

## ORIGINAL ARTICLE

## Activation of tumor suppressor LKB1 by honokiol abrogates cancer stem-like phenotype in breast cancer via inhibition of oncogenic Stat3

S Sengupta<sup>1,10</sup>, A Nagalingam<sup>1,10</sup>, N Muniraj<sup>1</sup>, MY Bonner<sup>2</sup>, P Mistriotis<sup>3</sup>, A Afthinos<sup>3</sup>, P Kuppusamy<sup>4</sup>, D Lanoue<sup>5</sup>, S Cho<sup>1</sup>, P Korangath<sup>1</sup>, M Shriver<sup>1</sup>, A Begum<sup>1</sup>, VF Merino<sup>1</sup>, C-Y Huang<sup>6</sup>, JL Arbiser<sup>2,7</sup>, W Matsui<sup>1</sup>, B Györfy<sup>8,9</sup>, K Konstantopoulos<sup>3</sup>, S Sukumar<sup>1</sup>, PA Marignani<sup>5</sup>, NK Saxena<sup>4,11</sup> and D Sharma<sup>1</sup>

Tumor suppressor and upstream master kinase Liver kinase B1 (LKB1) plays a significant role in suppressing cancer growth and metastatic progression. We show that low-LKB1 expression significantly correlates with poor survival outcome in breast cancer. In line with this observation, loss-of-LKB1 rendered breast cancer cells highly migratory and invasive, attaining cancer stem cell-like phenotype. Accordingly, LKB1-null breast cancer cells exhibited an increased ability to form mammospheres and elevated expression of pluripotency-factors (Oct4, Nanog and Sox2), properties also observed in spontaneous tumors in *Lkb1*<sup>-/-</sup> mice. Conversely, LKB1-overexpression in LKB1-null cells abrogated invasion, migration and mammosphere-formation. Honokiol (HNK), a bioactive molecule from *Magnolia grandiflora* increased LKB1 expression, inhibited individual cell-motility and abrogated the stem-like phenotype of breast cancer cells by reducing the formation of mammosphere, expression of pluripotency-factors and aldehyde dehydrogenase activity. LKB1, and its substrate, AMP-dependent protein kinase (AMPK) are important for HNK-mediated inhibition of pluripotency factors since LKB1-silencing and AMPK-inhibition abrogated, while LKB1-overexpression and AMPK-activation potentiated HNK's effects. Mechanistic studies showed that HNK inhibited Stat3-phosphorylation/activation in an LKB1-dependent manner, preventing its recruitment to canonical binding-sites in the promoters of Nanog, Oct4 and Sox2. Thus, inhibition of the coactivation-function of Stat3 resulted in suppression of expression of pluripotency factors. Further, we showed that HNK inhibited breast tumorigenesis in mice in an LKB1-dependent manner. Molecular analyses of HNK-treated xenografts corroborated our *in vitro* mechanistic findings. Collectively, these results present the first *in vitro* and *in vivo* evidence to support crosstalk between LKB1, Stat3 and pluripotency factors in breast cancer and effective anticancer modulation of this axis with HNK treatment.

Oncogene (2017) 36, 5709–5721; doi:10.1038/onc.2017.164; published online 5 June 2017

## INTRODUCTION

Liver Kinase B1/Serine/Threonine protein Kinase 11 (LKB1/STK11) functions as an important tumor suppressor protein as well as upstream kinase modulating various cellular functions such as maintenance of cellular polarity, regulation of cell-cycle, suppression of tumor-growth and promotion of apoptosis. LKB1 phosphorylates 14 AMP-dependent protein kinase (AMPK)-related kinases including AMPK, NIAK, SIK and MARK, and hence regulates multiple downstream signaling pathways.<sup>1,2</sup> Germ-line mutation of LKB1 are linked with Peutz–Jeghers syndrome, a dominantly inherited disorder distinguished by a propensity to gastrointestinal polyps, pigmented macules and increased risk of developing various cancers. Significant downregulation of LKB1 is

noted in many types of tumor tissues and reduced expression of LKB1 has been shown to promote cancer progression including the metastasis.<sup>2,3</sup> Recent studies indicate that LKB1 inactivation/loss may collaborate with activating oncogenes to drive tumor-progression in various cancer models.<sup>4,5</sup>

Signal transducer and activator of transcription 3 (Stat3) is a key mediator of cytokine signaling well established for its oncogenic role, manipulation of intracellular response to various extracellular cues, positive association with cell growth and angiogenesis as well as tumorigenesis. Various cancer types including breast cancer exhibit overexpression and constitutive-activation of Stat3. Upon phosphorylation, activated-Stat3 undergoes nuclear translocation, gets recruited to putative

<sup>1</sup>Department of Oncology, Johns Hopkins University School of Medicine and the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore MD, USA;

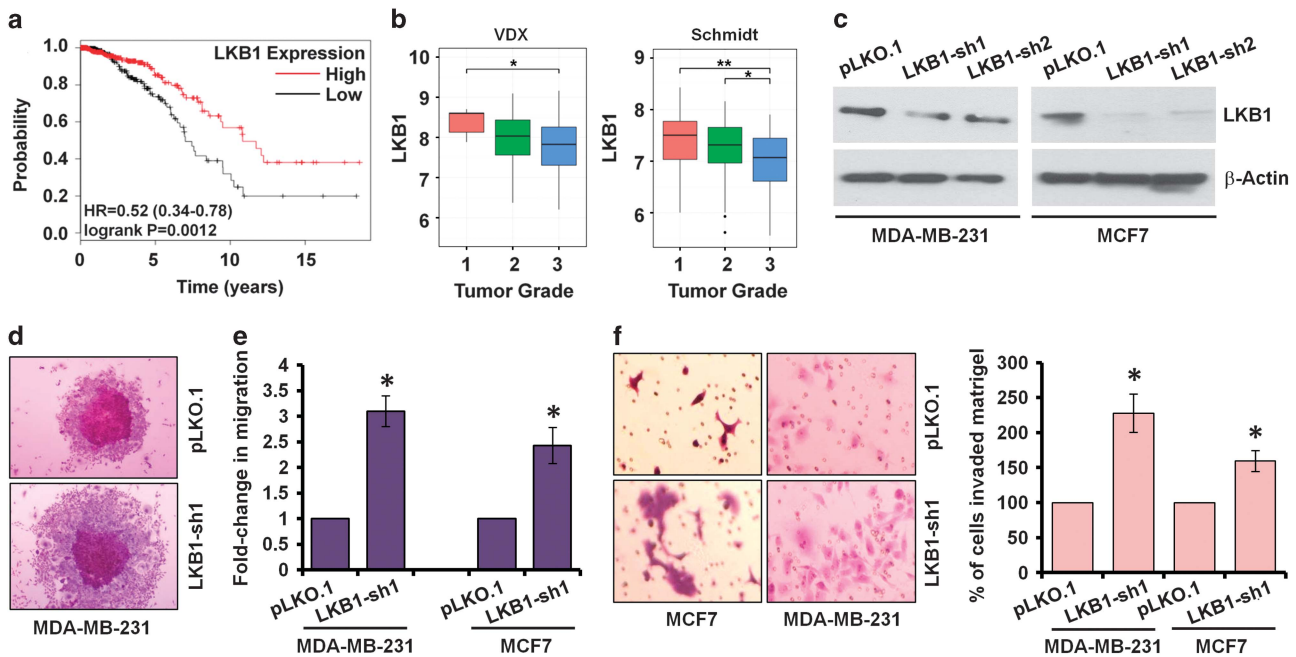
<sup>2</sup>Department of Dermatology, Emory University School of Medicine, Winship Cancer Institute, Atlanta, GA, USA; <sup>3</sup>Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore MD, USA; <sup>4</sup>Department of Medicine, University of Maryland School of Medicine, Baltimore MD, USA; <sup>5</sup>Department of Biochemistry and Molecular Biology, Dalhousie University, Nova Scotia Canada; <sup>6</sup>Division of Biostatistics and Bioinformatics, the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, USA; <sup>7</sup>Atlanta Veterans Administration Medical Center, Atlanta, GA, USA; <sup>8</sup>MTA TTK Momentum Cancer Biomarker Research Group, Budapest, Hungary and <sup>9</sup>Semmelweis University 2nd Department of Pediatrics, Budapest, Hungary. Correspondence: Dr NK Saxena, Department of Medicine, University of Maryland, School of Medicine, 660W Redwood St, Howard Hall, Rm 301, Baltimore, MD 21201, USA or Dr D Sharma, Department of Oncology, The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB 1, Rm 145, Baltimore, MD 21231, USA.

E-mail: nsaxena@medicine.umaryland.edu and saxenank@mail.nih.gov or dsharma7@jhmi.edu

<sup>10</sup>Co-first authors.

<sup>11</sup>Current address: National Cancer Institute, 9609 Medical Center Dr. Rockville, MD 20850, USA.

Received 27 June 2016; revised 9 April 2017; accepted 10 April 2017; published online 5 June 2017



**Figure 1.** Low LKB1 associates with poor prognosis and LKB1-silencing increases migration and invasion of breast cancer cells. **(a)** Kaplan-Meier plot of breast cancer patients in the TCGA BRCA data set binarized into high and low LKB1 expression using the median LKB1 expression as threshold. Patients with high LKB1 expression have longer overall survival (HR=0.52,  $P < 0.01$ ). **(b)** A statistically significant inverse relationship between tumor grade and LKB1 expression is observed in the VDX and Schmidt data sets suggesting that downregulation of LKB1 may play a role in increased tumor aggressiveness. **(c)** MCF7 and MDA-MB-231 cells were stably silenced for LKB1. Immunoblot analysis shows LKB1 expression levels in stable pools of LKB1-depleted (LKB1<sup>shRNA1-2</sup>) and vector control (pLKO.1) MCF7 and MDA-MB-231 cells. **(d)** MDA-MB-231-LKB1<sup>shRNA1</sup> and MDA-MB-231-pLKO.1 cells were subjected to spheroid-migration assay. **(e)** MDA-MB-231-LKB1<sup>shRNA1</sup>, MDA-MB-231-pLKO.1, MCF7-LKB1<sup>shRNA1</sup> and MCF7-pLKO.1 cells were tested in scratch-migration assay. Bar diagram represents fold-change in migration. \* $P < 0.001$ , compared with pLKO.1 cells. **(f)** MDA-MB-231-LKB1<sup>shRNA1</sup>, MDA-MB-231-pLKO.1, MCF7-LKB1<sup>shRNA1</sup> and MCF7-pLKO.1 cells were subjected to matrigel-invasion assay. Bar diagram represents % of LKB1<sup>shRNA1</sup>-cells invaded through matrigel in comparison to pLKO.1 cells. \* $P < 0.005$ , compared with pLKO.1 cells. HR, hazard ratio; TCGA, The Cancer Genome Atlas.

response-elements and activates expression of target genes. Stat3 functionally cooperates with various coactivator-complexes and histone acetyltransferases to create an open chromatin conformation. De-recruitment or blocking of Stat3-binding results in inhibition of expression of target genes. Many Stat3 target genes are key components implicated in regulation of cell growth, proliferation, apoptosis, migration, invasion, differentiation, early embryonic development as well as cancer stemness. The fact that cancer cells express molecular signatures similar to pluripotent embryonic stem cells indicate that the regulatory networks functionally important for embryonic stem cells may also be operational in maintenance of cancer stem-like phenotype. Although the molecular definition of the cancer stem-like phenotype is still emerging, three transcription factors Oct4, Nanog and Sox2 have been strongly implicated as master regulators of pluripotency.

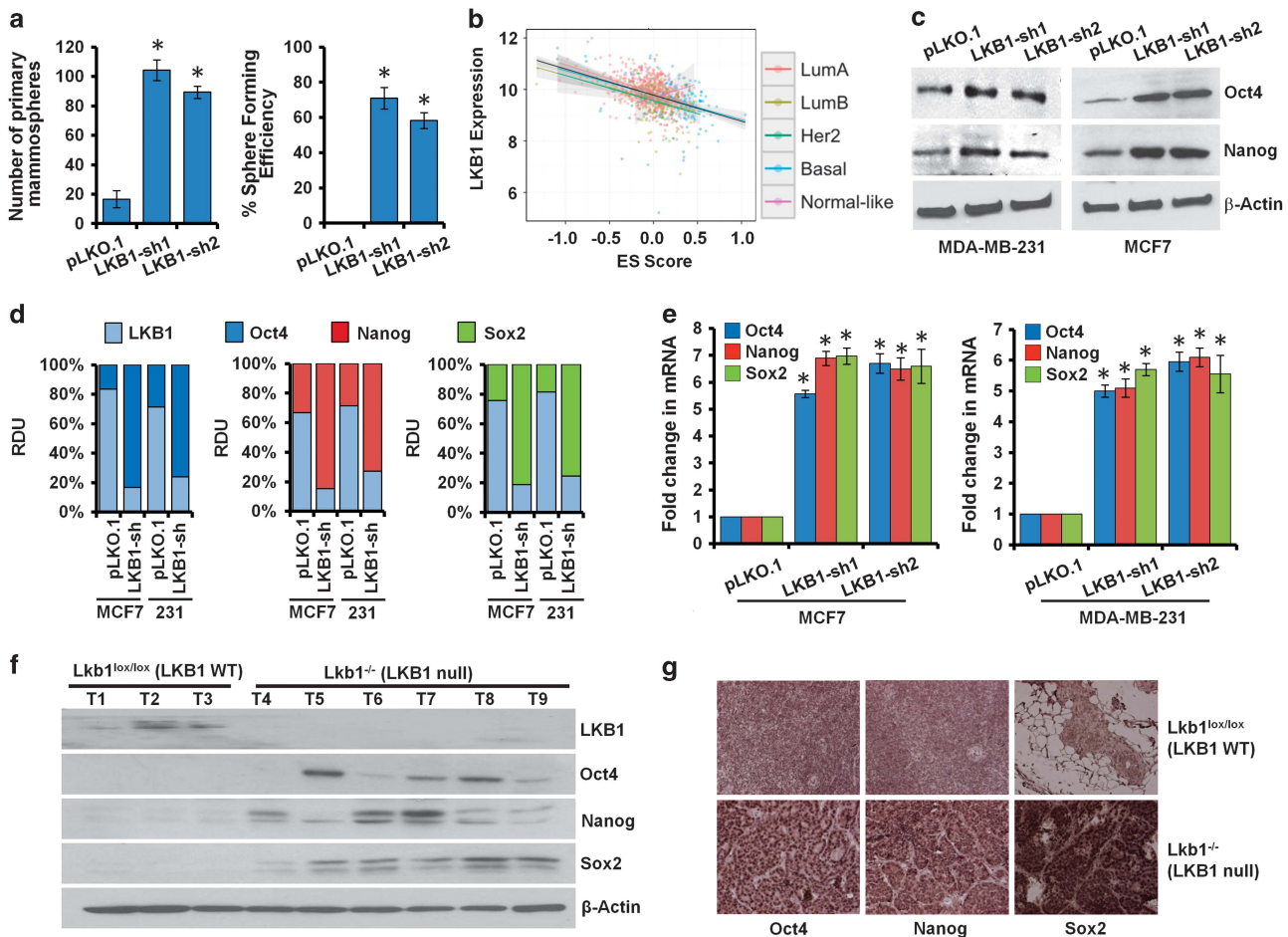
This study implicates LKB1 in aggressive progression of breast tumorigenesis. LKB1-loss leads to acquisition of highly migratory and invasive phenotype, supported by increased expression of pluripotency factors. LKB1-silencing resulted in elevated phosphorylated Stat3 which transcriptionally regulated pluripotency factors-Oct4, Nanog and Sox2. We discovered that honokiol (HNK), a bioactive compound isolated from *Magnolia Grandiflora*, can effectively inhibit pluripotency factors and cancer stem-like phenotype of breast cancer cells by elevating/activating LKB1. *In vivo* analyses of spontaneous tumors from Lkb1<sup>-/-</sup> mice and xenografts of LKB1-null breast cancer cells substantiate our *in vitro* findings. Our study uncovers a reciprocal crosstalk between LKB1 and Stat3 leading to modulation of pluripotent factors and stem-like phenotype and present HNK as an effective bioactive strategy that modulate this axis leading to tumor-inhibition.

## RESULTS

Loss of LKB1 associates with poor clinical prognosis and promotes an invasive phenotype

The association between LKB1 expression and survival of breast cancer patients was examined by Kaplan-Meier analysis and Cox regression of microarray-based gene-expression data from The Cancer Genome Atlas breast cancer data set dichotomized into high and low expression by median expression. A strong association between high LKB1 expression and longer overall survival (hazard ratio=0.52,  $P = 0.001$ ) was observed when LKB1 gene expression was compared in 781 patients (Figure 1a). Examination of the relationship between tumor grade and LKB1 expression using Bonferroni-adjusted Welch's *t*-test showed a stepwise, statistically significant inverse relationship between tumor grade and LKB1 expression in the VDX and Schmidt data sets, suggesting that downregulation of LKB1 may result in increased tumor aggressiveness (Figure 1b).

To directly investigate the impact of LKB1-downregulation on breast cancer, stable pools of breast cancer cells with LKB1-depletion were developed using LKB1<sup>shRNA</sup> lentiviruses and puromycin selection. LKB1 protein expression was analyzed in pLKO.1 and LKB1<sup>shRNA</sup> stable MCF7 and MDA-MB-231 cell pools to exhibit that LKB1 protein expression was indeed reduced in LKB1<sup>shRNA</sup> cells as compared to pLKO.1 control cells (Figure 1c). MDA-MB-231 cells are inherently very aggressive cells and, interestingly, LKB1-depletion further increased their migration and invasion potential. MDA-MB-231-LKB1<sup>shRNA</sup> cells exhibited increased migration from 3D-spheroids, increased chemotaxis response in scratch-migration and higher invasion through matrigel in comparison to MDA-MB-231-pLKO.1 cells (Figures 1d-f). MCF7 cells which usually exhibit non-metastatic



**Figure 2.** Silencing of LKB1 increases the expression of Oct4, Nanog and Sox2 in breast cancer cells. **(a)** MDA-MB-231-LKB1<sup>shRNA1-2</sup> and MDA-MB-231-pLKO.1 cells were subjected to mammosphere assay. \**P* < 0.001, compared with pLKO.1 cells. **(b)** Human embryonic stem cell score (hESC Score) calculated from a set of 68 embryonic stem-related genes is inversely correlated with LKB1 expression (Pearson's correlation coefficient = -0.40, *P* < 0.001). **(c, d)** Total protein was isolated from MDA-MB-231-LKB1<sup>shRNA1-2</sup>, MDA-MB-231-pLKO.1, MCF7-LKB1<sup>shRNA1-2</sup> and MCF7-pLKO.1 cells followed by immunoblot analysis for proteins as indicated. Actin was used as control. Bar diagram shows quantitation of western blot signals from multiple independent experiments. RDU (relative density units). **(e)** Total RNA was isolated from MDA-MB-231-LKB1<sup>shRNA</sup>, MDA-MB-231-pLKO.1, MCF7-LKB1<sup>shRNA</sup> and MCF7-pLKO.1 cells followed by real-time PCR analysis for Oct4, Nanog and Sox2 expression. \**P* < 0.001, compared with pLKO.1 cells. **(f)** Tumor lysates from Lkb1<sup>lox/lox</sup> (LKB1-WT) and LKB1<sup>-/-</sup> (LKB1-null) mice were subjected to immunoblot analysis. Expression of LKB1, Oct4, Nanog and Sox2 was examined as indicated. Actin was used as control. **(g)** Tumors from LKB1<sup>lox/lox</sup> and LKB1<sup>-/-</sup> mice were subjected to immunohistochemical (IHC) analysis using Oct4, Nanog and Sox2 antibodies.

behavior showed increased migration and matrigel-invasion upon LKB1-depletion (Figures 1e and f). These results show that LKB1-depletion results in increased invasion, migration potential of breast cancer cells and associates with poor clinical outcome in breast cancer patients.

Increased expression of pluripotency factors is detected in LKB1-silenced breast cancer cells

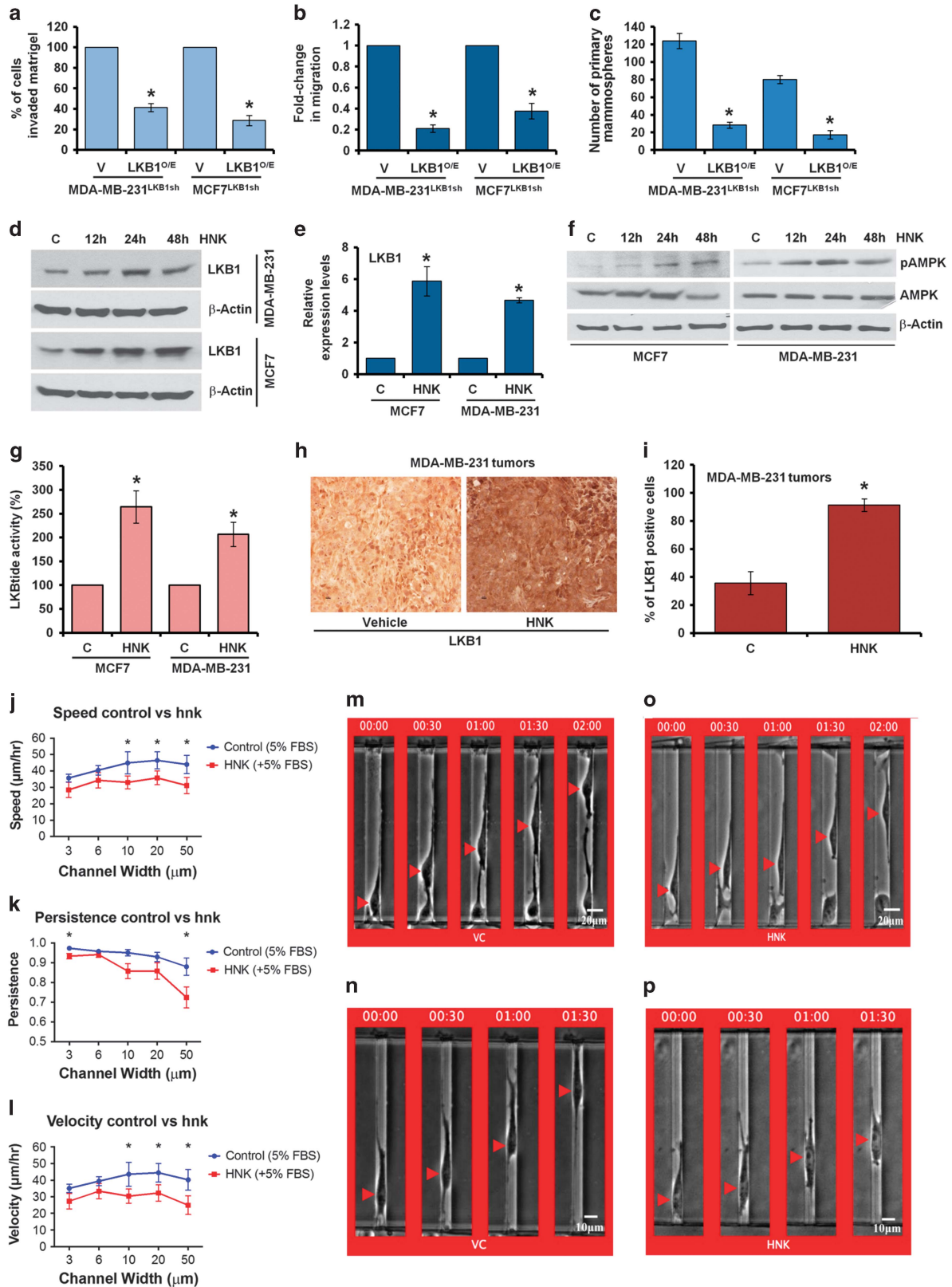
We next examined whether LKB1-loss also impacts the mammosphere-formation. MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> formed increased number of mammospheres (diameter > 100  $\mu$ m) in comparison to MDA-MB-231-pLKO.1 and MCF7-pLKO.1 cells. Primary mammospheres were dissociated and plated as single cells in mammosphere-media to further examine their self-renewing capabilities. Majority of LKB1-sh cells (~55–70%) formed secondary mammospheres (size ranging from 50 to 100  $\mu$ m) while pLKO.1 cells could not form secondary mammospheres (Figure 2a). Mammospheres are often enriched in breast cancer-stem-like cells displaying gene expression signatures characteristics of embryonic stem cells.<sup>6</sup> Since LKB1-depleted breast cancer cells showed enrichment of mammosphere-formation

potential, we queried the association between gene expression signature of human embryonic stem cell markers and LKB1 expression in 544 The Cancer Genome Atlas breast cancer samples. LKB1 expression and human embryonic stem cell-score (Pearson's correlation coefficient = -0.40) showed a significant inverse correlation (Figure 2b). Several inversely correlated mRNAs corresponded to genes involved in stemness and induced pluripotent stem cell (iPSC) inducers, Oct4, Nanog and Sox2. Analysis of LKB1-wild-type and LKB1-depleted breast cancer cells confirmed that LKB1-depletion is associated with increased expression of iPSC inducers. MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells expressed elevated levels of Oct4, Nanog and Sox2 proteins (Figures 2c and d) and mRNAs (Figure 2e) in comparison to MDA-MB-231-pLKO.1 and MCF7-pLKO.1 cells. Oct4, Nanog, Sox2 upregulation upon LKB1-depletion was validated using spontaneous breast tumors from Lkb1<sup>-/-</sup>/NIC mice and Lkb1<sup>lox/lox</sup> mice.<sup>7</sup> Indeed, immunoblot analysis of Lkb1<sup>-/-</sup>/NIC tumors exhibited increased expression of Oct4, Nanog and Sox2 whereas tumors from LKB1<sup>lox/lox</sup> mice showed extremely low expression of these iPSC inducers (Figure 2f). Immunohistochemical analysis of Lkb1<sup>-/-</sup>/NIC tumors exhibited significantly more tumor cells showing higher expression of Oct4,

Nanog and Sox2. Tumors from LKB1<sup>lox/lox</sup> mice did not show any significant change in Oct4, Nanog and Sox2 expression (Figure 2g) providing support to our *in vitro* findings. Together, these findings suggest that LKB1-loss associates with increased expression of iPSC inducers, Oct4, Nanog and Sox2.

Decreased metastatic properties are observed upon LKB1-overexpression; HNK treatment can increase LKB1 and impede migratory behavior of breast cancer cells

Owing to the evidence supporting the impact of LKB1-loss on the promotion of migratory and invasive potentials as well as



mammosphere-formation, we postulated that LKB1-overexpression would inhibit these properties. We tested this hypothesis by overexpressing LKB1 in LKB1-depleted cells followed by functional assays. In accord with our hypothesis, LKB1-overexpression in MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells resulted in reduced invasion of cells through matrigel (Figure 3a). This 'gain-of-function' approach also inhibited migration of MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells in a scratch-migration assay (Figure 3b). Mammosphere-formation of LKB1-depleted breast cancer cells was also notably reduced in MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells overexpressing LKB1 (Figure 3c). Next, we investigated the strategy to increase functional-LKB1 in breast cancer cells using non-toxic and effective bioactive compounds. We selected HNK which is a phenolic bioactive compound purified from an extract of seed cones from *Magnolia grandiflora*. To evaluate whether HNK can induce expression of LKB1 in breast cancer cells and to clarify its temporal effect, we treated breast cancer cells with 5 μM HNK. Immunoblot and RT-PCR analyses showed that LKB1 expression is augmented in a temporal manner in breast cancer cells (Figures 3d and e). We examined the phosphorylation of AMPK, a known substrate of LKB1, to evaluate LKB1 activity upon HNK treatment. Increased AMPK phosphorylation was noted upon HNK treatment as compared to untreated cells while total-AMPK protein levels remained unaltered (Figure 3f). LKB1tide-activity analyses corroborated that HNK induced functionally active LKB1 in breast cancer cells (Figure 3g). A recent study from our lab using female athymic nude mice harboring MDA-MB-231 tumors showed that HNK administration reduces breast tumor growth.<sup>8</sup> Tumor samples from the same study were utilized to evaluate the effect of HNK on LKB1 expression. Corroborating *in vitro* findings, tumors from mice treated with HNK showed increased levels of LKB1 expression (Figures 3h and i).

Next, we aspired to examine the effect of HNK on the migratory behavior of breast cancer cells. To this end, cells were seeded in a microfluidic device, which consists of an array of parallel microchannels of prescribed height (10 μm) and different widths (ranging from 50, 20, 10, 6 and 3 μm) coated with collagen type-I. This device enables real-time cell tracking in either unconfined (50- or 20-μm-wide channels) or partially confined (10 μm) or fully confined (3 μm) microenvironments. Our data reveal that HNK suppresses migration speed and velocity by ~25% in wider channels (≥10 μm), whereas smaller differences which did not reach statistical significance were noted in narrow channels (6- or 3-μm-wide) (Figures 3j–p, Supplementary Figure S1). Moreover, HNK repressed cell persistence, defined as the ratio of net cell displacement to the total length traveled by the cell, primarily in 50-μm-wide channels. Collectively, these results illustrate the

efficacy of HNK in mitigating cell motility in physiologically relevant microenvironments.

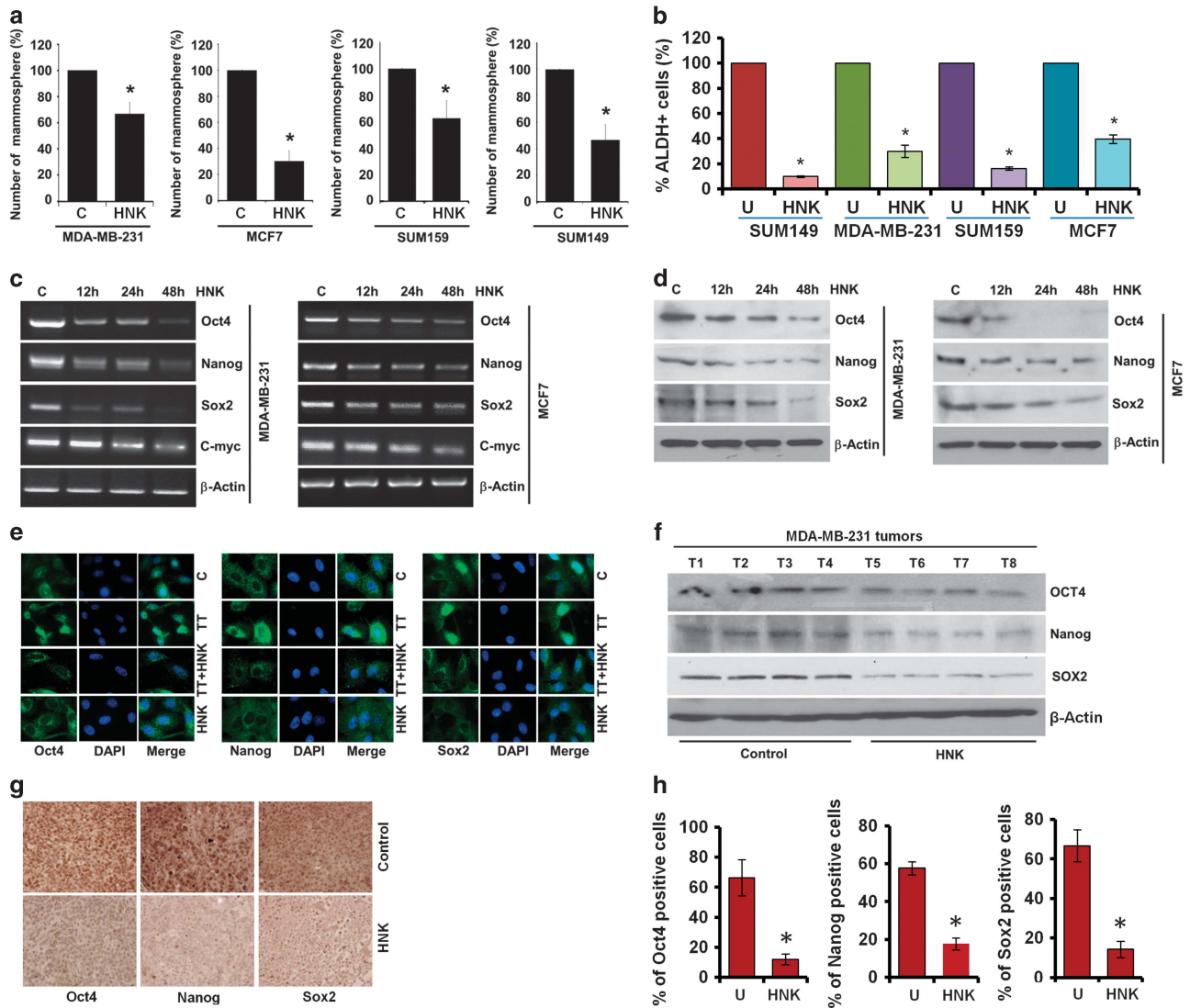
#### Inhibition of stem-like characteristics is achieved with HNK treatment

Mammosphere assay was utilized to determine the efficacy of HNK to affect stem-like characteristics of breast cancer cells. A significant reduction in mammosphere-forming efficiency was obtained in MCF7, SUM159, MDA-MB-231 and SUM149 breast cancer cells upon treatment with 5 μM HNK (Figure 4a). We questioned whether HNK was effective at inhibiting self-renewal capacity of stem-like breast cancer cells over successive mammosphere generations; primary-mammospheres formed in HNK-containing media were dissociated and seeded again as secondary-mammosphere followed by tertiary-mammospheres in the absence of HNK. Honokiol-treated primary-mammospheres formed significantly smaller number of secondary-mammospheres and no tertiary-mammospheres were formed (Supplementary Figure S2). In addition, the activity of aldehyde dehydrogenase (ALDH), a marker of cancer stem-cells was also significantly inhibited upon HNK treatment in SUM149, SUM159, MDA-MB-231 and MCF7 cells (Figure 4b). The expression of iPSC inducers was examined in breast cancer cells treated with 5 μM HNK for various time intervals. Honokiol decreased mRNA levels of iPSC inducers (Figure 4c) and inhibited protein expression of Oct4, Nanog and Sox2 in MCF7 and MDA-MB-231 cells (Figure 4d). Treatment with a combination of transforming growth factor beta (TGFβ) and tumor necrosis factor alpha (TNFα) has been shown to induce stemness in breast cancer cells.<sup>9</sup> MCF7 cells were treated with TGFβ/TNFα followed by HNK treatment. Immunofluorescence analysis showed that TGFβ/TNFα-induced nuclear localization of Oct4, Nanog and Sox2 was abrogated by HNK (Figure 4e). MDA-MB-231 xenografts from athymic nude mice treated with HNK or vehicle-control for 6 weeks were examined for the expression of iPSC inducers. Tumors from mice treated with HNK showed decreased expression of Oct4, Nanog and Sox2 in comparison to tumors from vehicle-treated mice (Figure 4f). Immunohistochemical analysis showed decreased number of tumor cells expressing Oct4, Nanog and Sox2 in tumor-xenografts from mice treated with HNK (Figures 4g and h). These results provide *in vivo* support to our *in vitro* findings that HNK treatment can effectively inhibit stem-like characteristics in breast cancer cells.

#### HNK treatment inhibits breast tumor-initiating cells *in vivo*

Our observations raised the possibility that HNK treatment also targets breast tumor-initiating cells *in vivo*. MDA-MB-231 xenografts were excised from mice treated with vehicle or HNK after 4 weeks and digested to single cells followed by secondary

**Figure 3.** LKB1 overexpression inhibits migration, invasion and mammosphere-formation of breast cancer cells. Honokiol treatment activates LKB1 and inhibits motility of breast cancer cells. (a–c) LKB1-depleted (LKB1<sup>shRNA</sup>) breast cancer cells were transfected with vector-control (V) or full length-LKB1 plasmid (LKB1<sup>OE5</sup>), and subjected to matrigel-invasion (a), scratch-migration (b) and mammosphere (c) assays. \**P* < 0.001, compared with vector-transfected cells. (d) Breast cancer cells were treated with 5 μM Honokiol (HNK) for indicated time-intervals. Total protein lysates were immunoblotted for LKB1 expression. β-actin was used as a control. (e) Total RNA was isolated from MCF7 and MDA-MB-231 cells treated with 5 μM Honokiol (HNK) for 24 h and expression level of LKB1 was analyzed. β-actin was used as loading-control. Bar graph shows fold change in LKB1 expression. \**P* < 0.05, C vs HNK treatment. (f) MCF7 and MDA-MB-231 breast cancer cells were treated with 5 μM Honokiol (HNK) for indicated time-intervals. Total lysates were immunoblotted for phospho-AMPK (pAMPK-Thr172) and total-AMPK expression. β-actin was used as a loading-control. (g) Breast cancer cells were treated with 5 μM Honokiol (HNK) and cell lysates were examined for catalytic activity towards LKB1tide peptide. \**P* < 0.01, C vs HNK treatment. (h) MDA-MB-231 xenograft tumors from mice treated with vehicle and honokiol (HNK) were subjected to immunohistochemical (IHC) analysis using LKB1 antibodies. (i) Bar diagrams show quantitation of IHC-analysis of LKB1. Columns, mean (*n* = 8); bar, s.d. \* significantly different (*P* < 0.005) compared with control. HNK suppresses MDA-MB-231 breast cancer cell migration in microfluidic device. The speed (j), persistence (k) and velocity (l) of MDA-MB-231 cells migrating through channels of prescribed widths are shown in the presence of HNK (5 μM) or vehicle control (VC). Micrographs indicate cells, shown by an arrowhead, migrating through 20 μm (m, n) and 10 μm (o, p) in width channels in the presence of HNK or VC. Data represent the mean ± s.e. of cells from *n* = 4 independent experiments. \**P* < 0.05 determined by paired *t*-test.



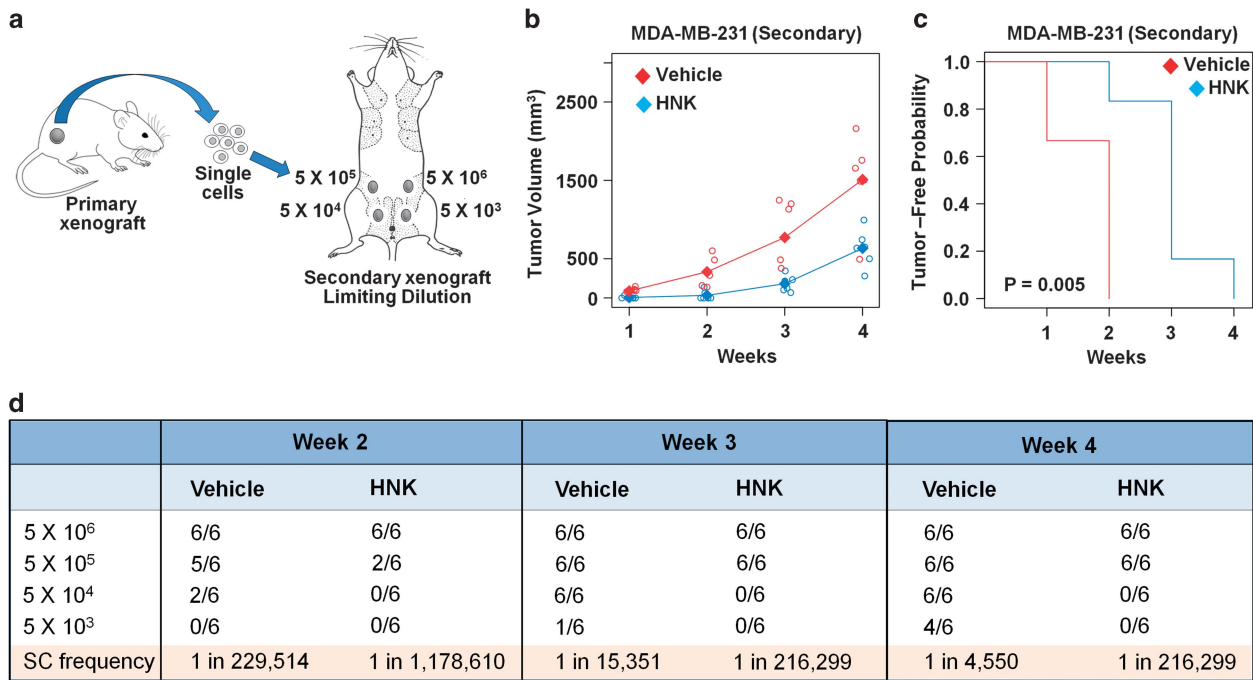
**Figure 4.** Honokiol inhibits stem-like characteristics of breast cancer cells. (a) Breast cancer cells were treated with 5  $\mu$ M honokiol (HNK) and subjected to mammosphere formation. Vehicle treated cells are denoted as (c). The graph shows the number of mammospheres. \* $P < 0.05$ , compared with untreated controls. (b) Breast cancer cells were grown as mammospheres in the absence or presence of 5  $\mu$ M honokiol, the activity of ALDH was analyzed by Aldeflour assay. The panels show the representative 2D plots of Aldeflour assay with or without HNK treatment and the graph shows the ALDH-positive population. \* $P < 0.001$ , compared with untreated controls. (c) MDA-MB-231 and MCF7 cells were treated with 5  $\mu$ M honokiol (HNK) for indicated time-intervals. Total RNA was isolated and expression levels of Oct4, Nanog, Sox2 and c-myc were examined.  $\beta$ -actin was used as loading-control. (d) MDA-MB-231 and MCF7 cells were treated with 5  $\mu$ M honokiol (HNK) for indicated time-intervals. Total protein lysates were immunoblotted for Oct4, Nanog and Sox2 expression.  $\beta$ -actin was used as a control. (e) Breast cancer cells were treated with vehicle control (control), TGF $\beta$ +TNF $\alpha$  (10 ng/ml of each), 5  $\mu$ M honokiol (HNK) or TGF $\beta$ +TNF $\alpha$  +HNK for 24 h, and subjected to immunofluorescence analysis of Oct4, Nanog and Sox2. Nuclei were stained with DAPI. (f) Total protein lysates were isolated from MDA-MB-231 xenograft tumors from mice treated with vehicle and honokiol (HNK) and subjected to immunoblot analysis. Expression of Oct4, Nanog and Sox2 was examined as indicated. (g) MDA-MB-231 xenograft tumors from mice treated with vehicle and honokiol (HNK) were subjected to immunohistochemical (IHC) analysis using Oct4, Nanog and Sox2 antibodies. (h) Bar diagrams show quantitation of IHC-analysis of Oct4, Nanog and Sox2. Columns, mean ( $n = 8$ ); bar, s.d. \* significantly different ( $P < 0.05$ ) compared with control.

transplants in limiting dilution (schema in Figure 5a). Honokiol treatment resulted in significantly smaller secondary tumor transplants and prolonged time to tumor compared to vehicle-treated group (Figures 5b and c, Supplementary Figures S3 and S4, Supplementary Table S1). Honokiol significantly decreased tumor-initiating frequencies of MDA-MB-231 tumor cells that were transplanted into secondary hosts at limiting dilutions (Figure 5d). The frequency of breast tumor-initiating cells in HNK-treated tumor cells was determined to be 1 in 1 178 610 compared to 1 in 229 514 in vehicle-treated mice at week 2 and 1 in 216 299 compared to 1 in 4550 in vehicle-treated mice at week 4 (Figure 5d). The effect of HNK on inhibiting tumor growth,

increasing tumor-free probability and reducing the frequency of breast tumor-initiating cells was also observed in an independent experiment set (Supplementary Figures S5 and S6 and Supplementary Tables S2 and S3). These data support the efficacy of the HNK treatment in targeting breast tumor initiating cells.

Inhibition of Oct4, Nanog and Sox2 by HNK is mediated by LKB1-AMPK axis

Our data showed that HNK can increase LKB1 expression as well as inhibit iPSC markers. Next, we investigated whether LKB1 is integral for HNK-mediated inhibition of Oct4, Nanog and Sox2. LKB1-



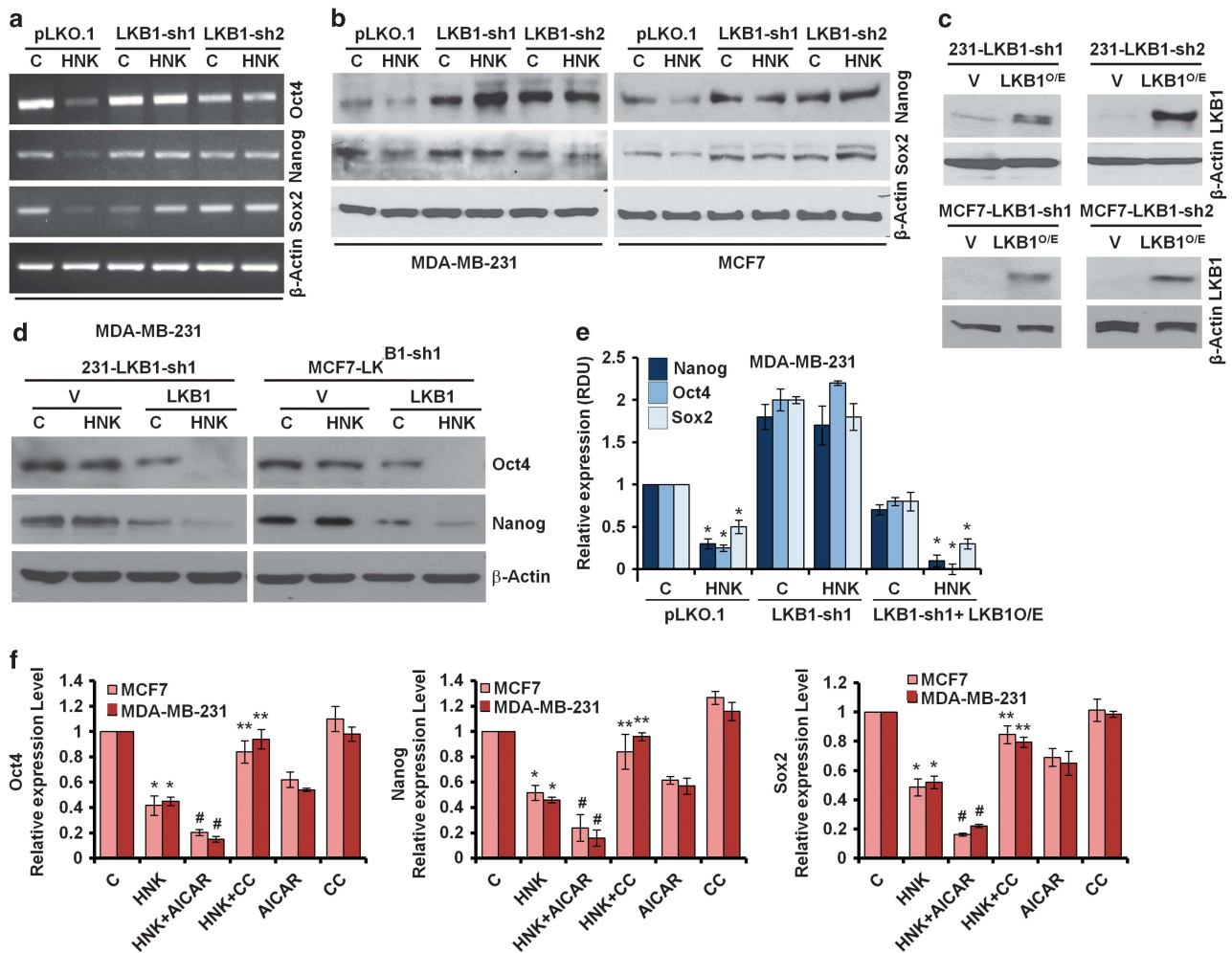
**Figure 5.** Honokiol decreases stemness *in vivo*. **(a)** Schematic outline of the tumorigenicity assays. **(b)** Tumor volume of MDA-MB-231 secondary xenografts established with  $5 \times 10^5$  cells. Plots show the values of tumor volume (open circles) and mean tumor volume (diamonds). Red-Vehicle, Blue-Honokiol. **(c)** Plots show Kaplan–Meier curves (Blue-Honokiol, Red-Vehicle) for time to detect tumors (100 mm<sup>3</sup> volume). **(d)** Honokiol decreases tumor incidence at limiting dilution. Tumor incidence at weeks 2, 3 and 4 of secondary transplants of MDA-MB-231 at limiting dilutions. The tumors/numbers of mice/group are shown. The bottom row indicates the estimated breast tumor-initiating/stem cell (SC) frequencies.

depleted and control breast cancer cells (MDA-MB-231-LKB1<sup>shRNA</sup>, MDA-MB-231-pLK0.1, MCF7-LKB1<sup>shRNA</sup> and MCF7-pLK0.1) treated with HNK were examined using RT–PCR and immunoblot analyses. We observed that HNK inhibited Oct4, Nanog and Sox2 expression in MDA-MB-231-pLK0.1 and MCF7-pLK0.1 cells. LKB1<sup>shRNA</sup> cells tended to exhibit higher expression of Oct4, Nanog and Sox2 which was not inhibited with HNK (Figures 6a and b). LKB1 was re-expressed in LKB1-null MCF7 and MDA-MB-231 cells as a ‘gain-of-function’ strategy (Figure 6c), treated with HNK and analyzed for the expression of iPSC markers. Honokiol inhibited the expression of iPSC markers in MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells transfected with full-length LKB1 construct (Figures 6d and e). LKB1<sup>shRNA</sup> and vector-control cells were treated with TGFβ/TNFα followed by HNK treatment. Honokiol inhibited expression of Oct4, Nanog and Sox2 in the presence of TT only in LKB1-vector control cells (Supplementary Figure S7). Interestingly, LKB1-null cells showed increased frequency of breast tumor-initiating cells (1 in 149 220) in comparison to LKB1-WT cells (1 in 340 386). Honokiol significantly decreased tumor-initiating frequencies of LKB1-WT tumor cells at limiting dilutions to 1 in 1 924 443 in comparison to vehicle-treated group while LKB1-null group remained unaltered (Supplementary Table S4). Next, we interrogated the role and requirement of functional AMPK in HNK-mediated inhibition of iPSC markers. Breast cancer cells were treated with 5-Aminoimidazole-4-carboxamide ribonucleotide which is an analog of adenosine monophosphate used to stimulate AMP-dependent protein kinase (AMPK) activity or compound C (an inhibitor of AMPK) in combination with HNK. We found that 5-aminoimidazole-4-carboxamide ribonucleotide treatment potentiated HNK-mediated inhibition of Oct4, Nanog and Sox2 expression while treatment with compound C abrogated the effect of HNK (Figure 6f), demonstrating the importance of AMPK in HNK-mediated inhibition of iPSC markers in breast cancer cells.

Abrogation of coactivator-Stat3 recruitment is important for HNK-mediated inhibition of Oct4, Nanog and Sox2 expression

Next, we examined the potential molecular underpinnings by which HNK inhibit expression of iPSC markers in breast cancer cells. Induced pluripotent stem cell markers act in a concerted mode in order to maintain the proliferation and self-renewal of cancer stem-like cells and their expression is tightly regulated to respond to extracellular cues and therapies. Previous studies have shown that phosphorylated-Stat3 gets recruited to the Oct4, Nanog and Sox2 promoter/enhancer region and activates their transcription.<sup>10–13</sup> We therefore hypothesized that HNK inhibits Oct4, Nanog and Sox2 via Stat3-inhibition. To test this notion, firstly, the expression of Stat3 and its phosphorylated form in breast cancer cells treated with HNK was examined. Honokiol decreased phosphorylated-Stat3 in MCF7, MDA-MB-231, SUM149 and SUM159 cells in a temporal manner while no change was observed in total-Stat3 (Figure 7a). To further examine whether the HNK-mediated inhibition of Oct4, Nanog and Sox2 in breast cancer cells is Stat3-dependent, we treated breast cancer cells with Stattic (Stat3-inhibitor) or alternatively, overexpressed constitutively-active Stat3 (CA-Stat3), in conjunction with HNK-treatment. Stattic treatment potentiated HNK-mediated inhibition of Oct4, Nanog and Sox2 while CA-Stat3-overexpression interfered with HNK’s effect (Figures 7b–d). Next, we confirmed that Stat3 gets recruited to Oct4, Nanog and Sox2 promoter regions in breast cancer cells. As expected, Stat3-recruitment was increased in the presence of CA-Stat3 and inhibited upon Stattic-treatment (Supplementary Figure S8).

Since we found the connection of LKB1 in HNK-mediated inhibition of Oct4, Nanog and Sox2, we questioned the association between LKB1 and Stat3, a putative regulator of these iPSC markers. Analyses of LKB1 expression and Stat3-activated genes showed a statistically significant inverse correlation (Pearson’s correlation coefficient = –0.44) (Figure 8a) indicating that LKB1 is negatively associated with Stat3-transactivation activity.



**Figure 6.** LKB1-AMPK axis plays an important role in honokiol-mediated inhibition of stemness transcription factors. **(a)** LKB1-depleted (LKB1<sup>shRNA 1-2</sup>) and vector control (pLKO.1) MDA-MB-231 cells were treated with 5  $\mu$ M honokiol (HNK). Total RNA was examined for the expression of Oct4, Nanog and Sox2 as indicated.  $\beta$ -actin was used as control. **(b)** Total protein lysates of LKB1-depleted (LKB1<sup>shRNA 1-2</sup>) and vector control (pLKO.1) breast cancer cells treated with 5  $\mu$ M honokiol (HNK) were analyzed for the expression of Nanog and Sox2 as indicated.  $\beta$ -actin was used as loading-control. **(c)** LKB1-depleted (LKB1<sup>shRNA 1-2</sup>) breast cancer cells were transfected with vector-control (V) or full length-LKB1 plasmid (LKB1<sup>O/E</sup>); total protein lysates were examined for LKB1 expression as indicated using immunoblot analysis. **(d)** LKB1-depleted (LKB1<sup>shRNA 1</sup>) breast cancer cells were transfected with vector-control (V) or full length-LKB1 plasmid (LKB1<sup>O/E</sup>) followed by treatment with 5  $\mu$ M honokiol (HNK). Total protein lysates were examined for Oct4 and Nanog.  $\beta$ -actin was used as loading-control. **(e)** Bar diagram shows quantitation of western blot signals. \* $P < 0.001$ , compared with untreated controls. **(f)** MCF7 and MDA-MB-231 breast cancer cells were treated with 5  $\mu$ M honokiol (HNK) in combination with AICAR (100  $\mu$ M) or compound C (CC, 20  $\mu$ M). Total RNA was examined for the expression of Oct4, Nanog and Sox2. \* $P < 0.005$ , compared with untreated controls; # $P < 0.05$ , compared with HNK-treated cells; \*\*\* $P < 0.001$ , compared with HNK-treated cells. AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide.

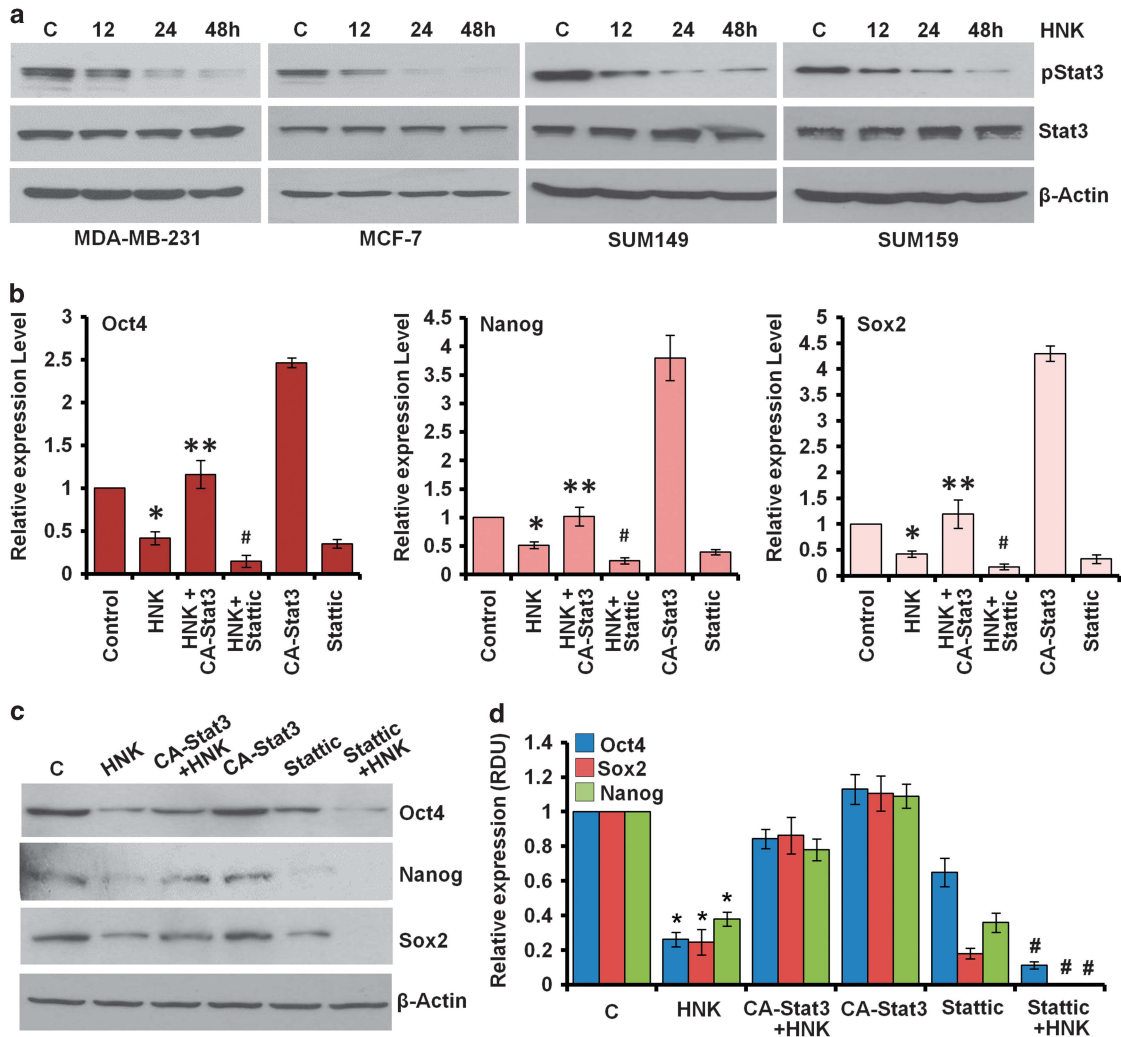
Interestingly, we found that HNK could not inhibit Stat3-phosphorylation in LKB1-null MDA-MB-231-LKB1<sup>shRNA</sup> and MCF7-LKB1<sup>shRNA</sup> cells in contrast to MDA-MB-231-pLKO.1 and MCF7-pLKO.1 cells (Figure 8b). Interestingly, re-introduction of LKB1 in LKB1-null cells resulted in reduction of Stat3-phosphorylation which was further inhibited with HNK (Figures 8c and d). Furthermore, we tested whether HNK could abrogate the recruitment of oncogenic-coactivator-Stat3 to Oct4, Nanog and Sox2 promoter-regions in the absence of tumor suppressor-LKB1. MCF7-LKB1<sup>shRNA</sup> and MCF7-pLKO.1 cells were treated with HNK and subjected to ChIP assay. Our data showed that HNK inhibited the recruitment of Stat3 to Oct4, Nanog and Sox2 promoter in MCF7-pLKO.1 cells while no change was observed in MCF7-LKB1<sup>shRNA</sup> cells. LKB1 overexpression alone in LKB1-null cells did not alter Stat3-binding to Oct4, Nanog and Sox2 promoter but it sensitized cells to HNK-mediated inhibition (Figures 8e and f). Collectively, these studies showed that HNK

inhibits Stat3-phosphorylation/coactivator-function and regulates Oct4, Nanog and Sox2 in an LKB1-dependent manner.

LKB1 is essential for HNK-mediated inhibition of mammosphere and breast tumor growth *in vivo*

We show that HNK inhibits mammosphere formation, stemness and expression of iPSC markers in breast cancer cells and also uncovered an imperative role of LKB1. We questioned the significance of LKB1 in HNK-mediated inhibition of mammosphere-formation. MDA-MB-231-LKB1<sup>shRNA</sup>, MDA-MB-231-pLKO.1, MCF7-LKB1<sup>shRNA</sup> and MCF7-pLKO.1 were treated with HNK and allowed to form mammospheres. Honokiol inhibited mammosphere-formation in pLKO.1 cells while LKB1<sup>shRNA</sup> mammospheres remained unaffected (Figures 9a and b). Next, the *in vivo* physiological significance of our *in vitro* findings was examined by evaluating whether LKB1 is essential for the anti-cancer potential of HNK. MDA-MB-231-LKB1<sup>shRNA</sup>, MDA-MB-231-pLKO.1 were utilized in





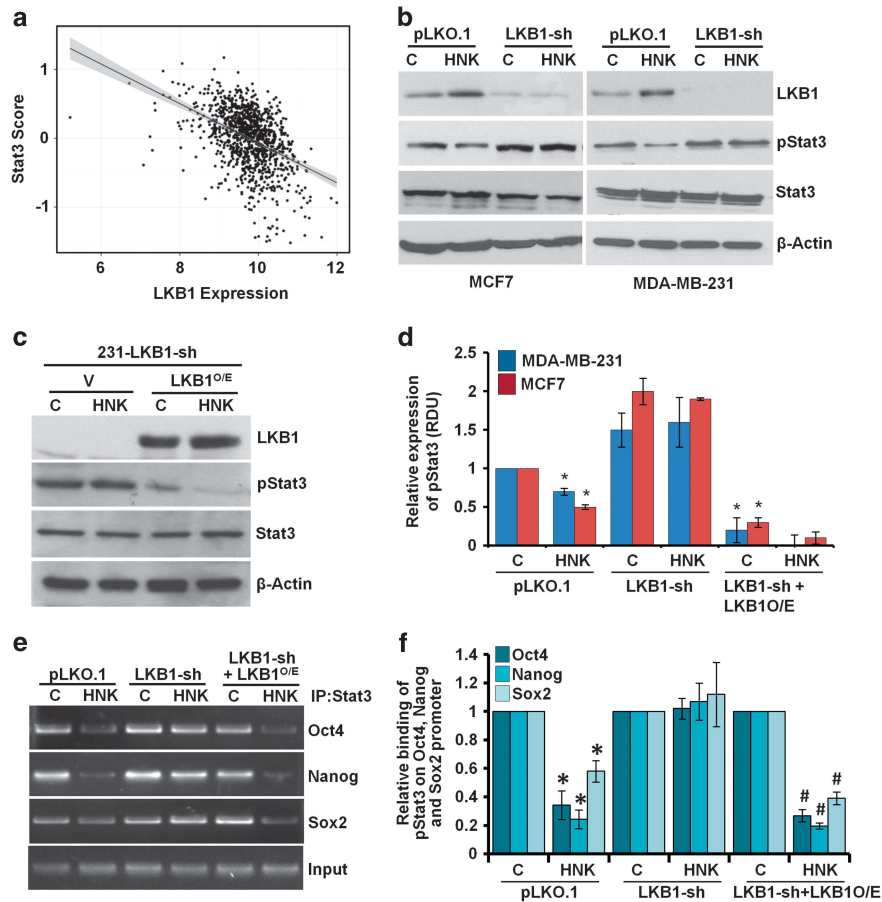
**Figure 7.** Stat3 gets recruited to the promoters of Oct4, Nanog and Sox2 and honokiol inhibits stemness transcription factors via inhibiting Stat3. **(a)** Breast cancer cells were treated with 5  $\mu$ M Honokiol (HNK) for indicated time-intervals. Total protein lysates were immunoblotted for phospho-Stat3 and total Stat3 expression.  $\beta$ -actin was used as a control. **(b)** MCF7 cells were transfected with Stat3-overexpression (Stat3 O/E) followed by 5  $\mu$ M Honokiol (HNK) treatment and co-treatment with HNK and Stattic as indicated. Total RNA was analyzed for the expression of Oct4, Nanog and Sox2. Bar graph shows fold change in gene expression. \* $P < 0.05$ , C vs HNK treatment; \*\* $P < 0.01$ , HNK+Stat3O/E vs HNK alone; # $P < 0.005$ , HNK+Stattic vs HNK alone. **(c, d)** MCF7 cells were transfected with vector or constitutively active Stat3 overexpression plasmid (CA-Stat3) followed by treatment with 5  $\mu$ M Honokiol (HNK). MCF7 cells were treated with 5  $\mu$ M Honokiol (HNK) with or without co-treatment with Stattic. Total protein lysates were analyzed for the expression of Oct4, Nanog and Sox2 as indicated.  $\beta$ -actin was used as control. Bar diagram shows relative density units of the western signals.

xenograft-athymic nude mice model. We observed that HNK treatment significantly inhibited breast tumor growth in MDA-MB-231-pLKO.1 (vector control group) whereas MDA-MB-231-LKB1<sup>shRNA</sup> group showed no HNK-mediated inhibition (Figure 9c). Immunohistochemical analysis showed that HNK-treated pLKO.1-tumors exhibit reduced number of tumor cells showing Ki-67 and pStat3 expression as compared to tumors from HNK-treated LKB1-sh group (Figures 9d and e). Tumors from pLKO.1 group exhibited low levels of pStat3 whereas LKB1-null tumors showed increased levels of pStat3 corroborating our *in vitro* findings showing a reciprocal association between LKB1 and pStat3 (Figure 9f). In conclusion, the *in vitro* and *in vivo* findings presented here propose an inverse relationship between tumor suppressor LKB1 and stem-like phenotype and iPSC markers, their regulation by Stat3-recruitment which is modulated by LKB1 and lastly, our studies present HNK as an effective bioactive strategy to inhibit iPSC markers and stemness in an LKB1-dependent manner in breast cancer cells.

## DISCUSSION

A convincing body of evidence has put forth that multiple tumor types, including breast cancer possess a subset of cells with stemness properties that typically display enhanced malignant and metastatic potential and are implicated in chemoresistance and tumor recurrence. Therefore, efforts have been ongoing to understand the mechanistic alterations underlying cancer stem-like phenotype in breast cancer. Stemness is not mediated by the absence or presence of an individual factor but relies on multiple regulatory genes that drive stemness networks.

Some of the key-regulators of stemness in cancer are Oct4, Nanog and Sox2, also known as iPSC markers, whose activation-targets are frequently overexpressed in poorly differentiated tumors and overlap with embryonic-stem-cell signatures.<sup>14</sup> Here, we report an inverse association between tumor suppressor LKB1 and iPSC markers. Investigating bioactive strategies to modulate LKB1 and inhibit breast cancer stemness we found that treatment with HNK not only increases LKB1 but also inhibits stem-like



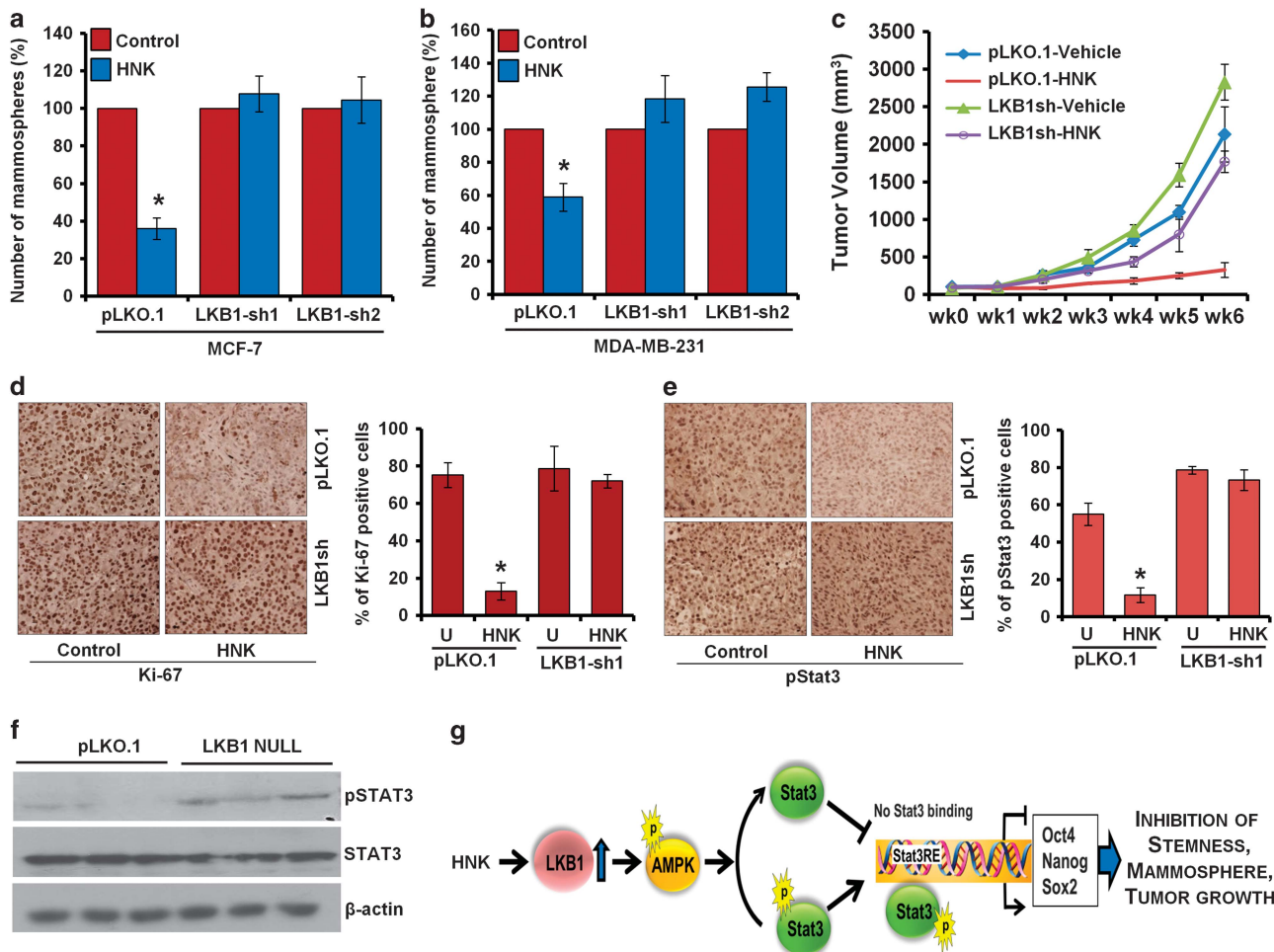
**Figure 8.** LKB1 is important for honokiol-mediated inhibition of Stat3 expression and recruitment to Oct4, Nanog and Sox2 in breast cancer cells. **(a)** STAT3-score calculated from a set of 24 STAT3-activated genes compiled by Azare *et al.*<sup>50</sup> is inversely correlated with LKB1 expression (Pearson correlation coefficient =  $-0.44$ ,  $P < 0.001$ ). **(b)** LKB1-depleted (LKB1<sup>shRNA</sup>) and vector control (pLKO.1) breast cancer cells were treated with 5  $\mu$ M honokiol (HNK). Total protein lysates were examined for the expression of LKB1, phospho-Stat3 and total Stat3 as indicated.  $\beta$ -actin was used as loading-control. **(c)** LKB1-depleted (LKB1<sup>shRNA</sup>) breast cancer cells were transfected with vector-control (V) or full length-LKB1 plasmid (LKB1<sup>O/E</sup>), total protein lysates were examined for LKB1, phospho-Stat3 and total Stat3 expression as indicated using immunoblot analysis.  $\beta$ -actin was used as loading-control. **(d)** Bar diagram shows quantitation of western blot signals from multiple independent experiments. Cells were treated as indicated \* $P < 0.001$ , compared with untreated controls. **(e, f)** Soluble chromatin was prepared from LKB1-depleted (LKB1<sup>shRNA</sup>), vector control (pLKO.1), LKB1-transfected LKB1<sup>shRNA</sup> (LKB1<sup>shRNA</sup>+LKB1<sup>O/E</sup>) MCF7 breast cancer cells treated with vehicle (c) or 5  $\mu$ M honokiol (HNK). Chromatin immunoprecipitation assay was performed using pStat3 antibody. IgG antibody was included as control. The purified DNA was analyzed by real-time quantitative PCR using primers spanning the Stat3-binding sites at Oct4, Nanog and Sox2 promoter. PCR products were also analyzed on agarose gels.

phenotype. Our study illustrates an interesting mechanism whereby HNK inhibits coactivation-function of Stat3 and blocks the expression of iPSC markers, Oct4, Nanog and Sox2 via LKB1. *In vivo* analyses of spontaneous tumors from LKB1<sup>-/-</sup> and HNK-treated LKB1-null xenografts provide further evidence of a negative association between LKB1 and Stat3-Oct4/Nanog/Sox2. Supported by these data, we present a schematic depicting a series of events where HNK-induced increase in LKB1 leads to dephosphorylation and inactivation of Stat3 which results in abrogation of Stat3-recruitment on the promoters of Oct4, Nanog and Sox2, eventually inhibiting stemness, mammosphere formation and *in vivo* tumor growth (Figure 8g).

While not commonly correlated with breast cancer, LKB1 inactivation/ LKB1 loss has been reported in high-grade ductal carcinoma *in situ* and high-grade invasive ductal carcinoma.<sup>15</sup> The fact that LKB1-loss is observed only in the ductal carcinoma *in situ* associated with invasion and not in pure ductal carcinoma *in situ* cases indicate that abrogation of LKB1 may promote invasive behavior. Reduced/loss of LKB1 expression, observed in stage III breast cancer cases in comparison to stage I cases, also correlates with established markers of unfavorable breast cancer

prognosis.<sup>16</sup> LKB1-null state exhibits elevated levels of matrix metalloproteinases including MMP2, MMP9 as well as increased expression of IGF-binding protein 5 (IGFBP5), prostaglandin-endoperoxide synthase 2 (COX2) and vascular endothelial growth factor (VEGF).<sup>17,18</sup> Interestingly, MMPs, vascular endothelial growth factor, IGFBP5 and COX2 are important components of tumor-progression networks that are known to be upregulated by Stat3 suggesting a negative association between Stat3 and LKB1. We report a significant inverse correlation between LKB1 expression and Stat3-coactivation function and expression of Stat3-activated genes in breast cancer. Importantly, our data also present the first evidence of the critical involvement of LKB1 in inhibition of stemness and iPSC markers-Oct4, Nanog and Sox2. These discoveries have potentially important implications in regards to increasing interest in targeting stemness and iPSC inducers through manipulation of tumor suppressor LKB1.

Anti-inflammatory, anxiolytic, anti-thrombocytic, anti-depressant, antioxidant, antispasmodic and antibacterial effects of cones, bark and leaves from *Magnolia* plant species have been known for many years<sup>19-22</sup>; their potential as cancer preventive and therapeutic agent has also been suggested.<sup>23,24</sup> The bioactive



**Figure 9.** LKB1 is integral for honokiol-mediated inhibition of mammosphere and breast tumor growth. **(a, b)** LKB1-depleted (LKB1<sup>shRNA 1-2</sup>) and vector control (pLKO.1) MCF7 and MDA-MB-231 breast cancer cells were treated with 5 μM honokiol (HNK) and subjected to mammosphere assay. \**P* < 0.001, compared with untreated controls. **(c)** LKB1-depleted (LKB1<sup>shRNA</sup>) and vector control (pLKO.1) MDA-MB-231 cells derived tumors were developed in nude mice and treated with vehicle and Honokiol (HNK). Tumor growth was monitored by measuring the tumor volume for 6 weeks. (*n* = 8–10); (*P* < 0.001), pLKO.1+HNK compared with LKB1<sup>shRNA</sup>+HNK. **(d)** Tumors from LKB1<sup>shRNA</sup>+Vehicle, pLKO.1+Vehicle, LKB1<sup>shRNA</sup>+HNK, and pLKO.1 +HNK groups were subjected to immunohistochemical (IHC) analysis using Ki-67 antibodies. Bar diagram shows the quantitation of Ki-67 expression in tumors from each treatment group. Columns, mean (*n* = 5); \**P* < 0.005, compared with control. **(e)** Tumors from LKB1<sup>shRNA</sup>+Vehicle, pLKO.1+Vehicle, LKB1<sup>shRNA</sup>+HNK, and pLKO.1 +HNK groups were subjected to IHC analysis using phospho-Stat3 (pStat3) antibodies. Bar diagram shows the quantitation of pStat3 expression in tumors from each treatment group. Columns, mean (*n* = 5); \**P* < 0.005, compared with control. **(f)** Total protein lysates from vector control (pLKO.1) and LKB1 null (LKB1<sup>shRNA</sup>) tumors were examined for the expression of pStat3 and Stat3 as indicated. β-actin was used as loading-control. **(g)** Schematic representation. Honokiol treatment increases the expression of tumor suppressor LKB1 which in turn activates AMPK phosphorylation. Increased LKB1 abrogates Stat3 phosphorylation and inhibits recruitment of Stat3 on Oct4, Nanog and Sox2 resulting in inhibition of the expression of Oct4, Nanog and Sox2 leading to inhibition of stemness, mammosphere and breast tumor growth.

compound responsible for the medicinal effects of Magnolia species is a phenolic compound, HNK. Using various *in vitro* and *in vivo* models, our research group has shown that HNK inhibits breast carcinogenesis.<sup>8,25</sup> Here, we show that HNK is effective in inhibiting mammosphere-formation, ALDH activity and expression of iPSC inducers; hence, appears to be a good candidate for further development as an effective inhibitor of stem-like phenotype in breast cancer cells.

Stat3-activation is important for transformation, multiple aspects of tumor progression and metastasis including epithelial mesenchymal transition in many types of cancers, including breast cancer; hence, Stat3 represents an attractive target for cancer therapy.<sup>26–29</sup> Various direct and indirect strategies such as phosphotyrosyl-peptides, dominant-negative mutants, antisense-methods and inhibition of upstream-kinases have been developed to abrogate Stat3-activation.<sup>30</sup> Few approaches, such as Tyrphostin AG490,<sup>31</sup> Anti-sense Stat3-ODN (oligodeoxynucleotide)<sup>32</sup> and

STAT21<sup>33</sup> have been evaluated in various model systems and functional assays. Translational use of Stat3 inhibitors has been limited despite these developments, mainly owing to limited efficacy, therapy-related toxicity and off-target effects. We present HNK as a bioactive strategy to block Stat3-phosphorylation and coactivation-function that may lead to a widespread inhibition of Stat3-target genes.

In conclusion, we uncovered an inverse relationship between LKB1 and iPSC inducers—Oct4, Nanog and Sox2. We put forth HNK as a bioactive strategy to induce LKB1 and inhibit stem-like phenotype of breast cancer cells via reducing expression of iPSC inducers, in part, mediated by Stat3-inactivation. Our results thus demonstrate the integral role of a previously unrecognized crosstalk between HNK and LKB1-Stat3-iPSC inducers in breast cancer. Furthermore, our findings can potentially open new avenues of research on the role of HNK as a novel inhibitor of stem-like phenotype.

## MATERIALS AND METHODS

### Cell culture and reagents

Human breast cancer cell lines (MCF7, MDA-MB-231, SUM149 and SUM159) were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA). Honokiol was extracted from seed cone of *Magnolia grandiflora*.<sup>34</sup> We have shown that 25  $\mu\text{M}$  HNK inhibit cell viability, and cell proliferation while 5  $\mu\text{M}$  HNK does not affect cell viability and proliferation.<sup>8,25,35</sup> Accordingly, we used 5  $\mu\text{M}$  HNK to investigate the effect of HNK on breast cancer stemness. Antibodies for Oct4, Nanog, Sox2, LKB1, phosphorylated AMPK, AMPK, phosphorylated-Stat3, Stat3 were procured from Cell Signaling Technology (Danvers, MA, USA). Antibody against  $\beta$ -Actin was procured from Sigma-Aldrich (St Louis, MO, USA). pcDNA3-Flag-LKB1 was a gift from Dr Lewis Cantley (Addgene).<sup>36</sup> Constitutively active (CA)-Stat3-C Flag pRc/CMV was a gift from Dr Jim Darnell (Addgene).<sup>37</sup> Stattic was procured from Sigma-Aldrich (St Louis, MO, USA). 5-Aminoimidazole-4-carboxamide ribonucleotide was purchased from Tocris Bioscience (Minneapolis, MN, USA) and Compound C was procured from EMD Millipore (Billerica, MA, USA).

### LKB1 stable knockdown cells

Breast cancer cells were transfected with pre-made lentiviral LKB1 short hairpin RNA (shRNA) constructs and negative control construct created in the same vector system (pLKO.1) (Open Biosystems, Huntsville, AL, USA) using Fugene or Lipofectamine. Mature Antisense 1: 5'-ATAAATA TTGACCGCACACTC-3'; Mature Antisense 2: 5'-AATTCCTCTTCACGT TGGC-3'. Following our previously published protocol,<sup>38</sup> paired LKB1 stable knockdown cells (MCF7 and MDA-MB-231) were generated.

### Western blotting, RNA isolation, transfection, RT-PCR, Chromatin immunoprecipitation (ChIP) and immunofluorescence

Whole cell lysates prepared using modified-RIPA buffer were subjected to immunoblotting.<sup>39</sup> Total cellular RNA was isolated using the TRIzol Reagent (Life Technologies, Inc., Rockville, MD, USA) and RT-PCR was performed using specific sense and antisense PCR primers. Full-length wild-type pcDNA3-Flag-LKB1 and constitutively active Stat3-C Flag pRc/CMV plasmid constructs were transfected using Fugene 6 (Promega Corporation, Madison, WI, USA) transfection reagent. ChIP analyses were performed using our published procedure.<sup>40</sup> Immunofluorescence analysis was utilized to visualize proteins and imaging was done using a Zeiss LSM510 Meta (Zeiss) laser scanning confocal system configured to a Zeiss Axioplan 2 upright microscope.

### Spheroid-migration, scratch-migration, microfluidic-migration, invasion and mammosphere assay

Intact tumor spheroids were selected as described,<sup>8</sup> treated with HNK and migrating cells from spheroids were examined under light microscope. For *scratch-migration*, a 1-mm-wide scratch was etched on the confluent cell layer and migration of cells was recorded at regular intervals.<sup>39,41</sup> A microfluidic device, consisting of an array of parallel microchannels combined with live-cell phase-contrast imaging was utilized and cell-speed, cell-velocity and persistence were calculated.<sup>42-44</sup> Matrigel invasion assay<sup>45</sup> was conducted using a Matrigel invasion chamber from BD Biocoat Cellware (San Jose, CA, USA). Mammosphere assays were performed as previously described and mammospheres ( $> 50 \mu\text{m}$ ) were counted.<sup>46</sup>

### LKB1<sup>-/-</sup> mice tumor samples

A model of human breast cancer was developed by breeding STK11<sup>lox/lox</sup> mice (FVB) with mice genetically engineered to express activated Neu/HER2-MMTV-Cre (FVB) under the endogenous Erbb2 promoter, referred to as NIC mice, to generate STK11<sup>-/-</sup>/NIC mice. STK11<sup>-/-</sup>/NIC mice exhibited significant reduction in the latency of tumor onset.<sup>5</sup> Tumor samples from this study were analyzed for the expression of LKB1, Oct4, Nanog and Sox2.

### LKB1 activity assay

LKB1 was immunoprecipitated from breast cancer cells treated with HNK using LKB1 antibody. Phosphotransferase activity of LKB1 was determined using protein complex (0.5–1.0  $\mu\text{g}$ ) in the presence of 0.1 mM [ $\gamma$ -<sup>32</sup>P]ATP at 30 °C for 15 min using the LKBtide peptide (150  $\mu\text{M}$ ) (EMD Millipore).<sup>47,48</sup>

### Fluorescence-activated cell sorting (FACS)-ALDH activity

Breast cancer cells were plated in ultra-low attachment dishes (Corning) at a density of 20 000 cells/ml in serum-free growth media containing 5  $\mu\text{g}/\text{ml}$  insulin and 1  $\mu\text{g}/\text{ml}$  hydrocortisone. Single cells were isolated from primary mammospheres and the Aldefluor kit (Stem Cell Technologies, Vancouver, BC, Canada) was utilized to measure ALDH enzymatic activity. Breast cancer cells were suspended in Aldefluor assay buffer containing ALDH1 substrate and incubated for 30 min at 37 °C. ALDH inhibitor diethylaminobenzaldehyde was included as negative control.

### Breast tumor samples

MDA-MB-231 cells xenografts were generated and treated with vehicle and HNK as previously described.<sup>25</sup> Breast tumors were measured routinely, collected post 4 weeks of treatment, weighed and analyzed. These tumors were utilized for examining the expression of Oct4, Nanog and Sox2 in immunoblot and immunohistochemistry analyses. All animal studies were conducted in accordance with the guidelines of Johns Hopkins University IACUC.

### Breast tumorigenesis assay

MDA-MB-231-pLKO.1 and MDA-MB-231-LKB1<sup>sh</sup> cells xenografts were generated; as previously described,<sup>25</sup> grouped in two experimental groups (8 mice/group). Mice were oral gavaged three times per week for 6 weeks with (1) Vehicle (saline and Intralipid); (2) HNK, at 3 mg/mouse/day in 20% Intralipid (Baxter Healthcare, Deerfield, IL, USA). The dose and route of HNK administration was in accordance with our previous studies showing *in vivo* efficacy of HNK.<sup>25</sup> Tumors were regularly measured, collected after six weeks of treatment, weighed and subjected to further analysis. These tumors were utilized for examining the expression of Ki67 and phospho-Stat3 in immunoblot and immunohistochemistry analyses. For immunohistochemistry, multiple nonoverlapping representative images from each tumor section from all the tumors of each group were captured using ImagePro software for quantitation of Oct4, Nanog, Sox2, Ki-67 and phospho-Stat3 expression. All animal studies were in accordance with the guidelines of Johns Hopkins University IACUC.

### Statistical analysis

Survival data were analyzed by using the Kaplan–Meier method and Cox's regression, and comparison of overall survival between groups was performed using logrank test. RNA-seq expression data were obtained from the The Cancer Genome Atlas network.<sup>49</sup> All experiments were performed thrice in triplicates. Continuous variables were summarized using mean  $\pm$  s.e. and were compared between groups using two-sided Welch's *t*-test. Results were considered to be statistically significant if  $P < 0.05$ . Statistical analysis of continuous variables was performed using R statistical software (The R Development Core Team) and Microsoft Excel.

## CONFLICT OF INTEREST

SS, AN, NM, MYB, PM, AA, PK, DL, PK, SC, MS, AB, VFM, C-YH, WM, BG, KK, SS, PAM, NKS and DS declare no conflict of interest. JLA is listed as an inventor on patents filed by Emory University. Emory has licensed its HNK technologies to Naturopathic Pharmacy. JLA has received stock in Naturopathic Pharmacy, which to the best of knowledge is not publically traded.

## ACKNOWLEDGEMENTS

This work was supported by NCI NIH, R21CA185943 (to NKS); OTKA K108655 (to BG); NCI NIH R01AR47901 (to JLA); The Nova Scotia Health Research Foundation (DL); The Canadian Breast Cancer Foundation and The Nova and The Dalhousie Medical Research Foundation (PAM); NCI NIH R01CA131294, NCI NIH R21CA155686, Avon Foundation, Breast Cancer Research Foundation (BCRF) 90047965 and The Fetting Fund (to DS).

## REFERENCES

- 1 Vaahoteri K, Makela TP. Molecular mechanisms of tumor suppression by LKB1. *FEBS Lett* 2011; **585**: 944–951.
- 2 Hardie DG. New roles for the LKB1–AMPK pathway. *Curr Opin Cell Biol* 2005; **17**: 167–173.

- 3 Jenne DE, Reimann H, Nezu J, Friedel W, Loff S, Jeschke R *et al*. Peutz-Jeghers syndrome is caused by mutations in a novel serine threonine kinase. *Nat Genet* 1998; **18**: 38–43.
- 4 Morton JP, Jamieson NB, Karim SA, Athineos D, Ridgway RA, Nixon C *et al*. LKB1 haploinsufficiency cooperates with Kras to promote pancreatic cancer through suppression of p21-dependent growth arrest. *Gastroenterology* 2010; **139**: 586–597. e581–586.
- 5 Andrade-Vieira R, Xu Z, Colp P, Marignani PA. Loss of LKB1 expression reduces the latency of ErbB2-mediated mammary gland tumorigenesis, promoting changes in metabolic pathways. *PLoS One* 2013; **8**: e56567.
- 6 Wong DJ, Liu H, Ridky TW, Cassarino D, Segal E, Chang HY. Module map of stem cell genes guides creation of epithelial cancer stem cells. *Cell stem cell* 2008; **2**: 333–344.
- 7 Andrade-Vieira R, Goguen D, Bentley HA, Bowen CV, Marignani PA. Pre-clinical study of drug combinations that reduce breast cancer burden due to aberrant mTOR and metabolism promoted by LKB1 loss. *Oncotarget* 2014; **5**: 12738–12752.
- 8 Avtanski DB, Nagalingam A, Bonner MY, Arbiser JL, Saxena NK, Sharma D. Honokiol inhibits epithelial-mesenchymal transition in breast cancer cells by targeting signal transducer and activator of transcription 3/Zeb1/E-cadherin axis. *Mol Oncol* 2014; **8**: 565–580.
- 9 Yan D, Avtanski D, Saxena NK, Sharma D. Leptin-induced epithelial-mesenchymal transition in breast cancer cells requires beta-catenin activation via Akt/GSK3- and MTA1/Wnt1 protein-dependent pathways. *J Biol Chem* 2012; **287**: 8598–8612.
- 10 Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, Nakashima K *et al*. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. *Proc Natl Acad Sci USA* 2006; **103**: 10294–10299.
- 11 Zhou JJ, Chen RF, Deng XG, Zhou Y, Ye X, Yu M *et al*. Hepatitis C virus core protein regulates NANOG expression via the stat3 pathway. *FEBS Lett* 2014; **588**: 566–573.
- 12 Foshay KM, Gallicano GI. Regulation of Sox2 by STAT3 initiates commitment to the neural precursor cell fate. *Stem Cells Dev* 2008; **17**: 269–278.
- 13 Do DV, Ueda J, Messerschmidt DM, Lorthongpanich C, Zhou Y, Feng B *et al*. A genetic and developmental pathway from STAT3 to the OCT4-NANOG circuit is essential for maintenance of ICM lineages in vivo. *Genes Dev* 2013; **27**: 1378–1390.
- 14 Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A *et al*. An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008; **40**: 499–507.
- 15 Fenton H, Carlile B, Montgomery EA, Carraway H, Herman J, Sahin F *et al*. LKB1 protein expression in human breast cancer. *Appl Immunohistochem Mol Morphol* 2006; **14**: 146–153.
- 16 Li J, Liu J, Li P, Mao X, Li W, Yang J *et al*. Loss of LKB1 disrupts breast epithelial cell polarity and promotes breast cancer metastasis and invasion. *J Exp Clin Cancer Res* 2014; **33**: 70.
- 17 Ylikorkala A, Rossi DJ, Korsisaari N, Luukko K, Alitalo K, Henkemeyer M *et al*. Vascular abnormalities and deregulation of VEGF in Lkb1-deficient mice. *Science* 2001; **293**: 1323–1326.
- 18 Rossi DJ, Ylikorkala A, Korsisaari N, Salovaara R, Luukko K, Launonen V *et al*. Induction of cyclooxygenase-2 in a mouse model of Peutz-Jeghers polyposis. *Proc Natl Acad Sci USA* 2002; **99**: 12327–12332.
- 19 Xu Q, Yi LT, Pan Y, Wang X, Li YC, Li JM *et al*. Antidepressant-like effects of the mixture of honokiol and magnolol from the barks of *Magnolia officinalis* in stressed rodents. *Prog Neuropsychopharmacol Biol Psychiatry* 2008; **32**: 715–725.
- 20 Oh JH, Kang LL, Ban JO, Kim YH, Kim KH, Han SB *et al*. Anti-inflammatory effect of 4-O-methylhonokiol, compound isolated from *Magnolia officinalis* through inhibition of NF-kappaB [corrected]. *Chem Biol Interact* 2009; **180**: 506–514.
- 21 Choi DY, Lee YJ, Hong JT, Lee HJ. Antioxidant properties of natural polyphenols and their therapeutic potentials for Alzheimer's disease. *Brain Res Bull* 2012; **87**: 144–153.
- 22 Leeman-Neill RJ, Cai Q, Joyce SC, Thomas SM, Bholra NE, Neill DB *et al*. Honokiol inhibits epidermal growth factor receptor signaling and enhances the antitumor effects of epidermal growth factor receptor inhibitors. *Clin Cancer Res* 2010; **16**: 2571–2579.
- 23 Prasad R, Kappes JC, Katiyar SK. Inhibition of NADPH oxidase 1 activity and blocking the binding of cytosolic and membrane-bound proteins by honokiol inhibit migratory potential of melanoma cells. *Oncotarget* 2016; **7**: 7899–7912.
- 24 Averett C, Arora S, Zubair H, Singh S, Bhardwaj A, Singh AP. Molecular targets of Honokiol: a promising phytochemical for effective cancer management. *Enzymes* 2014; **36**: 175–193.
- 25 Nagalingam A, Arbiser JL, Bonner MY, Saxena NK, Sharma D. Honokiol activates AMP-activated protein kinase in breast cancer cells via an LKB1-dependent pathway and inhibits breast carcinogenesis. *Breast Cancer Res* 2012; **14**: R35.
- 26 Sinibaldi D, Wharton W, Turkson J, Bowman T, Pledger WJ, Jove R. Induction of p21WAF1/CIP1 and cyclin D1 expression by the Src oncoprotein in mouse fibroblasts: role of activated STAT3 signaling. *Oncogene* 2000; **19**: 5419–5427.
- 27 Yu H, Jove R. The STATs of cancer—new molecular targets come of age. *Nat Rev Cancer* 2004; **4**: 97–105.
- 28 Buettner R, Mora LB, Jove R. Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. *Clin cancer res* 2002; **8**: 945–954.
- 29 Yue P, Turkson J. Targeting STAT3 in cancer: how successful are we? *Expert Opin Investig Drugs* 2009; **18**: 45–56.
- 30 Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis. *Oncogene* 2000; **19**: 2474–2488.
- 31 Burdelya L, Catlett-Falcone R, Levitzki A, Cheng F, Mora LB, Sotomayor E *et al*. Combination therapy with AG-490 and interleukin 12 achieves greater antitumor effects than either agent alone. *Mol Cancer Ther* 2002; **1**: 893–899.
- 32 Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS *et al*. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth in vitro. *J Clin Invest* 1998; **102**: 1385–1392.
- 33 Song H, Wang R, Wang S, Lin J. A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells. *Proc Natl Acad Sci USA* 2005; **102**: 4700–4705.
- 34 Bai X, Cerimele F, Ushio-Fukai M, Waqas M, Campbell PM, Govindarajan B *et al*. Honokiol, a small molecular weight natural product, inhibits angiogenesis in vitro and tumor growth in vivo. *J Biol Chem* 2003; **278**: 35501–35507.
- 35 Avtanski DB, Nagalingam A, Kuppusamy P, Bonner MY, Arbiser JL, Saxena NK *et al*. Honokiol abrogates leptin-induced tumor progression by inhibiting Wnt1-MTA1-beta-catenin signaling axis in a microRNA-34a dependent manner. *Oncotarget* 2015; **6**: 16396–16410.
- 36 Shaw RJ, Kosmatka M, Bardeesy N, Hurlley RL, Witters LA, DePinho RA *et al*. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. *Proc Natl Acad Sci USA* 2004; **101**: 3329–3335.
- 37 Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C *et al*. Stat3 as an oncogene. *Cell* 1999; **98**: 295–303.
- 38 Taliaferro-Smith L, Nagalingam A, Zhong D, Zhou W, Saxena NK, Sharma D. LKB1 is required for adiponectin-mediated modulation of AMPK-S6K axis and inhibition of migration and invasion of breast cancer cells. *Oncogene* 2009; **28**: 2621–2633.
- 39 Saxena NK, Taliaferro-Smith L, Knight BB, Merlin D, Anania FA, O'Regan RM *et al*. Bidirectional crosstalk between leptin and insulin-like growth factor-I signaling promotes invasion and migration of breast cancer cells via transactivation of epidermal growth factor receptor. *Cancer Res* 2008; **68**: 9712–9722.
- 40 Sharma D, Saxena NK, Davidson NE, Vertino PM. Restoration of tamoxifen sensitivity in estrogen receptor-negative breast cancer cells: tamoxifen-bound reactivated ER recruits distinctive corepressor complexes. *Cancer res* 2006; **66**: 6370–6378.
- 41 Saxena NK, Vertino PM, Anania FA, Sharma D. leptin-induced growth stimulation of breast cancer cells involves recruitment of histone acetyltransferases and mediator complex to CYCLIN D1 promoter via activation of Stat3. *J Biol Chem* 2007; **282**: 13316–13325.
- 42 Hung WC, Chen SH, Paul CD, Stroka KM, Lo YC, Yang JT *et al*. Distinct signaling mechanisms regulate migration in unconfined versus confined spaces. *J Cell Biol* 2013; **202**: 807–824.
- 43 Wang P, Chen SH, Hung WC, Paul C, Zhu F, Guan PP *et al*. Fluid shear promotes chondrosarcoma cell invasion by activating matrix metalloproteinase 12 via IGF-2 and VEGF signaling pathways. *Oncogene* 2015; **34**: 4558–4569.
- 44 Hung WC, Yang JR, Yankaskas CL, Wong BS, Wu PH, Pardo-Pastor C *et al*. Confinement sensing and signal optimization via Piezo1/PKA and Myosin II pathways. *Cell Rep* 2016; **15**: 1430–1441.
- 45 Saxena NK, Sharma D, Ding X, Lin S, Marra F, Merlin D *et al*. Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells. *Cancer Res* 2007; **67**: 2497–2507.
- 46 Avtanski DB, Nagalingam A, Kuppusamy P, Bonner MY, Arbiser JL, Saxena NK *et al*. Honokiol abrogates leptin-induced tumor progression by inhibiting Wnt1-MTA1-beta-catenin signaling axis in a microRNA-34a dependent manner. *Oncotarget* 2015; **6**: 16396–16410.
- 47 Boudeau J, Scott JW, Resta N, Deak M, Kieloch A, Komander D *et al*. Analysis of the LKB1-STRAD-MO25 complex. *J Cell Sci* 2004; **117**: 6365–6375.
- 48 Nath-Sain S, Marignani PA. LKB1 catalytic activity contributes to estrogen receptor alpha signaling. *Mol Biol Cell* 2009; **20**: 2785–2795.
- 49 Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature* 2012; **490**: 61–70.
- 50 Azare J, Doane A, Leslie K, Chang Q, Berishaj M, Nnoli J *et al*. Stat3 mediates expression of autotaxin in breast cancer. *PLoS One* 2011; **6**: e27851.

Supplementary Information accompanies this paper on the Oncogene website (<http://www.nature.com/onc>)