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Short communication

LKB1 expression is inhibited by estradiol-17β in MCF-7 cells

Kristy A. Brown[a,b,*], Kerry J. McInnes[a,1], Kiyoshi Takagi[c], Katsuhiko Ono[c], Nicole I. Hunger[a], Lin Wang[c], Hironobu Sasano[c], Evan R. Simpson[a,d]

[a] Prince Henry's Institute, Block E Level 4, Monash Medical Centre, 246 Clayton Road, Clayton, Melbourne, Victoria 3168, Australia
[b] Department of Physiology, Monash University, Clayton, Melbourne, Victoria 3168, Australia
[c] Department of Pathology, Tohoku University, School of Medicine, Sendai, Japan
[d] Department of Biochemistry and Molecular Biology, Monash University, Clayton, Melbourne, Victoria 3168, Australia

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ABSTRACT

The liver kinase B1 (LKB1) is encoded by the *STK11* gene and acts as a tumour suppressor and a regulator of energy homeostasis. LKB1 expression is reduced in primary breast tumours compared to normal breast epithelium. Although its expression in primary tumours does not appear to correlate with estrogen receptor (ER) status, it is differentially expressed in breast cancer cell lines where ER-negative cells have lower LKB1 expression than ER-positive cells. The present study aimed to examine the effects of estradiol on LKB1 expression and activity in the ER-positive breast cancer cell line MCF-7. Results demonstrate that estradiol causes a dose-dependent decrease in LKB1 transcript and protein expression and consistent with this, a significant decrease in the phosphorylation of the LKB1 target AMPK ($P \leq 0.05$). In order to assess whether effects of estradiol were due to effects on ERα binding to the *STK11* promoter, ChIP was performed. Results demonstrate that ERα binds to the *STK11* promoter in a ligand-independent manner and that this interaction is decreased in the presence of estradiol. Moreover, *STK11* promoter activity is significantly decreased in the presence of estradiol ($P \leq 0.05$). LKB1 transcript and IHC score were assessed in primary tumours of 18 patients and demonstrated no significant correlation with ER status ($n = 18$). Our results thereby provide a mechanism whereby LKB1 is decreased in ER-positive breast tumours.

1. Introduction

The observation that liver kinase B1 (LKB1), encoded by the *STK11* gene, can cause G1 cell growth arrest when over-expressed in breast cancer cells led to an increased interest in the newly identified tumour suppressor [1]. Interestingly, breast cancer cell lines have differential expression of LKB1 depending on their estrogen receptor (ER) status. The human breast cancer cell line MCF-7, which is ER-positive, expresses LKB1, whereas ER-negative cell lines, such as MDA-MB-435 and MDA-MB-231, have a reduced LKB1 expression [2]. AMP-activated protein kinase (AMPK) is now recognized as a master regulator of energy homeostasis and is tightly regulated by endocrine signals, including leptin, adiponectin, estradiol and phytoestrogens [3–5]. LKB1 activates AMPK by directly phosphorylating its α-catalytic subunit at Thr172. The activation of AMPK in liver and adipocytes results in decreased lipogenesis and increased fatty acid oxidation. Interestingly, a high rate of lipogenesis is essential for the proliferation of many tumour cells including breast cancer cells [6], suggesting that LKB1/AMPK must be down-regulated in breast cancer cells to allow this process to go forward. The present study aimed to examine the effect of estradiol on LKB1 expression and activity in the human breast cancer cell line MCF-7 and to relate these findings to expression in clinical samples.

2. Materials and methods

2.1. Plasmids

The LKB1prom reporter construct was generated by amplifying a 3998 bp fragment of the *STK11* promoter located −3002 to +996 using primers LKB1prom-F: 5′-ACT TTG GAA ATT CAG TGT GTA GGG CA-3′ and LKB1prom-R: 5′-CAA CAA AAA CCC CAA AAG GA-3′ from BAC clone #RP11-50C6 (BAC PAC Resources, Children's Hospital Oakland Research Institute). Further PCR using primers LKB1prom-XhoI-F: 5′-CGG GAA TCT CGA GAC TTT GGA AAT TCA GTG TGT AGG GCA-3′ and LKB1prom-HindIII-R: 5′-AAA GCG CAA

Abbreviations: LKB1, liver kinase B1; STK11, serine-threonine kinase 11; ER, estrogen receptor; AMPK, AMP-activated protein kinase; ChIP, chromatin immunoprecipitation.

* Corresponding author at: Prince Henry's Institute, Monash Medical Centre, P.O. Box 5152, Clayton, Victoria 3168, Australia. Tel.: +61 3 9594 3249; fax: +61 3 9594 6125.
E-mail addresses: kristy.brown@princehenrys.org (K.A. Brown), kerry.mcinnes@ed.ac.uk (K.J. McInnes).
[1] Present address: Endocrinology Unit, Centre for Cardiovascular Science, Queen's Medical Research Institute, University of Edinburgh, EH16 4TJ.

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GCT TCA ACA AAA ACC CCA AAA GGA-3′ resulted in the amplification of a product containing XhoI and HindIII restriction enzyme cleavage sites. After enzymatic digestion the PCR product was subcloned into the pGL3 basic vector (Promega) and the insert identity was confirmed by sequencing.

2.2. Cell culture, transfection and reporter gene assays

MCF-7 cells were seeded at 3×10^5/ml in six-well plates and maintained at no higher than 70% confluence in DMEM (Trace Scientific Ltd., Melbourne, Australia) supplemented with 10% (v/v) fetal-calf serum (Trace Scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 mM L-glutamine (Life Technologies, Inc., Auckland, New Zealand). Cells were transfected using the Nucleofector electroporation apparatus (Amaxa) as directed by the manufacturer. Briefly, 1×10^6 cells were trypsinised, washed and resuspended in 100 μl Solution V with 2 μg DNA and transfected using program E-014, with the LKB1prom vector as well as 10 ng of a renilla expression vector as a transfection control. Cells were plated in 24-well plates and incubated overnight. Prior to treatments, cells were serum-starved for 24 h in phenol-red free medium containing 0.1% BSA. After serum starvation, cells were treated with water-soluble 17β-estradiol (Sigma) at the concentrations indicated. Luciferase reporter assays were carried out using the Dual-Glo Luciferase Assay System (Promega) as described by the manufacturer.

2.3. Western blot analysis

Cells were washed in ice-cold PBS and lysed in ice-cold buffer as previously described [7]. Fifty micrograms of protein was denatured in buffer containing dithiothreitol, run on 8% polyacrylamide gels, and transferred to nitrocellulose for Western blotting. Western blotting was performed to assay phosphorylation of AMPK using antibodies to phosphopeptides based on the amino acid sequence surrounding Thr172 of the α-subunit of human AMPK (Cell Signaling, Beverly, MA). The level of phosphorylation was normalized to the level of total AMPK (Cell Signaling). A specific LKB1 antibody (Cell Signaling) was used to assess LKB1 protein levels. Proteins were visualized with an Alexa Fluor 680 goat anti-rabbit secondary antibody (Molecular Probes, Inc., Eugene, OR), and band intensities were quantified using the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE).

2.4. RT and real-time PCR

The RNeasy Mini kit (Qiagen) was used to extract total RNA and reverse-transcription was performed using AMV RT and random primers (Promega) as directed by the manufacturer. Briefly, 1.0 μg RNA was incubated with 0.5 μg random primers at 70 °C for 5 min, and RT reaction was incubated at 37 °C for 1 h. Quantification of human LKB1 and L32 transcript was performed on the RotorGene (Corbett) using primers hLKB1-F: 5′-GCC GGG ACT GAC GTG TAG A-3′, hLKB1-R: 5′-CCC AAA AGG AAG GGA AAA ACC-3′, hL32-F: 5′-CAG GGT TCG TAG AAG ATT CAA GGG-3′, hL32-R: 5′-CTT GGA GGA AAC ATT GTG AGC GAT C-3′. Cycling conditions were one cycle at 95 °C for 5 min, followed by a variable number of cycles of 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 20 s. Experimental samples were quantified by comparison with standards of known concentrations. All samples were normalised to L32 transcript levels.

2.5. Chromatin immunoprecipitation

ChIP was performed to examine protein binding to the LKB1 promoter after cells were treated with experimental agents for 45 min. Sample preparation was performed as previously described [7]. Briefly, serum-starved cells were grown to 50% confluency and treated for 45 min at 37 °C for study of binding of transcriptional regulators to the LKB1 promoter. Cells were then cross-linked using 1% formaldehyde for 5 min at room temperature and collected in PBS containing protease inhibitors. Cells were lysed and sonicated at 20% max power 6 times for 30 s pulses using a Sonics sonifier. After sonication, one tenth of the total sample was removed for input. ChIP was performed using the ChIP-IT express kit (Active Motif) as directed by the manufacturer. Briefly, 5 μg of DNA was immunoprecipitated overnight at 4 °C with 5.0 μg antibody (ERα and IgG; Santa Cruz Biotechnology). Protein/DNA complexes were eluted from the beads and treated with proteinase K solution at 37 °C for 1 h. A number of putative ERα, AP-1 and Sp1 binding sites were identified in the region 2.5 kb upstream of the LKB1 promoter transcription start site using several online tools such as *AliBaba2.1* (http://www.gene-regulation.com/pub/programs/alibaba2/index.html), *PROMO* (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) and *Prediction of Nuclear Hormone Receptor Response Elements* (http://asp.ii.uib.no:8090/cgi-bin/NHR-scan/nhr_scan.cgi). Real-time PCR was performed on the purified DNA as described above using primers designed −2287 to −2020 (LKB1-ChIP-F: 5′-CTG CCT TCT TCC TGT TTT GC-3′; LKB1-ChIP-R: 5′-TTC TCC TCC TCC TCC TCC TC-3′) for ERα binding to the LKB1 promoter. Images presented are representative of three separate experiments.

2.6. Breast cancer cases

This research was approved by Ethical Committee of Tohoku University (Approval number 2010-509). Eighteen cases of treatment naive primary breast cancer cases were retrieved from pathology files at Department of Pathology, Tohoku University School of Medicine, Sendai, Japan. Portions of tumour tissues were carefully dissected at the operation theatre following macroscopic evaluation of resected specimens and immediately frozen in liquid nitrogen with OCT compound and further stored at −80 °C for Laser Capture Microdissection (LCM) analysis and subsequent Real-Time PCR (RT-PCR) assay. Portions of the specimens were also immediately fixed in 10% neutral formalin for 18–36 h at room temperature and embedded in paraffin. 4 μM thick tumour sample tissue specimens were prepared by the specimens embedded into OCT compound using cryostat and stained with hematoxylin for detailed morphological analysis under light microscopy for laser dissection of each components. Tumour cells were carefully laser dissected and then collected under light microscopy. The dissected tumour cells components were then submitted for RNA extraction and RT-PCR assay with methods as described above. For IHC or immunohistochemistry, paraffin blocks were cut to 4 μM sections and deparaffinized. The sections were then submitted for antigen retrieval with microwave in citrate buffer (pH 6.0) for 20 min; following the block with normal goat serum for 30 min at 4 °C, the sections were incubated with a polyclonal anti-LKB1 antibody overnight (1:100 dilution, Cell signaling, USA). Envision staining system (DAKO Cp Ltd., Denmark) was used for subsequent staining and LKB1 immunoreactivity was visualized with 3,30-diaminobenzidine (Dojin Chemical Co. Ltd., Osaka, Japan). Reacted sections were then counterstained with hematoxylin.

In order to semiquantitate LKB1 immunoreactivity, relative immunointensity (+, ++) and ratio of immunoreactivity among carcinoma cells were added to classify the status of LKB-1 immunoreactivity into the following three categories. Tumours with no staining or ≤10% of cells with (+) staining were tentatively scored as 0, tumours with >10% of cells with (+) staining or ≤20%

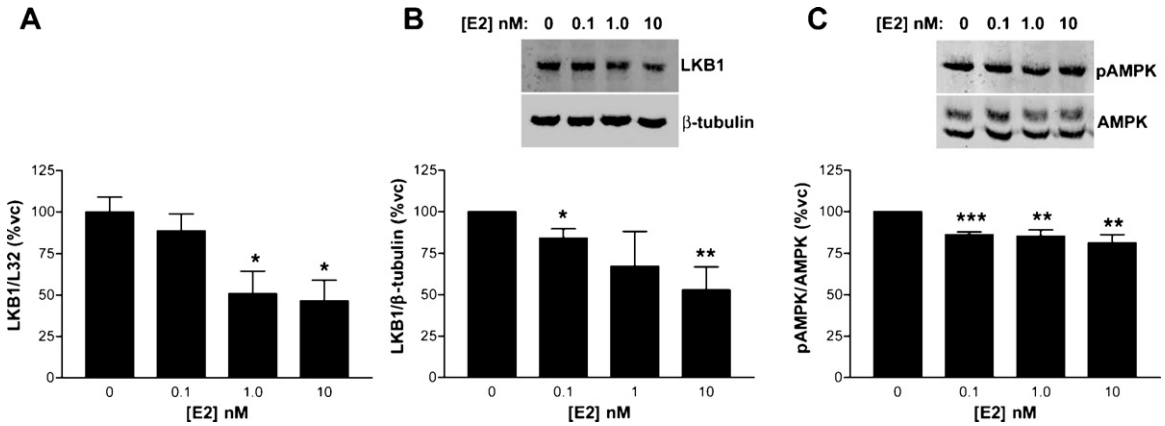

Fig. 1. Estradiol inhibits LKB1 expression and activity. Estradiol treatment of MCF-7 cells resulted in a dose-dependent decrease of LKB1 transcript (A) and protein (B) expression. (C) Estradiol treatment of MCF-7 cells resulted in a decrease in phosphorylation of AMPK. Graphs presented represent mean ± SEM. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. vc, vehicle control.

of cells with (++) staining as 1, and tumours with >20% of cells with (++) staining as 2.

ERα immunostaining status and other clinical parameters were retrieved from the charts of the patients.

2.7. Statistical analyses

For *in vitro* analysis, all experiments were performed at least three times and the data are reported as mean ± SEM. Statistical analyses were performed by two-tailed Student's *t* test. Kruskal Wallis non-parametric analyses were used to test correlations between LKB1 immunostaining score and different clinical parameters, Spearman non-parametric correlation for the analysis of the correlation between LKB1 and ERα. Immunostaining score. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. GraphPad Prism Version 3.00 was used.

3. Results

3.1. Estradiol decreases the expression of LKB1 in MCF-7 cells

MCF-7 cells are the most common breast cancer cell line employed to model ER-positive tumour cells. Their proliferation is largely dependent on the presence of E2, without which these cells cease to divide. The effect of estradiol on LKB1 expression was examined in MCF-7 cells after serum starvation. Treatment of MCF-7 cells with estradiol resulted in a dose-dependent decrease of LKB1 transcript and protein expression (Fig. 1A and B, respectively). This was accompanied by a similar decrease in phosphorylation of AMPK at Thr172 (Fig. 1C).

3.2. Estradiol decreases ERα binding to the LKB1 promoter

Chromatin immunoprecipitation assays performed using MCF-7 cells demonstrate that ERα binds to the LKB1 promoter. Interestingly, ERα binding to the LKB1 promoter is reduced when MCF-7 cells are treated with 10 nM E2 for 45 min (Fig. 2).

3.3. LKB1 promoter activity is decreased in the presence of estradiol

In order to assess the effect of estradiol on LKB1 promoter activity, a reporter construct was transfected into MCF-7. Consistent with effects on endogenous expression of LKB1 in MCF-7 cells, results demonstrate that estradiol caused a dose-dependent decrease in the activity of the LKB1 promoter in MCF-7 cells (Fig. 3).

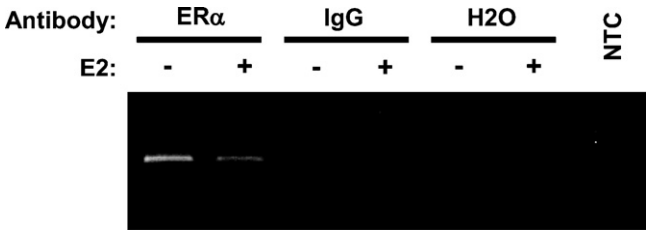

Fig. 2. ERα binds to the LKB1 promoter in the absence of estradiol. ChIP analysis demonstrated that ERα binding to the LKB1 promoter was reduced when MCF-7 cells were treated with 10 nM E2. The result is representative of three separate experiments.

3.4. Correlation between LKB1 and ER status

The results are summarized in Tables 1 and 2. There was no significant correlation between LKB1 IHC score/mRNA and other clinicopathological parameters examined in these cases (Tables 1 and 2, respectively). In addition, there were no significant correlations between LKB1 IHC score and ER Allred score as demonstrated by Spearman non-parametric test ($r = -0.186$, $P = 0.460$).

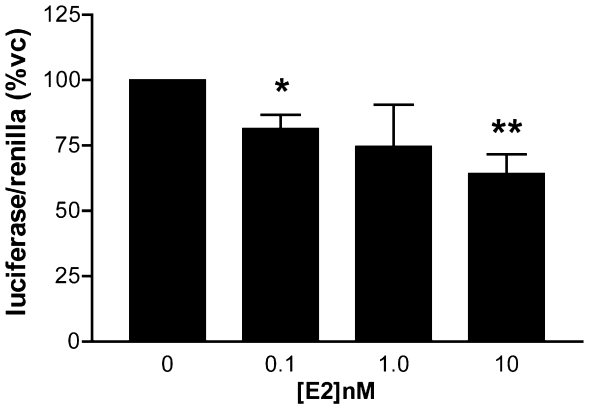

Fig. 3. Estradiol inhibits LKB1 promoter activity. A reporter construct containing 3003 bp of the LKB1 promoter was transfected into MCF-7. Results demonstrate that promoter activity was significantly decreased with increasing doses of estradiol in MCF-7 cells. Graphs presented represent mean ± SEM. Single, double, and triple asterisks indicate statistically significant differences: $^{*}p < 0.05$; $^{**}p < 0.01$. vc, vehicle control.

Table 1
LKB1 IHC in primary breast cancer tissues.

Parameters	*n*	Cases of different LKB1 scores			*P* value[†]
		0	1	2	
Age					
≤50	6	4	1	1	0.205
>50	12	3	7	2	
Nuclear grade					
I	8	3	3	2	0.068
II	5	4	1	0	
III	5	3	2	0	
Nottingham grade					
I	0	0	0	0	0.317
II	12	7	3	2	
III	2	0	2	0	
ER state[a]					
Negative	2	0	2	0	0.543
Positive	16	7	6	3	
PgR state[b]					
Negative	6	2	4	0	0.761
Positive	12	5	4	3	
HER-2 state[c]					
Negative	14	6	5	3	0.954
Positive	4	1	3	0	

[†] Kruskal Wallis non-parametric test was used for comparing LKB1 immunostaining score among different groups.
[a] The specimens with ER allred score ≤ 2 were classified into ER negative group, those with ER allred score > 2 were classified into ER positive group.
[b] The specimens with PgR allred score ≤ 2 were classified into PgR negative group, those with PgR allred score > 2 were classified into PgR positive group.
[c] The specimens with HER-2 scored 0 and 1 were classified into HER-2 negative group, those with ER scored 2 and 3 were classified into ER positive group.

4. Discussion

The regulation of LKB1 in various tissues has previously been examined (reviewed in [8,9]). The majority of these studies have focussed on the regulation of LKB1 phosphorylation by PKCζ and its resultant action on AMPK, leaving few indices as to the transcriptional regulation of the *STK11* gene. Results presented herein are therefore the first to describe the transcriptional regulation of LKB1 by estradiol and to identify ERα as a direct modulator of LKB1 promoter activity.

ChIP analysis in MCF7 cells showed binding of ERα to the *STK11* promoter, however consistent with the effects of 17β-estradiol on LKB1 expression, ERα binding to the *STK11* promoter was reduced in the presence of 17β-estradiol. It remains to be determined which site of the *STK11* promoter is involved in the ligand-independent binding of ERα to DNA and whether binding occurs in a similar manner in untransformed cells. Considering recent evidence identifying LKB1 as a tumour suppressor by virtue of its direct interaction with p53 [2,10,11], our results provide an additional mechanism by which estradiol can promote cell cycle progression in cells with a wild-type *TP53* gene.

LKB1 protein expression and pAMPK are decreased in primary breast tumours compared to normal breast epithelium [12,13]. Interestingly, we examined LKB1 mRNA and IHC score in primary tumours obtained from 18 Japanese patients with breast cancer. Results of this evaluation revealed no significant correlations of the LKB1 status with ER status in these patients. This is consistent with previous findings demonstrating that LKB1 and pAMPK IHC had no association with ER status [2,13]. Considering the effect of estradiol to inhibit LKB1 expression and activity in MCF-7 cells, the present study is the first to offer a mechanism whereby LKB1 expression is low in both ER-positive and ER-negative primary tumours, and why there is a discrepancy between untreated cell lines and primary tumours.

In parallel with this process, the inhibition of the LKB1/AMPK pathway will likely also result in the stimulation of *de novo* lipogenesis within the breast cancer cells, which is also an important factor contributing to breast cancer cell proliferation [14], and will prevent AMPK from inhibiting cancer cell proliferation through direct phosphorylation of TSC2 and mTORC1 (mammalian target of rapamycin complex 1), thereby preventing it from effectively shutting down protein synthesis and counteracting the stimulatory effects of Akt [15]. Furthermore, LKB1 has also been shown to negatively regulate aromatase [7], the enzyme responsible for converting androgens to estrogens. Consistent with these findings, it has been shown that metformin, a known LKB1-dependent stimulator of AMPK, inhibits proliferation of breast cancer cells in culture [16,17], aromatase expression in breast stroma [18] and inhibits spontaneous tumours from developing in PTEN deficient mice [19–21].

Table 2
LKB1 mRNA expression in primary breast cancer tissues.

Parameters	*n*	Relative LKB1 mRNA level (%, Mean ± SE)	*P* value[†]
Age			
≤50	6	1.876 ± 0.496	0.407
>50	12	3.672 ± 1.449	
Nuclear grade			
I	8	3.395 ± 1.699	0.657
II	5	1.604 ± 0.402	
III	5	4.029 ± 2.411	
Nottingham grade			
I	0	–	0.211
II	12	2.791 ± 1.147	
III	2	7.372 ± 6.054	
ER state[a]			
Negative	2	2.721 ± 1.039	0.904
Positive	16	3.118 ± 1.108	
PgR state[b]			
Negative	6	3.935 ± 1.957	0.553
Positive	12	2.643 ± 1.154	
HER-2 state[c]			
Negative	14	2.560 ± 0.988	0.345
Positive	4	4.871 ± 2.929	

[†] ANOVA was used for comparing relative LKB1 mRNA expression among multigroups; independent Student's *t* test was used for comparing relative LKB1 mRNA expression between two groups.
[a] The specimens with ER allred score ≤ 2 were classified into ER negative group, those with ER allred score > 2 were classified into ER positive group.
[b] The specimens with PgR allred score ≤ 2 were classified into PgR negative group, those with PgR allred score > 2 were classified into PgR positive group.
[c] The specimens with HER-2 scored 0 and 1 were classified into HER-2 negative group, those with ER scored 2 and 3 were classified into ER positive group.

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