism and participates in cardiomyocyte survival under ischaemia/hypoxia.<sup>6</sup> In contrast, our study revealed that AMPK does not play a role in the regulation of energy homeostasis in hypoxic MSCs. Indeed, chronic hypoxia did not activate AMPK in these cells. The absence of AMPK activation was linked to the lack of ATP decline and AMP accumulation. The metabolic resistance of MSCs to hypoxia indicates their independence from oxygen. This was confirmed by their efficient cell survival in the absence of oxygen, even if AMPK was not activated. Our results are in agreement with a recent study by Deschepper et al.,<sup>31</sup> comparing hypoxia in the presence and absence of glucose. They demonstrated that MSC death may occur under very long-term hypoxia exposure due to progressive glucose deficiency and not hypoxia *per se*. To understand the peculiar resistance of MSCs to hypoxia, we compared their metabolism with that of cardiomyocytes that typically shift from exclusive oxidative to glycolytic metabolism under hypoxia. The augmented production of lactate by MSCs under normoxia reveals that they are mainly glycolytic, producing energy independently of mitochondria and oxygen. Similar glycolytic status, which can be explained by the absence of mature mitochondria, and resistance to hypoxia have been also found in inducible pluripotent, embryonic, and/or neural progenitor stem cells.<sup>15,32–34</sup> It can be linked to the Warburg effect observed originally in cancer cells, where enhanced glycolysis is required to support cell proliferation.<sup>35</sup> MSC glycolysis is further increased under hypoxia via the up-regulated expression of Glut1 and iPFK2. In terms of cardiac cell therapy efficacy, the glycolytic characteristic of MSCs makes them resistant to transplantation in hypoxic areas independently of AMPK. However, once differentiated into oxidative (pre)cardiomyocytes, they should become sensitive to hypoxia. In this context, AMPK activation should induce protection, as for adult cardiomyocytes.

## 4.3 Sustained AMPK activation inhibits MSC proliferation

We showed that AMPK activation by A-769662 promoted MSC proliferation arrest without affecting viability. A-769662 blocked DNA synthesis and decreased the percentage of cells in the S/G2 phases. Cell cycle inhibition by the non-specific AMPK activators, AICAR and metformin, was discerned previously in various cell types, such as glioma,<sup>17</sup> neuronal stem,<sup>36</sup> and acute myeloid leukaemia cells.<sup>37</sup> Inversely, the expression of constitutively active AMPK reduced the growth of embryonic fibroblasts.<sup>16</sup> To the best of our knowledge, we are the first to demonstrate the anti-proliferative capacity of A-769662 on stem cells. By charting dose–response curves and studying AMPK-deleted MSCs, we also demonstrated that neither p70S6K/eEF2 nor p21 was involved in the AMPK-dependent anti-proliferative action of A-769662. In contrast, A-769662, at lower concentrations, increased p27 expression and inhibited cell proliferation, both disappearing in AMPKα1KO-derived MSCs. Experiments performing p27 knock-down confirmed the role of this protein in the AMPK-mediated MSC cycle arrest. The link between AMPK, p27, and proliferation has been already investigated.<sup>38–40</sup> The AMPK phosphorylation of p27 increases its stability and induces its accumulation in the cytoplasm.<sup>38</sup> This promotes autophagy that can be directly linked to cell proliferation inhibition.<sup>39,40</sup> Knowing that p27 accumulates in the cytoplasm of A-769662-treated

MSCs, we could speculate that a similar autophagic process occurred in our model.

As a future prospect and as shown previously for the differentiation of intestinal epithelial cells,<sup>19</sup> it would be interesting to see if such inhibition of proliferation favours MSC differentiation into cardiomyocytes in the presence of a differentiation cocktail. Our results are reinforced by a recent study disclosing that AMPK fosters the differentiation of endothelial progenitor cells.<sup>41</sup>

#### 4.4 A-769662 increases the expression of components of mitochondrial biogenesis

The present work also showed that A-769662 led to an AMPK-dependent increase in PGC1 $\alpha$  and mtTFA expression. Both are major elements of mitochondrial biogenesis, which could participate in the maturation of immature mitochondria necessary for transition from glycolytic to oxidative metabolism characteristic of MSCs differentiated into cardiomyocytes. However, we showed that AMPK activation alone is not able to shift MSC from glycolytic to oxidative metabolism, the presence of a complete differentiation cocktail being probably necessary.

### 5. Conclusions

Here, we report that: (i) undifferentiated MSCs, characterized by glycolytic metabolism, are particularly resistant to hypoxia independently of AMPK; (ii) pharmacological activation of AMPK by A-769662 reduces MSC proliferation via the regulation of p27 expression; and (iii) A-769662 treatment increases mitochondrial biogenesis via an AMPK-dependent pathway.

### Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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