Exogenous activation of LKB1/AMPK signaling induces G_1 arrest in cells with endogenous LKB1 expression

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Abstract. The tumor suppressor protein LKB1 is a serine/threonine kinase that plays a critical role in cell proliferation, and its inactivation has been linked to tumorigenesis in various cancer types. Current understanding of the LKB1 function is largely restricted to results from experiments on LKB1-deficient cancer cells, while the regulation and activity of endogenous LKB1 has been rarely investigated. In a previous study, we showed that LKB1 knockdown in two healthy cell lines accelerates cell cycle progression through the G_1/S checkpoint by inhibition of the p53 and p16 pathways. In the present study, we examined the effects of overexpression of LKB1 on two healthy and one cancer cell line. Administration of exogenous LKB1 activated LKB1/AMPK signaling and arrested the cell cycle at the G_1 phase in an LKB1-dependent manner. G_1 arrest induced by LKB1 was accompanied by the downregulation of cyclin D1 and cyclin D3, and the upregulation of p53, p21 and p16, while no differences were detected for CDK4, CDK6, cyclin E, p15 and p27. These results indicated that exogenous activation of LKB1/AMPK signaling inhibits the G_1/S cell cycle transition, even in cells with an endogenous expression of LKB1. Findings of the present study extend earlier observations on LKB1-inactivated neoplastic cells and provide novel insights into the growth-inhibitory effects of LKB1.

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Abbreviations: PJS, Peutz-Jeghers syndrome; HEK-293T cells, human embryonic kidney 293T cells; HUVECs, human umbilical vein endothelial cells; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; AMPK, AMP-activated protein kinase; CaMKK, Ca^{2+}/CaM-dependent protein kinase kinase

Key words: LKB1, tumor suppressor, Peutz-Jeghers syndrome, cell cycle, p53, p21, p16, cyclin D1, cyclin D3

Introduction

The serine/threonine kinase LKB1 is causally linked to the Peutz-Jeghers syndrome (PJS), which in turn increases the risk of developing cancer; LKB1 is one of the most commonly mutated genes associated with multiple sporadic cancer types (1,2). LKB1 is ubiquitously expressed in healthy adult and embryonic tissues (3,4). Studies in the past 14 years have established LKB1 as a critical tumor suppressor protein, regulating cell proliferation and survival (5,6). Currently, LKB1 is unanimously considered a central player of an important signaling network affecting numerous cell processes, whose deregulation contributes to pathologies such as PJS and carcinomas, although the underlying mechanisms remain elusive (7,8).

Among the various molecular mechanisms altered in human neoplasias, those involving control of the cell division cycle are believed to be fundamental for carcinogenesis (9). Modulation of the expression and the function of cell cycle regulatory proteins greatly contributes to growth inhibition (10). A number of studies have provided compelling evidence that reintroduction of LKB1 into human cancer cells with impaired LKB1 activity results in reduced growth by inhibiting the G_1/S transition (11-13). However, it is noteworthy that most of these experiments were performed in cancer cells with an LKB1-null or -deficient genetic background, and the regulation and activity of endogenous LKB1 were rarely investigated, considerably limiting our understanding of LKB1 function.

A previous study by our group demonstrated that LKB1 knockdown accelerates the G_1/S transition through the p53 and p16 pathways in two healthy cell lines, suggesting that abolishment of LKB1 expression in healthy cells contributes to the formation of malignancies (14). It is crucial to determine whether the enhanced expression of LKB1 can inhibit proliferation of cells with endogenous LKB1 expression. In the present study, we examined the effects of enhanced LKB1 expression on the cell cycle profile via transfection of cells with exogenous LKB1 and investigated the underlying mechanisms in two healthy cell lines, the human embryonic kidney 293T (HEK-293T) and the human umbilical vein endothelial cells (HUVECs), and in one human hepatocellular carcinoma cell line, HepG2. Our results reveal that an enhanced expression of LKB1 activates LKB1/AMPK signaling and confers

an observable G_1 arrest phenotype in cells with endogenous LKB1 activity.

Materials and methods

Materials. Cell culture media, supplements and the Lipofectamine 2000 reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Primary antibodies targeting AMP-activated protein kinase (AMPK), phospho-AMPK (Thr172), acetyl-coenzyme A carboxylase (ACC), phospho-ACC (Ser79), retinoblastoma protein (Rb), phospho-Rb (Ser807/811), cyclin-dependent kinase (CDK) 4, CDK6, cyclin D1, cyclin D3, cyclin E, p21, p27 and p15 were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies targeting LKB1, p53, p19, p16 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Unless otherwise indicated, chemicals and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of the highest grade.

Cell culture and transfection. Culturing of HEK-293T cells and HUVECs was performed as previously described (14). HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM). The cells were maintained in the presence of 10% fetal bovine serum, without antibiotics, at 37˚C in a humidified atmosphere containing 5% CO_2. Cultured cells from passages 3-10 were used. Transfection was performed with indicated plasmids using the Lipofectamine 2000 reagent, following the manufacturer's protocols.

Vector constructs. A plasmid for the eukaryotic expression of enhanced green fluorescent protein (EGFP) bearing a wild-type copy of the *LKB1* gene (LKB1-WT) and an EGFP empty plasmid were kindly provided by Dr Junying Yuan (Harvard Medical School). An LKB1 kinase-deficient EGFP plasmid (LKB1-K78M) was generated from LKB1-WT by converting the lysine residue at codon 78 into methionine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the primers: forward, 5'-CATAAGCTTGCCACCATGGAG GTGGTGGACCCGCA-3' and reverse, 5'-AATTCTAGA TCACTGCTGCTTGCAGGCCGAC-3'. Constructs were confirmed by sequencing in both directions.

Flow cytometric analysis. Exponentially growing cells were transfected with indicated plasmids for 48 h, detached and fixed with 80% ethanol overnight. Propidium iodide/RNase A (Sigma-Aldrich) was used to stain the nuclei. Cell cycle distribution was determined by flow cytometry using a FACSVantage SE cell sorter, and the percentage of cells at different phases of the cell cycle was analyzed using the CellQuest™ Pro software (both from BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Cells were harvested and lysed as previously described (14). The protein concentration was determined by the BCA method. Equal amounts of protein lysates were separated by SDS-PAGE electrophoresis on 8, 10, 12 or 15% gels and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). Protein loading was normalized with the housekeeping gene *GAPDH*. Membranes were blocked with 5% non-fat milk and incubated with specific primary antibodies targeting LKB1, AMPK, phospho-AMPK, ACC, phospho-ACC, Rb, phospho-Rb, CDK4, CDK6, cyclin D1, cyclin D3, cyclin E, p15, p16, p19, p21, p27, p53 and GAPDH. Detection of HRP on immunoblots was performed using the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL, USA). The protein signals were quantified using the Quantity One software (Bio-Rad, Hercules, CA, USA).

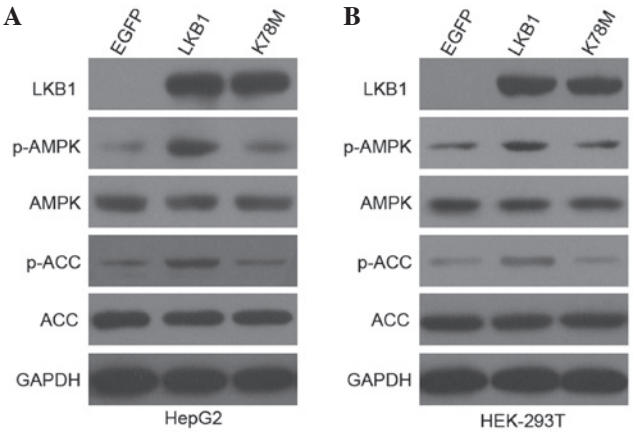

Figure 1. Plasmid-mediated expression of recombinant LKB1 activates LKB1/AMPK signaling in an LKB1 kinase-dependent manner. (A) Hepatocellular carcinoma HepG2 and (B) healthy HEK-293T cells were transfected with LKB1 wild-type and kinase-deficient constructs for 48 h. An LKB1-empty EGFP vector was also included. EGFP-tagged LKB1, total and phosphorylated AMPKα, total and phosphorylated ACC expression were analyzed by western blotting. GAPDH was used as a loading control. AMPK, AMP-activated protein kinase; HEK-293T, human embryonic kidney 293T; ACC, acetyl-coenzyme A carboxylase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Results

Enhanced expression of LKB1 activates LKB1/AMPK signaling in a kinase-dependent manner. Plasmids encoding EGFP-tagged wild-type LKB1 (LKB1-WT) were transfected into HepG2 and HEK-293T cells, with the LKB1-empty vector (EGFP) used as a negative control. An invariant nucleotide binding site mutant plasmid (LKB1-K78M), which confers deficiency in the kinase activity of LKB1 (15), was also included to test for kinase dependency. Consistent with previous reports (14,16), endogenous LKB1 was expressed in both HepG2 and HEK-293T cell lines (data not shown). As shown in Fig. 1, EGFP-tagged recombinant LKB1 was expressed at comparable levels in cells transfected with LKB1-WT and LKB1-K78M constructs, but not in cells transfected with the control EGFP vector. Thus, the enhanced expression of LKB1 protein was successfully achieved in HepG2 and HEK-293T cells.

A well-known substrate of LKB1 is AMPK, which is likely to mediate most, if not all, of the tumor suppressor effects of LKB1 (6,17). As expected (Fig. 1), transfection with wild-type but not kinase-inactive LKB1 into HepG2 and HEK-293T

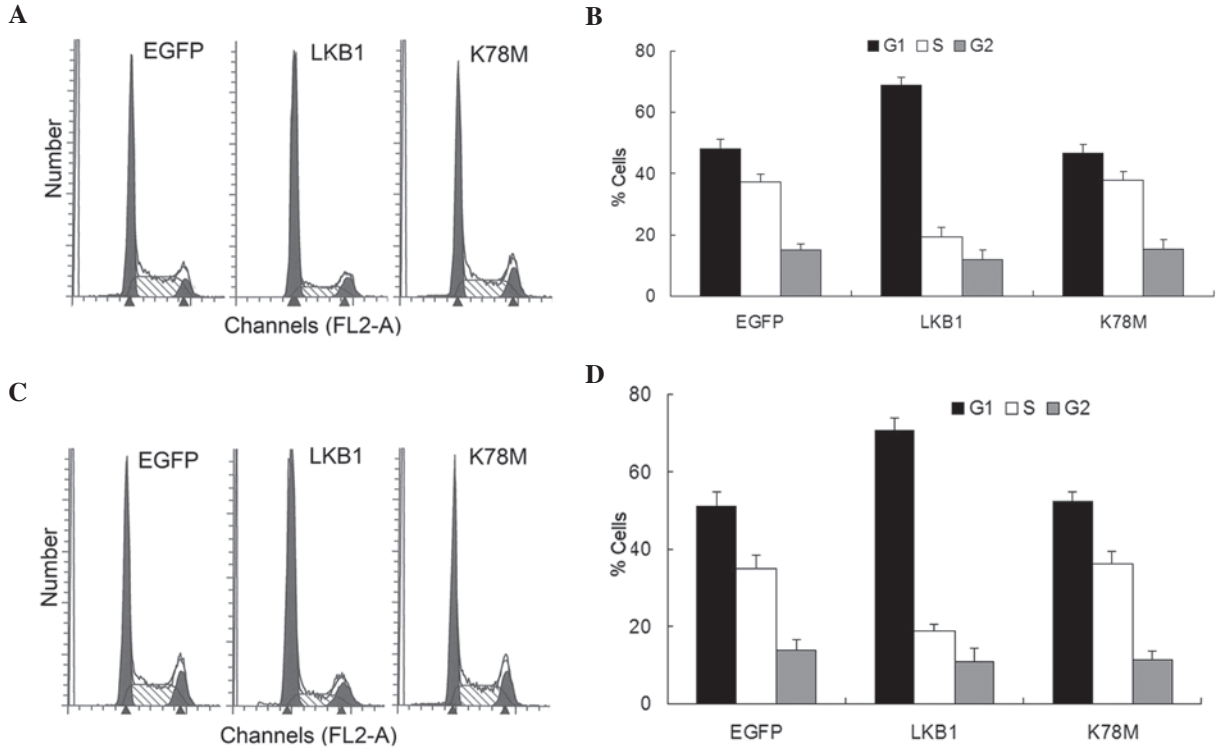

Figure 2. LKB1 overexpression increases the population of cells at the G_1 phase of the cell cycle. (A and B) Hepatocellular carcinoma HepG2 and (C and D) healthy HEK-293T cells were transfected with indicated vectors for 48 h, and then analyzed by flow cytometry. Cell cycle profiles (left panel) and phase distribution (right panel) are presented. Data are representative of three independent experiments. HEK-293T, human embryonic kidney 293T.

cells substantially increased phosphorylation of AMPKα at Thr172, and phosphorylation of ACC at Ser79, which is a useful indicator of increased AMPK activity *in vivo*. Total AMPK and ACC protein levels were not altered in transfected cells. These results indicate that the enhanced expression of LKB1 (via expression of exogenous LKB1 in addition to the endogenous one) enhanced LKB1/AMPK activation in HepG2 and HEK-293T cells in a LKB1 kinase-dependent manner.

Exogenous activation of LKB1/AMPK signaling arrests the cell cycle at the G_1 phase. Cell cycle control is primarily achieved at the 'restriction point', the transition from the G_1 to the S phase, beyond which mitogenic signaling is no longer required and the cell is committed to complete the replication cycle (18). Transfection with LKB1-WT into HepG2 and HEK-293T cells led to the accumulation of cells at G_1 and a reduction in the proportion of cells at the S phase (Fig. 2). Limited or no effect was noted in the LKB1-K78M-transfected cells (Fig. 2). These results agree with earlier reports that ectopic LKB1 expression in LKB1-deficient cancer cells blocks the G_1/S transition (11-13), and indicate that exogenous activation of LKB1/AMPK signaling is sufficient to induce G_1 arrest, even in cells with endogenous expression of LKB1.

Exogenous activation of LKB1/AMPK signaling reduces phosphorylation of Rb. Progression through the G_1 phase is characterized by the hyperphosphorylation of Rb, which releases E2F transcription factors to induce synthesis of proteins necessary for DNA synthesis (19). Compatible with the increase of cell populations at the G_1 phase, transfection with LKB1-WT, but not with LKB1-K78M, considerably reduced

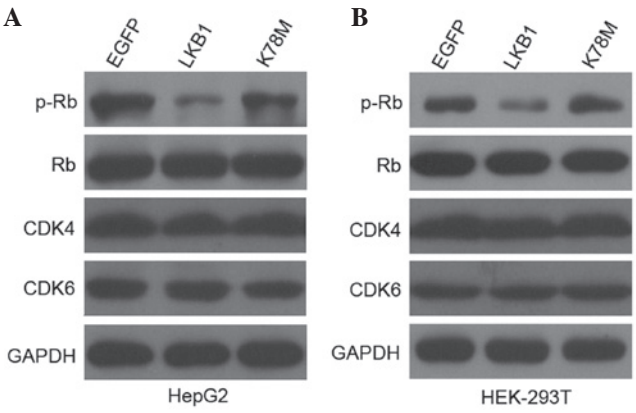

Figure 3. LKB1 overexpression reduces the phosphosphorylation of Rb, with no effect on the expression of CDK4 and CDK6. Western blot analysis of total Rb, phosphorylated Rb, CDK4 and CDK6 was performed in transfected (A) hepatocellular carcinoma HepG2 and (B) healthy HEK-293T cells. GAPDH was used as a loading control. CDK, cyclin-dependent kinase; Rb, retinoblastoma protein; HEK-293T, human embryonic kidney 293T; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

phosphorylation of Rb in HepG2 and HEK-293T cells, with no differences observed in the total Rb protein level (Fig. 3). LKB1 and Ca^{2+}/CaM-dependent protein kinase kinase (CaMKK) are essential activators of the AMPK signaling pathway (20). To examine the potential role of CaMKK activation in controlling the G_1/S transition, we pretreated HepG2 cells and HEK-293T cells with STO-609, a selective CaMKK inhibitor. No effect was observed on cell cycle distribution and Rb phosphorylation

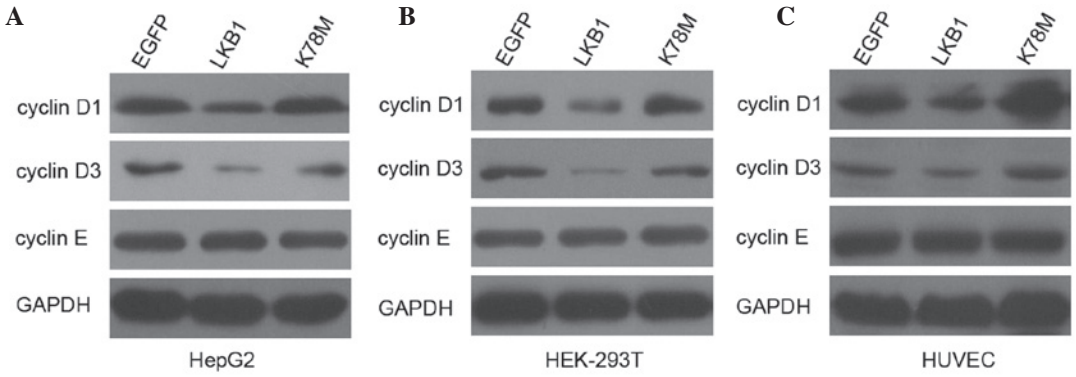

Figure 4. LKB1 overexpression reduces the protein levels of cyclin D1 and of cyclin D3, but does not alter the expression of cyclin E. (A) Hepatocellular carcinoma HepG2 cells, (B) healthy HEK-293T cells and (C) healthy HUVECs were transfected with the indicated vectors for 48 h. Cell lysates were subjected to western blotting to detect protein levels of cyclin D1, cyclin D3, and cyclin E. GAPDH was used as a loading control. HEK-293T, human embryonic kidney 293T; HUVECs, human umbilical vein endothelial cells.

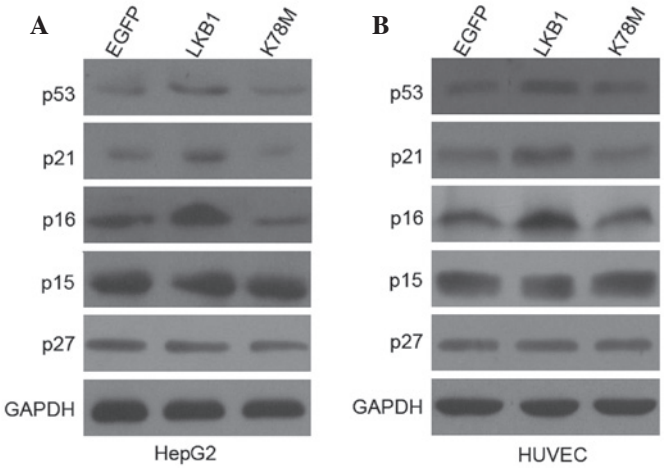

Figure 5. LKB1 overexpression increases the protein levels of p53, p21 and p16, but not p15 or p27. Expression of p53, p21, p16, p15 and p27 was assessed by western blot analysis in transfected (A) hepatocellular carcinoma HepG2 and (B) healthy HUVEC cells. GAPDH was used as a loading control. HUVECs, human umbilical vein endothelial cells.

(data not shown). These results indicate that activation of LKB1/AMPK signaling, but not of CaMKK/AMPK signaling, leads to hypophosphorylation of Rb, which in turn contributes to G_1 arrest in HepG2 and HEK-293T cells.

Exogenous activation of LKB1/AMPK signaling does not affect CDK4 and CDK6 protein levels. The cell cycle machinery comprises a variety of proteins that ensure the proper progression through distinct phases, with different CDKs proteins playing central roles, by phosphorylating Rb and leading to cell cycle entry into the S phase (21). To investigate whether the LKB1-induced G_1 arrest is due to changes in major G_1-phase CDKs, we examined the protein levels of CDK4 and CDK6. Transfection with LKB1-WT and LKB1-K78M into HepG2 and HEK-293T cells did not affect the expression of CDK4 or CDK6 (Fig. 3), indicating that neither CDK4 nor CDK6 are involved in the regulation of the G_1/S arrest mediated by LKB1, and excluding the possibility that hypophosphorylation of Rb results from the decreased expression of CDK4 or CDK6 in HepG2 and HEK-293T cells.

Exogenous activation of LKB1/AMPK signaling decreases the expression of cyclin D1 and cyclin D3, but not cyclin E. The enzymatic activity of CDKs is dependent on physical interactions with cyclins, which are the regulatory subunits of CDKs (22). We assessed whether enhanced LKB1 expression affects major G_1 regulatory cyclins in HepG2 and HEK-293T cells. As shown in Fig. 4, protein levels of cyclin D1 and cyclin D3 were markedly decreased following transfection with LKB1-WT, but not with LKB1-K78M. To confirm this finding, we extended the analysis to HUVECs. Transfection of HUVECs with LKB1 WT also decreased the expression of cyclin D1 and cyclin D3 (Fig. 4). These results indicate that activation of LKB1/AMPK signaling inhibits the expression of cyclin D1 and cyclin D3, which may reduce the activity of CDKs and lead to hypophosphorylation of Rb in HepG2 and HEK-293T cells. Protein profiles of cyclin E were also investigated, however, no change was detected in the level of this cyclin, indicating that cyclin E is not involved in the regulation of the G_1/S transition, affected by LKB1.

Exogenous activation of LKB1/AMPK signaling increases the expression of p53, p21 and p16, but not p15 or p27. Induction of CDK inhibitors (CKIs) is a well-characterized mechanism to inhibit G_1 CDK activity (23). p21 (WAF1/Cip1) is a CKI with a broad spectrum of activities, known to inhibit the activity of most CDKs, and generally considered a key downstream effector of the p53 tumor suppressor protein, since its expression is transcriptionally induced by p53 (24,25). p16 (INK4a) is also a critical CKI involved in G_1/S control, which binds directly to CDK4 and CDK6 and prevents the association with D-type cyclins (26,27). In the present study, the observation that the ectopic expression of LKB1 can considerably increase the protein levels of p53, p21, and p16 in HepG2 cells (Fig. 5) is in agreement with our previous study, which showed, to the best of our knowledge, for the first time that both p53 and p16 are the targets of LKB1, regulating G_1/S transition (14). Notably, transfection with LKB1-WT had no effect on p53, p21, and p16 expression in HEK-293T cells (data not shown). However in HUVECs, the ectopic expression of LKB1 elicited similar increases in the expression of p53, p21, and p16 compared with the ones observed in HUVECs (Fig. 5). These results indicate that in both cancer and healthy cells, the exogenous activation

of LKB1/AMPK signaling induces the expression of p53, p21 and p16, which in turn contributes to the inactivation of CDKs, and further leads to hypophosphorylation of Rb.

p15 (INK4b), p19 (INK4d) and p27 (Kip1) are important CKIs participating in cell cycle regulation (22,28). As shown in Fig. 5, no observable differences were detected in p15 and p27 expression following transfection with LKB1 constructs into HepG2 cells and HUVECs, indicating that neither p15 or p27 is involved in LKB1-mediated G_1 arrest. The protein level of p19 was not detectable in our study.

Discussion

The present study shows, to the best of our knowledge, for the first time that ectopic LKB1 expression activates the LKB1/AMPK signaling pathway and leads to G_1 arrest in healthy and cancer cells with endogenous LKB1 expression in a kinase-dependent manner, which is in agreement with our previous study demonstrating that the suppression of endogenous LKB1 activity in two healthy cell lines accelerates G_1/S transition (14). Our results indicate that G_1/S cell cycle arrest induced by exogenous LKB1 may not be limited to tumor cells with inactivated LKB1, and are in disagreement with results by Tiainen *et al*, who found that cells with endogenous LKB1 are resistant to the inhibitory effects of exogenous LKB1 on cell growth (12). This discrepancy may be explained, in part, by cell type-specific differences in signaling mediated by LKB1. Alternatively, the discrepancy could be due to differences in the delivery of LKB1. It is notable that the ectopic expression of the phosphatase and tensin homolog (PTEN) tumor suppressor protein strongly inhibited cell proliferation in both cancer and healthy cell lines maintaining endogenous wild-type PTEN expression, and this effect was also dependent on the kinase activity of PTEN (29). Germline mutations in LKB1 and PTEN lead to closely related diseases, including PJS and Cowden disease, suggesting that tumor suppressors LKB1 and PTEN exert negative effects on the growth of a wide variety of cell lines, and that these proteins may be involved in the same or related pathways that inhibit growth (30,31).

We found that the overexpression of LKB1 inhibited the synthesis of cyclin D1 and cyclin D3, but stimulated the synthesis of p53, p21 and p16. These effects were specific, as the expression of other components of the cell cycle machinery, including CDK4, CDK6, cyclin E, p15 and p27, was not affected. These results indicate a cell model whereby growth inhibition mediated by LKB1 depends on the combined action of cyclins and CKIs. The reduced expression of cyclin D1 and cyclin D3, and an increased expression of p53, p21 and p16 jointly inhibit the activity of CDKs, resulting in hypophosphorylation of Rb and consequently, cell cycle arrest at the G_1 phase. Notably, HEK-293T cells are an exception to this model, in that the expression of p53, p21 and p16 remained unaltered in response to LKB1 overexpression, indicating that the p53 and p16 pathways may not be involved in G_1 phase arrest in these cells. Supporting evidence for this hypothesis comes from the observation that inhibition of ubiquitin carboxy-terminal hydrolase (UCH) L1 expression arrested both HEK-293T and KR4 cell lines at the G_1 phase, while p53 upregulation was only observed in KR4 cells, but not in HEK-293T cells (32). One plausible explanation is that HEK-293T cells constitutively express the simian virus 40 (SV40) large T antigen, which inactivates the endogenous p53 protein and restricts its activity to the baseline level (33,34). The basal p53 level may be further downregulated, as shown in our previous study (14). However, due to the inhibition of SV40 large T antigen, p53 pathways were not activated. Since p21 is the major transcriptional target of p53 (24) and p16 and p53 may have coordinated expression (35,36), the detection of unaltered p21 and p16 protein levels in HEK-293T cells is expected. Additional studies are nevertheless required to validate the above-described model.

The finding that in HepG2 cells and HUVECs, the ectopic LKB1 expression leads to an increase in the expression of p53, p21 and p16 is inconsistent with early studies on LKB1-deficient cancer cells, which showed that either p53 or p16 is sufficient for the function of LKB1 in the cell cycle control (11-13), however, it is in agreement with our previous study reporting, to the best of our knowledge, for the first time that LKB1 targets both p53 and p16 pathways in healthy cells (15). Our results further suggest that in cells with endogenous LKB1 expression, there might be a cross-talk between p53 and p16 pathways, and that these pathways act as important components of LKB1 signaling, which is required for regulating the G_1/S transition. Nevertheless, which and how many molecules and cellular events mediate the link between LKB1 activity and the p53 and p16 pathways remains to be determined.

The results indicate that combined alterations in the levels of cyclins and CKI regulatory proteins may have synergistic effects, resulting in decelerated cell cycle progression and thereby, inhibiting cell proliferation in response to exogenous activation of LKB1 signaling. This may explain, at least in part, the association between concurrent aberrations in the activities of p53, p21, p16 and cyclin D proteins,as well as the aggressive clinical behavior observed in PJS patients, since increased neoplastic cell proliferation is a negative prognostic factor in related tumors (37-40). The extent to which these molecules individually contribute to the observed growth-arrest phenotype is still a subject of debate.

In summary, the results described in the present study extend earlier observations in LKB1-deficient neoplastic cells, by demonstrating that LKB1/AMPK activation via ectopic LKB1 expression can cause cell cycle arrest at the G_1 phase, even in cells with endogenous LKB1 expression. The results further suggest that, unlike LKB1-mediated growth inhibition in LKB1-null or -deficient genetic backgrounds, the antiproliferative activity of LKB1 on cells expressing endogenous LKB1 is related to a multifaceted regulation of multiple target molecules that are critically involved in cell cycle progression. Overall, this study has provided novel insights into the growth-inhibitory effect of the tumor suppressor protein LKB1.

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