Lactate Dehydrogenase B Is Critical for Hyperactive mTOR-Mediated Tumorigenesis

Xiaojun Zha, Fang Wang, Ying Wang, Shaozong He, Yanling Jing, Xueyan Wu, and Hongbing Zhang

Abstract

Mammalian target of rapamycin (mTOR) is a major downstream effector of the receptor tyrosine kinase (RTK)–phosphoinositide 3-kinase (PI3K)–v-akt murine thymoma viral oncogene homologue 1 (AKT) signaling pathway. Although this signaling network is frequently altered in cancer, the underlying mechanisms that cause tumorigenesis as a result of activated mTOR remain largely unknown. We report here that expression of lactate dehydrogenase B (LDHB), a critical enzymatic activator of glycolysis, was upregulated in an mTOR-dependent manner in TSC1−/−, TSC2−/−, PTEN−/−, or activated AKT1-expressing mouse embryonic fibroblasts (MEF). LDHB gene expression was transactivated by signal transducer and activator of transcription 3 (STAT3), a key tumorigenic driver in many cancers, acting as a downstream mTOR effector in both mouse MEFs and human cancer cells. LDHB attenuation blunted the tumorigenic potential of oncogenic TSC2-null cells in nude mice. We concluded that LDHB is a downstream target of mTOR that is critical for oncogenic mTOR-mediated tumorigenesis. Our findings offer proof of concept for targeting LDHB as a therapeutic strategy in cancers driven by aberrant activation of the RTK-PI3K-AKT-mTOR signaling cascade. Cancer Res; 71(1); 13–8. ©2011 AACR.

Introduction

Because of gain-of-function mutations of proto-oncogene epidermal growth factor receptor, phosphoinositide 3-kinase (PI3K), or v-akt murine thymoma viral oncogene homologue 1 (AKT), and by loss-of-function mutations of tumor suppressor phosphatase and tensin homologue (PTEN), LKB1, TSC1, or TSC2, the receptor tyrosine kinase (RTK)–PI3K–AKT–mammalian target of rapamycin (mTOR) signaling cascade is one of the most frequently activated signaling pathways in human cancers (1–5). mTOR, a serine/threonine kinase, integrates various inputs from upstream pathways and plays a central role in regulating cell growth and proliferation (2). However, the precise mechanisms downstream of the mTOR signaling leading to cancer development remain elusive.

In tumor cells, glucose is preferentially converted into lactic acid through aerobic glycolysis, which is known as the "Warburg effect" (6). Lactate dehydrogenase (LDH), the key glycolytic enzyme catalyzing the formation of lactic acid from pyruvate, is often activated in many types of cancer (7). It is a tetrameric enzyme composed of combinations of 2 subunits (LDHA and LDHB). LDHA is elevated and activated in many cancers, and plays a crucial role in tumor initiation, maintenance, and progression (8), whereas the significance of LDHB in tumor development remains more elusive and the regulation of LDHB expression is also less characterized.

The present study shows that mTOR is a positive regulator of LDHB and signal transducer and activator of transcription 3 (STAT3) is a transcriptional activator of LDHB, downstream of mTOR. Because LDHB is critical for hyperactive mTOR-mediated tumorigenesis, LDHB may be a druggable target for the treatment of diseases associated with aberrant mTOR signaling.

Materials and Methods

Reagents, antibodies, and plasmids are listed in Supplementary data.

Cell cultures

All the mouse embryonic fibroblasts (MEF) used here were reported elsewhere (9, 10). PC3, A549, PANC-1, HepG2, and MDA-MB-468 cell lines were from American Type Culture Collection. Bel-7402 was from the Cell Institute of Chinese Academy of Sciences. NTC/T2-null cells are a gift from Sandra Dabora (Brigham & Women’s Hospital; ref. 11). Production of retroviruses and lentiviruses and subsequent generation of stable gene expression cell lines were described elsewhere (10). For hypoxia, cells were incubated in a hypoxia incubator with a gas mixture containing 3% O2 and 5% CO2 balanced with nitrogen. For the starvation of amino acids, cells were
cultured in Krebs-Ringer bicarbonate buffer supplemented with 10% dialyzed FBS.

**Quantitative real-time PCR**

Total RNA was extracted from cells using Trizol (Invitrogen) and was reversely transcribed using the PrimeScript RT Reagent Kit (TaKaRa). cDNA was then used as template in the quantitative real-time PCR (qRT-PCR; Supplementary data).

**RNA interference**

siRNAs were synthesized and transfected into cells for mRNA knockdown (Supplementary data).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) was conducted using an EZ-ChIP kit (Upstate). The dissociated DNA from immunoprecipitated protein/DNA was used for PCR (Supplementary data).

**Cell proliferation assay**

Cell proliferation was measured using an MTT Assay Kit (BioDev-Tech; Supplementary data).

**Induction of subcutaneous tumors in nude mice**

Subcutaneous tumors were established as described previously (9). Six male mice were used in each cohort (Supplementary data).

**Statistical analysis**

qRT-PCR and cell proliferation were analyzed using Student’s t test, 2-tailed, with Excel software. The Kaplan–Meier log-rank test was used for analysis of mouse tumor
development and survival data with GraphPad Prism 5 software. Statistical significance was defined as \( P < 0.05 \).

Results and Discussion

Because TSC1 and TSC2 protein complex is the major suppressor of mTOR signaling, mTOR hyperactivation is responsible for multiorgan tumors in tuberous sclerosis complex (TSC) disease due to inactive mutations of either TSC1 or TSC2 (12). To investigate the effect of TSC2 deficiency, we compared the difference of the gene expression profiles between TSC2\(^{+/+}\) and TSC2\(^{+/−}\) MEFs. Among the differentially expressed genes, the mRNA abundance of LDHB but not LDHA was significantly increased in TSC2\(^{+/−}\) MEFs (Supplementary Table 1) and this finding was confirmed by qRT-PCR analysis (Fig. 1A, left and middle). Furthermore, the protein level of LDHB was also increased in TSC2\(^{+/−}\) MEFs (Fig. 1A, right). Similarly, LDHB expression was elevated in TSC1\(^{−/−}\) MEFs (Fig. 1B). Ectopic expression of human TSC2 in TSC2\(^{−/−}\) MEFs normalized the expression of LDHB but had no obvious influence on the expression of LDHA (Supplementary Fig. 1A and B). Furthermore, knockdown of TSC2 in wild-type MEFs dramatically increased LDHB expression (Supplementary Fig. 1C). Collectively, these data indicate that TSC1 and TSC2 negatively regulate LDHB expression.

In addition to genetic alteration of TSC2 gene itself, TSC2 may be inactivated by multiple upstream signaling events (1, 2, 4, 5, 13). Among them, AKT is a negative regulator of TSC2 and is frequently activated in cancers. Ectopic expression of constitutively activated AKT1 (myristoylated-AKT1) led to significantly increased LDHB expression and mTOR activation (hyperphosphorylation of S6; Fig. 1C). Loss of PTEN, which is a major suppressor of AKT signaling, also exhibited LDHB elevation and mTOR enhancement in addition to AKT activation (Fig. 1D).
mTOR can assemble into rapamycin-sensitive mTOR complex 1 (mTORC1) composed of mTOR, mLST8, Raptor (regulatory associated protein of mTOR), and PRAS40; and rapamycin-insensitive mTOR complex 2 (mTORC2) made of mTOR, mLST8, Rictor (Raptor-independent companion of mTOR), and SIN1 (2). Because loss of TSC1, TSC2, and PTEN or acquisition of active AKT all causes mTOR hyperactivation and LDHB overproduction, we speculated that AG490

Figure 3. STAT3 transactivates LDHB expression downstream of mTORC1. A, TSC2−/− and TSC1−/− MEFs were treated with or without 50 μmol/L of AG490 for 24 hours (left) or transfected with STAT3 siRNA for 48 hours (middle). TSC2−/− MEFs infected with STAT3DN or its control expressing retroviruses (V; right). Proteins were detected by immunoblotting. B, qRT-PCR was conducted to examine LDHB gene expression in TSC2−/− MEFs infected with STAT3DN or its control expressing retroviruses (V; left) and TSC2−/− MEFs infected with STAT3C or its control expressing retroviruses (right). Error bars, mean ± SD of triplicate samples. *, P < 0.05. TSC2−/− MEFs infected with retroviruses for STAT3C expression or its control viruses (V) were treated with or without 10 nmol/L of rapamycin (R) for 24 hours (middle). Proteins were detected by immunoblotting. C, schematic illustration of the 3 putative STAT3-binding sequences in the intron 1 of LDHB gene (left). TSC2−/− MEFs infected with STAT3C or its control expressing retroviruses (V; middle) and TSC1−/−, TSC1+/−, or rapamycin (10 nmol/L, 24 hours) treated TSC1−/− MEFs (right) were subjected to ChIP assay using an anti-phospho-STAT3 (Tyr705) antibody. Normal rabbit IgG antibody served as the negative control. PCR amplifications were conducted using primers surrounding the putative STAT3-binding sites. D, human cancer cell line PC3, Bel-7402, A549, PANC-1, HepG2, and MDA-MB-468 were treated with or without 20 nmol/L of rapamycin (R) for 48 hours and then subjected to immunoblotting.
mTOR was a positive regulator of LDHB expression. Suppression of mTORC1 by mTOR inhibitor rapamycin decreased the level of LDHB protein in both TSC1/C0/C0 and TSC2/C0/C0 MEFs (Fig. 2A). qRT-PCR analysis reveals that the suppression of LDHB by rapamycin occurred at transcriptional level (Supplementary Fig. 2). To further identify which one of the 2 mTOR complexes are involved in the regulation of LDHB expression, we assessed LDHB level in mTOR-, Raptor-, or Rictor-knockdown TSC2/C0/C0 MEFs. Reduction of mTOR or Raptor decreased the abundance of LDHB, whereas reduced Rictor did not alter the expression of LDHB (Fig. 2B), supporting the role of mTORC1 in the regulation of LDHB expression.

Hypoxia and amino acid starvation inhibit mTORC1 activity through TSC1/TSC2-dependent and -independent pathways, respectively (14, 15). To further ascertain the causative relationship between mTORC1 activation and LDHB expression, we checked the LDHB level in TSC1- or TSC2-null MEFs and the control cells under hypoxia or amino acid starvation. Both mTOR activity and LDHB protein were significantly decreased in the control cells but not in TSC2−/− MEFs under hypoxic conditions (Fig. 2C). In contrast, removal of amino acids abolished S6 phosphorylation and suppressed LDHB expression in both wild-type and TSC1−/− MEFs (Fig. 2D). Taken together, we identified that mTORC1, but not mTORC2, regulates LDHB expression. LDHB may thus be a potential biomarker for diseases associated with dysregulated mTOR signaling.

We next tested the candidacy of STAT3 as the bridge between mTORC1 signaling and LDHB expression, as STAT3 is a transcription factor and a downstream target of mTOR (10, 16). We have previously reported that the loss of TSC1/TSC2 increased total STAT3 and phospho-STAT3 Tyr705 and Ser727 levels and the activation of STAT3 is regulated by mTORC1 but not by mTORC2 (ref. 10; Supplementary Fig. 3). LDHB levels were dramatically decreased with the addition of the STAT3 inhibitor AG490 in TSC2−/− or TSC1−/− MEFs (Fig. 3A, left). Furthermore, knockdown of STAT3 led to the reduction of LDHB (Fig. 3A, middle). LDHB protein level was substantially downregulated in a dominant-negative STAT3 (STAT3DN)-transfected TSC2−/− MEFs (Fig. 3A, right). qRT-PCR analysis revealed that STAT3DN suppressed LDHB mRNA expression (Fig. 3B, left), suggesting that STAT3 regulates LDHB at the transcriptional level. Moreover, STAT3 protein level was significantly increased in constitutively active STAT3 (STAT3C)-transfected cells and could not be normalized with rapamycin (Fig. 3B, middle). In addition, STAT3 stimulated LDHB transcription (Fig. 3B, right). Therefore, STAT3 regulates the expression of LDHB downstream of mTORC1.

To investigate whether STAT3 directly promotes the transcription of LDHB, we identified 3 putative STAT3-binding sites in the LDHB promoter (Fig. 4A). Depletion of LDHB suppressed oncogenic TSC2-null cell tumorigenesis. A, 2 independent shRNAs that silence LDHB (shLDHB1 and shLDHB2) or a control shRNA (shScramble) were stably expressed in NTC/T2-null cells. Cell lysates were subjected to immunoblotting. B, the proliferation of NTC/T2-null cells expressing shScramble, shLDHB1, or shLDHB2 was examined by MTT assay. Error bars, mean ± SD of triplicate samples; *, P < 0.05. C, NTC/T2-null cells stably expressing shLDHB1 or shScramble were injected subcutaneously into nude mice and followed for tumor development (left) and survival (right). D, tumor lysates were examined for LDHB expression by immunoblotting.

**Figure 4.** Depletion of LDHB suppresses oncogenic TSC2-null cell tumorigenesis. A, 2 independent shRNAs that silence LDHB (shLDHB1 and shLDHB2) or a control shRNA (shScramble) were stably expressed in NTC/T2-null cells. Cell lysates were subjected to immunoblotting. B, the proliferation of NTC/T2-null cells expressing shScramble, shLDHB1, or shLDHB2 was examined by MTT assay. Error bars, mean ± SD of triplicate samples; *, P < 0.05. C, NTC/T2-null cells stably expressing shLDHB1 or shScramble were injected subcutaneously into nude mice and followed for tumor development (left) and survival (right). D, tumor lysates were examined for LDHB expression by immunoblotting.
sequences (−494/−486 TTCTGTGAA, −1,191/−1,183 TCCCCTAAA, and −2,280/−2,272 TATACTGAA) within the intron 1 of LDHB (Fig. 3C, left). ChiP revealed that STAT3 bound to 2 of the 3 predicted sites (−494/−486 TTCTGTGAA and −1,191/−1,183 TCCCCTAAA; Fig. 3C, middle). To address whether the binding of STAT3 onto the intron 1 of LDHB was regulated by mTORC1, we either treated TSC1−/− MEFs with rapamycin or restored TSC2 expression in TSC2−/− MEFs. Suppression of mTOR by both maneuvers decreased the binding of STAT3 on these 2 sites (Fig. 3C, right, and Supplementary Fig. 4). Taken together, STAT3 transduces mTORC1 signaling to LDHB by directly upregulating LDHB gene transcription.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

NTC/T2 null, a cell line with potent tumorigenicity derived from a subcutaneous tumor formed by the injection of TSC2-null MEFs with rapamycin or restored TSC2 expression in TSC2−/− MEFs. Suppression of mTOR by both maneuvers decreased the binding of STAT3 on these 2 sites (Fig. 3C, right, and Supplementary Fig. 4). Taken together, STAT3 transduces mTORC1 signaling to LDHB by directly upregulating LDHB gene transcription.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

NTC/T2 null, a cell line with potent tumorigenicity derived from a subcutaneous tumor formed by the injection of TSC2-null MEFs with rapamycin or restored TSC2 expression in TSC2−/− MEFs. Suppression of mTOR by both maneuvers decreased the binding of STAT3 on these 2 sites (Fig. 3C, right, and Supplementary Fig. 4). Taken together, STAT3 transduces mTORC1 signaling to LDHB by directly upregulating LDHB gene transcription.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

NTC/T2 null, a cell line with potent tumorigenicity derived from a subcutaneous tumor formed by the injection of TSC2-null MEFs with rapamycin or restored TSC2 expression in TSC2−/− MEFs. Suppression of mTOR by both maneuvers decreased the binding of STAT3 on these 2 sites (Fig. 3C, right, and Supplementary Fig. 4). Taken together, STAT3 transduces mTORC1 signaling to LDHB by directly upregulating LDHB gene transcription.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

NTC/T2 null, a cell line with potent tumorigenicity derived from a subcutaneous tumor formed by the injection of TSC2-null MEFs with rapamycin or restored TSC2 expression in TSC2−/− MEFs. Suppression of mTOR by both maneuvers decreased the binding of STAT3 on these 2 sites (Fig. 3C, right, and Supplementary Fig. 4). Taken together, STAT3 transduces mTORC1 signaling to LDHB by directly upregulating LDHB gene transcription.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.

To investigate whether mTOR-STAT3-LDHB signaling pathway exists in human tumors, we treated cancer cell lines PC3, Bel-7402, A549, Panc-1, HepG2, and MDA-MB-468 with rapamycin to check the expression of LDHB and STAT3. Inhibition of mTOR dramatically inactivated STAT3 and reduced the expression of LDHB in 5 of the 6 cell lines (Fig. 3D), indicating that mTOR is a positive regulator for STAT3 and LDHB in human cancer cells.
Lactate Dehydrogenase B Is Critical for Hyperactive mTOR-Mediated Tumorigenesis

Xiaojun Zha, Fang Wang, Ying Wang, et al.