



Inactivation of Wnt-LRP5 signaling suppresses the proliferation and migration of ovarian cancer cells

Jing Hong, Zeyu Xie, Zhihua Yang, Fangyao Yang, Hai Liao, Shuquan Rao, Xinhe Huang[^]

School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, China

Contributions: (I) Conception and design: S Rao, H Liao; (II) Administrative support: X Huang; (III) Provision of study materials or patients: J Hong, Z Xie, Z Yang, F Yang; (IV) Collection and assembly of data: J Hong, Z Xie, Z Yang; (V) Data analysis and interpretation: J Hong, Z Xie; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Xinhe Huang, School of Life Science and Engineering, Southwest Jiaotong University, No. 111, North 1st section, 2nd Ring Road, Chengdu 610031, China. Email: xinhehuang@swjtu.edu.cn.

Background: Ovarian cancer (OCa) is the most lethal gynecological malignant tumor, with few or no specific symptoms in its early stage. There are many signaling pathways involved in the process of OCa progression, among which the highly complex Wnt signaling pathway plays a unique role in the occurrence and development of OCa because of its functions of regulating gene expression, cell proliferation, migration, and invasion. Lipoprotein associated receptor protein 5/6 (LRP5/6) binds to activate this key pathway. Therefore, it is very important to study the mechanism of Wnt-LRP5 signaling pathway in the proliferation and migration of OCa.

Methods: In the present study, we have investigated the role of Wnt-LRP5 signaling pathway in OCa proliferation and migration for the first time using the dominant negative plasmid of LRP5 (DN-LRP5) and human OCa cells HO8910PM plus in a mouse model.

Results: Our data showed inactivation of LRP5 resulted in shift of epithelial-mesenchymal transition (EMT), rearrangement of the cytoskeleton, lowered activity of pro-proliferation and pro-migration cancer signaling pathways including Akt, p38 and NF- κ B, eventually decreased proliferation and migration of OCa cells HO8910PM *in vitro*. Moreover, *in vivo* OCa-DN-LRP5 mouse model developed significantly smaller tumors as determined by inoculation of HO8910PM-DN-LRP5 cells into nude mice.

Conclusions: Collectively, our results demonstrate the dominant role of Wnt-LRP5 in OCa proliferation and migration and its potential as a valuable therapeutic target.

Keywords: Wnt; lipoprotein associated receptor protein 5 (LRP5); ovarian cancer (OCa); proliferation; migration

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Introduction

Ovarian cancer (OCa) is one of the most common female malignant tumors with high mortality. It is the second most common female malignant tumor in terms of mortality (1). In the past 30 years, the 5-year survival rate of OCa patients is less than 30%. A major contributor to the high mortality rate is the fact that 70% of women with OCa initially

manifests with little or no specific symptoms at the early stage. Therefore, patients present with metastases and invasion throughout the peritoneal cavity (2). Due to lack of effective early detection methods, OCa is often diagnosed as advanced disease clinically. While the mortality of OCa remains highest, the etiology of OCa is still elusive.

Conserved Wnt signaling is generally active during

[^] ORCID: 0000-0003-4190-0737.

human development and growth activities, and plays an important role in physiological activities including embryonic development, organ growth and maintenance of various functions (3-6). Mutations in Wnt genes or components of the Wnt signaling pathway can cause various human diseases, such as developmental defects, and their abnormal functions can also cause cancer (7). In addition, there is growing evidence to show the cross-talk between Wnt signaling pathway and other signaling pathways including Notch (8) and Sonic Hedgehog (9) signaling pathways, which has implications for Wnt signaling pathway in therapeutic interventions of multiple cancers. As currently understood, Wnt proteins bind to receptors of the Frizzled proteins and lipoprotein receptor-related proteins 5/6 (LRP5/6) on the cell surface to further exert its physiological function (10). LRP5 is one member of low density lipoprotein receptor (LDLR) family and is found to be involved in fat metabolism mostly (11). As a co-receptor of the Wnt signaling pathway, LRP5 participates in the development of embryo (12), and especially the development of bone (13) and eye (14). Several lines of evidence show the important physiological function of LRP5, for instance, LRP5 triggers osteoporosis-pseudoglioma syndrome (OPPG), LRP5 is positively correlated with the activity of osteosarcoma cells (15), and mutations in LRP5 directly affect bone acquisition and eye development (16), cause insulin-dependent diabetes (17) and non-syndromic hearing loss (18).

Recently, LRP5 was found to be involved in multiple tumors progression though the exact mechanism is unknown. Rabbani *et al.* showed LRP5 regulated the process of epithelial-mesenchymal transition (EMT) and mediated the proliferation and migration of prostate cancer cells (19). Furthermore, LRP5 has proven to affect or directly interact with new tumor-associated factors such as STK40 (20), JAM3 (21), TG2 (22), Hsp90ab1 (23), in different cancer cells to regulate tumorigenesis and migration. In addition, a variety of natural products and synthetic compounds also exert their anticancer activity by acting on Wnt/LRP5 signaling (24,25), all of which suggests LRP5 could be a new anti-cancer target.

In our research, we have immediately studied the role of Wnt-LRP5 signaling pathway in OCa progression by transfection of the dominant negative plasmid of LRP5 (DN-LRP5) into highly metastatic human OCa cells HO8910PM. The effect of transfection of DN-LRP5 plasmid and abrogation of Wnt-LRP5 pathway was examined on OCa cells characteristics *in vitro* and on mouse tumor growth *in vivo*. We present the following article in

accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-3462>).

Methods

Cell culture

Human liver cell line (L02, Chang Liver), human liver cancer cell lines (HepG2, HepG2.2.1.5, Hep3B, 7721, PLC, Huh7, 97-H, LM3, FOCUS, SNU398, sk-hep-1), human colorectal cancer cell lines (LOVO, SW480, HCT116, SW620), human OCa cells (A2780, HO8910, SK-OV-3, HO8910PM), human lung cancer cell lines (H460, H446), human embryonic kidney cell line (HEK293T) were from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). HO8910PM was infected with DN-LRP5 lentiviral and selected with puromycin to obtain the stable clone of HO8910PM/DN-LRP5, then maintained in DMEM with 10% FBS and puromycin for future experiments.

MTT assay

The logarithmic growth phase HO8910PM and HO8910PM/DN-LRP5 cells were plated to 96-well culture plates with a density of 10^4 cells/mL in medium containing 10% FBS. MTT was added to the cell culture at specific time points with a final concentration of 1 mg/mL and incubated at 37 °C for another 3 h. The optical absorbance at 570 nm was measured to determine the change of cell density (26).

Western blotting analysis

Protein lysates (20–80 µg) were obtained by lysing HO8910PM and HO8910PM/DN-LRP5 cells, and then boiled at 100 °C for 5–10 minutes and resolved by 10% SDS-PAGE. Proteins were transferred to PVDF membranes, and sealed with 5% skim milk, the membranes were treated with specific primary antibodies. Then horseradish peroxidase-conjugated secondary antibodies were used to treat the blots for 1h. The blots were visualized through enhanced chemiluminescence.

Wound-healing analysis

HO8910PM and HO8910PM/DN-LRP5 cells were plated

to a 6-well culture plates with a density of 10^6 cells/mL. After adherence, draw a vertical parallel wound on the cell layer using a p10 tip, then the medium was removed and cells were rinsed 3 times with sterile PBS to remove shed cells and cell debris. Proliferation of the cells in DMEM medium containing 2% FBS were measured to assay the rate of wound healing (27).

Transwell migration assay

2×10^4 cells were mixed and added to the inside of the transwell, and the transwell was placed in a 24-well plate containing 500 μ L of complete medium, and cells were cultured for another 24 hours. Then the transwell was fixed with methanol for 25 min, stained with crystal violet for 45 min and washed with PBS three times, and the cell layer was gently scraped off with a medical cotton swab. Add 200 μ L of sterile water or PBS to the inside of the transwell after cleaning. Photograph the transwell with an inverted microscope and count the number of cells (28).

Actin staining

HO8910PM and HO8910PM/DN-LRP5 cells were grown on coverslips for 24 hours, then fixed with Ready-made 4% paraformaldehyde at room temperature for 10 minutes, diluted Triton X-100 with PBS to a final concentration of 0.1%, and then penetrated the cells 20 minutes, then fix with 5% bovine serum albumin (BSA) for 1 hour, and stain with FITC-Phalloidin for 2 hours at room temperature and dark conditions. After washing twice with PBS, it was counter-stained with DAPI for 10 min. Finally, the distribution of F-actin (29) was observed by laser confocal microscope.

Animal protocols

Male BALB/C nude mice were grown to 6 weeks of age for tumor formation experiments. The experiment was divided into two groups. The experiment was performed under a project license [No. SYXK(Chuan)2014-189] granted by institutional ethics board of Southwest Jiaotong University, in compliance with Sichuan guidelines for the care and use of animals. The prepared cell suspensions HO8910PM and HO8910PM/DN-LRP5 (5×10^7 cells/mL) were aspirated under sterile conditions, and 100 μ L of cell suspension was injected into the right neck of each nude mouse

(5×10^6 cells/mouse). Each nude mouse was labeled and kept under sterile conditions. The lengths of the long and short axes of tumors were measured every two days, and the tumor volume was calculated according to the formula $V = 0.5236 \times L_1 \times L_2 \times L_2$ (where L_1 is the long axis of the tumor, and L_2 is the short axis of the tumor).

Statistical analysis

Data were presented as the mean \pm SD. At least three independent experiments were performed. Comparisons of the experimental data were assessed using an independent two-sample *t*-test at $P < 0.05$ level of significance.

Results

DN-LRP5 suppresses the proliferation of HO8910PM cells

In the previous study, we found LRP5 had significantly increased expression in highly metastatic colorectal cancer cells SW620 and OCa cells HO8910PM, while little expression in non-metastatic colorectal cancer cell line SW480 and OCa cell line HO8910 through western blot experiments (Appendix 1, Figure S1), which suggests that LRP5 may regulate cell migration and invasion. Then we constructed dominant negative LRP5 in HO8910PM cell line to explore the functions of LRP5 (Appendix 1, Figure S2), as expected, we observed that HO8910PM/DN-LRP5 cells grew much slower than that of HO8910PM. Further growth rate measurements by MTT assay indicated DN-LRP5 significantly inhibited the proliferation of HO8910PM cells (Figure 1).

DN-LRP5 inhibits the migration of HO8910PM

To explore effects of DN-LRP5 on cell migration, HO8910PM and HO8910PM/DN-LRP5 cell lines were employed to perform the wound healing and the transwell experiments in a time course manner. The results showed the wound healing rate of HO8910PM cells was 93% at 48 h, which is much higher than a 32% of wound healing rate at 48 h for HO8910PM/DN-LRP5 cells (Figure 2A,B), indicating DN-LRP5 can significantly inhibit the migration of HO8910PM cells. Similarly, the transwell assay showed that HO8910PM/DN-LRP5 migrated much lower than HO8910PM, indicating that DN-LRP5 inhibited the migration of HO8910PM cells significantly with an inhibition rate of around 45% (Figure 2C,D).

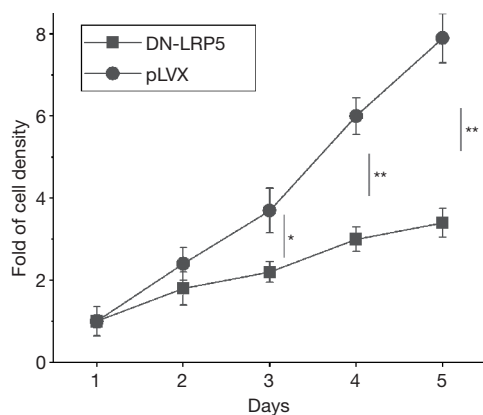


Figure 1 DN-LRP5 suppressed the proliferation of HO8910PM. The proliferation of HO8910PM and HO8910PM/DN-LRP5 was measured by MTT assay. DN-LRP5, dominant negative plasmid of LRP5. * $P < 0.05$; ** $P < 0.01$.

DN-LRP5 rearranges the cytoskeleton of HO8910PM

The arrangement of the cytoskeleton is closely related to the morphology and motility of the cells (30). Usually, the rearrangement of the cytoskeleton triggers changes in the structure of the microfilaments, resulting in an increase in the production of protrusive structures and an increase in the ability of tumor cells to move and migrate (31). As expected, HO8910PM cells had obvious protrusive structures (Figure 2E, left panel), showing high metastatic characteristics, while HO8910PM/DN-LRP5 showed no obvious protrusive structures, suggesting DN-LRP5 rearranged the cytoskeleton and inhibited the mobility of HO8910PM cells (Figure 2E, right panel).

DN-LRP5 regulates EMT process

EMT plays key roles in tumor metastasis. EMT molecular markers (for example, E-cadherin, Keratin 8/18, vimentin and fibronectin) are the hallmark events during EMT (32), accordingly, we detected the key molecules of EMT in both HO8910PM and HO8910PM/DN-LRP5 cells by western blotting, seeing the Appendix 1. As shown in Figure 3A,B and Figure S3A,B, HO8910PM/DN-LRP5 cells showed decreased expression of mesenchymal markers vimentin and fibronectin and increased expression of epithelial markers E-cadherin and Keratin 8/18, suggesting DN-LRP5 somehow reversed EMT process of HO8910PM cells and participated the regulation of cell migration.

DN-LRP5 regulates multiple cancer-associated pathways

Wnt signaling pathway is often implicated in regulation of other cancer signaling pathways, for example, the HGF/c-Met (33), the PI3 Kinase/Akt (34), the MAPK (35) and NF- κ B (36) pathways to play a pivot role in tumor formation and metastasis. With this context, we explored if DN-LRP5 has any effects on classical cancer signaling pathways including the Akt, p38, HGF/c-Met and NF- κ B, seeing the Appendix 1. As shown in Figure 3C and Figure S3C, DN-LRP5 significantly down-regulated the activity of the Akt, p38, HGF/c-Met and NF- κ B, while had little effects on the GSK3 β , the Stat3 and the Smad3 signaling pathways, suggesting Wnt-LRP5 cross-talked with those signaling pathways by a synergy manner possibly though the detailed mechanisms are elusive.

DN-LRP5 inhibits tumor proliferation of nude mice

Next, we were concerned about the effectiveness of DN-LRP5 on tumor inhibition in nude mice. With this purpose, nude mice were injected with HO8910PM and HO8910PM/DN-LRP5 cells at the same amount of cells respectively. From the fourth day after injection, the tumor volume was recorded every 2 days. The results showed that DN-LRP5 significantly inhibited tumor growth in nude mice in a time-dependent manner, with an inhibition rate high to around 90% eventually (Figure 4).

Discussion

The Wnt signaling pathway is super complex, containing 19 different Wnt ligands, 10 Frizzled receptors, several helper receptors, and numerous downstream target proteins, which have been evaluated by researchers for many years (37). Even though, the Wnt signaling pathway can be broadly classified into two categories, the canonical Wnt/ β -catenin pathway and non-canonical pathways, such as the Wnt/ Ca^{2+} pathway, the Wnt/PCP pathway, and the specificity of each pathway depends on different cell types, WNTs, SFRPs, WIF1, DKKs, Frizzled, co-receptors (LRP5/6), and regulation of Wnt signaling regulators in the cytoplasm (38). Except the developmental function of Wnt signaling pathway, recent studies have shown that inhibition of Wnt signaling activity can significantly inhibit the growth of tumor cells such as bladder cancer, rectal cancer, and liver cancer (39) though the detailed mechanisms are elusive.

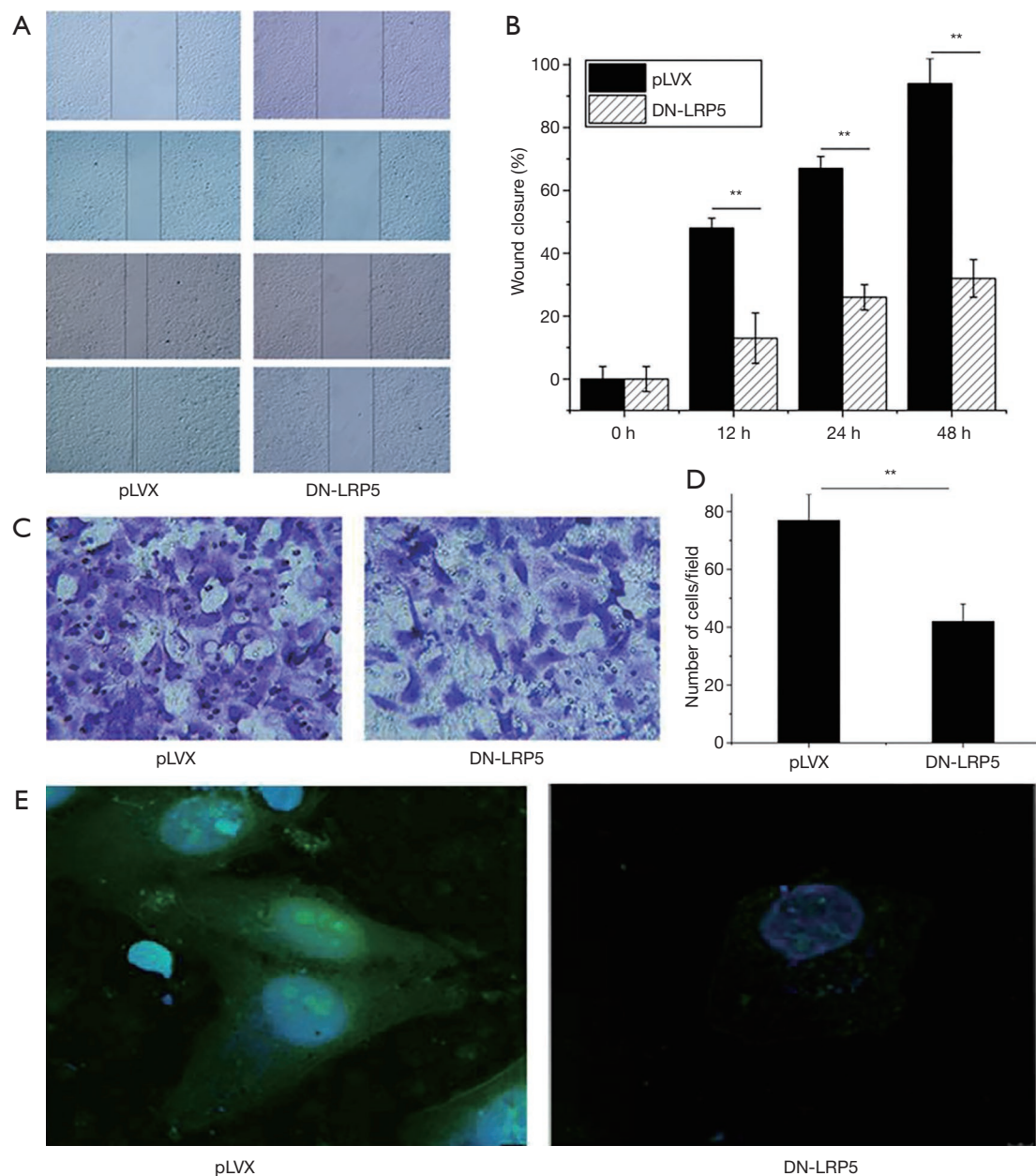


Figure 2 DN-LRP5 inhibited the migration of HO8910PM. (A) Migration of HO8910PM and HO8910PM/DN-LRP5 by straight scratch assay ($\times 40$). (B) Wound healing rate of HO8910PM and HO8910PM/DN-LRP5 at 0, 12, 24 and 48 h, respectively. (C) Invasion of HO8910PM and HO8910PM/DN-LRP5 cells in transwell by crystal violet staining ($\times 200$). (D) Statistical analysis for invasion of HO8910PM and HO8910PM/DN-LRP5 cells. (E) Cytoskeleton observation of HO8910PM and HO8910PM/DN-LRP5 cells by FITC-phalloidin staining assay ($\times 400$). **, $P < 0.01$. DN-LRP5, dominant negative plasmid of LRP5.

Here, we found Wnt-LRP5 is involved in progression of OCa cells, which adds a new possibility for OCa detection and treatment by targeting this classical signaling pathway.

As a co-receptor of Wnt signaling pathway, LRP5 was thought to be involved in the regulation of development (for

example, bone and eye development) mostly in previous research. The role of LRP5 in tumors regulation was rarely reported. In terms of the specific role of LRP5 in tumor cells, studies have shown that the dominant negative mutant of LRP5 overexpressing in prostate cancer PC-3 cells can

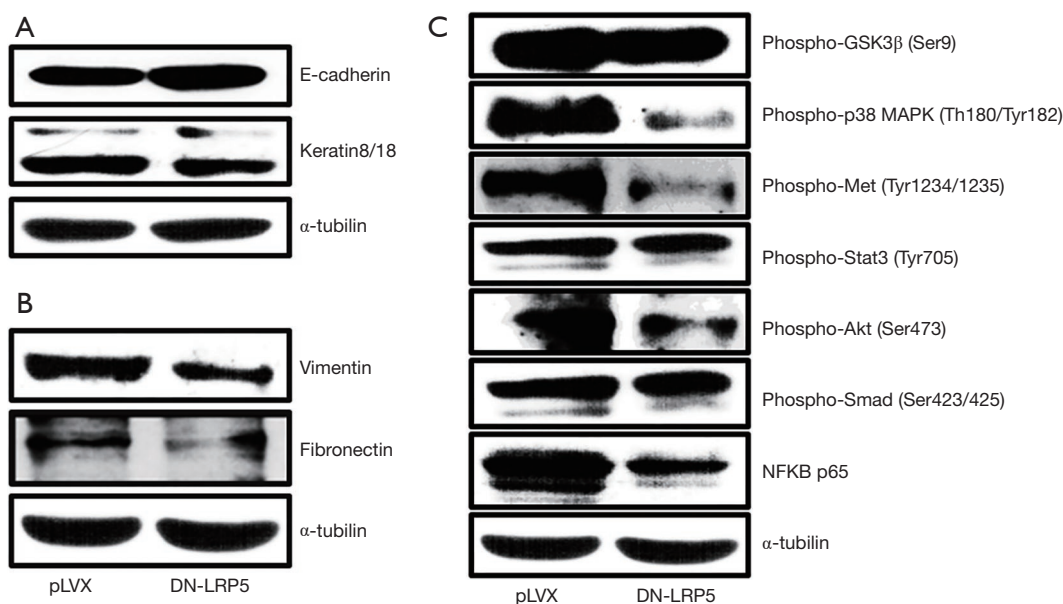


Figure 3 DN-LRP5 regulates EMT and multiple cancer-associated pathways. (A) Epithelial markers detection by western blotting; (B) mesenchymal markers detection by western blotting; (C) multiple cancer signaling pathways detection by western blotting. DN-LRP5, dominant negative plasmid of LRP5; EMT, epithelial-mesenchymal transition.

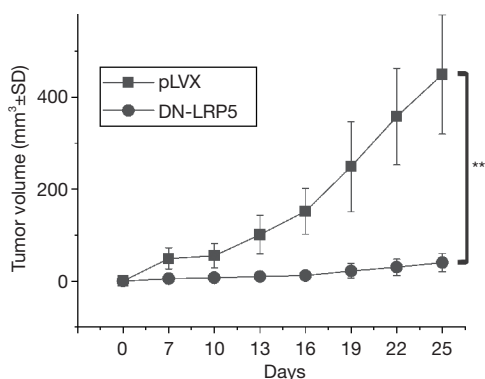


Figure 4 DN-LRP5 inhibits tumor proliferation of nude mice. Nude mice were injected with HO8910PM and HO8910PM/DN-LRP5 cells and tumor formation was recorded and measured every 2 days. Tumor volume = $0.5236 \times \text{long axis} \times \text{short axis} \times 2$. **, $P < 0.01$. DN-LRP5, dominant negative plasmid of LRP5.

inhibit the infiltration and migration of tumor cells and formation of subcutaneous xenograft tumors in nude mice, and the main mechanism is by up-regulating E-cadherin expression and inhibiting MMPs activity (40). After that, overexpression of DN-LRP5 also significantly inhibited the infiltration and migration of osteosarcoma cells HT1080 and SW872, as well as tumorigenicity in nude mice, via

down-regulation of hepatocyte growth factor receptor c-Met expression and inhibition of c-Met-mediated activity (41). In our current study, we found DN-LRP5 significantly inhibited the proliferation and migration of OCa cells *in vitro* (Figure 1) and tumor formation of mouse *in vivo* (Figure 4), our data plus the results from other group suggests LRP5 could be a new anti-tumor target.

In addition to the migration assay *in vitro*, the invasive phenotype of OCa may need to change the molecular structure from epithelial cells to mesenchymal cells. Down regulation of E-cadherin and up regulation of N-cadherin are associated with OCa progression, which is a common phenomenon in EMT sites during normal development and cancer (42). In our study, suppressing LRP5 expression in HO8910PM cells weakened cellular mobility, accompanying an obviously upregulation of epithelial markers E-cadherin and Keratin8/18 (Figure 3A) as well as downregulation of mesenchymal markers fibronectin and vimentin (Figure 3B), similar to the reversal of EMT (43). In summary, the reversal of EMT and the migration and actin staining assay *in vitro* suggested that LRP5 affected migration of OCa cells.

Activated phospho-Akt could promote cell proliferation via exerting anti-apoptotic effects through multiple downstream pathways including mTOR, Bcl-2, Caspase-9,

NF- κ B (34). Similarly, phosphorylation activation of p38 MAPK (35) and c-Met (33) and combination of NF- κ B and p65 (36) could activate the corresponding downstream pathway to promote cell proliferation and migration. The western blot results suggested that DN-LRP5 prevented phosphorylation activation of Akt, p38 MAPK and c-Met and combination of NF- κ B and p65 to inhibit cell proliferation and migration. This indicates that Wnt/LRP5 can cross talk with HGF/c-Met, Akt, p38 MAPK and NF- κ B signaling pathways to regulate cell proliferation and migration.

Our study successfully established a stable expression cell line of DN-LRP5 in OCa cells and proved that DN-LRP5 has a certain inhibitory effect on the growth and migration of OCa cells. This lays a foundation for further exploration of the anti-OCa and molecular mechanisms of DN-LRP5, thus building a framework for the development of anti-tumor drugs against Wnt signaling pathway.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <http://dx.doi.org/10.21037/tcr-20-3462>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tcr-20-3462>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-3462>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license [No. SYXK(Chuan)2014-189] granted by institutional ethics board of Southwest Jiaotong University, in compliance with

Sichuan guidelines for the care and use of animals.

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Increased expression of LRP5 in metastatic tumor cells

We detected the background expression of LRP5 protein in different cell lines by Western blot (Figure S1). LRP5 had little expression in colorectal cancer cell line SW480, but highly expressed in its high metastatic cell line SW620. Similarly, LRP5 barely expressed in ovarian cancer cell line HO8910, but highly expressed in its high metastatic cell line HO8910PM, all of which suggest that the expression of LRP5 may be related to cell migration.

Construction and verification of pLVX-IRES-ZsGreen1-DN-LRP5 and pLVX-puro-DN-LRP5 and verification of HO8910PM/DN-LRP5

To study the functions of LRP5 receptor protein and other related proteins in ovarian cancer cells both *in vitro* and *in vivo*, we shall knock down the expression of LRP5 by using dominant negative vector of LRP5. Accordingly, pLVX-puro-DN-LRP5 was constructed to obtain the LRP5 low expression cell line, while pLVX-IRES-ZsGreen1-DN-LRP5 was constructed to detect lentivirals infection efficiency.

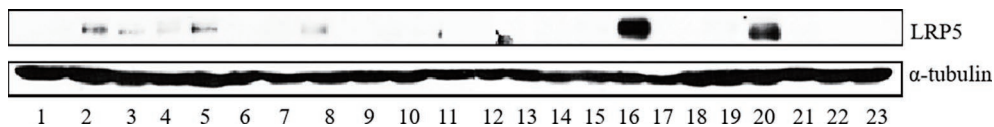


Figure S1 Increased expression of LRP5 in metastatic tumor cells. Human normal liver cells: 1: L02, 2: Chang's liver. Hepatocarcinoma cells: 3: HepG2, 4: HepG2.2.15, 5: Hep3B, 6: 7721, 7: PLC, 8: Huh7, 9: 97-H, 10: LM3, 11: FOCUS, 12: SNU398, 13: Sk-Hep-1. Colon carcinoma cells: 14: LoVo, 15: HCT116, 16: SW480, 17: SW620. OCa cells: 18: A2780, 19: HO8910, 20: HO8910PM, 21: SKOV3. Lung cancer cells: 22: H460, 23: H446.

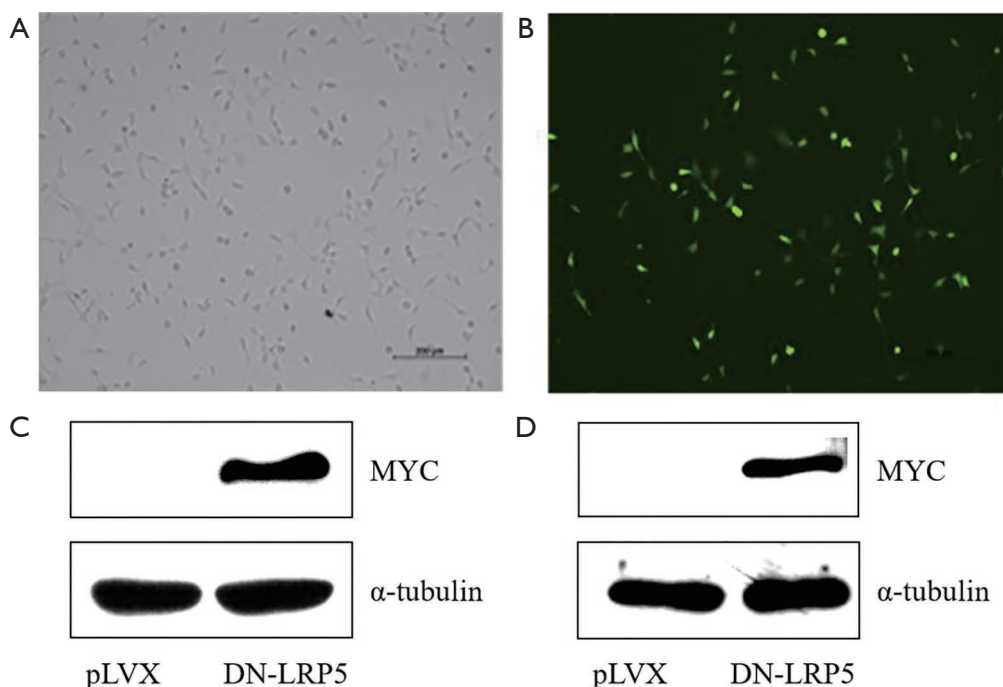


Figure S2 Verification of HO8910PM/DN-LRP5. (A) Light microscopy observation of HEK293T infected with pLVX-IRES-ZsGreen1-DN-LRP5 virals ($\times 100$); (B) fluorescence microscopy observation of HEK293T infected with pLVX-IRES-ZsGreen1-DN-LRP5 virals ($\times 100$); (C) Western blot analysis for expression of DN-LRP5 (myc) in pLVX-puro-DN-LRP5 virals in HEK293T; (D) Western blot analysis for expression of DN-LRP5(myc) in pLVX-puro-DN-LRP5 virals in HO8910PM. DN-LRP5, dominant negative plasmid of LRP5.

To obtain packaged recombinant lentivirus, HEK 293T cells were co-transfected with recombinant lentivirus expression plasmids and lentivirus packaging plasmids psPAX2 and pMD2.G. After infection, expression of a large number of green fluorescent proteins in HEK 293T cells was observed by fluorescence microscopy (Figure S2A,B), while expression of DN-LRP5 protein was detected by Western blot (Figure S2C).

To obtain the cell line stably expressing DN-LRP5, HO8910PM cells were infected with the recombinant lentivirus and resistant cell clones were selected with puromycin. Consistently, the expression of DN-LRP5 in positive clones was detected by Western blot (Figure S2D).

DN-LRP5 regulates EMT and multiple cancer-associated pathways

To see if DN-LRP5 are associated with EMT and some cancer-related pathways, multiple EMT or cancer-associated proteins are checked by Western blotting (Figure 3), and the quantitative densitograms of Figure 3 are shown in Figure S3.

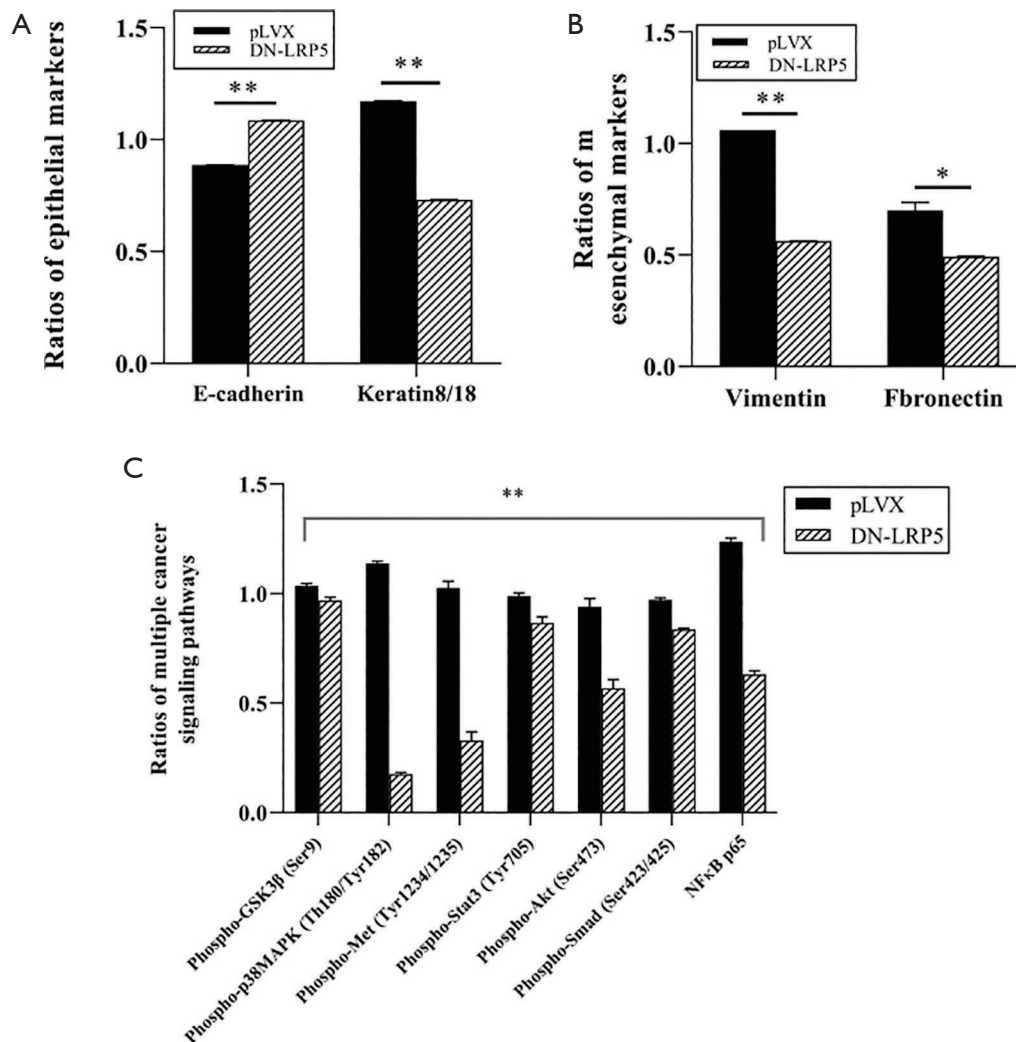


Figure S3 Western blotting densitograms of DN-LRP5 associated EMT and multiple cancer-associated pathways. (A) Ratios of epithelial markers verse tubulin; (B) ratios of mesenchymal markers verse tubulin; (C) ratios of multiple cancer signaling pathway proteins verse tubulin. DN-LRP5, dominant negative plasmid of LRP5; EMT, epithelial-mesenchymal transition.