Supplementary information for:

CDK5 is essential for TGF-β1-induced epithelial-mesenchymal transition and breast cancer progression

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Supplementary Materials and Methods

Plasmids and virus infection

The pBI-GFP-CDK5dn (dominant-negative CDK5 with a D144N kinase-dead mutant) expression plasmid was kindly provided by Dr. Barry D. Nelkin (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, USA). The CDK5dn constructs were subcloned into a lentiviral expression vector pWPXLd (Addgene) by the PCR using the same primers as CDK5. The human p35 expression construct was cloned into the vector pWPXLd by PCR using the following primers (forward primer 5'-AGCTTTGTTTAAACATGGGCACGGTGCTGTCC-3' and reverse primer 5'-CCGGAATTCTCACCGATCCAGGCCTAG-3') from MDA-MB-231 cDNA. The human Twist and Snail expression construct were cloned into the vector pWPXLd by PCR using the following primers (Twist, forward primer 5'-CGACGCGTTTAGTGACCGTCAGAATT-3' and reverse primer 5'-GGGTCATATGGATGCCACCCGG-3'; Snail, forward primer 5'-CGCGGATCCATGCCGCGCTCTTTCCTCGT-3' and reverse primer 5'-CCGGAATTCTCAGCGGGGACATCCTGAGC-3') from pcDNA4-Twist and pcDNA3-Snail expression plasmids respectively. The pcDNA4-Twist expression plasmid was kindly provided by Dr. Carlotta Glackin (Department of Neurosciences at Beckman Research Institute of City of Hope, Duarte, California, USA) and the pcDNA3-Snail expression plasmid was kindly provided by Dr. K-J Wu (Institute of Biochemistry and Molecular Biology,

National Yang-Ming University, Taipei, Taiwan).

The protocol of virus package and infection of the pWPXLd transfer vectors were the same as the pDSL-hpUGIP transfer vectors.

Trans-well assay under the condition of CDK5 inhibitor treatment

MDA-MB-231 and BT549 cells were treated with 10μM Rv for 24h. The following experimental procedures were the same as described in the main body of the text. MCF10A cells were infected with overexpression or control viruses. Seventy-two hours after infection, the stably transfected cells were added TGF-β1 and induced for forty-eight hours. The induced and non-induced cells were starved for 24h, and 5×10^4 cells in DMEM/F12 (with insulin, hydrocortisone and cholera toxin) media were plated into the upper chamber, and MCF10A complete medium was placed in the lower chamber. The chambers were coated with fibronectin. For migration assay, cells were stained with crystal violet after incubation for 24h. For invasion assay, the upper chambers were coated with Matrigel and stained after 60h. Randomly selected fields were photographed (Nikon ECLIPSE 80i) and stained cells were statistic analyzed.

Cell proliferation assay

Cell growth rates were assessed by the MTT assay. The control and transfected cells (MDA-MB-231, 2.5×10^3; BT549, 1×10^3) were seeded in 96-well plates. Rv was added after cells completed adhesion. After incubation for indicated time, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide solution was added to each well and incubated at 37 °C for 4h before removal of the culture medium. DMSO was then added and the cultures were shaken for 30 min at room temperature. Cell viability was determined by measuring the absorbance at 490 nm. The results were plotted as mean±SD of three separate experiments with ten determinations per experiment for each experimental condition.

Supplementary Figures

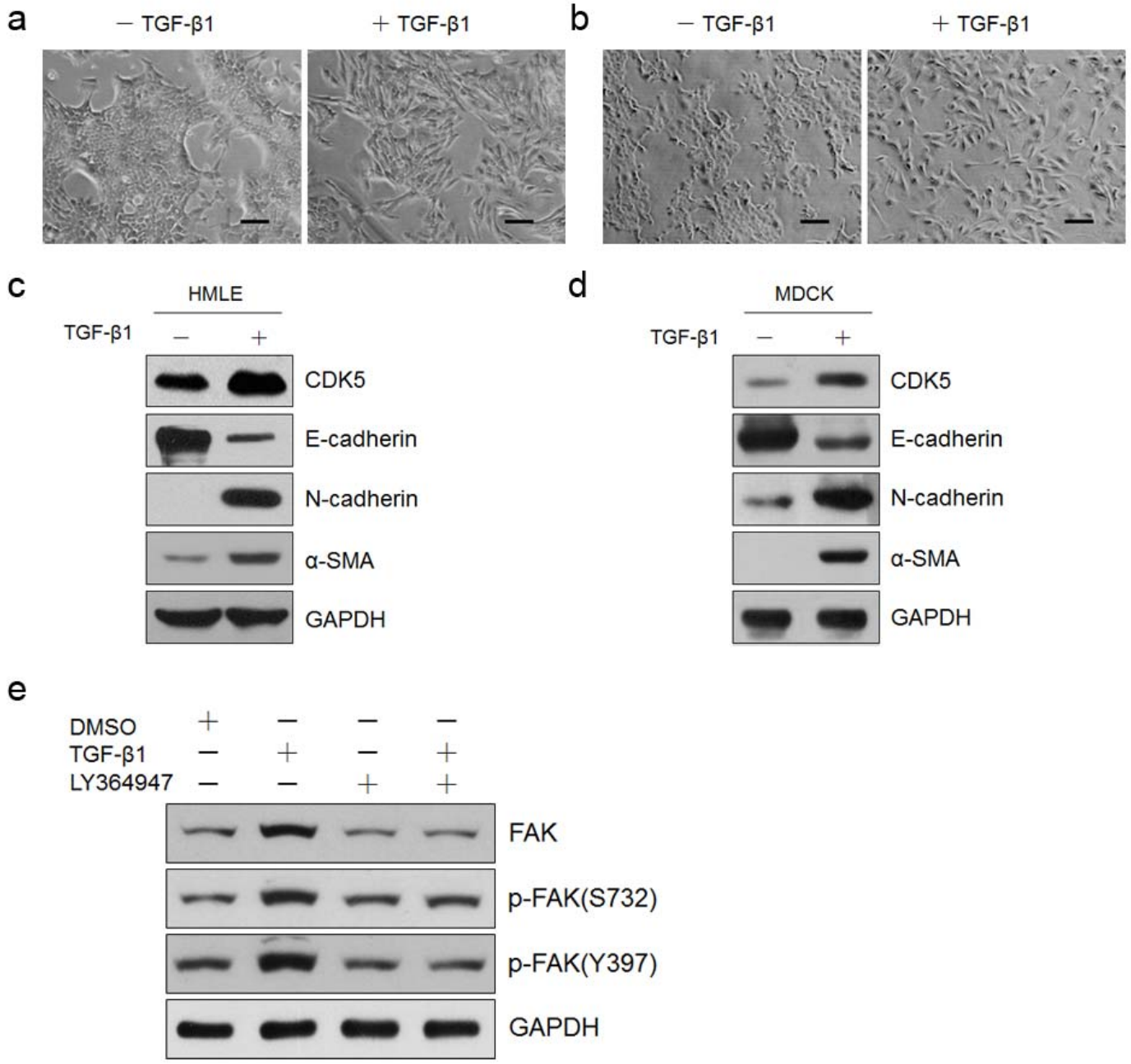

Figure S1. Upregulation of CDK5 and p35 and increase of CDK5 kinase activity during TGF-β1-induced EMT. (a) and (b) morphologic change of HMLE (a) and MDCK (b) cells cultured without or with TGF-β1 (5ng/ml, 48h), Scale bar=100μm. (c) and (d) immunoblotting analysis of the expression of CDK5 and the epithelial marker E-cadherin and the mesenchymal markers

N-cadherin and α-SMA. (e) immunoblotting analysis of the levels of FAK, p-FAK Y397 and p-FAK S732 under the conditions of different combination of DMSO, TGF-β1 and TGF-β1 inhibitor LY364947 in MCF10A cells.

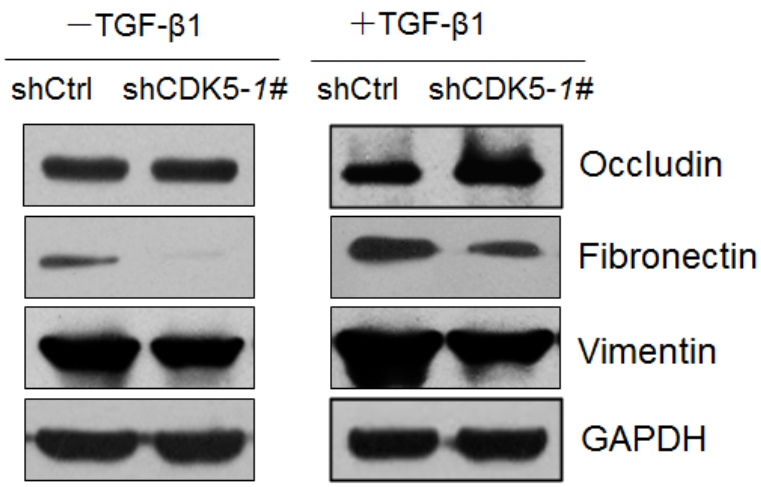

Figure S2. Knockdown of CDK5 inhibited TGF-β1-induced EMT.

Immunoblotting analysis of expression of the epithelial marker Occludin and the mesenchymal markers Fibronectin and Vimentin in MCF10A cultured without or with TGF-β1 after infection of shCDK5-*1#* or empty vector.

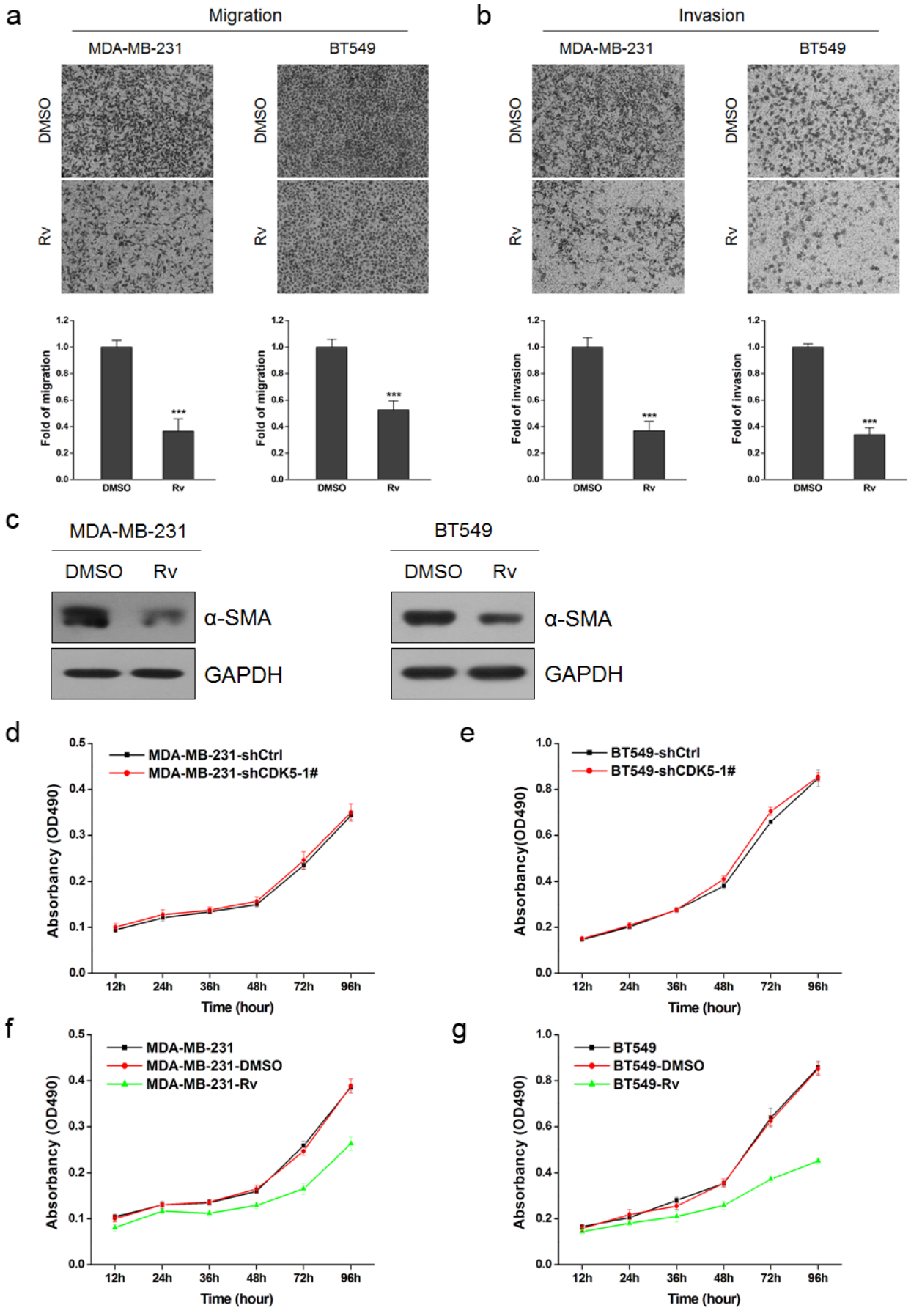

Figure S3. Inhibition of CDK5 kinase activity inhibited breast cancer cell

motility and tumorigenesis. (a) and (b) migration (24h; a) and invasion (48h; b) assays in MDA-MB-231 and BT549 cells after treatment with Rv or DMSO (control). The mean was derived from cell counts of 5 fields, and each experiment was repeated 3 times (***, $P<0.001$, compared with the control). Representative images of migrated and invaded cells are shown. (c) immunoblotting analysis of expression of the mesenchymal marker α-SMA in MDA-MB-231 and BT549 cells after treatment with Rv or DMSO. (d) and (e) proliferation of MDA-MB-231 cells (d) and BT549 cells (e) expressing control or shCDK5-*1#* vector were measured using MTT assays. (f) and (g) proliferation of MDA-MB-231 cells (f) and BT549 cells (g) cultured with Rv or DMSO and blank controls were measured using MTT assays.

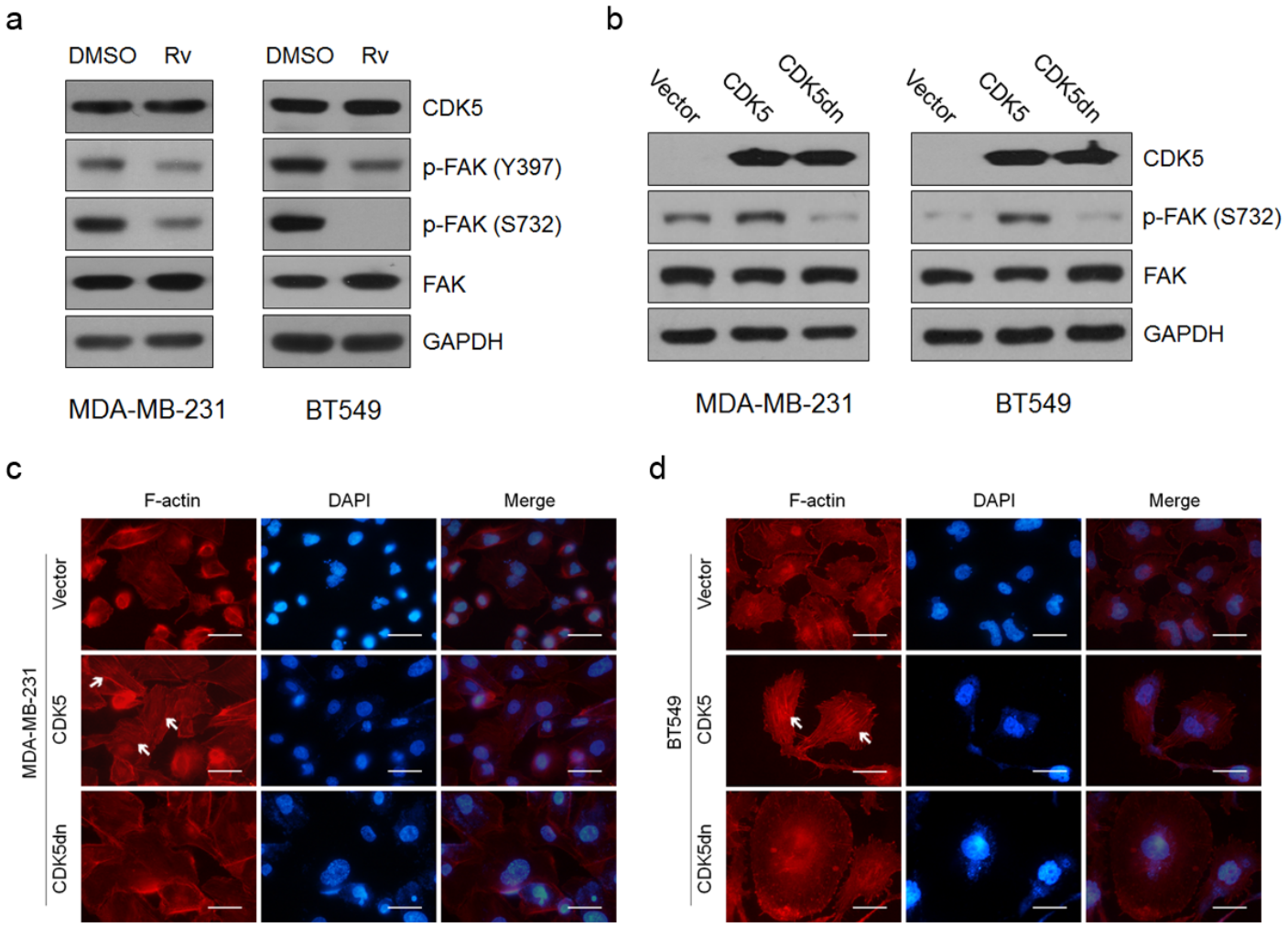

Figure S4. The kinase activity of CDK5 was essential for its function via phosphorylation of FAK at Ser-732 and affected F-actin remodeling. (a) immunoblotting analysis of expression of CDK5, FAK, p-FAK Y397 and p-FAK S732 in MDA-MB-231 (*left*) and BT549 (*right*) cells after treatment with Rv or DMSO. (b) immunoblotting analysis of expression of CDK5, FAK and p-FAK S732 in MDA-MB-231 (*left*) and BT549 (*right*) cells after infection of CDK5, CDK5dn or empty vector. (c) and (d) immunofluorescence staining for F-actin by phalloidine in MDA-MB-231 (c) and BT549 (d) cells after infection of CDK5, CDK5dn or empty vector. Scale bar=50μm. Arrows indicate the F-actin bundles.

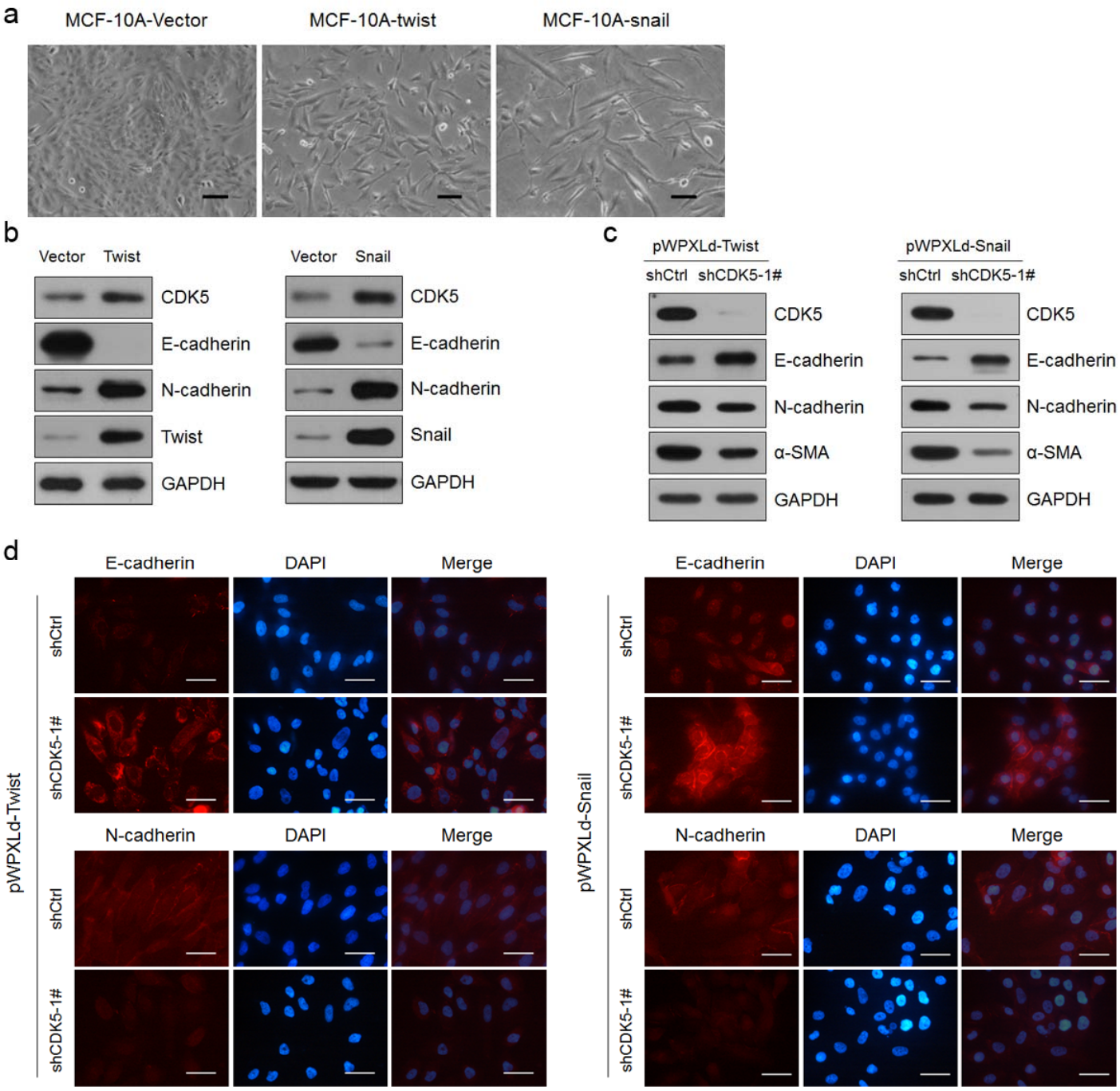

Figure S5. Knockdown of CDK5 inhibited Twist- and Snail-induced EMT.

(a) morphologic change of MCF10A cells overexpressing Twist or Snail, Scale bar=100μm. (b) immunoblotting analysis of the expression of CDK5 and the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and α-SMA in MCF10A overexpressing Twist (*Left*) or Snail (*Right*). (c)

immunoblotting analysis of expression of the epithelial marker E-cadherin and the mesenchymal markers N-cadherin and α-SMA in MCF10A overexpressing Twist (*Left*) or Snail (*Right*) after infection of shCDK5-*1#* or empty vector. (d) immunofluorescence staining for the epithelial marker E-cadherin and mesenchymal marker N-cadherin in MCF10A overexpressing Twist (*Left*) or Snail (*Right*) after infection of shCDK5-*1#* or empty vector. Scale bar=50μm.