Network pharmacology and experimental investigation of *Rhizoma polygonati* extract targeted kinase with herbzyme activity for potent drug delivery

Yingqiu Xie\textsuperscript{a,}\textsuperscript{*}, Chengling Mu\textsuperscript{b,}\textsuperscript{*,} Bexultan Kazybay\textsuperscript{a,}\textsuperscript{*}, Qinglei Sun\textsuperscript{c}, Aidana Kutzhanaova\textsuperscript{d}, Guldan Nazarbek\textsuperscript{a}, Na Xu\textsuperscript{a}, Lazzat Nurtaya\textsuperscript{a}, Qian Wang\textsuperscript{a}, Amr Amin\textsuperscript{e,f} and Xugang Li\textsuperscript{b}

\textsuperscript{a}Department of Biology, School of Sciences and Humanities, Nazarbayev University, Nur-Sultan, Kazakhstan; \textsuperscript{b}Sino-German Joint Research Center on Agricultural Biology, State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai’an, Shandong Province, China; \textsuperscript{c}Key Laboratory for Applied Technology of Sophisticated Analytical Instrument of Shandong Province, Shandong Analysis and Test Center, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China; \textsuperscript{d}Shandong Taishanghuangjing Biotechnology Co. Ltd., Tai’an, China; \textsuperscript{e}Biology Department, UAE University, Al Ain, United Arab Emirates; \textsuperscript{f}The College, The University of Chicago, Chicago, IL, USA

**ABSTRACT**

*Rhizoma polygonati* (Huangjing, RP) has been used for a long history with many chemical components in inducing anti-cancer, anti-aging, anti-diabetes, anti-fatigue, and more prevention of diseases or acts as nutrition sources in food. Here we investigated RP extract combination with kinase inhibitors in anti-cell growth and blockade in pathways targeting kinases. Experimental investigation and network pharmacology analysis were applied to test the potent kinase-mediated signaling. Herbzyme activity was determined by substrate with optical density measurement. Extract of processed RP inhibits cell growth in a much greater manner than alone when applied in combination with inhibitors of mTOR or EGFR. Moreover, processing methods of RP from Mount Tai (RP-Mount Tai) play essential roles in herbzyme activity of phosphatase suggesting the interface is also essential, in addition to the chemical component. The network pharmacology analysis showed the chemical component and target networks involving AKT and mTOR, which is consistent with experimental validation. Finally, EGFR inhibitor could be associated with nano-extract of RP-Mount Tai but not significantly affects the phosphatase herbzyme activity in vitro. Thus the processed extract of RP-Mount Tai may play a dual role in the inhibition of cell proliferation signaling by both chemical component and nanoscale herbzyme of phosphatase activity to inhibit kinases including mTOR/AKT in potent drug delivery of kinase inhibitors.

**Introduction**

*Rhizoma polygonati* (Huangjing, RP) is a ‘medicine food homology’ (MFH) known to be used in Traditional Chinese Medicine (TCM) for thousands of years with many natural products to maintain proper blood sugar levels, and exhibit many more pharmacological functions (Zhao et al., 2018, 2021). Recently it has been shown that RP can also be used for the prevention of COVID-19 (Mu et al., 2020, 2021). RP components include polysaccharides, alkaloids, lectins, saponins (Zhao et al., 2018). Traditionally, this herb was used to make medicine against obesity, fatigue, high temperature, and common cold (Zhao et al., 2018). Modern pharmacology also provides evidence of the efficacy of RP’s chemical components against diabetes, aging, Alzheimer’s disease, cardiovascular diseases, and viral infection (Zhao et al., 2021).

Recent research outcomes illustrate that RP’s ingredients could also be used against kinases including p38MAPK (Han et al., 2020), glycogen synthase kinase 3β (GSK-3β), extracellular signal-regulated kinase (ERK) (Peng et al., 2018). Consistently, we reported the potent herbzyme phosphatase-like herbzyme activity inhibiting cell growth, simulating the kinase targeting by RP herbzyme function (Benassi et al., 2021). Thus RP herbzyme was hypothesized to be linked to the crosstalk between kinase and phosphatase signaling pathways in cell growth.

It is well-documented that kinases play essential roles in the regulation of metabolic processes of cells and are further involved in critical differentiation, proliferation, apoptosis, and cell development (Fabbro et al., 2015). Studies also show that phosphatases are involved in these phosphorylation–dephosphorylation processes along with kinases. It has been recognized that phosphatase might be essential in targeting cancer cell growth by disrupting kinase signaling pathways (Heinrich et al., 2018). Therefore, we hypothesize that nanzymatic phosphatase activity of RP might inhibit or disrupt the kinase signaling pathways, hence blocking cellular
proliferation including cancer cells. It is additionally expected that RP ingredients could have a larger effect in combination with precision kinase inhibitor drugs against cancer cell growth. We further examined whether RP ingredients could have the potential to be used to inhibit cell growth in combination with kinase inhibitors which might be applied in treatments against other types of diseases in the future as well because many diseases are induced or related to kinase signaling for their progression.

Network pharmacology provided computational and systems tools to examine and explain the mechanisms underlying the effect of drugs by constructing compound-based target protein-protein interactions following the molecular modeling technology with calculations of stoichiometry to simulate compound ligand-target binding (Jiao et al., 2021). Here, both mentioned methods were used to identify and understand how RP extract can target cell growth by inhibiting kinases in potent drug delivery of kinase inhibitors.

Materials and methods

Collection of kinase-related gene sets

The targets of genes of kinases were retrieved from the GeneCards (https://www.geneCards.org) database, NCBI (https://www.ncbi.nlm.nih.gov) gene database, and GenCLiP3 (http://ci.smu.edu.cn/genclip3/GeneAssociation.php) database using ‘kinase’ as the keyword while searching and selecting the species as ‘Homo sapiens’ to obtain kinase-related human genes. Venny 2.1.0 (https://bioinfgp.cnb.csic.es/tools/venny/) software (Sun et al., 2019) was used to identify the targets of RP compounds against the kinase-related genes.

Construction of protein-protein interaction (PPI)

The potential targets were used to construct the target PPI network through the STRING database (Szklarczyk et al., 2019) (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string-db.org/) where the ‘Homo sapiens’ keyword was applied before running the Multiple Proteins service of the STRING. Further, Cytoscape 3.7.2 (Shannon et al., 2003) software (http://www.cytoscape.org/) was used to construct a protein interaction network and identify the most important RP’s protein targets for molecular docking analysis.

Network pharmacology analysis of target GO enrichment analysis and KEGG pathway enrichment analysis

To further understand the functions of core target genes and the main action pathways of the active substances of RP, the disease-related targets obtained from the above screening were entered into the DAVID database (Huang et al., 2009) (https://david.ncifcrf.gov/home.jsp). The list of genes of RP targets against kinases was uploaded to the DAVID searching tool. Here, the species section was selected as ‘homo sapiens’, and all target gene names were corrected to their official names (official gene symbol), and the threshold of $p < 0.05$ was set for GO enrichment before running the tool.

For KEGG enrichment pathway analysis the core target genes were imported into the KOBAS3.0 database (http://kobas.cbi.pku.edu.cn). Using $p < 0.05$ and sorting from small to large as the screening conditions, the enrichment analysis pathways were selected which then were visualized through online tool (http://www.bioinformatics.com.cn).

Virtual screening of the active ingredients of RP and online tools

The active ingredients of RP were obtained by searching the Traditional Chinese Medicine System Pharmacology database (TCMSP) (http://tcmspw.com/index.php) and the BenCaoZuJian database (http://herb.ac.cn/) (Fang et al., 2021). To confirm there is of RP ingredients the TCMS results were checked by the Swiss Target Prediction (http://www.swisstargetprediction.ch/) database. Additionally, the bioinformatics online mapping website (http://www.bioinformatics.com.cn) was used to visualize the analysis results.

Molecular modeling of RP compounds binding AKT1 and mTOR

The 3D structure of the effective active RP compounds was retrieved through the PubChem website (https://pubchem.ncbi.nlm.nih.gov/) in the SDF file format which was then converted into a PDB file using the Open Babel 2.3.2 software. The receptor protein was retrieved from the Protein Data Bank database (http://www.rcsb.org/pdb). For AKT1 docking, the PDB structure of 4EKL was used which is bound with GDC0068 by amino acids of Phe 22, Lys 40, Ala91, Glu95, Asp 153 (https://www.rcsb.org/sequence/4EKL). GDC-0068 is a highly selective pan-AKT inhibitor. The PYMOL software was further used to perform operations, such as water removal and ligand removal on the receptor protein, while
the Autodock 4.2.6, and AutoDock Vina 1.1.2 (Trott & Olson, 2010) were used to set the parameters of the receptor protein docking site including active pocket sites for molecular docking of receptor proteins and small ligand molecules after the molecules were converted into pdbqt format. For mTOR structure, PDB 4JSV was used as a reference structure.

**Herbzyme phosphatase assay**

The herb enzyme of phosphatase assay was tested with substrate NBT/BCIP (Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) and absorbance measurement (Benassi et al., 2021). The Calf-intestinal alkaline phosphatase (CIP, Life Technologies) was used as a positive control and vehicle water as a negative control for RP.

**Transmission electron microscopy (TEM) and scanning electron (SEM) microscopy**

TEM test was performed by the University core facility with the equipment of Transmission electron microscope JEOL JE-1400 plus. Samples were applied on a carbon grid (diameter: 3 mm) and dehydrated for at least 24 h. SEM investigation was operated by loading RP extract on papers of foil and air-

![Figure 1](image_url)

**Figure 1.** RP extract exhibits phosphatase activity by processing at high temperatures related to the nanoparticle effect. (A) TEM of processed RP extract at the nanoscale. (B,C) RP extract exhibits phosphatase activity by processing at high temperature measured by substrate NBT/BCIP (B) and correlates with quantum characteristics with fluorescence (C). *p<0.05; **p<0.01; ***p<0.001.
Results and discussion

Different processing generated RP Mount-Tai extract with nanoscale herbzymatic phosphatase activity

An ancient way of RP processing includes many times steam and sun-drying, which is unique to RP, compared to most herbs processing like boiling, or fresh use. We applied for a RP from a mountain area of Mount-Tai, China for further studies. We first reproducibly found the characteristics of ancient ways of processing generated nanoparticles in RP extract. We found the density plays an essential role in nanoparticle accumulation and high density may produce cluster simulating polymers of the nanostructures investigated by SEM (Benassi et al., 2021) and TEM (Figure 1(A)). Next, we found the nanoparticles of RP extract by commercial processing showed the highest activity of phosphatase compared to any other processed particles. RP baked at a high temperature of 230 °C can also generate enzyme activity, named by us, herbzyme, and a natural type of nanozyme (Figure 1(B)). The fluorescence investigation showed the high temperature of 230 °C processed RP extract has high fluorescence with high phosphatase activity (Figure 1(C)). The correlation of fluorescence with phosphatase activity in anciently processed RP and 230 °C processed RP suggesting the characteristics of the nanoparticles correlate with the nanozyme/herbzyme activity of phosphatase. Compared to the metal-based nanozyme, this natural processed herbzaomse seems cost-effective and easily absorbs with potential in drug delivery (Meng et al., 2021).

Given phosphatase plays a role in removing phosphorylation of target against kinases, we tested the relation between RP extract phosphatase function and kinase inhibitors in combination to inhibit cell growth in HT-1080 cells. We found that MET, PKC, Pim-1/2 kinases inhibitors did not show the synergistic association in inhibition of cell growth, while mTOR and EGFR kinases inhibitors showed the effect (Figure 2). Thus the RP extract mediated phosphatase activity may crosstalk with mTOR, EGFR related pathways for kinase inhibition. The downstream related targets include YAP, and a potent inhibitor of β-catenin, named as iCRT3, with MET kinase inhibitor/RP combination showed the significantly decreased cell growth compared to other target inhibition results, such as YAP (Figure 2).

Network pharmacology analysis of RP induced cell signaling against kinases including mTOR/AKT pathway

Next, we explored the whole RP chemical compound-targets correlations against kinases by network analysis. With the Venn diagram tools, we searched the intersection targets of kinase signaling and RP target pathways and we found a total of 163 (2.1%) targets (Figure 3(A)). Then we constructed and analyzed the protein-protein interaction networks (Figure 3(B)) of these intersection targets via the STRING platform and Cytoscape 3.7.2 software, respectively. The PPI network contains a total of 162 nodes and 1985 edges. The nodes in the network graph represent proteins. The degree value represents the number of lines connected to the same node, which is used to evaluate the importance of each node in the network. The larger the node and the darker the color, the greater the degree value. Each edge represents the interaction relationship between proteins, and the more lines, the greater the degree of association. It can be seen from Figure 3(B) that the degree values of INS, AKT1, TP53, PTGS2, CAT, VEGFA, JUN, SRC, PPARG, MMP9, HSP90AA1, STAT3, ESP1, CASP3, MTOR, and FOS are significantly higher than those of other targets, implying that they play an important role in the PPI network. Among them, AKT1, INS, and MTOR have the largest nodes and the darkest colors, which may be the most important potential targets for their effects. Because the AKT1 and MTOR are major players of PI3K-AKT signaling pathway that have many functions including cell cycle progression, cell survival, and apoptosis, and also were regulated by RP compound polysaccharides in anti-neurotoxicity (Afify et al., 2021; Huang et al., 2021).

The targets corresponding to the RP compounds obtained from the TCMSP website were uploaded into the DAVID database, and 312 GO entries were screened out according to \( p \leq 0.05 \). Among them, there are 228 biological processes (BP), accounting for 73%; 30 cellular components (CC), accounting for 9%; and 54 molecular functions (MF), accounting for 17%. The important biological processes were recorded as a response to an antibiotic, protein kinase B signaling, fibroblast proliferation, response to a toxic substance, apoptotic process, response to hypoxia, response to estradiol, response to lipopolysaccharide, nitric oxide biosynthetic process, cellular response to hypoxia, intracellular receptor signaling pathway, aging, oxidation-reduction process, cell proliferation, and steroid hormone-mediated signaling pathway (Figure 4). And the potential targets of RP compounds...
showed enrichment for MFs, such as chromatin binding, protein heterodimerization activity, cyclin binding, oxidoreductase activity, NADP binding, protein binding, protein kinase binding, identiccal protein binding, protein homodimerization activity, transportation factor binding, steroid binding, drug binding, heme binding, enzyme binding, steroid hormone receptor activity (Figure 4). From here, it can be claimed that the mechanism of the RP compounds involved in the kinase signaling may be related to the activity of steroid hormone receptor activity, oxidoreductase activity, cyclin binding activity, and other protein activities. At the same time, involved cellular components include a vast array of cellular structures starting from the extracellular space, membrane, and cytosol to the nucleus, chromatinns, mitochondria, and endoplasmic reticulum drawing the path of kinase signaling cascade from outside of the cell to the DNA (Figure 4). Such results might
imply that the RP compounds affect the kinase signaling at many cascade points illustrating their great potential to disturb and target the kinases involved, especially, the cellular growth and proliferation.

Further, the key common targets of the RP compounds and different KEGG diseases and the RP ingredients were input into the KOBAS 3.0 database, and a total of 133 diseases were identified. We used $p \leq 0.05$ to screen 57 diseases and selected the top nine pathways for display (Figure 5) including head and neck cancers, somatic breast cancer, cancers of the breast and female genital organs, skeletal diseases, cancers of male genital organs, colorectal cancer, cancers of endocrine organs, cervical cancer, and breast cancer. These records illustrate the RP compounds mainly target cancer compared to other diseases, which might be due to the kinases that are important for cancer cell growth. Moreover, the constructed disease-compound-target relationship diagram below also shows that the main ingredients are diosgenin, methylprotodioscin, baicalein, beta-sitosterol, DFV, liquiritigenin, and 3'-Methoxydaidzein. While diosgenin could target all nine KEGG diseases and baicalein 7 of them, others seem to have the potential to target more specific diseases. These top seven RP ingredients were further investigated via molecular docking.
For further analysis, the GEPIA website was used. The gene list of potential targets of RP compounds was uploaded into this service which generated the gene expression map of nine genes (CASP8, BCL2L1, FOS, PPARG, CAT, MAPK14, PTGS2, TP53, and CASP3) against 31 sample genes (Figure 6). There, while FOS, CAT, and MAPK14 were downregulated in the tumor, the BCL2L1 and TP53 were upregulated. The proteins of the latter two genes are involved in the PI3K-AKT-mTOR pathway. And because BCL2L1 and TP53 are responsible for cellular apoptosis, their upregulation in tumors means that the RP compounds can cause cancer cell death and consequently inhibit their proliferation.

Figure 5. KEGG enrichment pathway analysis of the potential targets of RP compounds against kinases involved in cancer signaling. The diagram shows the KEGG diseases and the RP compounds corresponding to the intersection targets establishing the relationship between the compound and the various cancer types.

Figure 6. Gene expression of RP target genes in different cancer types. The potential targets of RP compounds were uploaded to the GEPIA website to obtain gene expression under normal (control group) conditions and cancer (Tumor) conditions. The gene expression level is indicated on the right side with blue being low expression (minimal) and red being high expression (maximum).
Molecular docking of the screened components of *Rhizoma polygonati* with AKT1 and mTOR

Molecular docking technology would allow to find the best binding pattern between molecules through spatial matching and energy recognition. Usually, the spatial matching of ligand and receptor binding is the basis for its function, and energy recognition is the key to maintain relatively stable binding. In general, the Vina system docking score value of less than certain energy (calculated by kcal/mol) indicates the binding between the docking molecule and the target (http://autodock.scripps.edu/qa-help/manual/autodock-4-2-user-guide/AutoDock4.2_UserGuide.pdf) (Morris et al., 2009; Quiroga & Villarreal, 2016).

For the molecular docking, we used the AKT1 and mTOR protein targets which were identified to have a critical role as demonstrated by the network pharmacology analyses and seven RP ingredients identified to target main KEGG pathways. The results demonstrated that both AKT1 and mTOR have a strong binding affinity with RP ingredient molecules which were chosen for the molecular docking from the KEGG analysis as indicated by their docking scores, except for mannose, though it also showed relatively good binding affinity.

### Table 1. Molecular docking scores of AKT1 and mTOR with RP compounds*

| Molecule name | Docking score (kcal/mol) | | | |
|---------------|-------------------------|---|---|---|
|               | AKT1                    | mTOR | | |
| Diosgenin     | −8.8                    | −9.8 | | |
| Sitosterol    | −7.6                    | −8.5 | | |
| Baicalein     | −8.1                    | −8.6 | | |
| DFV           | −8.5                    | −8.2 | | |
| Mannose       | −5.3                    | −5.5 | | |
| Positive control | −8.7** LY294002 | −10.4*** Rapamycin | | |

*The docking scores of AKT1 and mTOR against six molecules are provided in kcal/mol. Controls: **LY294002; ***Rapamycin.

![Figure 7. Molecular docking of diosgenin with the targets of RP compounds, AKT1, and mTOR. The binding mode between the small diosgenin ligand molecule and the receptor protein AKT1 (A) and the receptor protein mTOR (B). In both pictures, the 3D docking model (left) is enlarged in the binding pocket site (right side within the dashed box). The AKT1 and mTOR are depicted in gray with their amino acids which are interacting in purple.](image)

Y. XIE ET AL.

2194
(about $-5$ kcal/mol). Also, similar to AKT inhibitor LY294002, the RP's compound diosgenin has the strongest binding affinities with AKT1 ($-8.7$, $-8.8$ kcal/mol, respectively). In addition, similar to mTOR inhibitor Rapamycin, diosgenin also showed strong binding to mTOR ($-10.4$, $-9.8$ kcal/mol, respectively). See Table 1 for details.

The molecular docking performed with diosgenin with AKT1 and mTOR target proteins displays that the binding pockets of both AKT1 and mTOR receptor proteins have many amino acid residues that form various bonds with diosgenin (Figure 7). Although the size of AKT1 is much smaller than mTOR, they have a similar number of residues that interact with diosgenin. AKT1 has Phe-161, Leu-181, Lys-179, Glu-191, His-194, Thr-195, Asp-274, Lys-276, Asp-292, Gly-311, and Thr-319, while mTOR has been recorded with Gly-1897, Asn-1898, Leu-1900, Glu-1937, Pro-1940, Leu-1900, Thr-2207, Arg-2224, and Leu-2207. It can be observed that there are hydrogen bonds near Lys-276 of AKT1 (Figure 7(A)) and Asn-1898 in mTOR (Figure 7(B)), however, it is not clear which residues are involved. Thus, the binding pockets of both proteins were further studied.

As shown in Figure 8(A) the results of the additional analysis show that AKT1 compound forms multiple hydrogen bonds with the amino acids at the active site of the protein (LYS-276, LYS-179, THR-195, ASP-292), which can effectively bind small diosgenin molecule, allowing the compound to form a stable complex with it. In addition, the compound has high hydrophobicity and has strong hydrophobic interaction with surrounding hydrophobic amino acids (Thr-160, Phe-161, Leu-181, Lys-179, His-194, Thr-195, Glu-198, Phe-225, Gly-294, Leu-295, Asp-274, Lys-276, Asp-292, Gly-311, and Thr-319), which indicates that the compound matches the protein's active pocket in a high degree and that their bonding is stable (Figure 8(A)). Additionally, the mTOR compound matches well with the diosgenin molecule as well. The binding sites include 14

Figure 8. The interaction of the diosgenin (ligand) with targets of RP compounds, AKT1 (A) and mTOR (B), within their binding pockets.
a complex structure (Yang et al., 2013). Both above reported 2012). For the mTOR structure, PDB 4JSV was used as a refer-

Asp-53 (https://www.rcsb.org/sequence/4EKL) (Lin et al.,

which is bound with a highly selective pan-Akt inhibitor,
mTOR pathway.

cancer that uses kinase signaling, specifically, the PI3K-AKT-
drug that could be used against various diseases including
tosis processes. This means that diosgenin is a good candi-
date for further experimental validation to design a potent
drug that could be used against various diseases including

Overall, the obtained results of the paper indicate that
the RP compounds can be effectively used to target the kin-
ase signaling pathways regulating cell growth. The thorough
examination of the RP compounds using network pharmacol-
ogy and molecular docking demonstrated that one of its
ingredients—diosgenin can fit well into the binding pocket of
important target proteins, AKT1 and mTOR, which play a
critical role in the cellular proliferation, survival, and apop-
tosis processes. This means that diosgenin is a good can-
didate for further experimental validation to design a potent
drug with kinase signaling, specifically, the PI3K-AKT-
mTOR pathway.

For AKT1 docking, the PDB structure of 4EKL was used
which is bound with a highly selective pan-Akt inhibitor,

For the mTOR structure, PDB 4JSV was used as a refer-
ence structure, which has been shown binding the ligand in
a complex structure (Yang et al., 2013). Both above reported
binding patterns of ligand binding to targets (Lin et al., 2012;
Yang et al., 2013) are different from our modeling of
diosgenin (ligand) binding to the targets of RP compounds,
AKT1, and mTOR, within their binding pockets. However, the
binding affinity showed a comparable in the contrast to
LY294002 and Rapamycin. Thus the potent diosgenin bind-
ing to mTOR or AKT needs to be further investigated by
dynamics both in vitro and in vivo.

The results suggest that diosgenin can interfere with kin-
ase pathways which reregulate cell proliferation. This claim is
confirmed by other reports (Shishodia & Aggarwal, 2006;
Chiang et al., 2007; Srinivasan et al., 2009). Diosgenin is a
natural product of steroidal sapogenin found in plants that
inhibits cellular proliferation and induces tumor cell apop-
tosis through regulation of mTOR, AKT, JNK kinases signaling
but direct binding and dephosphorylation are unknown (Shishodia & Aggarwal, 2006). It has been shown in breast
cancer cells that diosgenin inhibits AKT activity which is an
evidence of diosgenin inhibiting kinase especially AKT or
mTOR (Chiang et al., 2007; Srinivasan et al., 2009). Thus our
data is consistent with publications and their original data

RP extract may against kinases through multiple pathways
but one of the main signaling is AKT/mTOR. Network
pharmacology and docking showed the consistent mTOR
pathways targeted by RP. Thus, RP extract might be poten-
tially used as a drug delivery carrier presented by a complex
of the natural compound with kinase inhibitors to target kin-
ases with intrinsic phosphatase herbzyme activity. Different
from other types of drug delivery systems with potent com-
bined kinase inhibitors, the RP extract compound may also
exert a herbzyme of phosphatase function to target kinases.
Thus the dual role of RP nano-extract shows the greatest
potential of drug delivery by natural products complex.

Conclusion

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Note
1. Heatmap was plotted by http://www.bioinformatics.com.cn, an online platform for data analysis and visualization.

Author contributions
Yingqiu Xie developed the plan, designed the whole project, performed and supervised some experiments, analyzed data, and wrote the paper. Qian Wang processed the RP. Qinglei Sun and Na Xu performed SEM and wrote a part of the paper. Cuiping Ma and Xingang Li performed bioinformatics analysis with some figures and wrote part of methods, figure legends, and results. Aidana Kutzhanova, Guldan Nazarbek, and Lazzat Nurty performed some experiment, and data analysis.

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