Peer Review File

Article information: http://dx.doi.org/10.21037/tcr-20-1601

Reviewer A

Intro

Comment 1: The intro is misleading. It sets the reader up to expect that EZRIN will be central to the paper, when in fact EZRIN barely appears in the paper. Rationale for investigating STK10 is insufficiently justified; the reader is left expecting that the paper will identify the upstream activator of EZRIN, which the paper does not do.

Reply 1: Thank you for the rectification. According to your advice, we have modified our text. We have deleted the sentences related to EZRIN.

Changes in the text: Sentences related to EZRIN have been deleted (see page 4, line 74).

Comment 2: The intro also states that STK10 knockdown attenuated cell migration and invasion (lines 90-91), when the data presented clearly shows that STK10 knockdown promoted cell migration and invasion.

Reply 2: Thanks a lot for your reminding. We are so sorry for this mistake. *STK10* deficiency promotes cervical cancer cell migration and invasion. But these statements are results and should not be included in the Introduction, so we deleted these sentences.

Changes in the text: Sentences related to results have been deleted (see page 4, line 82).

Comment 3: Finally, the experiments are not designed to investigate cervical cancer development as stated in line 86 (the process of going from HPV infection to cancer, or pre-neoplasia to cancer); rather, they are designed to investigate processes occurring in cells derived from established cancers. This is an important distinction.

Reply 3: Thank you very much for this question. We have modified our text as advised. We deleted the phrase “the development of”.

Changes in the text: In the present study, to investigate the function of STK10 in cervical cancer (see page 4, line 79).

Methods

Comment 1: In general, too many methodological details are provided where “according to manufacturer’s protocol” or “according to the method of [citation]” could be indicated.

Reply 1: Thank you so much for your advice. We have modified the methods according to your suggestion. We have added more details about the methods of “CCK-8 cell viability assay” and “BrdU incorporation and apoptosis detection”.

Changes in the text:

Each group contained four duplicates. (see page 6, line 123).

The relative cell viability is the ratio of absorbance at the time relative to 0 h. (see page 6, lines 125-126).

HeLa and Caski cells were seeded to a 6-well plate with 5×10^5 cells per well. Each group contained three duplicates. The cells were digested with 0.25% trypsin after 24 h, dyed with fluorochrome-conjugated AnnexinV and propidium iodide (PI) staining solution and analyzed by flow cytometry. (see page 7, lines 132-136).

Comment 2: What is lacking from the methods is details that are pertinent, such as how the STK10 KO clone(s) were established. Were bulk cultures used in experiments (as it appears), or were limiting dilutions done to isolate individual STK10 KO clones? The use of biological replicate clones in the experiments would strengthen the data greatly. Also, were parental cells transfected with control vectors containing a puromycin selectable marker so that they were treated to the same selection process as the STK10 knockout cells, to rule out off-target responses to puromycin selection?

Reply 2: Thank you so much for your advice. STK10 KO clones are bulk cultures and not individual clones. But we have confirmed the absence of STK10 at protein level by western blot analysis. The control cells were transfected with PX459 control plasmids and selected with puromycin as the same as the *STK10* KO clones. We have modified the methods of "Transfection and puromycin selection" as advised.

Changes in the text:

A total of 4 μg *STK10* KO plasmids and PX459 plasmids (control) were transfected into

8×10^5 cells with Lipofectamine 3000 reagent (Thermo Fisher) (see page 5, lines 97-98).

After 2 days, the cells (including the control) were incubated in fresh medium with 1 μg/mL puromycin. (see page 5, lines 99-100).

Western blot analysis was used to identify the effect of knockout. The HeLa *STK10* KO and Caski *STK10* KO cell lines that were not the single clone were established, and DNA sequencing by a specific sequencing primer (5′-GTGCTCCGAAACAGGGC-3′) identified the genomic changes. (see page 5, lines 101-105).

Comment 3: HeLa cells are a highly lab adapted, extremely high-passage cell line that is no longer recognizable as homo sapiens. It was good that the authors replicated many experiments with Caski cells, but the RNA-Seq analysis was only conducted on HeLa. It is thus difficult to know how to apply the findings from this experiment.

Reply 3: Thanks for your question. We used RNA-Seq analysis in HeLa cells results to look for possible altered genes, and then validated these genes in HeLa and Caski cells using qRT-PCR.

Changes in the text: none.

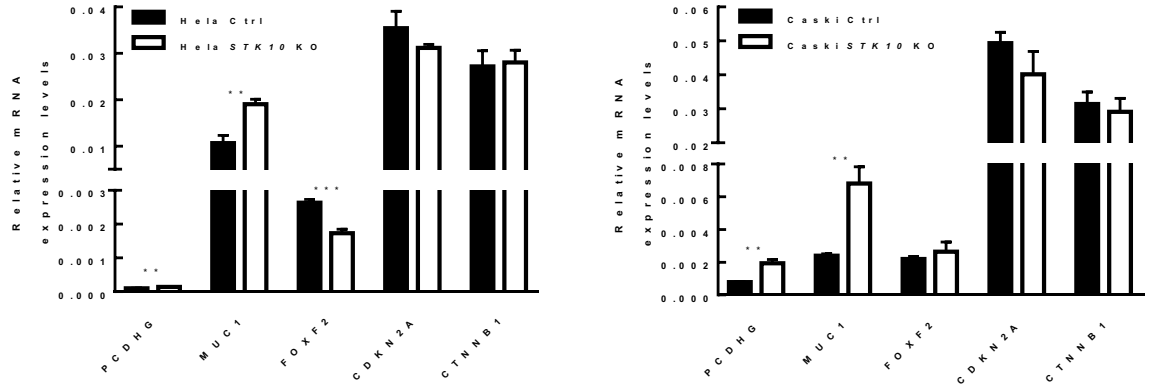
Comment 4: Beta-actin for the normalization control in RT-qPCR experiments using cells with clear changes in migration and invasion properties seems an odd choice.

Reply 4: Thank you very much for your suggestion. qRT-PCR results with GAPDH as normalization control have been obtained, which is the same as before. We have modified the figure 6 and methods as advised.

Changes in the text:

The figure 6 E, F have been replaced with new graphs (as shown below) (see figure6).

The primer sequence of GAPDH in the methods "RNA extraction and qRT-PCR



analyses" has been modified (see page 9, lines 191-192).

GAPDH-qRT-PCR-F: 5′-TGACTTCAACAGCGACACCCA-3′

GAPDH-qRT-PCR-R: 5′-CACCCTGTTGCTGTAGCCAAA-3′

Comment 5: What was the actual p-value cutoff after Bonferroni correction?

Reply 5: Thanks for your question. The actual p-value cutoff after Bonferroni correction was 0.05.

Changes in the text: none.

Comment 6: Were STK10 cells still under puromycin selection when the experiments

were conducted? If not, how long after stopping selection, or how many passages, did the cells undergo before beginning the experiments?

Reply 6: Thank you for your question. Cells resisted puromycin can be selected after one week of puromycin screening. There is no need to maintain puromycin screening because the cells genomic changes are heritable.

Changes in the text: none.

Results

Comment 1: The “noise” in the sequencing analysis (figure 1) suggests that these were bulk cultures and not individual clones.

Reply 1: Thank you very much for the question. Yes, these are bulk cultures. But we have identified the deficiency of STK10 by western blot analysis.

Changes in the text: none.

Discussion

Comment 1: The first paragraph does not discuss the paper and doesn’t belong in the discussion section.

Reply 1: Thanks a lot for your advice. We have deleted the first paragraph and replaced with a paragraph that is relevant to the content (see page 14, lines 288-294).

Changes in the text:

In this study, we assessed the role of STK10 in the development of cervical cancer using CRISPR-Cas9-mediated *STK10* KO HeLa and Caski cervical cancer cell lines. The proliferation, apoptosis, migration, and invasive activity of these cells were respectively detected by Brdu incorporation, AnnexinV/PI staining, wound healing assay, and transwell assays without and with matrigel. The results showed that STK10 depletion does not affect cell proliferation or apoptosis, but promotes the adhesion, migration, and invasion of cervical cancer cells. (see page 14, lines 288-294).

Comment 2: There is no discussion of the data in the context of the existing literature.

Reply 2: Thank you so much for your suggestion. We added some discussion related to literature as advised (see pages 14-16, lines 303-334).

Changes in the text:

STK10 is expressed in about 17 cancer types, including cervical cancer. Previous studies have shown that STK10 may affect LFA-1-mediated lymphocyte adhesion[19], while other studies report a mutation (R634H) in peripheral T-cell lymphoma, in which the arginine residues of STK10 were replaced with histidines, with the subsequent decline in the pro-apoptotic activity of STK10. Also, other somatic mutations in STK10 have been reported, suggesting that STK10 may act as a suppressor gene[20].

We have experimentally demonstrated that STK10 plays a vital role in tumor cell migration and invasion. To reveal the underlying mechanism, we determined the

phosphorylation and expression levels of ERM proteins, which have been demonstrated to be essential in the metastasis of various cancers[10,21-23]. However, neither phosphorylation nor the expression level of all three ERM proteins is affected by the target deletion of STK10. Thus, STK10 participates in the biology of cervical cancer independently of the ERM pathway. To uncover the underlying mechanism, in the present study, we conducted an RNA-seq analysis for HeLa cells with and without STK10 and found the expression of 374 genes was changed in the cells lacking STK10.

Most importantly, more than half of these genes are related to tumor cell migration or invasion. PCDHG is a member of the protocadherin family and plays a vital role in mediating cell adhesion and synaptic development[24]. FOXF2 and CDKN2A expression can significantly inhibit tumor cell migration and invasive ability[25,26]. The downregulation of CTNNB1 can reduce the capacity for intercellular adhesion and promote cell migration and invasion [27]. Compared with the control group, the expression levels of *MUC1* in the *STK10* KO HeLa and Caski cells were increased. *MUC1* (Mucin-1) is a member of the mucoprotein family; the expression levels of *MUC1* are upregulated in a variety of metastatic tumors[28-30]. STK10 is an upstream kinase, acting as MAP kinase kinase kinase kinases (MAP4Ks). Maybe MUC1 is not the substrate of STK10 directly. However, we can conclude that the expression of *MUC1* increases after *STK10* depletion in cervical cancer cells, and the expression levels of other migration-related genes are also changed, jointly promoting the adhesion, migration, and invasion of cervical cancer cells, in a pathway that is ERM-independent. (see pages 14-16, lines 303-334).

Comment 3: There is no discussion of the translational relevance of the findings, which is conspicuously missing for an article that has been submitted to the journal "Translational Cancer Research".

Reply 3: Thank you so much for your suggestion. We added some discussion related to translational medicine in the last paragraph (see page 16, lines 335-340).

Changes in the text:

Although more precise studies are needed to clarify the functional role of *STK10* in the pathogenesis of cervical cancer, the current study has demonstrated for the first time a possible role of *STK10* in the metastasis of cervical cancer and provided some new, useful information on the biology underlying cervical cancer, which will be of great benefit to patients through improved prevention, diagnosis, and treatment of this disease (see page 16, lines 335-340).

Reviewer B

Comment 1: What is the Clinicopathological characteristics of patients with cervical cancer according to STK10 expression.

Reply 1: Thank you very much for your advice. We obtained some data about tissue microarrays of human cervical cancer. Scores of the results were graded by IHC staining, and the product of the staining intensity score and the staining positive rate

score was taken as the total score for grouping. 0~4(low expression group), 5~8(middle expression group) and 9~12(high expression group).

The expression level of STK10 protein in human cervical cancer tissues was analyzed with clinical indicators such as patients' age, pathological classification, T stage, N stage, M stage and recrudescence. The results showed that there was no correlation between the expression level of STK10 protein in cervical cancer tissues and different clinicopathological parameters of cervical cancer patients ($P > 0.05$). As shown in the table below.

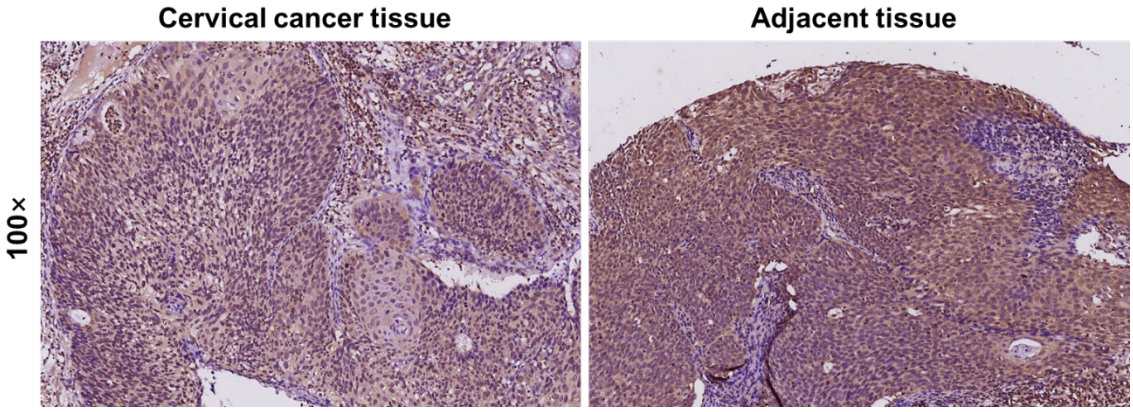
Changes in the text: none.

Parameters	n	Expression of STK10		*P*
		Middle	Low	
Age				
＞55	21	14	7	0.933
≤55	68	46	22	
Pathological classification				
Ⅰ-Ⅱ	9	7	2	0.636
Ⅲ	67	47	20	
T stage				
T1-T2	70	46	24	0.511
T3-T4	19	14	5	
N stage				
N0	71	47	24	0.626
N1	18	13	5	
M stage				
M0	89	60	29	
M1	0	0	0	
Recrudescence				
Yes	29	19	10	0.791
No	60	41	19	

Comment 2: STK10 expression should be verified in cervical cancer issues.

Reply 2: Thank you very much for your advice. IHC staining was used on human cervical cancer tissues and adjacent tissues. Scores of the results were graded by IHC staining, and the product of the staining intensity score and the staining positive rate score was taken as the total score for grouping. The results were as follows. The mean score of STK10 expression in cervical cancer tissues and adjacent tissues was 6.8 and 7.2, respectively. Images of representative IHC are shown below.

Changes in the text: none.



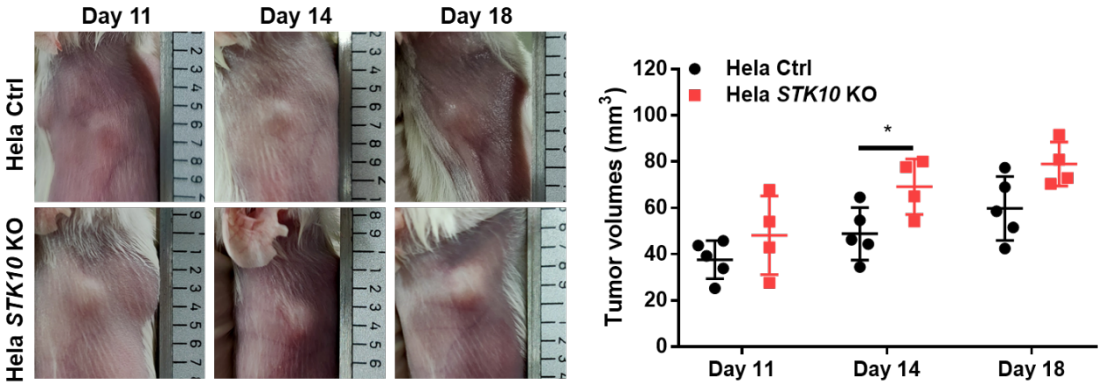
Comment 3: Animal experiments need to be supplemented.

Reply 3: Thanks a lot for your suggestion. For establishing human cervical cancer xenograft model in NPSG mice (NOD-*Prkdc*^{scid}*Il2rg*^{null}, super immunodeficient mice), HeLa cells (1×10^6 cells in 100 μl medium) were subcutaneously injected into 8-week-old female NPSG mice. Tumors were measured using a digital caliper and the tumor volume was calculated by the formula: $(\text{width})^2 \times \text{length}/2$.

Representative images showing the growth of HeLa control and *STK10* KO tumors in NPSG mice. Experiments were performed using 5 mice for each group. The results

showed that the mean tumor volume of *STK10* KO was slightly larger than that of the control group. This experiment is still under way, and it will take another month to complete the experiment. More experiments are needed on the molecular mechanisms affecting tumorigenesis after STK10 knockout, which may be the focus of the next paper. All experimental manipulations were approved by the Animal Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

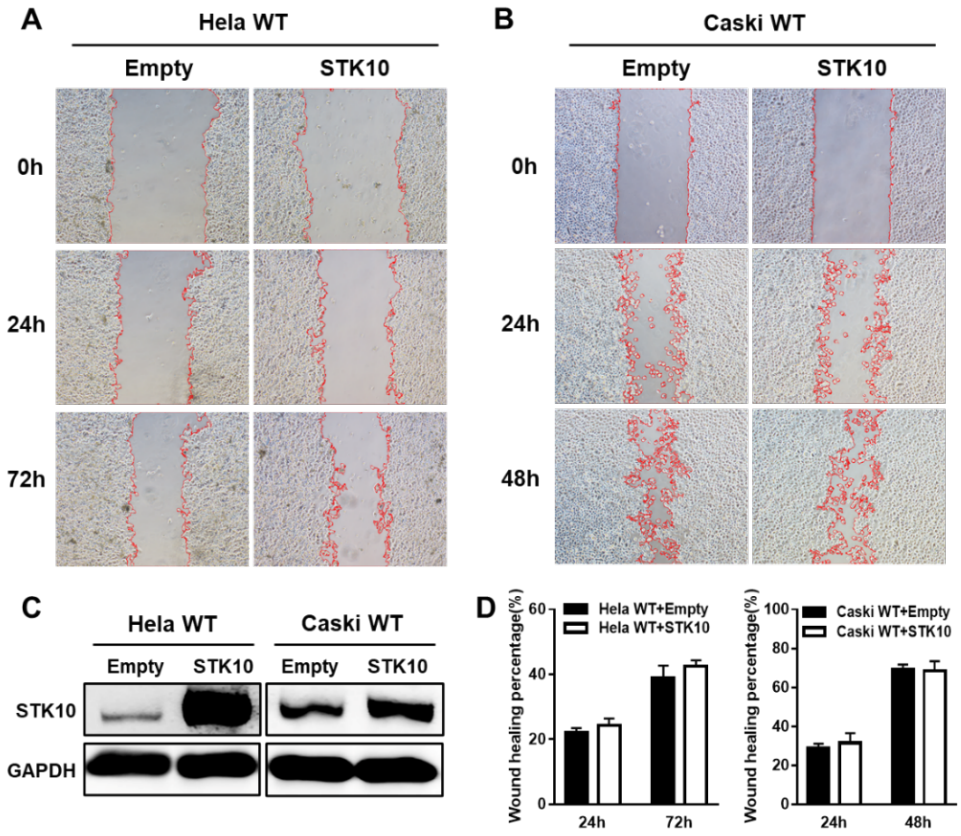
Changes in the text: none.



Comment 4: STK10 overexpression experiments should be made.

Reply 4: Thank you so much for your suggestion. We have added an overexpression experiment as you suggested. Overexpression of STK10 in HeLa and Caski wild-type cells did not affect the ability of migration. We think the main reason is that there is already a high level of STK10 expression in the wild-type cell. When it was transfected with plasmids, the exogenous STK10 is expressed in large quantities and the cell will keep its function stable due to the biological robustness effect.

Changes in the text: none.



Comment 5: Cell scratch test should be made.

Reply 5: Thank you for your advice. We have added a wound healing assay as you suggested. As shown in Figure 4C, D, wound closure of the STK10 depletion cells were accelerated.

Changes in the text:

Methods

Wound healing assay

HeLa and Caski cells were seeded to a 6-well plate with 5×10^5 cells per well. When the cells reached 100% confluence, they were starved with medium containing 1% FBS for 24 h. A 200-μl pipette tip was used to create a wound through the center of the

confluent cell layer, which was then washed twice with medium. Cells were treated with medium containing 1% FBS. Photographs of the wound were taken at 0, 24, 36, and 48 h. Image-J software was used to quantify the wound area. Wound healing percentage (area at 0 h – the area at the relevant time)/ area of 0 h × 100%) was used to represent the cells' migratory capacity. (see page 8, lines 163-171).

Results

Depletion of* STK10 *and migration of cervical cancer cells

To explore the role of STK10 in the tumorigenesis and metastasis of cervical cancer, we assessed cell migration in vitro. The transwell assay showed that the number of cells that migrated out increased in the *STK10* KO group compared with the control group ($P<0.0001$; *Figure 4A, B*). As shown in *Figure 4C, D*, wound closure of the STK10-depleted cells accelerated. Collectively, these results suggested that the depletion of STK10 could promote the migration of cervical cancer cells and indicated that STK10 might play an essential role in the metastasis of cervical cancer.

(see page 12, line 248-255).

Figure legends

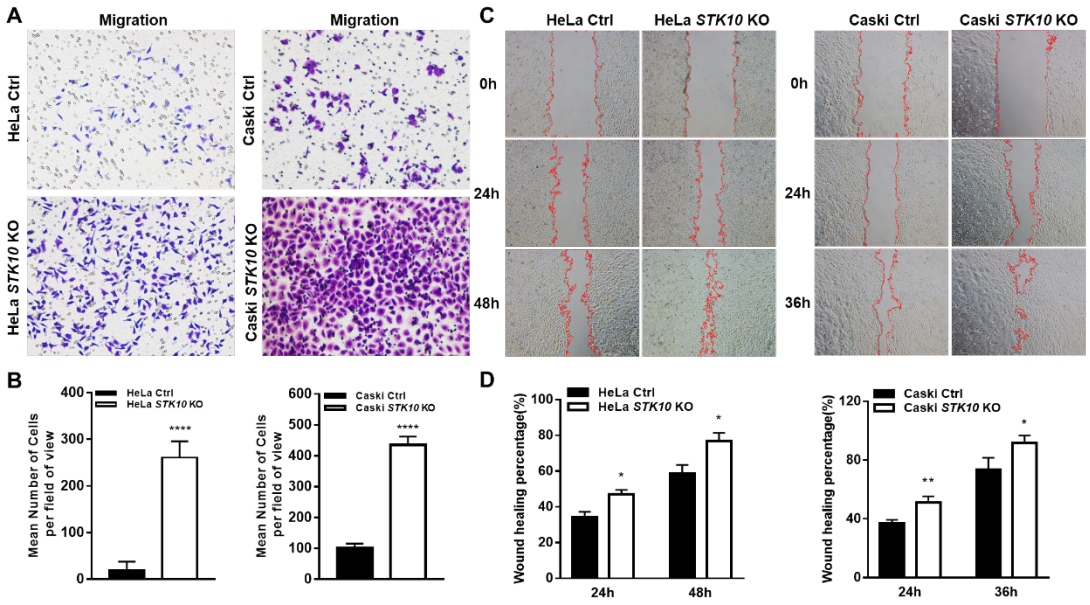


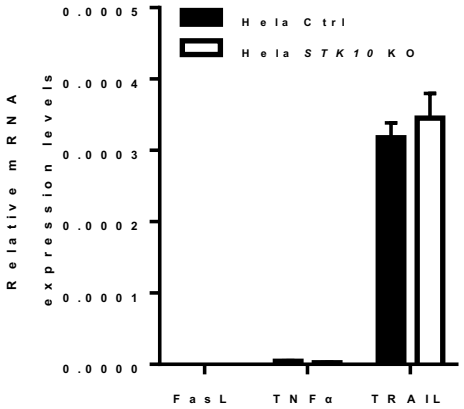
Figure 4. *STK10* depletion promotes the migration of cervical cancer cells *in vitro*. (A, B) Representative figures and data of the transwell assay for indicated cells to detect migration ability (n = 3 in each group). (C, D) Wound healing assay images and data at 0, 24, 36, or 48 h for indicated cells to determine their migratory capacity (n = 3 in each group). All experiments were performed at least in triplicate. (see page 22, lines 476-480).

Comment 6: Does STK10 affect death receptor mediated apoptosis? Additional pro-apoptotic factors (eg. FasL, TNFa, TRAIL) should be added to test the effect of STK10 on death receptor mediated apoptosis.

Reply 6: Thank you very much for your question. The expression levels of apoptosis related genes were detected by qRT-PCR in HeLa cells with GAPDH as normalization control. Levels of FasL and TNFα are so low that they are almost undetectable. There was no significant difference in expression of TRAIL. As mentioned in the paper,

AnnexinV/PI staining assay indicated that STK10 deficiency does not affect the apoptosis of cervical cancer cells.

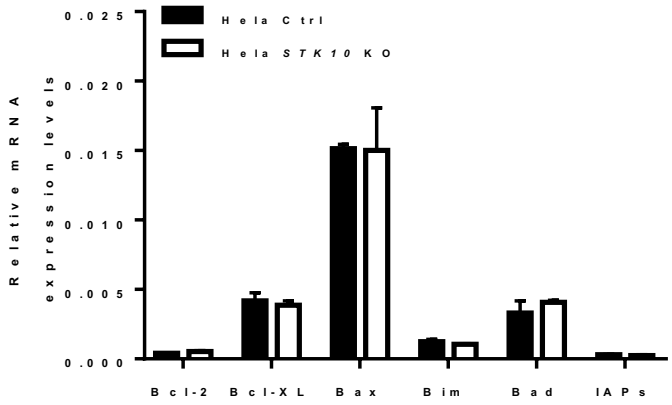
Changes in the text: none.



Comment 7: Does STK10 affect the expression of other apoptosis related mitochondrial proteins, for example: Bcl-2, Bcl-XL, Bax, Bim, Bad, IAPs?

Reply 7: Thanks a lot for this question. The expression levels of apoptosis related genes were detected by qRT-PCR in HeLa cells with GAPDH as normalization control. There was no significant difference in expression of Bcl-2, Bcl-XL, Bax, Bim, Bad and IAPs. As mentioned in the paper, AnnexinV/PI staining assay indicated that STK10 deficiency does not affect the apoptosis of cervical cancer cells.

Changes in the text: none.

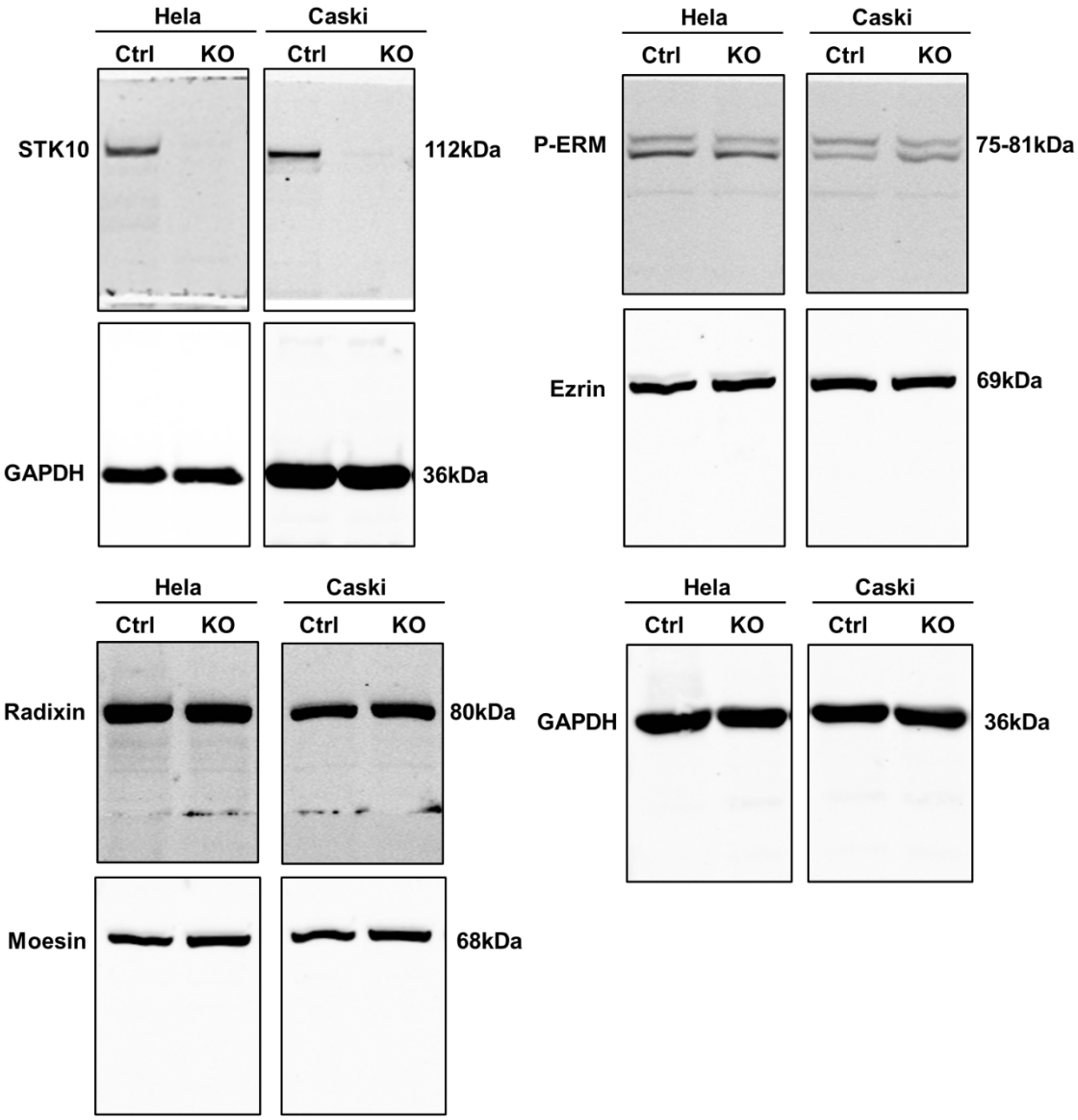


Comment 8: Original WB images were required.

Reply 8: Thanks a lot for this question. The following figure is the original WB images.

If all the original images are needed, we can upload them.

Changes in the text: none.

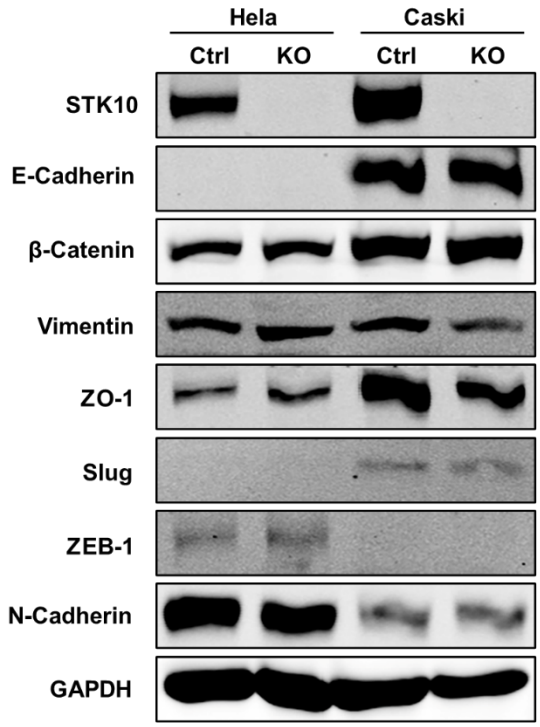


Comment 9: What is the detailed mechanism of STK10 regulating the cell migration of cancer cells. Does STK10 affect EMT signaling?

Reply 9: Thank you so much for you question. As shown in the figure below, Western blot analysis results showed that the major proteins in the EMT pathway showed no significant consistent changes after STK10 knockout, and maybe STK10 did not affect

cell migration through the EMT pathway. More experiments are needed to explore the mechanism.

Changes in the text: none.



Comment 10: Kaplan–Meier survival analysis of cervical cancer patients with high or low STK10 should be supplied.

Reply 10: Thank you very much for your advice. As shown in the figure below, Kaplan–Meier survival curve was used to analyze the relationship between the expression level of STK10 in human cervical cancer and recurrence. Low STK10 expression was more likely to recur but there was not statistically difference between them ($P > 0.05$).

Changes in the text: none.