ROCKII Ser\textsuperscript{1366} phosphorylation reflects the activation status

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ROCK (Rho-associated protein kinase), a downstream effector of RhoA, plays an important role in many cellular processes. Accumulating evidence has shown the involvement of ROCK activation in the pathogenesis of many diseases. However, a reagent capable of detecting ROCK activation directly is lacking. In the present study, we show autophosphorylation of ROCKII in an \textit{in vitro} kinase reaction. The phosphorylation sites were identified by MS, and the major phosphorylation site was found to be at the highly conserved residue Ser\textsuperscript{1366}. A phospho-specific antibody was generated that can specifically recognize ROCKII Ser\textsuperscript{1366} phosphorylation. We found that the extent of Ser\textsuperscript{1366} phosphorylation of endogenous ROCKII is correlated with that of myosin light chain phosphorylation in cells in response to RhoA stimulation, showing that Ser\textsuperscript{1366} phosphorylation reflects its kinase activity. In addition, ROCKII Ser\textsuperscript{1366} phosphorylation could be detected in human breast tumours by immunohistochemical staining. The present study provides a new approach for revealing the ROCKII activation status by probing ROCKII Ser\textsuperscript{1366} phosphorylation directly in cells or tissues.

Key words: autophosphorylation, marker, RhoA, Rho-associated protein kinase (ROCK).

INTRODUCTION

ROCKs (Rho-associated protein kinases) belong to the AGC serine/threonine protein kinase family [1] and play key roles in many cellular processes, including cell migration, apoptosis, centrosome duplication and cytokinesis [2–7]. Two ROCK isoforms have been identified in mammals: ROCKI and ROCKII [8,9]. They are composed of a kinase domain in the N-terminus, an RBD (Rho-binding domain) and a PH (pleckstrin homology) domain in the C-terminal region, which folds back on to the kinase domain to form an autoinhibitory loop that maintains ROCK in an inactive state [10]. The binding of GTP-bound RhoA to the RBD of ROCK disrupts this negative interaction, switching on ROCK activation [8,11], and its RhoA-binding affinity is regulated by Src-dependent phosphorylation [12]. In addition to RhoA binding, ROCK can be activated through proteolytic cleavage of the C-terminal inhibitory domain in apoptotic cells [13–15] or by intracellular second messengers, such as arachidonic acid [16,17]. ROCK kinase activity is also regulated through interaction with several proteins, such as stimulation by Polo-like kinase-1 and nucleophosmin [7,18], and repression by Gem, Rad and Morgana [19,20]. Thus various signalling pathways modulate ROCK activity.

ROCK activation leads to phosphorylation of various substrates, one of which is MLC (myosin light chain) [1,3]. ROCK phosphorylates MLC directly and regulates the amount of phosphorylated MLC indirectly by inactivating MLCP (MLC phosphatase) through the phosphorylation of its MBS (myosin-binding subunit) [21,22]. As a result, myosin II ATPase is activated to generate actomyosin contractility, thus contributing to RhoA-induced stress fibres and focal adhesions in fibroblasts [23,24]. Cancer cells have been shown to respond to microenvironmental cues, such as matrix stiffness or inflammation signals, to increase ROCK-mediated actomyosin contractility as a driving force in tumour growth and invasion in tissues [25–29]. Given that stiffer tissues associated with hepatocarcinoma and breast cancer development involve ROCK activation, inhibition of ROCK reduces metastasis in hepatoma and breast tumour models [30–32]. Similar effects have also been shown in other cancer types, such as leukaemia, melanoma, and prostate and lung cancer [30,33–35]. In addition to cancers, many clinical trials using the ROCK inhibitor fasudil have shown significant therapeutic effects on cardiovascular and neurological diseases [2,36–38]. All of these results suggest the importance of ROCK activity in the pathogenesis of many diseases. However, whether ROCK activity is higher in these diseased tissues than in normal counterparts remains unknown.

Despite the importance of ROCK functions, a reagent that can assess ROCK activation directly in tissues or cells is lacking. Currently, ROCK activation can be detected directly by \textit{in vitro} kinase assay [39] or indirectly by determining the inhibitor-sensitive phosphorylation of its downstream substrates, such as MLC, MBS, LIMK (LIM kinase) and α-adducin [40]. However, none of these markers reflects ROCK activation status in cell and tissue samples. In the present study, we identified ROCK inhibitor-sensitive autophosphorylation sites in an \textit{in vitro} kinase reaction and generated a phospho-specific antibody to reveal ROCKII activation. We found that Ser\textsuperscript{1366} phosphorylation of ROCKII reflects its kinase activation and provides a direct way for assessing the extent of ROCKII activation in disease progression.

Abbreviations used: DTT, dithiothreitol; GFP, green fluorescent protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; IHC, immunohistochemical; HEK, human embryonic kidney; LC, liquid chromatography; LPA, lysophosphatidic acid; MBS, myosin-binding subunit; MEF, mouse embryonic fibroblast; MLC, myosin light chain; MS/MS, tandem MS; xPase, x protein phosphatase; RBD, Rho-binding domain; ROCK, Rho-associated protein kinase; siRNA, small interfering RNA; WT, wild-type.

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EXPERIMENTAL

Plasmids and reagents

Specific mutations were individually introduced to WT (wild-type) pEF-myc-ROCKII (6-1388) (from K. Kaibuchi, Nara Institute of Science and Technology, Ikoma, Japan) using the QuickChange® site-directed mutagenesis kit (Stratagene). The cDNA of ROCKI was originally from J. Hamelin (INSERM U461, Faculté de Pharmacie, Châteay-Malabry, France) and subcloned into pCMV2 with FLAG tag. The phospho-MLC2 (Thr18/Ser19) antibody was from Cell Signaling. Antibodies were purchased from Santa Cruz Biotechnology; anti-(lysophosphatidic acid); nocodazole and anti-MLC antibody against human ROCKII was obtained from Dharmacon; LPA (lysophosphatidic acid); polyethylene glycol (PEG); siRNA (small interfering RNA) subcloned into pCMV2 with FLAG tag. Y27632 was from U461, Faculté de Pharmacie, Châteay-Malabry, France) and cDNA of ROCKI was originally from J. Hamelin (INSERM U461, Faculté de Pharmacie, Châteay-Malabry, France) and subcloned into pCMV2 with FLAG tag. 

Cell culture and transient transfection

Normal MEFs (mouse embryonic fibroblasts) and HEK (human embryonic kidney)-293T cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum) in a humidified atmosphere of 5% CO2/95% air at 37°C. For transient transfection experiments, HEK-293T cells were transfected by PolyJet reagent (SignaGen Laboratories). For siRNA transfection experiments, cells were transfected by Lipofectamine® 2000 reagent (Invitrogen).

Immunoprecipitation and in vitro kinase reaction

myc–ROCKII-expressing cells were harvested in an immunoprecipitation buffer (1% Nonidet P40, 5% glycerol, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF and protease inhibitor cocktail). After pre-clearance, the lysates were incubated with anti-myc antibody at 4°C for 60 min, and then precipitated with Protein A–agarose beads for 30 min. The immunoprecipitates were pre-incubated with or without 100 μM Y27632, which was followed by incubation with a 1× kinase buffer [50 mM Tris/HCl, pH 7.4, 10 mM MgCl2, 1 mM EGTA, 0.5 mM DTT (dithiothreitol), 5 mM NaF, 0.1 mM Na3VO4 and 20 μM ATP] containing 5 μCi of [γ-32P]ATP at 30°C for 20 min. The kinase catalytic activity of myc–ROCKII was measured as described previously [41]. Briefly, the immunoprecipitates were pre-incubated with or without 1 μM GTP[S] (guanosine 5′-γ-thio)triphosphate)-loaded GST–RhoA in 1× kinase buffer for 5 min. The kinase reaction was started by adding 2.5 μg of myelin basic protein and 5 μCi of [γ-32P]ATP. After incubation at 30°C for 20 min, 5 μl of reaction mixture was spotted on to P81 paper (Whatman), and the papers were washed five times with 0.75% phosphoric acid. Incorporation of 32P into substrate was determined by scintillation counting. The immunoprecipitates from non-transfected cells (MOCK) was used as background. The protein levels of myc–ROCKII detected by Western blotting with anti-myc antibody.

In-gel digestion and LC (liquid chromatography)–MS/MS (tandem MS)

The protein band corresponding to myc–ROCKII was excised from the gel for proteinase digestion as described by Tsay et al. [42]. In brief, proteins were digested with Lys-C, Arg-C or Asp-N (multiple enzymes used to increase sequence coverage), and the peptides were extracted with 0.1% formic acid. Electrospray ionization–ion-trap–MS/MS was performed using a Thermo Finnigan LTQ Orbitrap tandem mass spectrometer interfaced with an Agilent 1100D HPLC system (Proteomes Research Center, Yang-Ming University). The spectra for the eluate were acquired as successive sets of scan modes described by Tsay et al. [42], which included (i) identification of phosphopeptides on selected ion tracings on the basis of their elution behaviour, (ii) mapping phosphoamino acids within the sequence by CID (collision-induced dissociation) experiments, and (iii) using the selected ion tracking method to quantitatively determine the phosphorylation states of myc–ROCKII proteins.

RhoA–ROCK interaction assay

Interaction between RhoA and ROCKII was measured as described previously [41]. Briefly, cells expressing myc–ROCKII were harvested and lysed with buffer (0.2% Nonidet P40, 20 mM Tris/HCl, pH 7.4, 20 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 2 mM Na3VO4, 1 mM PMSF, 10% glycerol and protease inhibitor cocktail). Pre-cleared supernatants were incubated with different amounts (0–5 μg) of GTP[S]-loaded GST–RhoA protein for 15 min and followed by incubation with glutathione beads at 4°C for another 20 min. After extensive washing, myc–ROCKII pulled down by GTP[S]–GST–RhoA was detected by Western blotting with anti-myc antibody.

Phospho-specific antibody generation

The polyclonal anti-(pSer1366 ROCKII) antibody was raised using phosphopeptide containing pSer1366 of ROCKII conjugated with KLH (keyhole-limpet haemocyanin) as an antigen to immunize rabbits. Antisera were collected and sequentially affinity purified by phosphopeptide- and non-phosphopeptide-conjugated columns (GTX122651; ICON Biotechnology).

IHC (immunohistochemical) staining

Specimens used for immunohistochemistry followed ethical standards of the Helsinki Declaration of 2008 and the Institutional Review Board of Taipei Veterans General Hospital. The tumour part and non-tumour part of the breast tissue blocks were embedded in Tissue-Tek OCT™ compound, snap-frozen and sectioned into 5 μm cryosections. The sections were fixed with 4% formaldehyde for 15 min and placed in boiling citrate buffer for 10 min. Endogenous peroxidase activity was quenched with 3% H2O2 for 10 min and this was followed by incubation with 5.5% (v/v) paraformaldehyde for 15 min and placed in boiling citrate buffer for 10 min. Endogenous peroxidase activity was quenched with 3% H2O2 for 10 min and this was followed by incubation with 5.5% (v/v) normal goat serum in TBST (Tris-buffered saline containing 0.1% Triton X-100) for blocking. Sections were subsequently incubated with primary antibody against pSer1366 ROCKII (1:200 dilution; 5 μg/ml) in the presence or absence of the phosphopeptide or non-phosphopeptide (0.5 μg/ml). After extended washing, sections were incubated with HRP (horseradish peroxidase)-conjugated anti-rabbit antibody and then developed with DAB (diaminobenzidine) for 3 min. Slides were counterstained with haematoxylin, which was followed by dehydration then mounting.

RESULTS

Identification of Y27632-sensitive phosphorylation sites within ROCKII

Many protein kinases are autophosphorylated when they become active [43–45]. To determine whether this also occurs in ROCKII, myc–ROCKII protein overexpressed in HEK-293T cells was
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Figure 1  Identification of the autophosphorylation sites of ROCKII

(A) Myc–ROCKII overexpressed in HEK-293T cells was immunoprecipitated with anti-myc antibody and pre-incubated with or without 100 μM Y27632 for an in vitro kinase reaction with 20 μM ATP plus 5 μCi of [γ-32P]ATP. After 20 min, the reaction was stopped and proteins were applied to SDS/PAGE for autoradiography. The protein level of myc–ROCKII was detected by Western blotting (WB) with anti-myc antibody. Molecular masses are indicated in kDa. (B) For LC–MS/MS analysis, the reaction without radioactively labelled ATP was performed and the gel was stained with Coomassie Blue. The protein band corresponding to myc–ROCKII was excised for in-gel digestion. Molecular masses are indicated in kDa. (C) The tandem mass spectra of phospho-peptides.

immunoprecipitated and subjected to an in vitro kinase reaction containing [γ-32P]ATP in the presence or absence of the ROCK inhibitor Y27632 [46]. Radioactively labelled ROCKII was detected and Y27632 treatment abolished the phosphorylation signal (Figure 1A). To identify phosphorylation sites, immunoprecipitated ROCKII was incubated with non-radioactive ATP in a kinase reaction, followed by SDS/PAGE separation. The protein band corresponding to myc–ROCKII (Figure 1B) was digested with Lys-C, Arg-C or Asp-N to increase the total sequence coverage (78.3 %) for LC–MS/MS analysis. Three Y27632-sensitive phosphopeptides were found (Figure 1C). Judging from the increased mass of 79.96 Da in the spectra, each peptide is monophosphorylated at Thr1365/Ser1366, Ser1374 or Ser1379.

Characterization of autophosphorylation sites on ROCKII

We tested the contribution of Thr1365, Ser1366, Ser1374 and Ser1379 in the phosphorylation of myc-ROCKII carrying an alanine mutation in these sites. As shown in Figure 2(A), the S1366A mutant gave a clear reduction in the intensity of [γ-32P]ATP labelling in contrast with no effect for the T1365A mutation, ruling out Thr1365 in phosphorylation. Mutation at either Ser1374 or Ser1379 reduced the radioactive labelling intensity, but not as much as the S1366A mutation. Four alanine substitutions (4A) almost completely abolished the radioactive labelling signal of myc–ROCKII. In summary, ROCKII is autophosphorylated at Ser1366, Ser1374 and Ser1379, with the highest distribution at Ser1366.

We then performed a GTP[S]–GST–RhoA pull-down assay to determine whether in vitro autophosphorylation has an effect on ROCKII binding to the active form of RhoA. The WT and 4A mutant of myc–ROCKII exhibited similar RhoA-binding ability (Figure 2B). Furthermore, the in vitro kinase assay also indicated that unphosphorylatable (4A) and phosphomimetic (4E) myc–ROCKII mutants have similar catalytic activities when stimulated with GTP[S]-loaded GST–RhoA (Figure 2C). Thus in vitro phosphorylation of ROCKII at these sites has no effect on RhoA binding or catalytic function.
Figure 2 Characterization of ROCKII autophosphorylation

WT and various mutants of myc–ROCKII were expressed in HEK-293T cells for the following assays. (A) Autophosphorylation in vitro. Proteins were immunoprecipitated with anti-myc antibody for the in vitro kinase reaction as described in Figure 1(A). (B) GST–RhoA pull-down assay. An equal amount of protein in each lysate was incubated with the indicated amount of GTP[S]-GST–RhoA protein followed by glutathione–Sepharose bead pull-down. The myc–ROCKII pull-down was determined by Western blotting with anti-myc antibody. GST–RhoA protein was revealed by Coomassie Blue staining. One representative result is shown at the top and the quantification of amounts of myc–ROCKII in the pull-down materials relative to the total input from three independent experiments is shown at the bottom. (C) Comparison of RhoA-dependent kinase activation of 4A and 4E myc–ROCKII. Immunoprecipitated myc–ROCKII proteins were stimulated with or without 1 μM GTP[S]-loaded RhoA and the catalytic activity was determined by an in vitro kinase assay. Data from three independent experiments are expressed as the fold increase relative to 4A mutant without RhoA incubation. 4A, alanine substitution for Thr 1365, Ser1366, Ser1374 and Ser1379; 4E, glutamate substitution for Thr 1365, Ser1366, Ser1374 and Ser1379. Results are means ± S.D. for three independent experiments.

An antibody to specifically recognize phosphorylation of Ser\(^{1366}\) in ROCKII

To determine whether phosphorylation at Ser\(^{1366}\) is a mark of active ROCKII in biological samples, we generated anti-(pSer\(^{1366}\) ROCKII) antibody, which was capable of detecting the in vitro autophosphorylation of immunoprecipitated WT but not S1366A myc–ROCKII by Western blotting, and the signal was neutralized by phosphorylated peptide but not non-phosphorylated peptide (Figure 3A). Treatment of WT myc–ROCKII immunoprecipitates with λPPase (λ protein phosphatase) abolished the antibody detection signal (Figure 3B), indicating its specificity in detecting phosphorylated ROCKII. Furthermore, the signal level of Ser\(^{1366}\)-phosphorylated myc–ROCKII was markedly decreased in cells treated with Y27632 (Figure 3C).

We also found that ROCKI was autophosphorylated in the in vitro kinase reaction (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430145add.htm). The sequence of human ROCKI corresponding to the region surrounding Ser\(^{1366}\) of ROCKII shows 50% identity (Figure 3D). We immunoprecipitated FLAG–ROCKI and FLAG–ROCKII proteins expressed in cells for Western blot analysis using anti-(pSer\(^{1366}\) ROCKII) antibody. The result showed no cross-reactivity of anti-(pSer\(^{1366}\) ROCKII) antibody to ROCKI (Figure 3E). All of these results suggest that the antibody specifically recognizes ROCKII Ser\(^{1366}\) phosphorylation.

Detecting endogenous ROCKII activation by probing Ser\(^{1366}\) phosphorylation

Next, we tested whether Ser\(^{1366}\) phosphorylation on endogenous ROCKII can be detected in response to RhoA activation in cells. We found that the level of Ser\(^{1366}\) phosphorylation of ROCKII in HEK-293T cells was increased by expression of constitutively active GFP (green fluorescent protein)–RhoAV14. In contrast, neither GFP–RhoAV14E40L, a constitutively active mutant of RhoA defective in its interaction with ROCK [47], nor a dominant-negative form of GFP–RhoAN19 altered the level of ROCKII Ser\(^{1366}\) phosphorylation (Figure 4A). Depletion of the endogenous ROCKII by siRNA transfection diminished the staining signal, confirming that the signal derived from ROCKII (Figure 4B). These results demonstrated that specific activation of endogenous ROCKII by RhoA leads to an increase in Ser\(^{1366}\) phosphorylation. Thus RhoA-mediated ROCKII activation in cells can be specifically detected by Western blotting with the anti-(pSer\(^{1366}\) ROCKII) antibody.

We also examined the level of Ser\(^{1366}\) phosphorylation of ROCKII in MEFs that were serum-starved and treated with LPA to activate the RhoA signal through a G-protein-coupled receptor [23]. We found that LPA treatment for 15 min increased Ser\(^{1366}\) phosphorylation of ROCKII and co-treatment with Y27632 prevented Ser\(^{1366}\) phosphorylation as expected, coinciding with MLC phosphorylation (Figure 5A). Similarly, treatment with
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Figure 3 Validation of anti-(pSer1366 ROCKII) antibody

HEK-293T cells were transfected with the ROCK expression constructs as indicated. (A) Myc–ROCKII proteins were immunoprecipitated with anti-myc antibody and probed with anti-(pSer1366 ROCKII) antibody (1:1000 dilution; 1 μg/ml) with or without peptide (0.2 μg/ml) competition as indicated. (B) WT myc–ROCKII immunoprecipitates were incubated with or without λPPase at 30°C for 20 min before SDS/PAGE separation and Western blot analysis. (C) The transfected cells were treated with or without 20 μM Y27632 for 2 h before cells were harvested. Proteins were immunoprecipitated and detected by Western blot analysis. (D) The amino acid sequence alignment of human ROCKI and the region surrounding Ser1366 in ROCKII. Residues corresponding to Ser1366 of ROCKII are highlighted white on black and indicated by an asterisk. (E) FLAG-tagged ROCKI and ROCKII were immunoprecipitated with anti-FLAG antibody and detected by Western blot analysis (WB) as indicated. MOCK, non-transfected cells. IP, immunoprecipitation.

nocodazole, which disrupts microtubules and results in RhoA activation via GEF-H1 [48], also enhanced ROCKII Ser1366 phosphorylation as seen in MLC phosphorylation (Figure 5B). Taken together, the level of Ser1366 phosphorylation of ROCKII can reflect the status of RhoA-mediated ROCKII activation in cells.

Having demonstrated ROCKII Ser1366 phosphorylation in a cellular setting, we next used this anti-(pSer1366 ROCKII) antibody to test whether ROCKII Ser1366 phosphorylation could be detected in tissues by IHC staining. Since several reports have suggested the involvement of ROCK activation in breast cancer [30,32], the frozen breast tissues from two patients with breast cancer were used to test the IHC application of anti-(pSer1366 ROCKII) antibody. We found that the ROCKII Ser1366 phosphorylation signal was detectable and pronounced in the patient specimens from the tumorous tissue, whereas the signal in the non-tumorous tissue was weak (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/443/bj4430145add.htm). The staining signal could be abolished by competition with phosphorylated Ser1366 peptide, indicating the specificity of detection. Thus anti-(pSer1366 ROCKII) antibody may be useful for assessment of ROCKII activation in various settings such as breast cancer.

DISCUSSION

In the present study, we carried out an in vitro kinase reaction of ROCKII and identified three Y27632-sensitive phosphorylation sites: Ser1366, Ser1374 and Ser1379. Among them, Ser1366 was demonstrated to be the major autophosphorylation site. Using
anti-(pSer\textsuperscript{1366} ROCKII) antibody, we showed that the level of ROCKII Ser\textsuperscript{1366} phosphorylation correlates with RhoA activation in cells. Altogether, our data suggest that Ser\textsuperscript{1366} phosphorylation may serve as a marker of ROCKII activation. The IHC staining also detected a significant increase in Ser\textsuperscript{1366} phosphorylation of ROCKII in breast cancer tissues compared with their non-tumorous counterparts. Nevertheless, more samples for analysis are needed to validate its potential as a breast cancer marker.

ROCK belongs to the AGC kinase family, and the consensus substrate sequence for phosphorylation is R/KXS/T or R/KXXS/T \cite{[1,3]}. Sequences surrounding Ser\textsuperscript{1366} and Ser\textsuperscript{1379} fit the consensus substrate sequences, thereby becoming phosphorylated as a result of ROCKII activation. It is likely that Ser\textsuperscript{1379} phosphorylation is another potential indicator of ROCKII activation. Unlike the autophosphorylation of PAK1 (p21-activated kinase 1) on Thr\textsuperscript{423}, which is required for full kinase activation \cite{[49]}, we found that kinase activity of ROCKII is not affected by introducing mutations at the autophosphorylation sites to unphosphorylatable alanine or phosphomimetic glutamate. This raises a concern if Ser\textsuperscript{1366} phosphorylation status is sustained even when its catalytic function has been switched off. The transient induction of ROCKII Ser\textsuperscript{1366} phosphorylation in MEFs in response to LPA or nocodazole stimulation suggests that this phosphorylation status is not persistent. The pattern, indeed, is similar and correlated to that of MLC phosphorylation (Figure 5). Since MLC is also a substrate of MLC kinase, the increase in Ser\textsuperscript{1366} phosphorylation level can be a more direct readout of ROCKII activation.

We have reported previously that Tyr\textsuperscript{722} phosphorylation affects the binding affinity of ROCKII for RhoA \cite{[41]}. In the present study, we found that autophosphorylation does not affect myc–ROCKII binding with RhoA. However, whether these autophosphorylations have a functional effect on protein targeting or protein–protein interaction \textit{in vivo} remains to be investigated. Also unknown is whether these phosphorylation events take place intracellularly or intercellularly.

The importance of ROCK in pathogenesis is shown by using its specific inhibitors to interfere with disease progression in clinical trials and animal experiments. Liu et al. \cite{[32]} reported that the levels of ROCKI and ROCKII transcripts of metastatic cells in bone cores were significantly higher than cells from primary tumours at the orthotopic site in a “human breast cancer metastasis to human bone” mouse model. Given that application of a ROCK inhibitor reduced cells metastasizing to bone \textit{in vivo}, the increase in ROCK function makes a critical contribution to cancer progression \cite{[32]}. In the present study, we also detected Ser\textsuperscript{1366} phosphorylation in human breast cancer tissues, indicating its feasibility as a marker in clinical diagnosis. Moreover, the surrounding sequence covering Ser\textsuperscript{1366} of ROCKII is highly conserved among different species of mammals, allowing probing of Ser\textsuperscript{1366} phosphorylation in tissues in the mouse model experiments. In fact, we were able to detect positive signals by IHC staining in cirrhosis of the liver in rat (\cite{[50]} and results not shown). Given the existence of another ROCK isoform, it is possible to apply the same approach to define phosphorylation sites for assessing the activation status of ROCKI in cell and tissue samples. The specific antibodies against phosphorylated ROCKs that mark activation will provide valuable tools for evaluation of ROCK activation in disease progression.

**AUTHOR CONTRIBUTION**

Chih-Hsuan Yang performed the \textit{in vitro} experiments and the identification of ROCKII autophosphorylation sites. Hsiang-Hao Chuang carried out the experiments for mutational analysis and Western blot analysis for antibody validation. Yeo-Yuang Tsay carried out the MS analysis. Chih-Yi Hsu and Ling-Ming Tseng contributed to the IHC experiment. Zee-Fen Chang and Hisiao-Hui Lee conceived and planned the experiments. Hisiao-Hui Lee wrote the paper. All authors read and approved the final paper.

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SUPPLEMENTARY ONLINE DATA

ROCKII Ser\textsuperscript{1366} phosphorylation reflects the activation status

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Figure S1  Autophosphorylation of ROCKI

The FLAG–ROCKI protein was expressed in HEK-293T cells and immunoprecipitated with anti-FLAG antibody. Equal amount of immunoprecipitated complexes were pre-incubated with 100 \mu M Y27632 for 20 min and subjected to an in vitro kinase reaction with 5 \mu Ci of \( \gamma ^{32} \text{P} \)ATP. After 20 min, the reaction was stopped and proteins were then applied to SDS/PAGE followed by autoradiography. The protein levels of FLAG–ROCKI were determined by Western blotting (WB) with anti-FLAG antibody.

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Figure S2  ROCKII Ser1366 phosphorylation in human breast tumours

IHC staining results of pSer1366 ROCKII in frozen breast tissues. The tumorous and non-tumorous tissue blocks from two breast cancer patients were sectioned and stained with anti-(pSer1366 ROCKII) antibody in the presence of the phosphopeptide or non-phosphopeptide as indicated. IHC staining with normal rabbit IgG in tumorous specimens is shown as a negative control. Haematoxylin was used for counterstaining. The images were acquired with a ×10 (upper panels) or ×40 (lower panels) lens. Both samples are oestrogen receptor-positive invasive ductal cancer. #1 is lymph node-negative and #2 is lymph node-positive.

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