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Research paper

Detection of endogenous LRP6 expressed on human cells by monoclonal antibodies specific for the native conformation

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ABSTRACT

LRP6 is a cell surface molecule that plays a critical role in the Wnt signaling pathway, and is implicated in numerous human diseases. Studies of cellular signaling mediated by LRP6 have relied on overexpression experiments, due to the lack of good monoclonal antibodies (mAbs) reactive with native LRP6 ectodomain. By using native recombinant LRP6 ectodomain fragment produced in mammalian expression system, we succeeded in developing a panel of anti-human LRP6 mAbs. Selected mAbs were capable of staining endogenous LRP6 on cell surface by using flow cytometry and immunofluorescence microscopy, and enriching detergent-solubilized LRP6 from cell lysate by immunoprecipitation.

1. Introduction

The low density lipoprotein (LDL) receptor superfamily includes a large number of cell surface receptors implicated not only in lipoprotein metabolism but also in a diverse set of phenomena such as regulation of cell surface protease activity, intracellular signaling during embryonic development, and pathogen uptake (Herz, 2001; Schneider and Nimpf, 2003; Strickland et al., 2002). LDL receptor-related protein (LRP)5 and LRP6 are two highly homologous receptors that constitute a unique subgroup among the LDLR gene family in that they lack NPXY endocytosis signal in the cytoplasmic tail (Strickland et al., 2002). Functionally, LRP5/6 plays critical role in the Wnt pathway by both serving as a co-receptor for the Wnt protein and a receptor for the Wnt antagonist Dkk (Kikuchi et al., 2007). Mutation or variation within LRP6 gene has been linked to human diseases such as coronary artery disease and late-onset Alzheimer disease, making this class of receptor highly relevant to human health (De Ferrari et al., 2007; Mani et al., 2007).

In the current working model, cell surface LRP5/6 undergo clustering upon Wnt stimulation, leading to the phosphorylation of multiple PPPSP motifs present in the intracellular domain of LRP6 by GSK3 and CK1 (Bilic et al., 2007; Davidson et al., 2005). This provides the docking sites for the scaffolding protein axin and further recruits GSK3 (Zeng et al., 2008). It was recently shown that the phosphorylated LRP6 cytoplasmic domain directly inhibits GSK3, leading to the dephosphorylation and stabilization of β-catenin (Piao et al., 2008; Wu et al., 2009). It was also postulated that endocytosis of activated LRP6 via caveolin rather than clathrin-mediated pathway is critical in the signaling, consistent with the lack of NPXY motifs in LRP5/6 (Yamamoto et al., 2006).

Despite the wealth of information we can obtain through tagged receptor overexpression studies, we still need means to detect endogenous, untagged receptors for studies using tissues and cells under the physiologic conditions. For LRP5/6, this has been hampered by the lack of good monoclonal antibodies that recognize native receptor. All commercially

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available antibodies are raised against synthetic peptide derived from LRP5/6 sequence, and their usage is limited to the Western blotting after SDS-PAGE because of the inability to recognize native conformation. Recently, Khan et al. reported a highly sensitive monoclonal antibody recognizing a cytoplasmic domain of LRP6 and successfully applied it for functional and biochemical analyses of LRP6 in nontransfected cells (Khan et al., 2007). However, there is no monoclonal antibody capable of binding to the cell surface LRP5/6 in its native conformation, precluding the easy evaluation of receptor expression in various tissues/cell lines and the assessment of receptor trafficking. In the current study, we have succeeded in establishing several anti-human LRP6 mAbs recognizing native cell surface LRP6, by using highly pure recombinant LRP6 ectodomain fragment as antigen.

2. Materials and methods

2.1. cDNA constructs

The cDNAs coding for human and mouse full-length LRP6 were obtained from Drs. S. Sokol (Beth Israel Deaconess Medical Center, Boston, MA) and F. Hess (Merck Research Laboratories, West Point, PA), respectively. To generate expression plasmid for LRP6 ectodomain fragments, full-length human LRP6 coding region was first transferred to pcDNA3.1 vector (Invitrogen), truncated at either Gly-1225 (corresponds to the last residue of the 4th EGF module) or Cys-1344 (corresponds to the last residue of the 3rd LA module), and C-terminally tagged with octahistidine, resulting in the plasmids LRP6ΔLA -His or LRP6ec-His, respectively. Octahistidine tag was preceded by a tobacco etch virus (TEV) protease site and followed by a basic "velcro" peptide to facilitate tag removal and detection by anti-velcro antibody (Takagi et al., 2001). To generate N-terminally FLAG-tagged LRP6 constructs, the signal sequence of both human and mouse full-length LRP6 was replaced by a prolactin signal sequence followed by a FLAG tag, taken from FLAG-tagged human PAR-4 construct (a gift from S. R. Coughlin). A series of human/mouse chimeric receptor constructs were prepared by swapping these two at unique restriction enzyme site. To this end, HindIII site (present in both human and mouse) was used to produce h195m, PshAI site (present only in human sequence but was introduced in the corresponding position of mouse sequence by mutagenesis) was used to produce h359m and m359h, and EcoRV site (present in both sequences) was used to produce h641m.

2.2. Cell culture and transfection

293T cells and HelaS3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Transient transfection was performed by using Fugene6 (Roche) according to the manufacturer's instructions.

2.3. Recombinant production of LRP6 ectodomain fragments

HEK293SGnT1- cells (kindly provided by G. Korhana) were used for the stable expression of LRP6ΔLA-His or LRP6ec-His. Cells were co-transfected with either of the LRP6 plasmid (neomycin resistance) and a mouse Mesd plasmid (puromycin resistant, gift from Dr. B. Holdener) (Hsieh et al., 2003) by electroporation, plated on 96-well plates and selected for resistance to 1 mg/ml G418 (Gibco) and 10 μg/ml puromycin (Sigma). The clone with the highest secretion level of LRP6 fragment, as judged by Ni-NTA pulldown experiments, was cultured in DMEM + 10% FCS containing the selection antibiotics described above using BelloCell™ high density cell culture system (CESCO Bioengineering). Recombinant LRP6 fragments were purified from the culture supernatants by direct Ni-NTA agarose chromatography. The sample was treated with hexahistidine-tagged TEV protease at room temperature, and passed through Ni-NTA agarose column to remove cleaved tag and the enzyme. The final purification was achieved by gel filtration chromatography on a Superdex 200 column (2.5 × 60 cm, GE Healthcare), and the purified proteins were concentrated to ~1 mg/ml and stored at −80 °C until used. Typically, 0.5–1 mg of pure proteins was obtained from 1 l of culture supernatants, and the entire purification process was completed within 4 days.

2.4. Production of monoclonal antibody

Balb/c mice were immunized subcutaneously with LRP6ec protein emulsified in complete Freund's adjuvant. Injections (25 μg in incomplete Freund's adjuvant) were repeated 4 times with an interval of 2 weeks. Spleen cell from immunized mice were fused with the SP2/0 mouse myeloma cell line using polyethylenegrycol method. Hybridomas were selected in complete RPMI1640–10% fetal calf serum (FCS) with HAT supplement and subjected to limiting dilution culture in 96-well plates, followed by culture in HT medium. Supernatants were screened for reactivity toward native LRP6ec coated onto microtiter plates with ELISA, and multiple positive hybridomas were obtained after cloning by limiting dilution. Antibody subclass was determined by mouse antibody isotyping kit (Serotec). Fab fragments were prepared from purified IgG as described previously (Sangawa et al., 2008).

2.5. Flow cytometry

293T cells were transiently transfected with various LRP6 chimeric constructs together with plasmid encoding mouse Mesd (5/1 wt/wt). After 48 h, the cells were washed, collected by centrifugation, resuspended in DMEM containing 1% FCS (DMEM/FCS), and dispensed into a V-bottom 96-well plate. The cells were then incubated with DMEM/FCS containing 10 μg/ml anti-LRP6 mAb and 5 μg/ml rabbit anti-FLAG polyclonal antibody (Sigma) on ice for 1 h, washed twice with DMEM/FCS, and resuspended in DMEM/FCS containing FITC-conjugated goat anti-mouse IgG (Zymed, 1/100) and phycoerythrin-conjugated donkey anti-rabbit IgG (Chemicon, 1/200). After 30 min incubation on ice, the cells were washed once and suspended in PBS, and analyzed on a Cell Lab Quanta instrument (Beckman-Coulter).

2.6. Immunoprecipitation/immunoblotting

HelaS3, 293 T, and LRP6/Mesd-transfected 293 T cells were treated with or without conditioned media collected from Wnt3a-expressing L cells (gift from S. Takada)(Shibamoto

et al., 1998) for 1 h and solubilized in lysis buffer (50 mM Tris–HCl, pH7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF,1 mM Na_3VO_4, 0.1 mM leupeptin,1 mM PMSF). Lysates were incubated with 5 μg/ml A59 and Protein G-beads, and the precipitated materials were subjected to SDS-PAGE and transferred to membrane. Membranes were blocked in 5% BSA and incubated with anti-phospho-LRP6 (Ser1490) (Cell Signaling Technology), probed with peroxidase-conjugated anti-rabbit IgG (Sigma) followed by visualization with enhanced chemiluminescence reagent.

2.7. Immunofluorescence microscopy

HelaS3 cells were seeded onto 18-mm glass coverslips coated with poly-L lysine. The cells were fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 20 min at room temperature. After blocking with 5% BSA in PBS, they were then incubated with 15 μg/ml A59 at 4 °C for overnight. After washing three times with PBS, cells were incubated with Alexa 488-conjugates anti-mouse IgG (Invitrogen) and Hoechst33342 (Invitrogen) for 2 h at room temperature. The coverslips were mounted using Vectashield (Vector Laboratories). Fluorescence images were obtained using a digital fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

2.8. Luciferase reporter assay

293T cells seeded in 24-well plates were transfected with 25 ng of TOPFlash or FOPFlash (Upstate) together with 2.5 ng of the *Renilla* luciferase construct, pRL-TK (Promega). Eighteen hours after the transfection, conditioned media of L cells expressing Wnt3a or control L cells were added and incubated for an additional 6 h prior to the harvest and cell lysis. Reporter activities were measured using a dual luciferase reporter assay system (Promega). Each assay was performed in duplicate.

3. Results and discussions

3.1. Recombinant production of LRP6 ectodomain fragments

Production of monoclonal antibody specific for native proteins depends critically on the availability of pure protein preparation with structural and functional integrity, especially during the step of screening. Thus our effort was first directed toward obtaining high-quality recombinant soluble ectodomain fragment of LRP6. It has been known that normal biosynthesis of LRP5/6 proteins in cells require a special chaperon protein Mesd/Boca (Hsieh et al., 2003; Culi and Mann, 2003). Mesd is required for the correct folding of YWTD β-propeller domain of many, if not all, LDLR family members (Culi et al., 2004), and co-transfection of Mesd with soluble ectodomain fragments of LRP6 facilitated efficient secretion of these fragments (Koduri and Blacklow, 2007). We established stable cell lines expressing two different LRP6 ectodomain fragments by co-transfection with Mesd. The longer construct (LRP6ec) spans residues 1–1363 of human LRP6 corresponding to the entire ectodomain segment, and the shorter construct (LRP6ΔLA) spans residues 1–1248 and lacks lipoprotein-binding LA modules (Fig. 1A). Although both fragments were efficiently secreted into the culture medium, initial attempts to purify these fragments from concentrated culture supernatants were unsuccessful due to the disulfide-bonded aggregate formation of LRP6 together with contaminating proteins. The formation of intermolecular disulfide-bonded aggregates has been observed earlier which was reduced, but not eliminated, in the presence of Mesd (Hsieh et al., 2003). We reasoned that LRP6 was prone to disulfide-mediated aggregation even after the Mesd-assisted successful secretion into the medium, and decided to apply unprocessed culture supernatant directly to the Ni-NTA resin to minimize the contact of LRP6 protein with contaminating proteins. This simple modification in the procedure, in combination with the minimized total purification time (<3 days) contributed to the successful purification of monomeric fragments with no apparent contamination (Fig. 1B). It is clear from the non-reducing SDS-PAGE that both LRP6ec and LRP6ΔLA preparation are devoid of oligomers, and disulfide-bonded intermolecular aggregates do not form once LRP6 was separated from contaminating proteins. The migration positions for each fragments were consistent with their estimated molecular sizes (153 and 140 kDa + carbohydrate chains). Earlier reports have suggested that cell surface LRP6 undergo homodimerization mediated by their extracellular region (Liu et al., 2003). Even though the molecular sizes for LRP6ec and LRP6ΔLA estimated by gel filtration (300 and 250 kDa, respectively) are compatible with the homodimer configuration, negative-stain electron microscopy of purified fragments revealed monomeric molecules with extended conformation (manuscript in preparation). We conclude that LRP6 ectodomain does not have strong tendency to form homodimer and speculate that dimerization may occur only at cell surface where the receptors are confined in the 2D plane and thus very weak and transient interaction become detectable.

3.2. Production of anti-LRP6 mAbs and their epitope mapping

The purified LRP6ec was used as an immunogen and monoclonal antibodies were developed using a standard procedure. Nine monoclonal antibodies, all IgG1 subclass, that recognize purified LRP6ec protein in ELISA were obtained (Table 1). Among them, seven mAbs also reacted with the LRP6ΔLA that lacked LA module region. In contrast, two mAbs (clones A10 and A47) failed to recognize LRP6ΔLA, suggesting that epitope for these two antibodies lie in the most membrane-proximal region containing three LA modules. All mAbs were shown to recognize conformational epitopes on the native protein, because they could not react with SDS-denatured LRP6 in the Western blotting format (data not shown). Interestingly, A10 and A47 were able to detect LRP6 in immunoblot when the samples were run under non-reducing condition, suggesting that the native conformation of the Cys-rich LA module is partially maintained in the SDS-denatured samples.

In order to map epitope locations for the 7 mAbs within the large β-propeller region of LRP6, we used human/mouse chimeric receptor strategy. As all mAbs bound human but not mouse LRP6 expressed on 293T cells, epitope location can be narrowed down by checking reactivity of each mAb toward various human/mouse chimeric receptors. Chimeric constructs, all containing N-terminal FLAG tag, were transiently expressed on 293T cells and FLAG-positive cell population

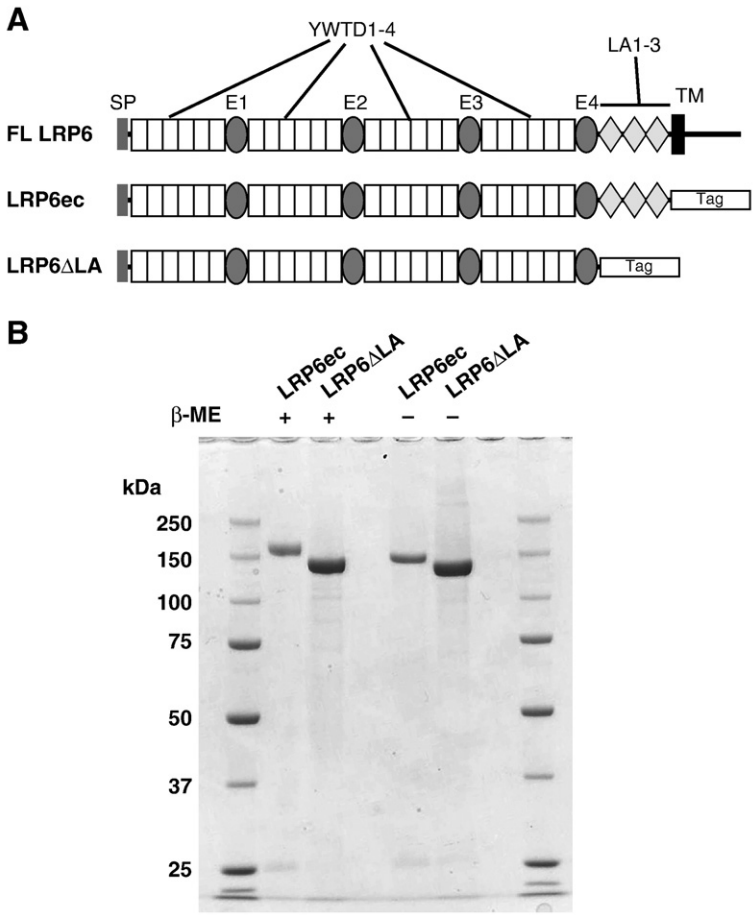

Fig. 1. Production of recombinant soluble ectodomain fragments of human LRP6. (A) Schematic representation of full-length and truncation constructs of LRP6. Signal peptide (SP), EGF repeats (E1 to E4) separated by YWTD propeller domains (YWTD1-4), LA modules (LA1-3), and transmembrane domain (TM) are denoted. "Tag" sequence, appended to the C-termini of the truncated fragments (LRP6ec and LRP6ΔLA), contain a TEV protease site, an octahistidine, and a basic "velcro" sequence. (B) SDS-PAGE analysis of purified LRP6 fragments. Tag-cleaved ectodomain fragments were subjected to a 10% SDS-PAGE in the presence (+) or absence (−) of reducing agent β-mercaptoethanol (β-ME). Note that both proteins are highly pure and devoid of any contaminants as well as oligomeric species.

was analyzed for anti-LRP6 mAb binding. As shown in Fig. 2, all mAbs reacted with h641m mutant, indicating that the epitope for all mAbs are located within the N-terminal half of the LRP6 ectodomain. B5 also bound m359h but not h359m, suggesting that it recognizes segment spanning residues 359–641. Another group of mAbs (A11, A12, and A59) bound h359m but not h195m, narrowing down the epitope to residues 195–359. The remaining three (L35, A81, and A91) recognize 1–195 as judged by the reactivity toward h195m mutant. In summary, we obtained a panel of anti-human LRP6 mAbs directed toward different regions of LRP6 ectodomain covering the 1st and the 2nd propeller domains as well as the membrane-proximal LA modules.

Table 1
List of monoclonal antibodies established in this study.

Name	Subclass	Reactivity		LA domain requirement
		LRP6ec	LRP6ΔLA	
A10	IgG1	+++	−	Yes
A11	IgG1	+++	+++	No
A12	IgG1	+++	+++	No
A47	IgG1	++	−	Yes
A59	IgG1	+++	+++	No
A81	IgG1	++	++	No
A91	IgG1	+	+	No
B5	IgG1	++	++	No
L35	IgG1	+++	+++	No

3.3. Detection of endogenous LRP6 on human cells by flow cytometry

Next we decided to utilize the mAbs to detect endogenous LRP6 expressed on cells. We focused on an mAb A59, because of its high affinity toward LRP6 suggested by the highest staining intensity in the FACS analysis (Fig. 2). We analyzed A59 binding to two human cell lines (HelaS3 and 293T) that had been used in Wnt-dependent signaling studies and hence should express endogenous LRP6 (Yamamoto et al., 2006; Khan et al., 2007). As shown in Fig. 3, HelaS3 exhibited positive staining with A59 (5-fold increase in the mean fluorescent intensity (MFI) over control IgG). In contrast, signals from 293T cells were only marginally increased (1.8-fold), making

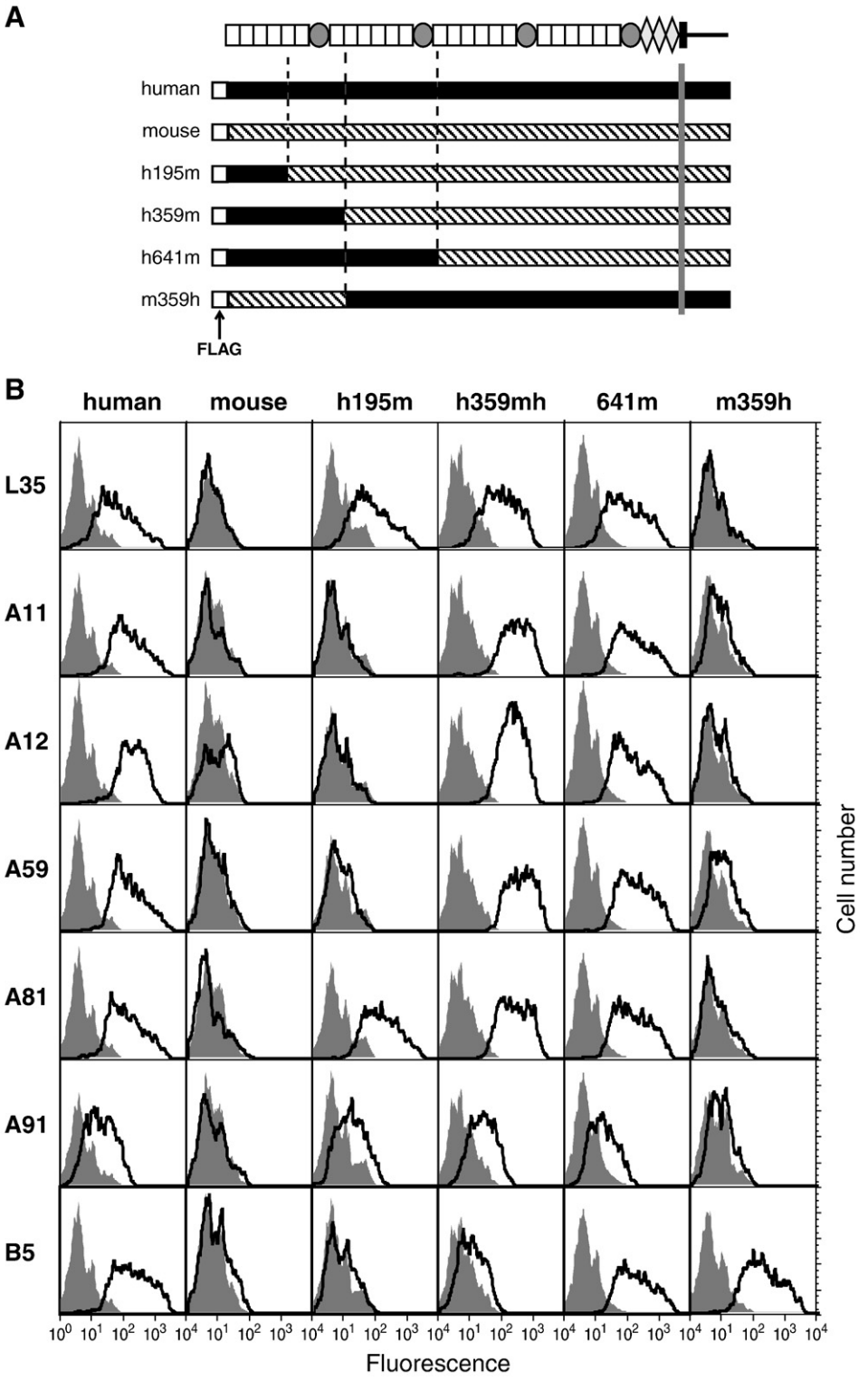

Fig. 2. Epitope mapping of mAbs using human/mouse chimeric LRP6. (A) The chimeric constructs used for epitope mapping. Constructs are comprised of human (black) and mouse (hatched) LRP6 sequences and an N-terminal FLAG tag. (B) Flow cytometric analysis of chimeric LRP6. 293T cells expressing indicated LRP6 mutants were analyzed for staining with various anti-LRP6 mAbs (solid lines) or control IgG (gray area). Shown are the histograms for cell populations that are positive for FLAG staining.

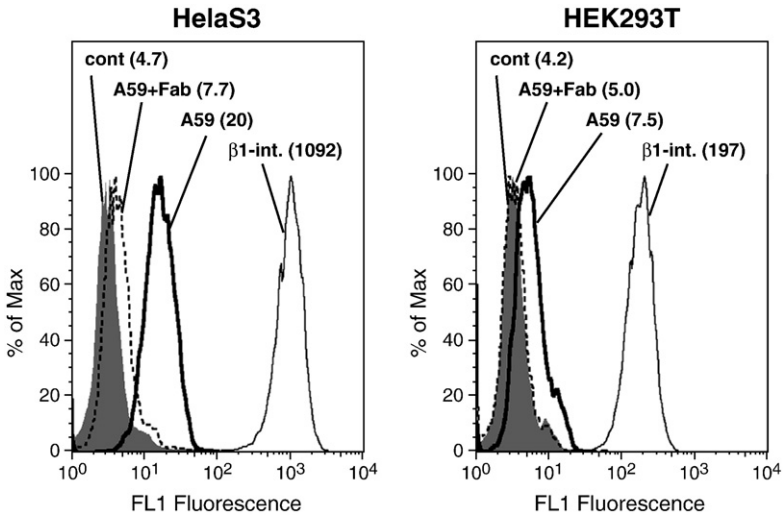

Fig. 3. A flow cytometric analysis of endogenous cell surface LRP6 using mAb A59. HelaS3 or 293T cells were incubated with anti-LRP6 mAb A59 in the absence (thick solid lines) or presence (thin dashed lines) of 10-fold molar excess of A59 Fab fragment and stained with FITC-labeled secondary antibody specific for mouse IgG Fc region. Staining with anti-human β1 integrin mAb TS2/16 (thin lines) and control IgG (gray area) are also shown. Values in parenthesis denote mean fluorescence intensity for each histogram.

it difficult to tell if it represented specific binding. We therefore prepared Fab fragment of A59 and used it to block the IgG binding. As clearly shown in Fig. 3 (dotted lines), 10-fold molar excess addition of A59 Fab resulted in the histograms indistinguishable from control staining (gray), confirming that the increased fluorescence does represent specific A59 binding. The very weak A59 binding signal, however, suggests that the expression level of LRP6 on 293T cells is extremely low. We therefore compared the binding of A59 with that of TS2/16, a high affinity mAb against β1 integrin that is abundantly expressed on these cells. In both cell lines, β1 integrin expression was ~70-fold higher than LRP6. Since the expression level of common adhesion receptor β1 integrin on human cells such as K562 is in the range of ~200,000/cell (Tsuchida et al., 1997), LRP6 expression on 293T is estimated to be ~3000 molecules/cell, assuming that A59 has affinity comparable to TS2/16 ($K_D = 0.3$ nM). This underscores the difficulty in detecting endogenous LRP6 using standard techniques.

3.4. Immunoprecipitation of endogenous LRP6

In addition to flow cytometry, immunoprecipitation is also an essential immunological detection method that had been difficult to apply for endogenous LRP6 because commercially available antibodies do not react with native receptors. Immunoprecipitation can be used to analyze associating molecules, and to simply enrich low abundance proteins from cell lysate. Although Kahn et al reported that they could directly detect phosphorylated LRP6 from Wnt3a-treated 293T cell lysate by using anti-phospho-Ser1490 antibody (Khan, 2007), we could not detect reliable signal in similar experiment (data not shown). When the LRP6 protein was enriched by immunoprecipitation with A59 prior to the Western blotting, however, very faint but reproducible reactivity was observed with Wnt3a-treated 293T cell lysate (Fig. 4, lane 4). The signal was much more pronounced when HelaS3 cells or LRP6-transfected 293T cells were used (Fig. 4). The signal intensity of the bands in different cells roughly corresponded to the expression level evaluated by flow cytometry. Curiously, we observed that anti-phospho-Ser1490 reactive band appeared in the blot of untreated cells as well as Wnt3a-treated cells, although the intensity was significantly lower. Whether this indicates the presence of basal phosphorylation of Ser1490 in the absence of Wnt activation or the cross reactivity of the anti-phospho-Ser1490 antibody to nonphosphorylated LRP6 remains unknown at this point.

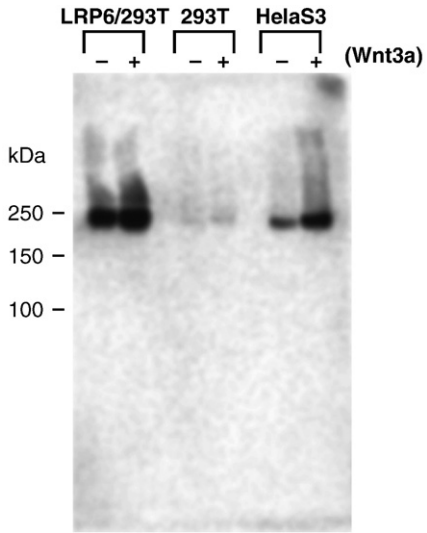

Fig. 4. Detection of phosphorylated LRP6 in Wnt3a-treated cells. LRP6 protein was immunoprecipitated from lysates of nontransfected or LRP6/Mesd-transfected cells incubated with or without Wnt3a-containing conditioned medium by using mAb A59, and probed with anti-phosphoLRP6 Ser1490 antibody.

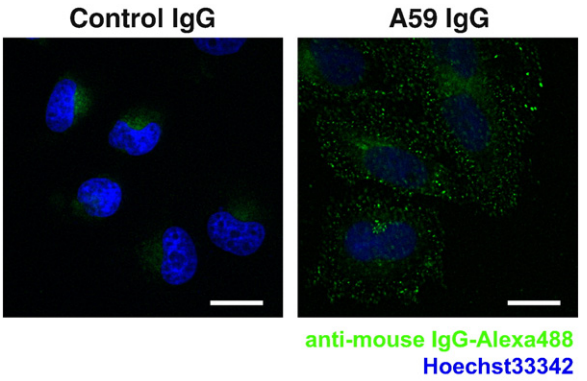

Fig. 5. Immunostaining detection of endogenous LRP6 in HeLaS3 cells using A59. HeLaS3 cells were fixed, incubated with A59, and then stained with Alexa 488-conjugated anti-mouse IgG and Hoechst33342. Bar: 20 μm.

3.5. Immunofluorescence localization of endogenous LRP6 on HelaS3 cells

Another very important utility of mAbs reactive with native receptors is the immunofluorescent imaging. So far all the LRP6 cellular localization studies relied on either epitope-tagged or GFP-tagged LRP6 overexpression. We stained HelaS3 cells using A59 and analyzed endogenous cell surface LRP6 localization for the first time. As expected from the low expression level, we could observe fairly low but specific staining on the surface of HelaS3 cells (Fig. 5). Interestingly, the A59 staining was not uniform and appeared as small speckles indicative of a cluster formation. Attempts to stain intracellular pool of LRP6 by permeabilizing the cells with Triton X-100 prior to the staining did not succeed because such treatment somehow inhibited the overall staining (data not shown).

3.6. anti-LRP6 mAbs does not inhibit Wnt signaling

Within the extracellular region of LRP6, it is postulated that the receptor agonist (i.e., Wnt proteins) binds to the N-terminal half comprising of the first and the second propeller domains (Liu et al., 2003), while the antagonist (i.e., Dkk) binds to the C-terminal half (Mao et al., 2001). So we sought if any of the mAbs directed against N-terminal half of LRP6 compete with Wnt and hence inhibit Wnt-induced signaling. Since LRP6 overexpression in 293T cells resulted in an exaggerated TCF transcription activity independent of Wnt addition, we evaluated the effect of mAbs on the Wnt3a-induced TCF signaling in untransfected 293T cells using TOPFLASH reporter assay system. As shown in Fig. 6, Wnt3a treatment of cells for 6 h results in an ~2-fold increase in the basal activity. When mAbs were present at 50 μg/ml during the incubation period, no inhibition was observed. Instead, some of the mAbs even enhanced the luciferase induction. Although mechanism for this enhancement is not clear, none of the mAbs are likely to have ability to compete with Wnt binding to LRP6.

In conclusion, we have succeeded in developing several mAbs that can recognize native human LRP6 ectodomain region. There are some commercially available mAbs that claim they bind native LRP6 protein. When we tested those mAbs for the reactivity toward the native LRP6 protein, however, all of them failed to show meaningful binding in experiments using FACS, immunoprecipitation, and ELISA. This is not surprising because most mAbs were developed using LRP6-derived synthetic peptides and anti-peptide mAbs generally does not recognize natively folded proteins unless the epitope is well exposed. The availability of mAbs capable of recognizing native LRP6 on cell surface enables analysis of LRP6 trafficking in cells without relying on the overexpression, which may cause unwanted signal perturbation. Moreover, they can be used to screen for the presence of LRP6 proteins on cells or body fluids, opening the way to explore its possible quantitative correlation to various disease states.

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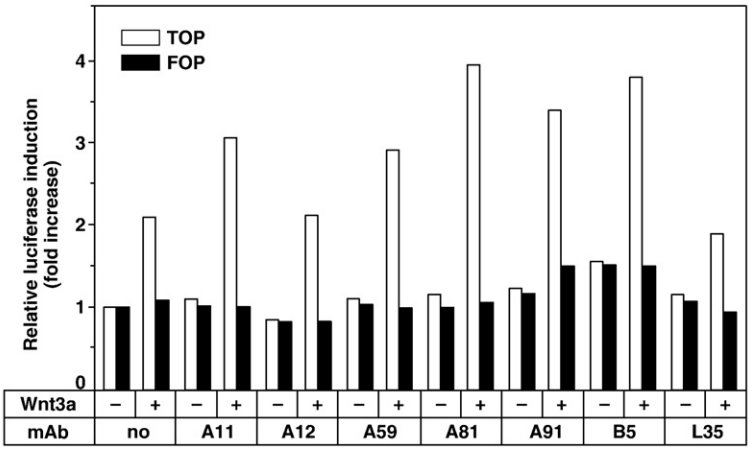

Fig. 6. Effects of anti-LRP6 mAbs on the Wnt3a-induced TCF signaling. 293T cells were transfected with TCF reporter (i.e., TOP) or control (i.e., FOP) luciferase construct, together with the second *Renilla* luciferase construct. Eighteen hours after the transfection, cells were incubated for additional 6 h with 50 μg/ml anti-LRP6 mAbs together with control or Wnt3a-containing conditioned media. Luciferase activity of TOPflash or FOPflash-transfected cells were normalized against *Renilla* luciferase activity and expressed as the relative increase from the control value obtained in the absence of Wnt3a and mAbs.

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