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Role of Wnt co-receptor LRP6 in triple negative breast cancer cell migration and invasion

Jinlu Ma[1,2], **Wenyan Lu**[2], **Dongquan Chen**[3], **Bo Xu**[2], **Yonghe Li**[2,*]

[1]Department of Radiation Oncology, the First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, Shaanxi, 710061, China

[2]Department of Oncology, Drug Discovery Division, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35255, USA

[3]Division of Preventive Medicine and Comprehensive Cancer Center; Department of Medicine; University of Alabama at Birmingham; Birmingham, AL USA

Abstract

The low-density lipoprotein receptor-related protein 6 (LRP6) is an essential Wnt co-receptor of the Wnt/β-catenin signaling pathway. Although studies have shown an increased expression of LRP6 in several types of cancer, its function in tumor development and progression remains to be elucidated. We herein demonstrated that LRP6 expression is up-regulated in human triple negative breast cancer (TNBC) patients and human TNBC cell lines, and that knockdown of LRP6 expression and treatment of recombinant Mesd protein (a specific inhibitor of LRP6) significantly decreased cell migration and invasion of TNBC MDA-MB-231 and BT549 cells. Interestingly, the effects of LRP6 knockdown and Mesd treatment on TNBC cell migration and invasion were more prominent than on TNBC cell proliferation/viability. Mechanistically, LRP6 knockdown and Mesd treatment inhibited Wnt/β-catenin signaling and decreased the expression of S100A4, a mediator of cancer metastasis and a specific target of Wnt/β-catenin signaling, in TNBC cells. Together, our data suggest that LRP6 promotes TNBC cell migration and invasion by regulating the expression and function of S100A4 via the Wnt/β-catenin signaling pathway.

Keywords

LRP6; Mesd; S100A4; migration; invasion; TNBC cells

The low-density lipoprotein receptor (LDLR)-related protein 6 (LRP6), a member of the LDLR family (Schneider and Nimpf, 2003), is an indispensable coreceptor of the canonical Wnt/β-catenin signaling pathway (He et al., 2004). Wnt proteins can activate the canonical Wnt/β-catenin signaling pathway only in the presence of LRP6 (He et al., 2004). Upon binding of Wnt to its cell-surface receptors Frizzled (FZD) and LRP6, β-catenin, an essential transcriptional co-activator of the Wnt/β-catenin signaling pathway, is stabilized and then

*Corresponding author at: Department of Oncology, Drug Discovery Division, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA. Tel.: +1 205 581 2750; fax: +1 205 581 2093, yli@souternresearch.org (Y. Li).

translocates into the nucleus where it interacts with T-cell factor/lymphoid enhancing factor (TCF/LEF) to induce the expression of downstream target genes (He et al., 2004).

Deregulation of Wnt/β-catenin signaling is involved in the pathogenesis of many diseases including cancer (Clevers and Nusse, 2012; Madan and Virshup, 2015). LRP6 is up-regulated in a broad panel of cancers, including breast cancer (Lindvall et al., 2009; Liu et al., 2010; Yang et al., 2011), prostate cancer (Liu et al., 2012), hepatocellular carcinoma (Tung et al., 2012) and retinoblastoma (Wang et al., 2014). Activation of Wnt/β-catenin signaling is associated with a poorer prognosis in breast cancer patients (Lin et al., 2000), and is preferentially found in triple negative breast cancer (TNBC) which is distinguished by negative immunohistochemical assays for expression of the estrogen and progesterone receptors (ER/PR) and human epidermal growth factor receptor-2 (HER2) (Dey et al., 2013; Geyer et al., 2011; Khramtsov et al., 2010). Accordingly, it was found that LRP6 is significantly up-regulated in TNBC (Lindvall et al., 2009; Liu et al., 2010; Yang et al., 2011). In mice, mammary gland development and MMTV-Wnt1-induced mammary tumorigenesis are delayed in $LRP6^{+/-}$ mice (Lindvall et al., 2009), while MMTV-LRP6 transgenic mice develop hyperplasia in their mammary glands due to LRP6-mediated Wnt/β-catenin signaling activation (Zhang et al., 2010). Moreover, transcriptional knockdown of LRP6 and inhibition of LRP6 on the cell surface in TNBC cells significantly decreased Wnt/β-catenin signaling, cell proliferation and tumor growth *in vivo* (Lin et al., 2013; Liu et al., 2010). Together, these studies clearly indicate that LRP6 plays a critical role in breast cancer development and progression.

TNBC is clinically characterized as more aggressive and less responsive to standard treatment with a poorer overall patient prognosis (Carey et al., 2010). Women with TNBC have an increased likelihood and earlier appearance of distant recurrence, as compared with those with non-TNBC subtypes (Dent et al., 2009). The S100 calcium binding protein A4 (S100A4) is a direct transcriptional target of the Wnt/β-catenin signaling pathway (Stein et al., 2006), and has been established as a mediator of cancer metastasis (Dahlmann et al., 2016; Garrett et al., 2006; Mishra et al., 2012). S100A4 promotes breast cancer motility and invasion (Jenkinson et al., 2004; Wang et al., 2012), induces metastasis in rodent models for breast cancer (Ambartsumian et al., 1996; Davies et al., 1996; Lloyd et al., 1998), and is associated with poor patient survival in breast cancer patients (Platt-Higgins et al., 2000; Rudland et al., 2000). While LRP6/β-catenin signaling is highly activated in TNBC, the role of LRP6 in TNBC metastases remains to be elucidated. In the present study, we present evidence that LRP6 regulates TNBC cell migration and invasion by altering the expression of S100A4.

MATERIALS AND METHODS

MATERIALS

Preparation of recombinant mouse Mesd protein has been described before (Li et al., 2005; Lu et al., 2010). Plasmid pGST-E-cadherin was provided by Dr. Gail Johnson (University of Rochester). The Super8XTOPFlash luciferase construct was provided by Dr. Randall T. Moon (University of Washington, Seattle). A β-galactosidase-expressing vector was from Promega. Polyclonal anti-LRP6 was from Santa Cruz Biotechnology. Monoclonal anti-

phospho-LRP6, anti-LRP5, anti-axin2 and anti-S100A4 were purchased from Cell Signaling Technology. Monoclonal anti-β-catenin was from BD Biosciences. Monoclonal anti-actin was from Sigma. Peroxidase labeled anti-mouse antibody and ECL system were purchased from Amersham Life Science. The luciferase and β-galactosidase assay systems were from Promega. Tissue culture media, fetal bovine serum (FBS), and plastic-ware were obtained from Life Technologies, Inc. Proteinase inhibitor cocktail Complete™ was obtained from Boehringer Mannheim.

ANALYSIS OF LRP6 EXPRESSION WITH BREAST CANCER DATASET OBTAINED FROM THE CANCER GENOME ATLAS (TCGA)

TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga) was used to download expression data of breast invasive carcinoma samples (n=1,070). The RNAseqV2 level 3 data that includes fragments per kilobase of exon per million fragments mapped (FPKM)-normalized gene level data were used before statistics. In addition, idf file and sdrf file were also downloaded for sample mapping and annotation. The clinical outcome data was downloaded for correlation and model building. Group wise comparison as well as ANOVA was used to select genes of significance. False Discovery Rate (FDR)-corrected p values were used for multiple hypothesis testing purpose.

CELL CULTURE

All cell lines were obtained from American Type Culture Collection (Manassas, VA), where the cell lines were authenticated by STR profiling before distribution. All cell lines were expanded upon receipt to prepare frozen cell stocks that were cultured *in vitro* for no longer than 3 months after thawing. MDA-MB-231, MDA-MB-435s, HS578T, BT549, T-47D, ZR-75-1 and MCF-7 cells were cultured in DMEM medium (GIBCO-Invitrogen, Carlsbad, CA) containing 10% of FBS, 2 mM of L-glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin, and grown under standard cell culture conditions at 37°C in a humidified atmosphere with 5% CO2. The MCF-10A human breast epithelial cells were cultured in DMEM/Ham's F-12 (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor (EGF), 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, and 5% chelex-treated horse serum.

SiRNA DEPLETION OF LRP6

For RNAi experiments, ON-TARGETplus double-stranded siRNA oligomers against human LRP6 and non-specific scrambled siRNA control (Stealth RNAi™ siRNA Negative Control, Med GC) were purchased from Thermo Scientific. siRNA LRP6-1, siRNA LRP6-2 and control siRNA were Thermo Scientific catalog numbers J-003845-09, J-003845-11 and 12935300, respectively. Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) with a final siRNA concentration of 50 nM according to the manufacturer's instructions.

WESTERN BLOTTING

Cells in 6-well plates were lysed in 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM PMSF) at 4°C for 10 min. Equal quantities of protein were subjected to SDS-PAGE under reducing conditions. Following transfer to

immobilon-P transfer membrane, successive incubations with a primary antibody, and a horseradish peroxidase-conjugated secondary antibody were carried out for 60–120 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned by Hp Scanjet 5590.

CYTOSOLIC FREE β-CATENIN ANALYSIS WITH THE GST-E CADHERIN BINDING ASSAY

The GST-E-cadherin binding assay was conducted exactly as previously described (Lin et al., 2011). Uncomplexed cytosolic free β-catenin present in 100 μg of total cell lysate was subjected to SDS-PAGE and detected using the monoclonal antibody to β-catenin.

LUCIFERASE REPORTER ASSAY FOR WNT/β-CATENIN SIGNALING

Cancer cells were plated into 24-well plates. After overnight culture, the cells were transiently transfected with the Super8XTOPFlash luciferase construct and β-galactosidase-expressing vector. After 24 h incubation, cells were treated with Mesd at the indicated concentrations. Cells were then lysed 24 h later and both luciferase and β-galactosidase activities.

CELL MIGRATION AND INVASION ASSAYS

Breast cancer cell migration and invasion assays were performed as described previously, with minor modifications (Li et al., 1998; Song et al., 2009). The migration assay was carried out in 6.5-mm diameter transwell chambers with pore size of 8.0 μm from BD Biosciences. MDA-MB-231 and BT549 cells (1×10^5) in 200 μl of the serum-free DMEM medium with 0.1% BSA and 2 mM L-glutamine were placed in the upper compartment of the transwell chambers. The lower compartment was filled with 600 μl of complete medium (DMEM medium with 10% FBS and 2 mM L-glutamine). After incubation for 15 h at 37°C, cells on the lower surface of the filter were fixed in 4% paraformaldehyde and stained with crystal violet. The images were scanned and counted at 100 × magnification.

Invasion of cells through Matrigel was determined using 24-well BD invasion chambers (8.0-μm pore size with polycarbonate membrane; BD Biosciences) according to the manufacturer's instructions. MDA-MB-231 and BT549 cells (5×10^4) in 100 μl of the serum-free DMEM medium with 0.1% BSA and 2 mM L-glutamine were placed in the upper compartment of the invasion chambers, and the lower compartment was filled with 600 μl of complete medium. After incubation for 22 h at 37°C, cells on the lower surface of the filter were fixed, stained and counted at 100 × magnification.

CELL PROLIFERATION/VIABILITY ASSAY

Cells were seeded at 2000 cells/well in opaque-walled 96 well plate 24 hours prior to Mesd and siRNAs exposure. At the indicated time, cell proliferation/viability was determined by CellTiter-Glo luminescent cell viability assay kit according to the manufactures protocol.

STATISTICAL ANALYSIS

The unpaired two-tailed student's t-test was used to determine statistically significant differences between treatment effects and calculated using GraphPad Prism version 5 for

Windows (GraphPad Software, La Jolla California USA). Significance was defined as $P < 0.05$.

RESULTS

LRP6 IS UP-REGULATED IN TNBC CELLS

It has been reported that LRP6 expression is up-regulated in TNBC (Lindvall et al., 2009; Liu et al., 2010; Yang et al., 2011). Through the TCGA Data Portal, we characterized LRP6 expression in breast cancer patients. We found that the level of LRP6 expression is higher in ER, PR and HER2 negative than in corresponding ER, PR, and HER2 positive breast tumors (Figure 1A), and confirmed that the level of LRP6 expression is higher in TNBC than in non-TNBC (Figure 1B).

LRP5 is a closely related paralog of LRP6, but LRP5 is a much less potent transducer of Wnt/β-catenin signaling (Chin et al., 2015; MacDonald et al., 2011). We found that the level of LRP5 expression is not associated with ER and PR status (Figure S1A). Although the level of LRP5 expression is higher in TNBC than in non-TNBC, the change of LRP5 expression is much smaller than that of LRP6 (Figure S1B). Together, these data suggest that LRP6 is more important than LRP5 in activation of Wnt/β-catenin signaling in TNBC.

To further characterize LRP6 expression in breast cancer, we performed Western blotting to examine LRP6 expression in non-cancerous human breast epithelial cells line MCF-10A, three ER positive breast cell lines and four TNBC cell lines. As expected, LRP6 was highly expressed in 4 TNBC cell lines MDA-MB-231, HS578T, MDA-MB-435s and BT549, while low LRP6 expression was found in MCF-10A and three ER positive breast cell lines (MCF-7, ZR-75–1 and T-47D) (Figure 1C). As LRP6 is most abundant in MDA-MB-231 and BT549 cells, the rest of our experiments were focused on these two TNBC cell lines.

KNOCKDOWN OF LRP6 INHIBITS WNT/β-CATENIN SIGNALING IN TNBC CELLS AND SUPPRESSES CELL MIGRATION AND INVASION

We next examined the effects of modulating LRP6 expression on TNBC cell migration and invasion. Using two independent siRNAs targeting distinct regions of LRP6, we knocked down LRP6 expression in TNBC MDA-MB-231 and BT549 cells (Figure 2A). While the levels of LRP5 expression were not changed, the levels of LRP6 phosphorylation and cytosolic free β-catenin were greatly reduced in LRP6 siRNA-transfected TNBC cells (Figure 2A). When MDA-MB-231 and BT549 cells were transiently transfected with the Wnt/β-catenin signaling reporter Super8XTOPFlash, knockdown of LRP6 resulted in significant inhibition of the Super8XTOPFlash activity in TNBC cells (Figure 2B). In addition, levels of axin2, a specific target of Wnt/β-catenin signaling, were also significantly decreased in LRP6 siRNA-transfected TNBC cells (Figure 2A), indicating that LRP6 knockdown induces inhibition of Wnt/β-catenin signaling in TNBC cells. Importantly, knockdown of LRP6 significantly suppresses TNBC cell migration and invasion. Knockdown of LRP6 led to 61–70% inhibition of TNBC cell migration when compared to corresponding control cells (Figure 2C & 2D). Similar results were obtained in

invasion assays. Knockdown of LRP6 induced 51 – 62% inhibition of TNBC cell invasion when compared to corresponding control cells (Figure 2E & 2F).

We then examined the effects of LRP6 knockdown on TNBC cell proliferation/viability. Consistently with the study by Liu et al. (Liu et al., 2010), we found that the cell proliferation/viability was decreased by 14–47 % after transfection of LRP6 siRNA-1 or LRP6 siRNA-2 for 72–96 h in MDA-MB-231 cells and for 48–96 h in BT549 cells (Figure 3). LRP6 knockdown for 48 h in MDA-MB-231 had no significant effects on cell proliferation/viability (Figure 3A). The migration and invasion assays were conducted after LRP6 knockdown for 48 h in TNBC cells. Clearly, LRP6 knockdown in TNBC cells induced more profound effects on cell migration and invasion than on cell proliferation/viability.

MESD PROTEIN INHIBITS LRP6 PHOSPHORYLATION AND WNT/β-CATENIN SIGNALING IN TNBC CELLS AND SUPPRESSES CELL MIGRATION AND INVASION

Mesd is a specialized chaperone for LRP6 (Culi and Mann, 2003; Hsieh et al., 2003). We have demonstrated that recombinant Mesd protein binds to LRP6 with a high affinity and is able to inhibit Wnt- and Rspondin-induced Wnt/β-catenin signaling in LRP6-expressing cells (Li et al., 2005; Lin et al., 2011; Lin et al., 2013; Lu et al., 2010). To confirm the role of LRP6 in TNBC cell migration and invasion, we examined the effects of recombinant Mesd protein on MDA-MB-231 and BT549 cell migration and invasion. It was found that the treatment of Mesd protein at 2 or 4 μM for 24 h significantly decreased the levels of LRP6 phosphorylation, cytosolic free β-catenin, axin2 expression, and activity of the Wnt/β-catenin signaling reporter Super8XTOPFlash in TNBC cells (Figure 4A & 4B), indicating that Mesd treatment induces inhibition of Wnt/β-catenin signaling in TNBC cells. Importantly, Mesd protein significantly inhibited TNBC cell motility and invasion in a dose-dependent manner. Mesd protein at 2 μM and 4 μM led to about 50% and 85% inhibition, respectively, of TNBC cell migration (Figure 4C & 4D). Similarly, Mesd protein at 2 μM and 4 μM led to about 25% and 60% inhibition, respectively, of TNBC cell invasion (Figure 4E & 4F).

Consistently with our previous study (Lin et al., 2013), we also found that Mesd protein significantly inhibited TNBC cell proliferation/viability after treatment at 2 and 4 μM for 96 h (Figure 5). However, Mesd protein had no significant effects on TNBC cell proliferation/viability after treatment at 2 and 4 μM for 24–72 h (Figure 5). In the migration and invasion experiments, TNBC cells were pretreated with Mesd protein for 24 h, and examined by the migration and invasion assays with the treatment of Mesd protein for 15 h and 22 h, respectively. These results indicate that treatment of Mesd protein in TNBC cells induced more profound effects on cell migration and invasion than on cell proliferation/viability.

LRP6 MODULATES TNBC CELL MIGRATION AND INVASION BY REGULATING S100A4 EXPRESSION

S100A4 is a specific target of the Wnt/β-catenin signaling pathway (Stein et al., 2006), and has a well-established metastasis-promoting activity (Dahlmann et al., 2016; Garrett et al., 2006; Mishra et al., 2012). It has been demonstrated that S100A4 depletion effectively

blocked MDA-MB-231 cell motility and invasion, whereas S100A4 overexpression induced opposite effects (Wang et al., 2012). Therefore, we investigated whether the depletion of LRP6 and treatment of Mesd protein inhibit S100A4 expression in TNBC cells. As shown in Figure 6A, LRP6 knockdown significantly reduced S100A4 expression in MDA-MB-231 and BT549 cells. Similarly, Mesd protein inhibited S100A4 expression in a dose-dependent manner in MDA-MB-231 and BT549 cells (Figure 6B). Taken together, these data suggest that LRP6-mediated cell migration and invasion are associated with the regulation of S100A4 expression in TNBC cells.

DISCUSSION

As an essential Wnt co-receptor, LRP6 is up-regulated in many types of cancer (Lindvall et al., 2009; Liu et al., 2010; Liu et al., 2012; Tung et al., 2012; Wang et al., 2014; Yang et al., 2011), and overexpression of LRP6 promotes cancer cell proliferation *in vitro* and tumor growth *in vivo* (Li et al., 2004; Tung et al., 2012). Recent studies further suggest that LRP6 is associated with cancer cell motility, invasion and metastasis (Bernaudo et al., 2016; Deng et al., 2015; Marastoni et al., 2014; Nagaoka et al., 2013; Ren et al., 2015; Tung et al., 2012; Wang et al., 2014; Wen et al., 2015; Zhang et al., 2013). It has been demonstrated that the tumor metastasis suppressor gene-1 (NDRG1) interacts with LRP6, followed by blocking of LRP6 phosphorylation and Wnt/β signaling in breast and prostate cancer cells, and therefore, impairs the metastatic progression of tumor cells (Liu et al., 2012). Indeed, LRP6 expression is significantly up-regulated in prostate patients with metastatic disease compared to those without metastasis, and the elevated level of LRP6 is associated with a significantly increased risk of recurrent disease (Liu et al., 2012). Moreover, it has been reported that miR-183 suppresses retinoblastoma cell migration and invasion by targeting LRP6 (Wang et al., 2014), and that kallistatin, a plasma protein, inhibits cell motility via binding to LRP6 and suppressing LRP6 phosphorylation and Wnt/β-catenin signaling in TNBC cells (Zhang et al., 2013). In the present study, we confirm that LRP6 is up-regulated in TNBC patients and TNBC cell lines. More importantly, we demonstrated that knockdown of LRP6 expression and treatment of Mesd protein, a specific inhibitor of LRP6, significantly decreased cell migration and invasion of TNBC MDA-MB-231 and BT549 cells, providing direct evidence that LRP6 plays a critical role in TNBC cell motility and invasion.

Both LRP5 and LRP6 are essential Wnt co-receptors of the Wnt/β-catenin signaling pathway (He et al., 2004). While LRP5 has a Wnt-independent role in glucose uptake and growth for mammary epithelial cells (Chin et al., 2015), LRP6 is a more potent transducer of Wnt/β-catenin signaling (Chin et al., 2015; MacDonald et al., 2011). Furthermore, LRP5 knockdown induced caspase-dependent apoptosis in TNBC cells, whereas LRP6 knockdown had no such effect (Maubant et al., 2014). In the present study, we demonstrated that LRP6 knockdown or LRP6 inhibition by Mesd in TNBC cells induced more profound effects on cell migration and invasion than on cell proliferation/viability. Together, these findings suggest that LRP5 and LRP6 have different functions in TNBCs, with LRP6 playing an important role in cell motility.

Activation of Wnt/β-catenin signaling is required both FZD and LRP6 on the cell surface (He et al., 2004). It was reported that FZD7 was upregulated in TNBC and TNBC derived

cell lines, and that FZD7 knockdown resulted in inactivation of Wnt/β-catenin signaling in TNBC cells, leading to inhibition of TNBC cell proliferation, migration and invasion *in vitro* and suppression of TNBC cell growth *in vivo* (Yang et al., 2011). It appears that aberrant Wnt/β-catenin signaling in TNBC cells is caused by overexpression of both LRP6 and FZD7 on the cell surface.

S100A4, a member of the S100 family of calcium-binding proteins, is able to activate and integrate pathways both intracellular and extracellular to generate a phenotypic response characteristic of cancer metastasis (Mishra et al., 2012). S100A4 is up-regulated in various types of cancers, and S100A4 expression levels in tumors are considered as a biomarker for the prognosis of both metachronous metastasis and survival of cancer patients (Dahlmann et al., 2016; Garrett et al., 2006; Mishra et al., 2012). Particularly, S100A4 is able to enhance TNBC cell motility and invasion *in vitro* and induces lung and brain metastasis *in vivo* (Sartorius et al., 2016; Wang et al., 2012), and the elevated levels of S100A4 expression are associated with the poor prognosis in human patients (Platt-Higgins et al., 2000; Rudland et al., 2000). Importantly, S100A4 is a direct transcriptional target of the Wnt/β-catenin signaling pathway, and a transcriptionally active β-catenin enhances the S100A4-induced migration and invasion of colorectal cancer cells (Stein et al., 2006). Wnt/β-catenin signaling is up-regulated in TNBC (Dey et al., 2013; Geyer et al., 2011; Khramtsov et al., 2010), and is positive associated with TNBC lung and brain metastasis (Dey et al., 2013). Moreover, the functional blockade of the Wnt/β-catenin pathway by either pharmacological Wnt inhibitors or β-catenin depletion results in inhibition of TNBC cell migration and invasion (De et al., 2016; Dey et al., 2013). In this study, we demonstrated that knockdown of LRP6 expression and treatment of Mesd protein inhibit Wnt/β-catenin signaling and S100A4 expression in TNBC cells, suggesting that S100A4 is associated with Wnt/β-catenin signaling-mediated TNBC cell migration, invasion and metastasis.

Mesd was original discovered as a specialized chaperone for LRP6 (Culi and Mann, 2003; Hsieh et al., 2003). We have demonstrated that recombinant Mesd protein is able to bind to mature LRP6 on the cell surface with a high affinity and acts as a specific inhibitor of LRP6 and Wnt/β-catenin signaling in cancer cells (Li et al., 2005; Lin et al., 2011; Lin et al., 2013; Lu et al., 2010). Mesd protein suppresses LRP6 phosphorylation and Wnt/β-catenin signaling in TNBC cells, inhibits TNBC cells proliferation *in vitro* and tumor growth *in vivo* (Lin et al., 2013; Liu et al., 2010). In this study, we demonstrated that Mesd protein blocks LRP6 phosphorylation and Wnt/β-catenin signaling in TNBC cells, and inhibits TNBC cells migration and invasion. Together, these results support the notion that, as a specific inhibitor of LRP6, recombinant Mesd protein has a therapeutic value in TNBC.

In summary, we have demonstrated that Wnt co-receptor LRP6 is up-regulated in human TNBC patients and cell lines, and that LRP6 knockdown via siRNA and LRP6 inhibition by recombinant Mesd protein not only inhibit TNBC cell proliferation/viability but also suppress TNBC cell migration and invasion. Furthermore, LRP6-mediated TNBC cell migration and invasion is associated with modulation of S100A4 expression in TNBC cells. These observations reveal a novel role of LRP6 in TNBC cell migration and invasion and further suggest LRP6 as a therapeutic target for TNBC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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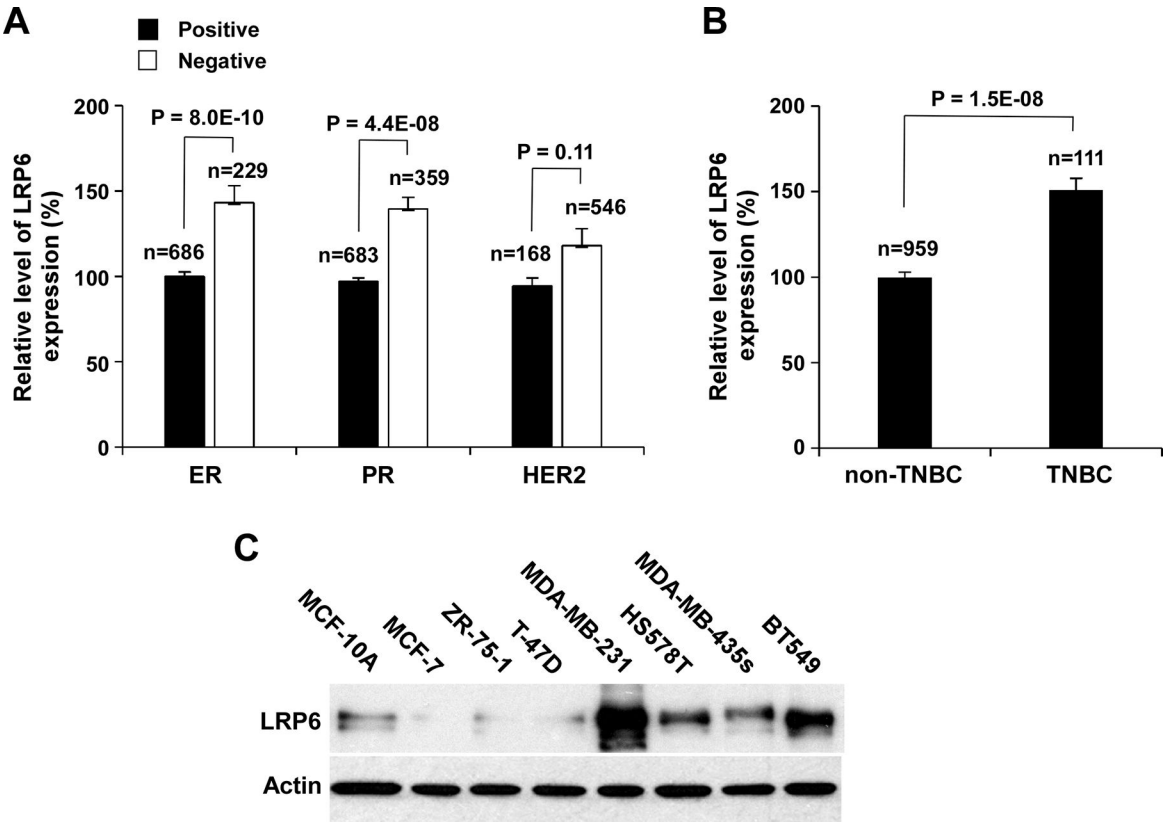

Fig. 1.

LRP6 is upregulated in TNBC cells. (A, B) LRP6 expression in breast cancer as related to TNBC markers. Data were collected from TCGA Data Portal and analyzed as described in Materials and Methods, and were presented as relative levels to the LRP6 expression level in ER positive breast cancer (A) or the LRP6 expression level in non-TNBC (B). All the values are the average with the s.d. indicated by error bars. (C) LRP6 expression in non-cancerous human breast epithelial cell line MCF-10A, three ER positive breast cell lines and four TNBC cell lines was examined by Western blotting. Samples were also probed with the anti-actin antibody to verify equal loading.

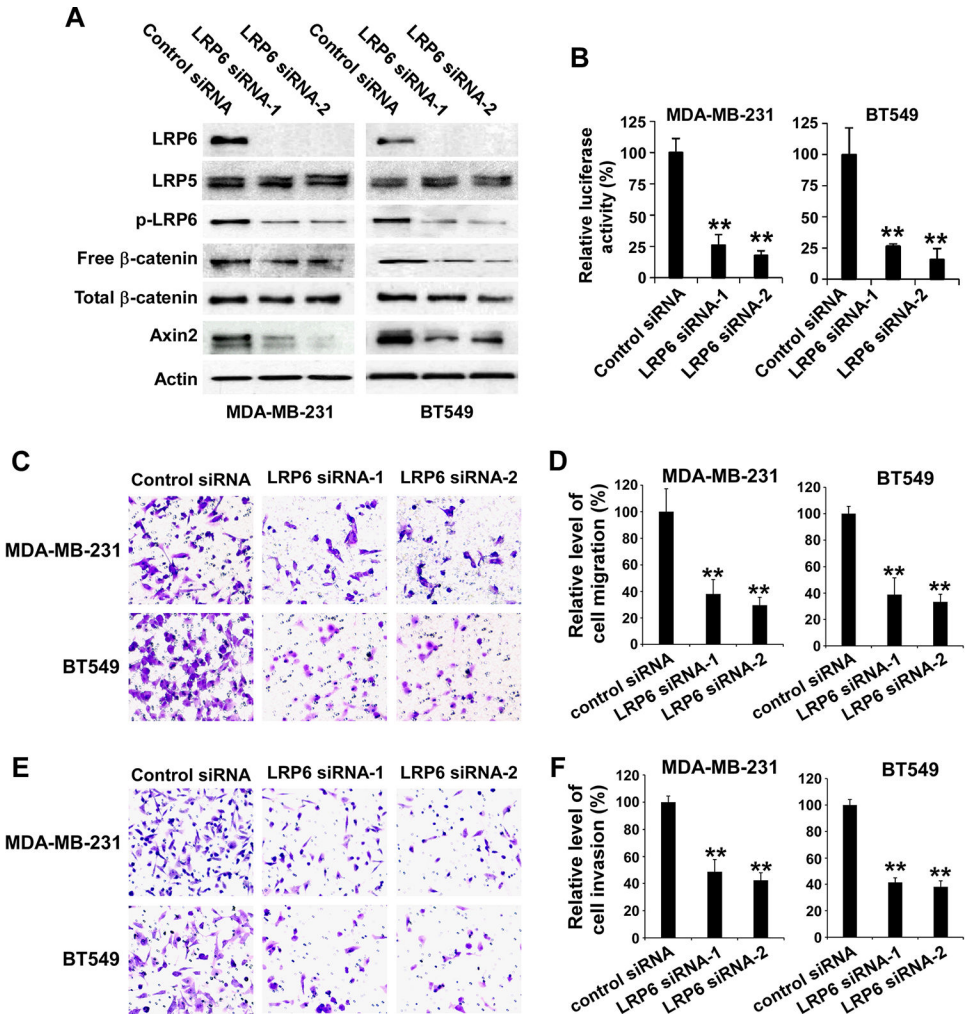

Fig. 2.

Depletion of LRP6 results in inhibition of Wnt/β-catenin signaling in TNBC cells and suppression of TNBC cell migration and invasion. (**A**) MDA-MB-231 and BT549 cells in 6-well plates were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After incubation of 48 h, the levels of human LRP6, LRP5, phospho-LRP6 (p-LRP6), cytosolic free human β-catenin, total cellular human β-catenin, and axin2 were examined by Western blotting. All the samples were also probed with anti-human actin antibody to verify equal loading. (B) MDA-MB-231 and BT549 cells in 24-well plates were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After incubation of 24 h, cells were transfected with the Super8XTOPFlash luciferase construct and β-galactosidaseexpressing vector in each well. The luciferase activity was then measured 24 h later with normalization to the activity of the β-galactosidase. Values are the average of triple determinations with the s.d. indicated by error bars. (C-F) MDA-MB-231 and BT549 cells were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After incubation of 48 h, the transwell migration assay (C, D) and Matrigel invasion assay (E, F) were performed. (C) Representative images of cell migration. (D) Relative levels of cell migration are significantly decreased following LRP6

knockdown in MDA-MB-231 and BT549 cells. (E) Representative images of cell invasion. (F) Relative levels of cell invasion are significantly decreased following LRP6 knockdown in MDA-MB-231 and BT549 cells. Data shown are representative of three independent experiments. All the values are the average of quadruplicate determinations with the s.d. indicated by error bars. **P < 0.01 versus control cells.

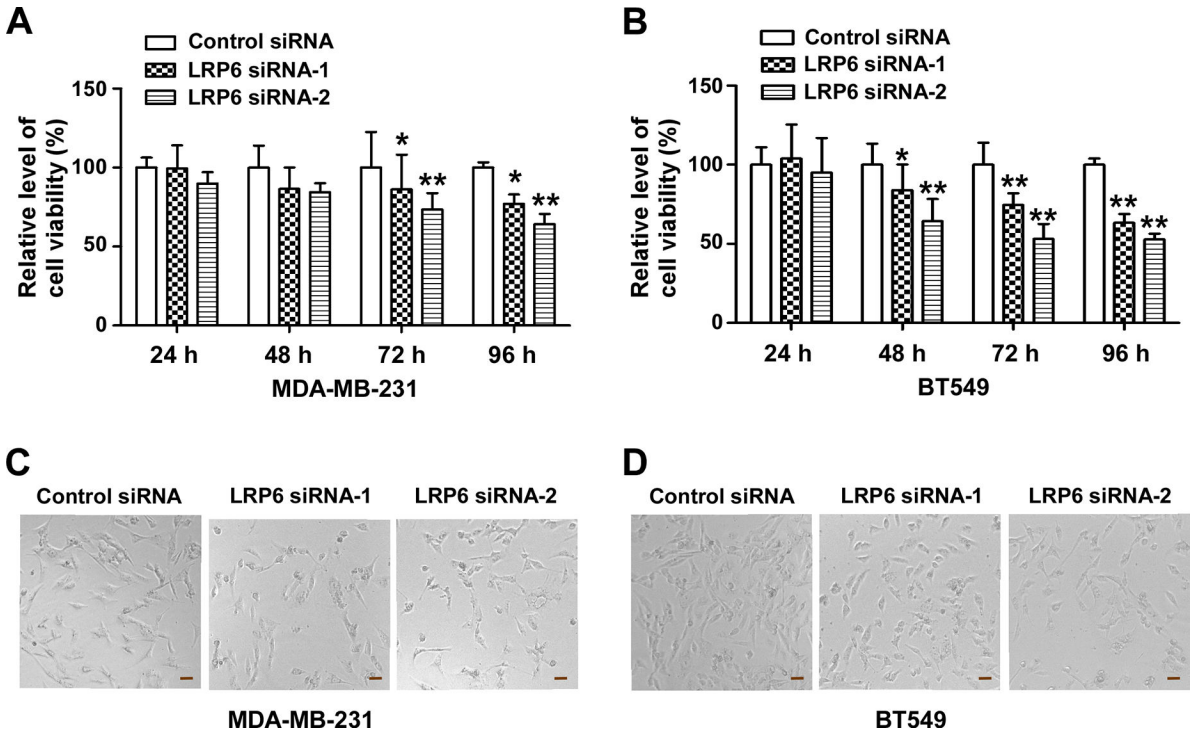

Fig. 3.

Depletion of LRP6 suppresses TNBC cell proliferation/viability. (A, B) MDA-MB-231 and BT549 cells in 96-well plates were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After 24, 48, 72 or 96 h of transfection, cell viability was measured by the Cell Titer Glo Assay system. Data shown are representative of three independent experiments. All the values are the average of triplicate determinations with the s.d. indicated by error bars. *P < 0.05, **P < 0.01 versus control cells. (C, D) MDA-MB-231 and BT549 cells were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After 96 h incubation, images were taken using phase contrast microscopy. Scale bar: 40 μm.

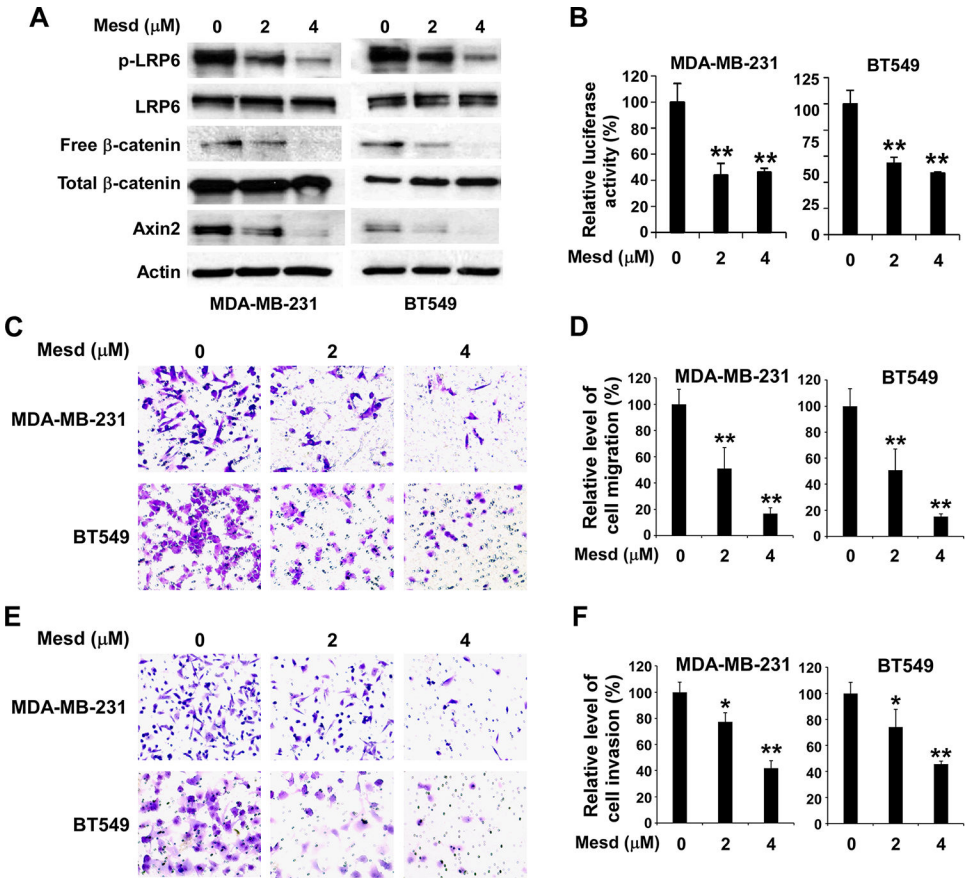

Fig. 4.

Mesd inhibits Wnt phosphorylation and Wnt/β-catenin signaling in TNBC cells and suppresses TNBC cell migration and invasion. (A) MDA-MB-231 and BT549 cells in 6-well plates were treated with Mesd at the indicated concentrations for 24 h. The levels of human LRP6, LRP5, phospho-LRP6 (p-LRP6), cytosolic free human β-catenin, total cellular human β-catenin, and axin2 were examined by Western blotting. All the samples were also probed with anti-human actin antibody to verify equal loading. (B) MDA-MB-231 and BT549 cells in 24-well plates were transiently transfected with the Super8XTOPFlash luciferase construct and β-galactosidase expressing vector in each well. After 24 h incubation, cells were treated with Mesd at the indicated concentrations. The luciferase activity was then measured 24 h later with normalization to the activity of the β-galactosidase. Values are the average of triple determinations with the s.d. indicated by error bars. (C-F) MDA-MB-231 and BT549 cells were treated with Mesd at the indicated concentrations. After incubation of 24 h, the transwell migration assay (C, D) and Matrigel invasion assay (E, F) were performed. (C) Representative images of cell migration. (D) Relative levels of cell migration are significantly decreased by Mesd treatment in MDA-MB-231 and BT549 cells. (E) Representative images of cell invasion. (F) Relative levels of cell invasion are significantly decreased by Mesd treatment in MDA-MB-231 and BT549 cells. Data shown are representative of three independent experiments. All the values are the average of quadruplicate determinations with the s.d. indicated by error bars. * $P < 0.05$, ** $P < 0.01$ versus control cells.

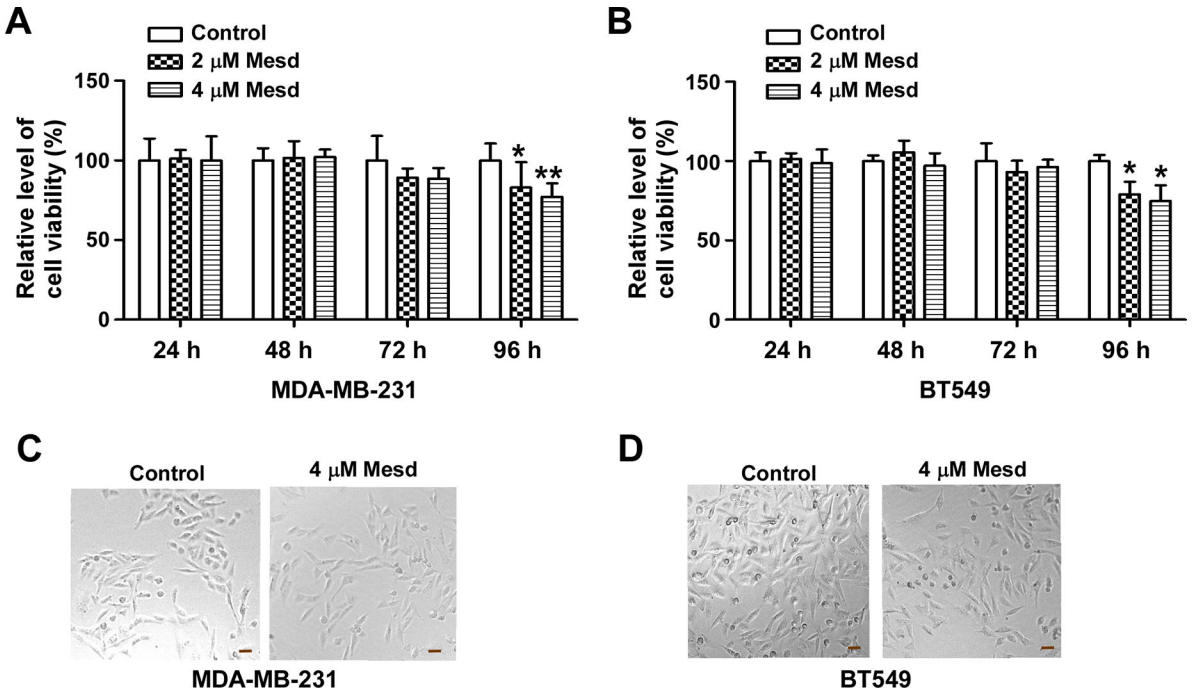

Fig. 5.

Mesd inhibits TNBC cell proliferation/viability. (A, B) MDA-MB-231 and BT549 cells in 96-well plates were treated with Mesd at the indicated concentrations. After incubation for 24, 48, 72 or 96 h, cell viability was measured by the Cell Titer Glo Assay system. Data shown are representative of three independent experiments. All the values are the average of triplicate determinations with the s.d. indicated by error bars. *P < 0.05, **P < 0.01 versus control cells. (C, D) MDA-MB-231 and BT549 cells were treated with Mesd (4 μM) for 96 h, and images were taken using phase contrast microscopy. Scale bar: 40 μm.

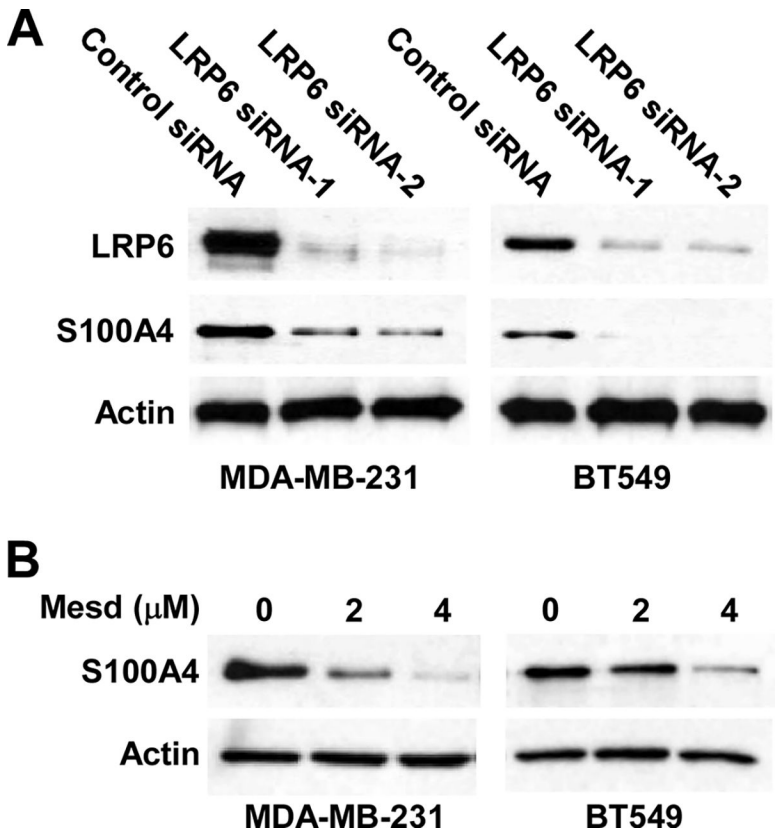

Fig. 6.

LRP6 depletion and Mesd treatment inhibit S100A4 expression in TNBC cells. (A) MDA-MB-231 and BT549 cells in 6-well plates were transiently transfected with 50 nM of LRP6 siRNA-1, LRP6 siRNA-2 or control siRNA. After incubation of 48 h, the levels of S100A4 expression were examined by Western blotting. (B) MDA-MB-231 and BT549 cells in 6-well plates were treated with Mesd at the indicated concentrations for 24 h. The levels of S100A4 were examined by Western blotting. Data shown are representative of three independent experiments. All the samples were also probed with anti-human actin antibody to verify equal loading.