Hypoxia upregulates Malat1 expression through a CaMKK/AMPK/HIF-1α axis

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Abstract. Increased expression levels of the long non-coding RNA metastasis-associated lung adenocarcinoma transcript 1 (Malat1) have been associated with enhanced proliferation and metastasis of several cancer cell types. Hypoxia, a hallmark characteristic of solid tumors, has been linked to an increase in the activity of the ATP-generating AMPK protein. Since Malat1 was recently shown to be upregulated during hypoxia, the objective of this study was to determine the contribution of AMPK in the mechanistic pathways regulating Malat1 expression in low oxygen conditions. Compared to those cultured in 21% O_2 conditions, HeLa cells incubated in 1.5% O_2 expressed more Malat1 transcripts. This observation was mimicked in HEK293T cells using a synthetic reporter construct containing 5.6 kb of the human Malat1 promoter, suggesting that hypoxia directly impacted Malat1 gene transcription. Interestingly, pharmacological stimulation of AMPK increased Malat1 promoter transactivation in 21% O_2 conditions, whereas inhibition of either AMPK or its upstream activator CaMKK completely abolished the augmentation of Malat1 under hypoxia. Pharmacological modulation of LKB1, another major regulator of AMPK, had no impact on Malat1 promoter transactivation, suggesting that calcium inputs are important in the control of Malat1 expression by AMPK. Overexpression of hypoxia-inducible factor-1α (HIF-1α) increased Malat1 expression in 21% O_2 conditions, whereas pharmacological inhibition of HIF-1α blocked the impact of hypoxia on the Malat1 promoter. Taken together, these findings strongly suggest that Malat1 expression is regulated in hypoxic conditions by a CaMKK/AMPK/HIF-1α axis. More research is needed in physiological settings to test the clinical relevance of this pathway.

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Introduction

During cancer development, cell proliferation rates exceed blood vessel formation, leading to hypoxia in solid tumor microenvironment, which is a hallmark of highly proliferative tumor cells (1). Proliferating cancer cells proliferate using 'the Warburg effect', which consists in increased glucose uptake and metabolism through anaerobic glycolysis instead of oxidative phosphorylation (2), a process in part controlled by the AMP-activated protein kinase (AMPK), a master regulator of cellular energy pools (3). In addition, AMPK is tightly linked to cancer by its ability to induce the phosphorylation of the tumor suppressor p53, leading to DNA synthesis inhibition (4) and cell cycle arrest through inhibition of the mammalian target of rapamycin (mTOR) (5).

Hypoxic exposure leads to an increase in hypoxia-inducible factor 1α (HIF-1α), a transcription factor essential in cell adaptation/survival (6). Whereas the HIF-1α protein is rapidly degraded under normal (21%) oxygen conditions, hypoxia increases HIF-1α levels and transcriptional activity, which then promotes the expression of a number of genes necessary for cancer cell survival (7).

Metastasis-associated lung adenocarcinoma transcript 1 (Malat1), also named nuclear-enriched abundant transcript 2, is a conserved long non-coding RNA (lncRNA) ubiquitously expressed (8). High levels of Malat1 were initially associated to the severity of lung metastasis (8), but this observation has since been extended to many other types of tumors (9-13). Malat1 appears to stimulate cell proliferation at the expense of differentiation and senescence (13-15). Malat1$^{-/-}$ mouse xenografts show a nearly 80% lower tumor development *in vivo* (10), possibly through modification of serine/arginine splicing factors (16).

Genetic loss of Malat1 does not affect mouse viability (17); however, Malat1 has been suggested to modulate angiogenesis *in vivo* (18). Interestingly, hypoxia upregulates Malat1 *in vitro* (18,19), and mice directly exposed to hypoxia also show increased Malat1 expression levels in specific tissues such as proximal tubules (19). Based on these findings, it is thus likely that the increase in Malat1 expression is part of an adaptive response to hypoxia. The aim of this study was thus to mechanistically investigate the pathways that contribute to the transcriptional stimulation in Malat1 levels in cells under hypoxia. Our results show that AMPK, through its upstream

calcium/calmodulin-dependent protein kinase kinase (CaMKK), is a major node triggering Malat1 transcription upon hypoxia in a HIF-1α-dependent manner.

Materials and methods

Cell culture and reagents. HeLa and HEK293T cells were from ATCC (Manassas, VA, USA). Cells were grown in DMEM containing 1 g/l glucose, 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin. Compounds (Sigma-Aldrich, Oakville, ON, USA) were suspended in the appropriate vehicle (DMSO or medium without FBS). A pCDNA3 expression plasmid containing the human HIF-1α cDNA was purchased from Addgene (Cambridge, MA, USA).

Oxygen conditions. Hypoxic conditions were maintained in a humidified variable aerobic workstation (Coy Laboratory) at 37˚C. To induce hypoxia, oxygen concentrations were reduced from 21 to 1.5%, while carbon dioxide remained at 5%. Oxygen sensor continuously monitored and adjusted the oxygen level during experiments.

Cloning of the human Malat1 promoter. The Malat1 promoter was amplified from human genomic DNA by PCR using primers 5'-TGTGGGAGCTTTTCAGTATTC-3' and 5'-CTGG AATGGCCAGCCTATAA-3', effectively resulting in a fragment containing a sequence of 5.6 kb directly upstream of the Malat1 gene initiation site. The fragment was first subcloned in TOPO®XL vector (Thermofisher) according to the manufacturer's instructions, and then cloned in *Kpn*I/*Xho*I-digested pGL3 luciferase reporter vector (Promega). Validity of this construct was confirmed by sequencing.

Gene reporter assays. HEK293T cells were seeded in 24-well plates at 80% confluence. Six hours later, cells were transfected for 12 h with 250 ng of the reporter vector (hMALAT1_ prom-pGL3 or p(HA)HIF-1α-pCDNA3) and 50 ng of a β-galactosidase expression vector as described previously (20). Transfected cells were FBS-starved for 2 h before any pharmacological treatment. Luciferase activity and β-galactosidase activity were measured as described previously (20) using a Luminoskan™ Ascent microplate luminometer or a Multiskan Spectrum (Thermo Scientific), respectively. Luciferase activity levels were normalized against β-galactosidase activity levels. The figures represent the mean fold activation ± SEM of at least three independent gene reporter experiments.

RNA isolation and quantitative PCR. Whole cell RNA extracts were prepared as recommended by the manufacturer (GE Healthcare). DNA reverse transcription was prepared with 0.5 μg of total RNA using qScript™ cDNA Synthesis kit (Quanta Biosciences). Quantitative PCR were performed on a 7900HT Applied Biosystems, using Sybr™-Green detection (Sigma-Aldrich), normalized to a housekeeping gene. The following nucleotide pairs were used to amplify Malat1: forward, GTAATGGAAAGTAAAGCCCTGAAC and reverse, CCCCGGAACTTTTAAAATACCTCT.

Western blot analysis. Whole cell proteins were extracted by lysis with an extraction buffer containing NP40 0.04%, Tween 0.02%, sodium orthovanadate 1.5 mM and 10% protease inhibitors (Roche), and incubated 10 min on ice. After centrifugation, the supernatant was collected and considered as whole cell extract. Protein concentrations were determined with DC™ Protein Assay Reagent (Bio-Rad). Proteins (50 μg) per lane were loaded onto a 7.5% SDS-polyacrylamide gel, and then blotted with Trans-Blot® Turbo™ (Bio-Rad) on PVDF membranes. Membranes were saturated in fat-free dry milk for 1 h, and then incubated with primary antibodies overnight at 4˚C in recommended buffer at recommended dilutions (anti-HIF-1α from R&D, anti-phospho-Thr172 AMPK and anti-AMPK total from Cell Signaling, anti-β-actin from Millipore). Membranes were then incubated with secondary antibodies coupled with HRP for 1 h (from Santa-Cruz for anti-goat-HRP, from GE Healthcare for anti-mouse-HRP and anti-rabbit-HRP). Signals were detected with ECL™ Western Blotting Detection Reagents (GE Healthcare) on Kodak film.

Statistical analyses. Data are presented as mean ± SEM of at least three independent experiments performed in triplicate. Data were analyzed by one or two-way ANOVA as appropriate. A value of $p<0.05$ was considered statistically significant.

Results

Hypoxia induces Malat1 expression through AMPK. In adenocarcinoma HeLa cells, hypoxia (1.5% O_2) induced a time-dependent increase in Malat1 RNA levels (Fig. 1A). To determine that this effect was due to an impact on Malat1 gene transcription, this experiment was repeated in HEK293T cells transfected with a construct containing 5.6 kb of the human Malat1 promoter cloned upstream of the luciferase gene. In this setting, hypoxia stimulated Malat1 promoter transactivation to a similar time-dependent extent, resulting in 2- and 4-fold increases after 24 and 48 h of incubation in low oxygen conditions, respectively (Fig. 1B). This suggested a direct impact of hypoxia on the Malat1 promoter.

Remarkably, the induction of the Malat1 promoter by hypoxia was completely blocked by compound C (Fig. 1C), an ATP-competitive inhibitor of AMPK (21), suggesting that AMPK mediates the effect of 1.5% O_2 conditions on Malat1 expression. Consistent with this concept, pharmacological activation of AMPK with the 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) was sufficient to stimulate Malat1 promoter transactivation in normal oxygen conditions (21% O_2) (Fig. 1D). This effect was preventable by co-treatment with compound C (Fig. 1D). Interestingly, the anti-diabetic drug metformin, an indirect AMPK activator shown to lower carcinogenesis (22), also induced the activation of the Malat1 promoter, albeit to a lower extent (Fig. 1E). Taken together, these findings indicate that hypoxia stimulates Malat1 expression through the activation of AMPK.

Inhibition of CaMKK blocks hypoxia-induced Malat1 promoter transactivation. The activity of AMPK is mainly regulated by two upstream kinases, namely the calcium-dependent kinase CaMKK and LKB1, itself activated by EPAC (23,24). In 21% O_2 conditions, treatment of HEK293T cells with the specific EPAC activator 8-CTP-2me-cAMP (25) did not result in the expected increase in Malat1 promoter

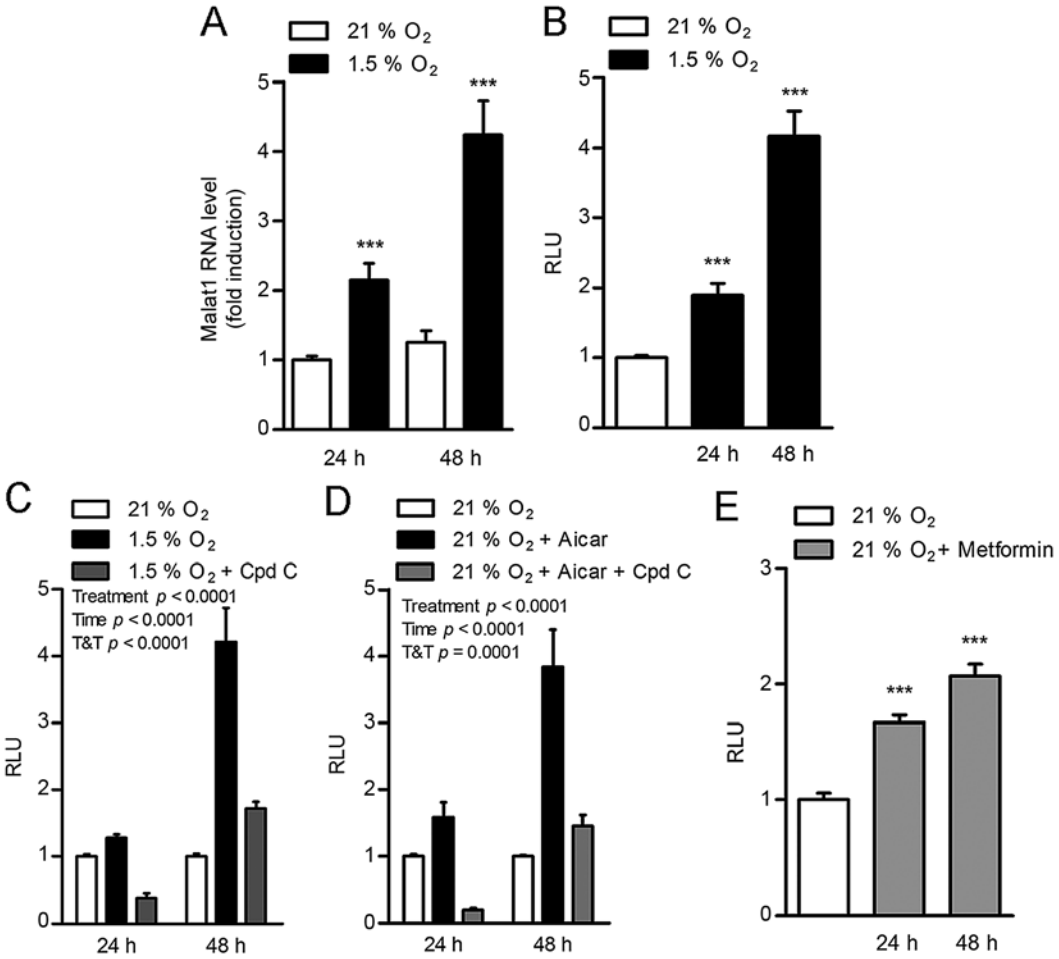

Figure 1. Hypoxia induces Malat1 expression through AMPK. (A) HeLa cells were exposed to normoxic (21% O_2) or hypoxic (1.5% O_2) conditions for 24 and 48 h. Malat1 gene expression levels were normalized against those of L27 expression levels. n=3, ***p<0.0001. (B) HEK293T cells were transfected with a luciferase-reporter gene construct containing 5.6 kb of the human Malat1 promoter and co-transfected with an expressing vector for β-galactosidase to correct for transfection efficiency. Cells were incubated under normoxic (21% O_2) or hypoxic (1.5% O_2) conditions for 24 and 48 h. n=3, ***p<0.0001. (C) HEK293T cells, transfected as above, were incubated with compound C (10 μM) 30 min before exposure to hypoxia. Luciferase activity was determined as above. n=3. (D) HEK293T cells transfected as above and incubated in normoxic conditions were treated with 1 mM AICAR, with or without compound C. Luciferase activity was determined as above. n=3. (E) HEK293T cells were transfected as above and incubated in normoxic conditions with 5 mM Metformin. Luciferase activity was determined as above. n=3, ***p<0.0001.

transactivation (Fig. 2A). In contrast, incubation with the pharmacological CaMKK inhibitor STO-609 (26) completely prevented the induction of the Malat1 promoter under hypoxia (Fig. 2B). These findings suggest that hypoxia induces the CaMKK/AMPK cascade to stimulate Malat1 expression.

Hypoxia induces Malat1 via the induction of HIF-1α. Analysis of protein extracts from cells incubated under 21% and 1.5% O_2 conditions indicated that hypoxia induced the phosphorylation of AMPK at its Thr-172 residue within 60 min (Fig. 3A). In the same conditions, an increase in HIF-1α was observed within 120 min under hypoxia (Fig. 3A). This suggests that the stimulation of HIF-1α protein levels occurs after AMPK-activating events. Supporting this hypothesis, the hypoxia-induced upregulation of HIF-1α levels was blocked in cells incubated with the CaMKK inhibitor STO-609 (Fig. 3B). This further indicates that the increase in HIF-1α levels is downstream of the CaMKK/AMPK complex.

To investigate the possibility of a direct impact of HIF-1α in the hypoxia-induced stimulation of Malat1 expression,

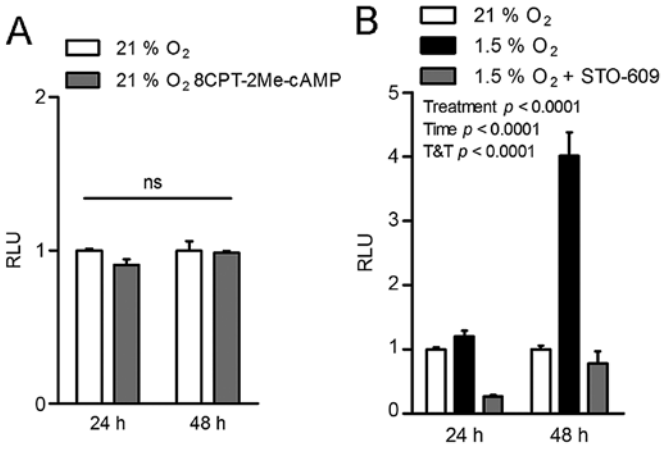

Figure 2. Inhibition of CaMKK blocks hypoxia-induced Malat1 promoter transactivation. (A) HEK293T cells were transfected as above and incubated in normoxic conditions with the selective EPAC activator, 8-pCPT-2'-O-Me-cAMP (30 μM). Luciferase activity was determined as above. n=2. (B) HEK293T were transfected as above and treated with STO-609 (25 μM) 30 min before exposure to hypoxia. Luciferase activity was determined as above. n=3.

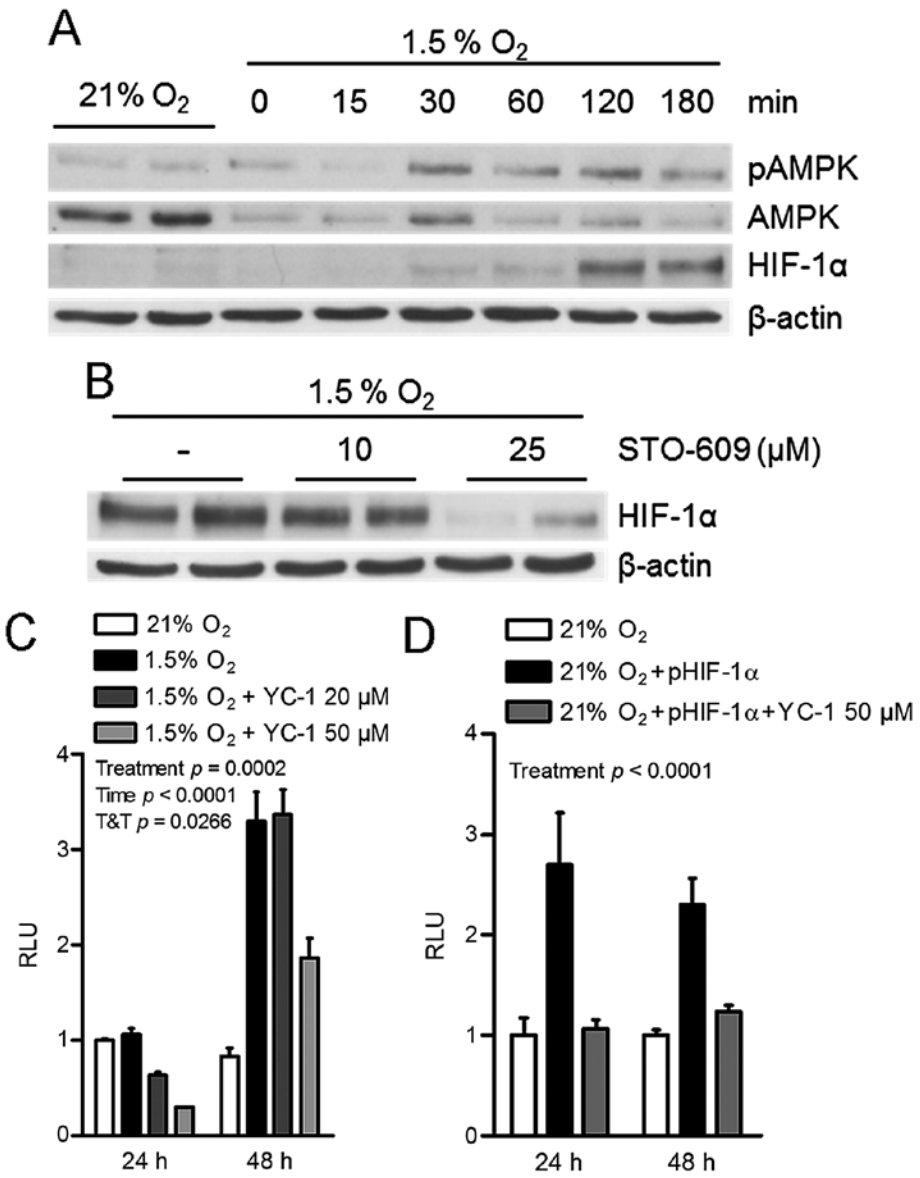

Figure 3. Hypoxia induces Malat1 via the induction of HIF-1α. (A) HEK293T cells were incubated in normal (21%) or low (1.5%) oxygen conditions. Cells were harvested at the indicated time-points. Protein extracts were submitted to western blotting. The pAMPK-Ab recognizes modifications at the Thr172 site. β-actin serves as loading control. Representative blot of three experiments done in duplicate. (B) HEK293T were incubated in 1.5% oxygen and treated with increasing doses of STO-609. β-actin serves as loading control. Representative blot of two experiments done in duplicate. (C) HEK293T cells were transfected with a luciferase-reporter gene construct containing 5.6 kb of the human Malat1 promoter and co-transfected with an expressing vector for β-galactosidase to correct for transfection efficiency. Cells were incubated under normoxic (21% O_2) or hypoxic (1.5% O_2) conditions for 24 and 48 h and co-treated with the HIF-1α inhibitor YC-1 (20-50 μM). n=3. (D) HEK293T cells were transfected with a luciferase-reporter gene and β-galactosidase constructs as above, co-transfected with an expression plasmid for HIF-1α or empty plasmid as control, and co-treated with YC-1 (50 μM) or vehicle. Luciferase activity was determined as above. n=3.

HEK293T cells containing the 5.6-kb human Malat1 promoter reporter construct were treated with YC-1, a pharmacological inhibitor of HIF-1α activity (27). Whereas hypoxia increased Malat1 promoter transactivation in control cells as expected, this effect was dose-dependently attenuated in cells treated with YC-1 (Fig. 3C). Consistent with this finding, transient overexpression of HIF-1α under 21% O_2 conditions was sufficient to transactivate the Malat1 promoter (Fig. 3D). This effect was completely abolished by co-treatment with YC-1 (Fig. 3D).

Discussion

The lncRNA Malat1 appears ubiquitously present in cells during nonpathogenic conditions (8), however, several studies have reported its high levels in many cancer types. Yet, the metabolic pathways regulating its transcription in these conditions remain elusive. This study focused on hypoxia, a powerful physiologic input in solid tumors. This study strongly suggests that hypoxia triggers a robust increase in Malat1 expression through the enhanced activity of the CaMKK/AMPK/HIF-1α axis.

Although most studies on the stimulating impact of Malat1 on cancer cell proliferation and migration have been obtained in normoxic conditions (18), upregulation of Malat1 expression during hypoxia has been recently observed *in vitro* and *in vivo* (19). Our study confirmed that hypoxia is an initial signal leading to increased Malat1 expression level (Fig. 1). Oxygen deprivation triggers several cellular processes required for survival, including modulation of energy sensors such as

AMPK (3). Indeed, in hypoxic environments, AMPK is phosphorylated (28), triggering the activation of its downstream effector acetyl coenzyme A carboxylases 1 and 2 (ACC1/2). Such adaptive phenomenon is not observed in AMPK-null mouse embryo fibroblasts (MEFs) (28), highlighting the importance of AMPK in the regulation of energy upon hypoxia. This study also found that AMPK phosphorylation occurs early after oxygen deprivation, and that this event is necessary for a full augmentation in Malat1 expression (Fig. 1). Thus, it is likely that Malat1 overexpression is part of the global adaptive response to low oxygen conditions.

The upstream mechanisms leading to hypoxia-driven AMPK activation are unclear. In response to low energy status, AMPK activation has been linked to phosphorylation of LKB1, a serine/threonine kinase also associated with tumor suppression (29). However, incubation of cells with the LKB1 upstream kinase activator 8-CTP-2me-cAMP suggests that LKB1 does not robustly modify Malat1 expression in our model (Fig. 2A), which is consistent with the absence of impact of PKA activators on the same system (data not shown).

More recently, other studies indicated that CaMKK may play an important role in hypoxia-induced AMPK stimulation (30). Indeed, knockdown of LKB1 in MEFs cultured in hypoxic conditions had no impact on ACC1/2 phosphorylation, suggesting that LKB1 is not the upstream kinase leading to AMPK activation under hypoxia (30). In contrast, knockdown of CaMKK in the same system clearly diminished AMPK and ACC1/2 phosphorylation status (30). This is in agreement with the fact that hypoxia increases intracellular calcium concentrations (31), which has been recently shown to be regulated by the effects of STIM1-mediated store-operated calcium entry (32). Our study is consistent with these studies, since the specific CaMKK inhibitor STO-609 abolished the activation of Malat1 under hypoxia (Fig. 2). The possible control of Malat1 by STIM1 remains to be investigated.

Calcium plays major functions in the regulation of gene expression. Notably, calcium chelation modulates HIF-1α activity (33-35). Moreover, calcium entry rapidly stimulates CaMKK-induced p300 phosphorylation, which stabilizes HIF-1α (32,36). Our study also corroborates that hypoxia-induced HIF-1α protein stabilization is under CaMKK regulation, as it occurred after AMPK phosphorylation (Fig. 3), and that cells treated with STO-609 did not show high levels of HIF-1α under hypoxic conditions (Fig. 3). More importantly, this study shows that Malat1 overexpression upon hypoxia is dependent of HIF-1α, and that an increase in HIF-1α levels is sufficient to stimulate Malat1 transcription to a similar extent as did hypoxia (Fig. 3). Further supporting the large contribution of HIF-1α in the increase in Malat1 in low oxygen conditions, loss of HIF-1α activity by YC-1 treatment completely blocked hypoxia-induced Malat1 transcription. Interestingly, bioinformatics analysis indicated four putative HIF-1α binding sites, corresponding to the consensus hypoxia response element ([A/G]CGTG), within 5.6 kb of the human Malat1 promoter, at positions -2246, -1687, -1317, and -259 from the initiation start of the Malat1 coding sequence. The relative importance of each of these binding sites in the transactivation of the Malat1 promoter by HIF-1α remains to be tested.

Interestingly, a recent study reported a possible involvement of p53 as a transcriptional repressor of Malat1 expression in early stage of hematopoietic cell proliferation (37). It is established that oxygen deprivation modulates the levels and activity of p53, a major transcription regulator of cellular fate under intensive stress (38). Depending on oxygen availability (39), it is thus possible that p53 influenced the action of HIF-1α in this study, since reciprocal transcriptional effects by HIF-1α and p53 have been reported (1,40). At the molecular level, this is a likely event as they share and compete for the nuclear cofactor p300 (41).

In conclusion, this study was conducted to understand the mechanisms regulating Malat1 expression levels upon hypoxia, an important characteristic of cancer tissues. This study indicates that the enhanced transcription of the Malat1 gene upon low oxygen conditions is under the control of HIF-1α, itself regulated by the activation of the CaMKK/AMPK complex, a master regulator of cellular energy. Our results also support and extend published literature that calcium influx is an early signal in the adaptive response to hypoxic stress. Finally, since Malat1 induces angiogenesis in cancer (18,42), it would be of interest to determine its role in physiological processes in which increased tissue mass is associated with high demand for oxygen and energy substrates, such as exercise-induced myogenesis, or cold-induced brown adipose tissue hyperplasia.

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