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Metformin impairs the growth of Liver Kinase B1-intact cervical cancer cells

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Abstract

Objective—Metformin is one of the most widely used drugs for the treatment of type 2 diabetes. Recent investigations demonstrated that application of metformin reduces cancer risk. The present study aimed to determine the role of liver kinase B1 (LKB1) in the response of cervical cancer cells to metformin.

Methods—LKB1 expression and the integrity of LKB1-AMPK signaling were determined with immunoblot in 6 cervical cancer cell lines. Cellular sensitivity to metformin was analyzed with MTT assay.

Results—Metformin inhibited growth of cervical cancer cells, C33A, Me180, and CaSki, but was less effective against HeLa, HT-3, and MS751 cells. Analyzing the expression status and the integrity of LKB1-AMPK-mTOR signaling, we found that cervical cancer cells sensitive to metformin were LKB1 intact and exerted an integral AMPK-mTOR signaling response after the treatment. Ectopic expression of LKB1 with stable transduction system or inducible expression construct in endogenous LKB1 deficient cells improved the activation of AMPK, promoted the inhibition of mTOR, and prompted the sensitivity of cells to metformin. In contrast, knock-down of LKB1 compromised cellular response to metformin. Our further investigation demonstrated that metformin could induce both apoptosis and autophagy in cervical cancer cells when LKB1 is expressed.

Conclusions—Metformin is a potential drug for the treatment of cervical cancers, in particular to those with intact LKB1 expression. Administration of cell metabolism agonists may enhance LKB1 tumor suppression, inhibit cell growth, and reduce tumor cell viability via the activation of LKB1-AMPK signaling.

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Conflict of Interest There are no conflicts of interest to report

Keywords

cervical cancer; metformin; LKB1; AMPK

Introduction

Cervical cancer is among the most common cancers worldwide, with over 510,000 new cases and 288,000 deaths each year [1]. Human Papilloma Virus (HPV) infection is thought to be necessary for the development of cervical cancer, as HPV DNA sequences are detectable in more than 99% of cervical tumors [2]. The introduction of HPV vaccines is a major advance, but current vaccines can prevent only 70% of cases [1]. HPV initiates tumor progression by abrogating cell cycle control and apoptosis through the viral oncoproteins E6 and E7, which inactivate p53 and RB respectively [1-3]. HPV infections are often asymptomatic and transient, but promote *in situ* cervical pre-cancers though most spontaneously regress, indicating that HPV by itself is not sufficient to cause cervical cancer [2]. The definitive identification of host biological factors driving the progression of pre-cancers to invasive cancers remains elusive. Another major impediment to improving the survival of women with cervical cancer is the development of resistance to current therapies [3]. Chemoresistance, whether intrinsic or acquired, is likely determined by genetic, epigenetic, or cellular metabolic elements that influence tumor biology [4, 5]. Therefore, it is imperative to characterize additional genetic or cellular metabolism abnormalities in cervical cancers and to identify molecular targets that contribute to therapy insensitivity.

The tumor suppressor Liver Kinase B1 (LKB1) is mutationally inactivated in Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder characterized by gastrointestinal polyps, mucocutaneous pigmentation, and a markedly increased risk for malignant tumors [6-8]. Inactivating mutations in LKB1 are also found in cancer patients without PJS, such as those with sporadic lung adenocarcinoma, ovarian and breast cancer, and pancreatic cancer [8-11]. A recent report showed that at least 20% of primary cervical cancers harbor somatic mutations of LKB1 [12]. Homozygous deletion of LKB1 locus and the generation of novel fusion transcripts involving LKB1 and its neighboring genes are frequently discovered in cervical cancer cells [12, 13]. Wingo et al found that LKB1 inactivation in primary tumors is associated with accelerated disease progression and may play a role in cervical cancer tumorigenesis [12]. Physiologically, LKB1 is involved in multiple cellular functions including embryo development, cell polarity, cell cycle arrest, apoptosis, and cell energy metabolism [7, 8, 14, 15]. Recent work showed that LKB1 also regulates the function and dynamics of hematopoietic stem cells [16-18]. LKB1 phosphorylates at least 13 members of the AMP-activated protein kinase (AMPK) superfamily [19, 20]. AMPK is a master regulator of cellular energy metabolism that maintains cell energy homeostasis by stimulating catabolic pathways and inhibiting energy-consuming anabolic processes [21]. Mounting evidence showed that activation of AMPK not only reprograms metabolism, but also enforces a metabolic checkpoint on the cell cycle through its effects on p53 and mTORC1 signaling [22]. AMPK promotes the maintenance of a resting cell phenotype in mature tissues which do not require proliferation to keep its function (like vascular smooth muscle cells in a large vessel) and also helps protect cells from transformation by oncogenic stimulation [22]. Our published data demonstrated that AMPK activation suppresses cell proliferation in a variety of cell types [23]. Thus, AMPK is a critical mediator of LKB1-associated tumor suppression and could be targeted for cancer therapeutics.

Metformin is one of the most widely used drugs for the treatment of type 2 diabetes [24]. Its primary function is to decrease hepatic glucose production, mainly by inhibiting gluconeogenesis through the activation of AMPK [25-27]. Interestingly, population-based

studies recently showed that metformin treatment was associated with a dose-dependent reduction in cancer risk [27]. The LKB1-AMPK-mTOR axis is believed to mediate the tumor suppression of metformin [28-30]. Inactivation of LKB1-AMPK signaling reverses the inhibitory effects of metformin in breast and ovarian cancer cells [28, 29]. Metformin decreases insulin resistance and indirectly reduces insulin levels, a beneficial effect that could also contribute to tumor suppression considering that insulin promotes cancer cell growth [30, 31]. Further, metformin can induce cell cycle arrest and decrease the oncoprotein level of epidermal growth factor receptor 2 (Her2) in breast and pancreatic cancer cells, respectively [32-36]. Hirsch et al [37] reported that when used in combination with traditional cytotoxic chemotherapy, metformin not only targeted bulk proliferating tumor cells, but also acted to eliminate cancer-initiating stem cells to prevent the relapse of cancer.

Several metformin-based clinical trials in human solid tumors are ongoing [28, 38]. These trials, which focus on breast, endometrial, kidney, lung, and prostate cancers [28, 29, 38], are mainly designed to evaluate the biological effects of metformin in the presence or absence of conventional treatments and to identify the potential dose-limiting toxicity. There are very few reports or clinical trials addressing the potential use of metformin in cervical cancer. Based on the mechanisms by which metformin regulates tumor suppression [27, 31-38], it is likely to inhibit cancer growth in multiple systems, including cervical cancers. To gain insight into the potential role of metformin in cervical cancer cells, we investigated the cytotoxic effect of metformin on multiple cervical cancer cell lines. Furthermore, we evaluated the outcome based on the expression levels of LKB1, an approach that has not been previously explored.

Materials and Methods

Cell lines

Human cervical cancer cell lines, HeLa, C33A, CaSki, HT-3, Me180, and MS751, were kindly provided by Dr. DJ Buchsbaum in the University of Alabama at Birmingham. HeLa cells were cultured in Dulbecco modified Eagle medium (DMEM) with L-glutamine and 10% fetal bovine serum (FBS) in a humidified incubator containing 5% CO₂ at 37°C. C33A cells were cultured in MEM containing L-glutamine, 10% FBS, 1% Non-essential amino acids, and 1% sodium pyruvate. CaSki cells were maintained in RPMI1640 with L-glutamine, 10% FBS, 1% sodium pyruvate, and 10mM HEPES. HT-3 and Me180 cells were cultured in McCoy's medium with L-glutamine and 10% FBS. MS751 cells were cultured in EMEM with L-glutamine, 10% FBS, 1% Non-essential amino acids, and 1% sodium pyruvate.

Antibodies and chemicals

Caspase-7 antibody was purchased from BD Biosciences (San Diego, CA). Antibodies against pan- and phosphorylated-AMPK, phosphorylated-ACC (S79) were purchased from Millipore (Upstate) Inc. (Lake Placid, NY). Anti-LKB1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-S6 (S240/244), phospho-p53 (S15), p21, and phosphorylated- AKT (S473) were purchased from Cell Signaling Technology, Inc. (Boston, MA). Beta-actin and GAPDH polyclonal antibodies were obtained from Bethyl Laboratories (Montgomery, TX). Metformin and cisplatin were purchased from Sigma Aldrich (St. Louis, MO).

Cell viability assay

Cells were seeded into 96-well plates at 3×10^3 cells per well and allowed to adhere overnight. Cell viability was determined after 24 to 72 h in the presence or absence of

metformin using the MTT assay. Plates were read with a SpectraMax 190 microplate spectrophotometer (Beckman Coulter, Inc.) at a wavelength of 540/570 nm.

Flow cytometry analysis of DNA content

For analysis of the DNA content, cells were treated, collected, and then prepared as described previously [39]. Cell apoptosis was determined by the percentage of sub-G1/G0.

Establishment of LKB1 over-expression cell lines

HeLa cells, a cell line with endogenous LKB1 deficiency, were transfected with pcDNA3/c-myc or pcDNA3/c-myc-LKB1. The cells were then subjected to G418 selection (400 μ g/ml) for 2 weeks. The established cells lines were validated with Western blot detection of LKB1 expression. HeLa cells were also transfected with pMEP4-GFP (control) or pMEP4-GFP-LKB1 plasmids, and selected with hygromycin (100 μ g/ml) for two weeks. Pools of hygromycin-resistant stable clones were collected. Expression of LKB1 was determined by induction with 100 μ M Zinc for 16 h followed by immunocytochemistry analysis with LKB1 antibody.

LKB1 stable knockdown using lentiviral short hairpin RNA

Five premade lentiviral LKB1 short hairpin RNA (shRNA) constructs and a negative control construct that was created in the same vector system (pLKO.1) were purchased from Open Biosystems (Huntsville, AL). Lentiviral helper plasmids (pCMV-dR8.2 dvpr and pCMV-VSV-G) were obtained from Addgene (Cambridge, MA). Transient lentivirus stocks were prepared following the manufacturer's protocol as previously described [39].

Immunoblotting

Cell lysis and immunoblotting were performed as described previously [39, 40]. A total of 50 μ g of protein was used for the immunoblotting. The blotting membrane was incubated with first antibody (1:1000 dilution) for 3 hrs and second antibody (Bio-Rad, 1:3000 dilution) for 1 hr. β -actin, α -tubulin, or GAPDH were used for the loading control.

Results

Metformin inhibits growth of cervical cancer cells

To determine whether metformin exerts a cytotoxic impact on cervical cancers, we exposed cervical cancer cell lines to various doses of metformin and assessed the effects of metformin on cell viability. Though the sensitivity to the treatment was diverse, metformin treatment from 1.25 mM to 20mM exhibited a dose-dependent cytotoxicity to cervical cancer cells (Figure 1A). The half maximal inhibitory concentration (IC₅₀, concentration at which 50% of cells survive) for C33A, CaSki, and Me180 was between 2.5 mM to 10 mM while the IC₅₀ for HeLa, MS751, and HT-3 was higher than 20 mM (Figure 1A). To further validate the effect of metformin, cells were exposed to 5 mM metformin at varying times. Metformin reduced the viability of cervical cancer cells in a time-dependent manner (Figure 1B). C33A, CaSki, and Me180 cells were much more sensitive to the treatment than HeLa, MS751, and HT-3 cells. Our results suggest that metformin possesses a cytotoxic function in some cervical cancer cell lines, such as C33A, CaSki, and Me180, as it has been shown to in cell lines and xenograft models originated from other tissues, such as those from the breast and prostate [27, 32].

Metformin activates AMPK and inhibits mTOR in LKB1-intact cervical cancer cells

Previous studies demonstrated that tumor suppressive function of metformin largely results from its activation on LKB1-AMPK signaling [27, 38, 41]. To determine whether the

sensitivity differences in response to the treatment of metformin among cervical cancer cells was associated with the activation status of LKB1-AMPK signaling, we exposed the cells to metformin and detected signaling molecules from this pathway by immunoblot. Strikingly, the cell lines (C33A, CaSki, and Me180) highly sensitive to metformin were LKB1 positive and exhibited an integral AMPK-mTOR signaling response to the treatment (Figure 2). Metformin enhanced AMPK activation, indicated by the increased phosphorylation of AMPK α and acetyl-CoA carboxylase (ACC), a substrate of AMPK with a rate-limiting enzyme activity [42] (Figure 2). Consistent with enhanced AMPK activation, metformin decreased the activity of mTOR as reflected by reduced S6 phosphorylation in the LKB1 expressing cells, though levels of LKB1 were unchanged (Figure 2). In contrast, metformin insensitive cell lines (HeLa, MS751, and HT-3) were LKB1 negative and exhibited a compromised response in terms of the AMPK-mTOR signaling (Figure 2). In these cells, metformin had marginal effects on AMPK activation (phosphorylation of AMPK α and ACC) and inhibition of mTOR signaling (reduction of the phosphorylation of S6) (Figure 2). In addition, metformin neither exerted a direct impact on DNA damage nor exhibited a cooperative effect with DNA damage drug cisplatin (CDDP) as judged by phosphorylation of p53 (p53-S15) and expression of p21 (Figure 2). Thus, our data suggest that LKB1 plays a role in the response of cervical cancer cells to metformin.

LKB1 sensitizes cervical cancer cells to metformin

We and other groups previously reported that activation of AMPK and inhibition of mTOR could inhibit tumor cell growth [23, 42, 43]. To determine whether LKB1-mediated activation of AMPK is associated with cellular sensitivity to metformin, we ectopically expressed LKB1 in HeLa cells (Figure 3A). As observed previously, upon treatment with metformin, control HeLa/pcDNA3/c-myc cells exhibited only a marginal increase in AMPK activation (Figure 3A). Ectopic expression of LKB1 (HeLa/pcDNA3/c-myc-LKB1) increased the basal levels of AMPK activation and reduced the phosphorylation of mTOR and its downstream target, S6 (Figure 3A). Treatment with metformin substantially enhanced AMPK activation, as indicated by the increased phosphorylation of AMPK and ACC, and induced further down-regulation of mTOR and S6 phosphorylation (Figure 3A). The IC₅₀ for HeLa/pcDNA3/c-myc-LKB1 cells was between 2.5 and 5 mM while the IC₅₀ for control HeLa/pcDNA3/c-myc cells was higher than 20 mM (Figure 3B). These results, together with our aforementioned data, suggest that LKB1 not only determines the AMPK-mTOR signaling response to metformin but also sensitizes cervical cancer cells to the treatment.

The levels of ectopic LKB1 expression might affect cell survival unrelated to its tumor suppressive function [2, 8, 9]. Thus, physiologically relevant levels of LKB1 expression in endogenous LKB1 deficient cells, such as HeLa, MS751, and HT-3, would be more desirable than massive overexpression. Therefore, we introduced a metal inducible system (pMEP4) to express LKB1 at endogenous levels by adding a proper dosage of inducible agent (zinc sulfate). We established stable clones with inducible expression of GFP-LKB1 (HeLa/pMEP4-GFP-LKB1) or GFP (HeLa/pMEP4-GFP, control) in HeLa cells. These cells expressed a moderate level of GFP-LKB1/GFP under the treatment of 100 μ M zinc sulfate as detected by immunoblot (Figure 4A), immunofluorescence (Figure 4B), and immunocytochemistry (Figure 4C). Of note, due to the presence of trace amounts of metal ions in the serum of cell culture, cells without induction may occasionally exhibit background expression of the proteins (Figure 4A, lane 3). We treated the cells with a range of metformin doses. Cell viability was only modestly affected in HeLa/pMEP4-GFP control cells regardless of induction, however, induced expression of LKB1 substantially enhanced cell sensitivity to metformin (Figure 4D). The IC₅₀ for HeLa/pMEP4-GFP-LKB1 cells was around 5 mM while the IC₅₀ for control HeLa/pMEP4-GFP cells was higher than 20 mM (Figure 4D), further suggesting that LKB1 sensitizes the cells to metformin treatment.

Lack of LKB1 desensitizes cervical cancer cells to metformin

To further validate the role of LKB1 in metformin-induced disturbance of cervical cancer cell survival, we established an shRNA-mediated LKB1 knock-down cell line in C33A (Figure 5A), and exposed the cells to metformin. Knock-down of LKB1 abrogated metformin-triggered activation of AMPK and suppression of mTOR (Figure 5B). Most importantly, the LKB1 knock-down cells (C33A/shR-LKB1) but not control transfectants (C33A/Ctrl-shR) were protected from metformin-induced loss of cell viability. C33A/shR-LKB1 cells were resistant to metformin with an IC_{50} higher than 8 mM (Figure 5C), while LKB1 intact C33A/Ctrl-shR cells remained susceptible to metformin treatment with an IC_{50} of 2 mM. Taken together, our results suggest that LKB1 is necessary for metformin-mediated AMPK-mTOR signaling response and cytotoxicity of metformin in cervical cancer cells.

Metformin induces both cell apoptosis and autophagy

To determine the mechanism(s) by which metformin induces cell death in cervical cancer cells, we exposed HeLa/myc and HeLa/pcDNA3/c-myc-LKB1 cells to 5 mM metformin for 24 hours and detected apoptosis with caspase-7 activation. Treatment with metformin led to an increase in the active form of caspase-7 in HeLa/pcDNA3/c-myc-LKB1 cells compared to control HeLa/pcDNA3/c-myc cells (Figure 6A). To confirm apoptotic cell death being induced by metformin under LKB1 expression, we treated C33A/Ctrl-shRNA and C33A/shRNA-LKB1 cells with 5 mM metformin for 48 h and subsequently fixed the cells for flow cytometry analysis of sub-G1/G0 cell population [40]. As expected, metformin substantially enhanced cell apoptosis in LKB1 intact C33A/Ctrl-shRNA cells (Figure 6B). In contrast, the percentage of cells in sub-G1/G0 phase in LKB1 shRNA cells treated with metformin was dramatically reduced to around one third of that in LKB1 intact cells (Figure 6B). Together, our results suggest that metformin induces cancer cell apoptosis in the presence of LKB1.

In addition to apoptosis, AMPK activation can also lead to autophagy-associated cell death [7, 23, 44, 45]. To determine whether metformin can induce autophagy in cervical cancer cells, and whether the autophagy is associated with LKB1 expression, we transfected HeLa/pcDNA3/c-myc and HeLa/pcDNA3/c-myc-LKB1 cells with pEGFP-LC3. Upon metformin treatment, HeLa/pcDNA3/c-myc-LKB1 cells exhibited a redistribution of GFP-LC3 from a diffuse cytoplasmic distribution to discrete vesicular structures (punctate fluorescence, Figure 6C), an indication of autophagy [46]. The GFP-LC3 punctate was induced as early as 4 h after metformin treatment and the number of the cells with punctate GFP-LC3 increased in a time-dependent manner (data not shown). Cells without LKB1 expression (HeLa/pcDNA3/c-myc) exhibited fewer GFP-LC3 vesicles (Figure 6C). This redistribution was confirmed biochemically by immunoblot analysis of LC3 (Figure 6A). Metformin-treated LKB1 positive cells underwent conversion from the LC3-I isoform to the LC3-II isoform, which is specific for autophagy [47] (Figure 6A). Taken together, our data suggest that metformin can induce both apoptotic and autophagy in cervical cancer cells under the presence of LKB1 expression.

Discussion

Metformin is a biguanide derivative that has been widely used for treating diabetes mellitus [48]. Recent epidemiological, preclinical, and pilot clinical studies have shown that metformin is a promising anticancer drug as well [27-34, 48, 49]. However, most of the studies have focused on breast, prostate, and lung cancers [27-29, 33]. There are very few reports examining the role of metformin in cervical cancer. To gain insight into the possible role of metformin in cervical cancer, we investigated the cytotoxic effect of metformin on several cervical cancer cell lines with various levels of LKB1 expression. We found that

metformin impaired the viability of LKB1 intact cervical cancer cells by inducing both apoptosis and autophagy in cervical cancer cells. Consistent with functional assays, our immunoblot analysis showed that metformin enhanced AMPK activation and inhibited mTOR signaling as indicated by reduced S6 phosphorylation (Figure 2, 3). We demonstrated that LKB1-AMPK-mTOR signaling is necessary for the response to metformin.

The identification of predictive biomarkers represents a critical issue in the contexts of both prevention and personalized treatment, but few data are available for metformin treatment. Our current study showed that cervical cancer cells lacking LKB1 were less sensitive to metformin, and that ectopic expression of LKB1 in these cells restored the sensitivity to metformin, suggesting that LKB1 indeed mediated the effect of metformin in cervical cancer cells. With regard to the central role of LKB1-AMPK-mTOR signaling in cell energy metabolism, cancer, and mediation of metformin [7, 8, 50], LKB1 could be an ideal candidate for the prediction of metformin response. Thus, our research not only offers mechanistic insight into the use of metformin to treat cervical cancer, but also provides new information toward the development of LKB1 as a biomarker.

AMPK is a major target and mediator of LKB1 [7, 50]. AMPK activation is largely dependent on phosphorylation by LKB1 [8, 50]. Thus, AMPK activation by metformin and other agonists would have no biological effect in the context of LKB1 deficiency [22]. Although this is the case in most systems, it is possible that in some cervical cancer cells, LKB1 may act through other targets independent of AMPK signaling, such as additional kinases of the AMPK family [7-9]. Thus, it will be interesting to investigate the effects of additional LKB1 substrates in the treatment of metformin and the role of these potential targets in mediating cytotoxicity. In addition, a recent study demonstrated that metformin prevents tobacco carcinogen-induced lung tumorigenesis through the inhibition of mTOR by reducing the phosphorylation of insulin-like growth factor-I receptor/insulin receptor (IGF-1R/IR), Akt, and extracellular signal-regulated kinase (ERK) [51]. Whether LKB1 also plays a role in metformin-induced suppression of these kinases and whether metformin exerts a similar mechanism in cervical cancer cells remain unknown. On the other hand, in addition to the activation by LKB1, AMPK is partially phosphorylated and activated by CAMKK and TAK1 [52-57]. Nafz et al [58] demonstrated that AICAR, another agonist of AMPK, induces HPV suppression in cervical cancer cells and triggers cell apoptosis in the absence of LKB1. Previous reports showed that HPV E6 activates Akt and enhances the degradation of TSC2 [59-61], leading to the activation of mTOR. Therefore, activated AMPK, such as those under treatment with AICAR and metformin, may inactivate mTOR via the inhibition of HPV, such as in HeLa and MS571 cells, and, in contrast, exhibit a relatively minor effect on cells with both HPV and LKB1 negative, such as HT-3 (Figure 2).

Studies with *in vitro* cell culture systems can provide a greater flexibility for screening a wider range of concentrations and combinations of metformin with standard therapeutic agents (Figures 1, 2, and data not shown). Thus, agents found to selectively target different LKB1 expression backgrounds could then be further tested *in vivo*. In addition, given our imperfect understanding of complex signaling pathways regulated by metformin and the metabolism of the drug itself, *in vivo* studies, such as cervical cancer xenograft models, are also needed to ultimately validate the potential role of metformin in cervical cancers. In addition, cancer cell lines have been cultured *in vitro* for passages and are subjected to selective bias for growth; thus, results obtained from cells lines need to be interpreted with caution. To avoid this potential pitfall, creating a primary xenograft of cervical cancer for further exploring the effects of drugs, such as metformin, in the future may also warrant a preclinical value.

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Highlights

- Metformin is a potential drug for the treatment of cervical cancers.
- The integrity of LKB1-AMPK signaling is necessary for the response to metformin.

Figure 1. Metformin inhibits growth of cervical cancer cells

Proliferating human cervical cancer cell lines, C33A, CaSki, Me180, HeLa, MS751, and HT-3, were cultured in medium as described Materials and Methods. The cells were exposed to 0-20 mM metformin for 72 h (A) or 5 mM metformin for 0-72 h (B). Four hours before the termination of the experiments, 100 μ g/ml MTT was added to the cell culture medium. Cell viability was determined by MTT assay. The number in cells without treatment was set as 100%. Data represent average of three independent experiments $\pm$ SD. Met: metformin (The abbreviation is used throughout the figures).

Figure 2. Metformin activates AMPK and inhibits mTOR in LKB1-intact cervical cancer cells
 Cervical cancer cells were exposed to 5 mM metformin and / or 1 μg/ml cisplatin for 16 h. After the treatments, whole cell extract was isolated and subjected to immunoblot analysis. Expression of LKB1-AMPK-mTOR signaling molecules were detected. Fifty microgram whole cell lysates were used for the detection. GAPDH was served as a loading control.

Figure 3. LKB1 sensitizes cervical cancer cells to metformin

HeLa/pcDNA3-c-myc (HeLa/myc) or HeLa/pcDNA3-c-myc-LKB1 (HeLa/myc-LKB1) cells were established as described in Materials and Methods. **A**, Cells were exposed to 5 mM metformin for 16 h. Expression of LKB1 and AMPK-mTOR signaling molecules were detected. Fifty mg whole cell lysates were used for the detection. α -tubulin was served as a loading control. **B**, HeLa/myc or HeLa/myc-LKB1 cells were exposed to different dosages of metformin for 72 h. Cell viability was determined by MTT assay. Data represent average of three independent experiments $\pm$ SD.

Figure 4. Inducible expression of LKB1 enhances cellular sensitivity to metformin

A-C, Establishment of LKB1 inducible cell line. HeLa cells were transfected with pMEP4-GFP (control) or pMEP4-GFP-LKB1 plasmids, and selected with hygromycin (100 μ g/ml) for two weeks. Pools of hygromycin-resistant stable clones were collected. Expression of LKB1 was determined by induction with 100 μ M Zinc for 16 h followed by immunoblot (**A**), fluorescence (**B**), and immunocytochemistry detection with anti-LKB1 antibody (**C**). **D**, Cell viability after treatment of metformin in cells with inducible expression of LKB1. HeLa/pMEP4-GFP and HeLa/pMEP4-GFP-LKB1 cells were pre-treated with Zinc for 4 h for induction. The cells were then exposed to different dosages of metformin for 72 h. Cell viability was determined by MTT assay. Data represent average of three independent experiments $\pm$ SD.

Figure 5. Lack of LKB1 desensitizes cervical cancer cells to metformin

A, Human cervical cancer C33A/control-shRNA and C33A/shR-LKB1 stable cell lines were established as described in Materials and Methods. Knock-down of LKB1 was verified with Western blot. **B**, The cells were exposed to 0, 5, and 10 mM metformin for 16 h. LKB1-AMPK-mTOR signaling molecules were detected by Western blot. Fifty mg whole cell lysates were used for the analysis. GAPDH was served as a loading control. **C**, The cells were exposed to 0- 8 mM metformin for 72 h. Cell viability was determined by MTT assay. Data represent the average of three independent experiments $\pm$ SD.

Figure 6. Metformin induces cell apoptosis and autophagy

A, HeLa/myc or HeLa/myc-LKB1 cells were exposed to 5 mM metformin for 24 h. After the treatment, whole cell lysates were collected for Western blot detection of cell apoptosis with caspase-7. The lysates were also analyzed for the expression of autophagosome marker, LC3. **B**, Apoptosis induced by metformin. C33A/Ctrl-shRNA and C33A/shRNA-LKB1 cells were treated with 5 mM metformin for 48 h. After the treatment, the cells were fixed and stained with 50 μ g/ml of PI. Flow cytometry (FCM) analysis was carried out to detect sub-G1 (apoptotic cells) and other cell-cycle distributions. Percentage of cells in sub-G1/G0 phase was displayed. Data represent average of three independent experiments $\pm$ SD. Student's *t*-test was applied for the statistical analysis. **C**, Autophagosomes / autolysosomes in metformin treated cell. HeLa/myc or HeLa/myc-LKB1 cells were transfected with GFP-LC3. Twenty four hours after the transfection, cells were treated with 5 mM metformin for another 24 h. GFP fluorescence in the cells were then captured with NIKON ECLIPSE TE2000-E fluorescence microscope. Arrows indicate the autophagosomes/autolysosomes.