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Expression, purification and functional characterization of Wnt signaling co-receptors LRP5 and LRP6

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ABSTRACT

Activation of the Wnt signaling cascade plays a pivotal role during development and in various disease states. Wnt signals are transduced by seven-transmembrane Frizzled (Fz) proteins and the single-transmembrane LDL-receptor-related proteins 5 or 6 (LRP5/6). Genetic mutations resulting in a loss or gain of function of LRP5 in humans lead to osteopenia and bone formation, respectively. These findings demonstrate the genetic link between LRP5 signaling and the regeneration of bone mass. Herein we describe for the first time the production and characterization of soluble ectodomains of LRP5 and LRP6, (EC-LRP5, EC-LRP6). We have produced these proteins in amounts that are compatible with both *in vitro* and cell-based assays to study their binding properties. Purified EC-LRP5 and EC-LRP6 were able to interact with Wnt signaling components Dkk1 and Dkk2 and their functionality was confirmed in cell-based Wnt signaling assays. Hence, tools are now available to explore LRP5/6 interaction with other proteins and to screen for synthetic or natural compounds and biologics that might be novel therapeutics targeting the Wnt pathway.

Introduction

Wnt are secreted, cystein-rich glycoproteins that regulate various differentiation events during embryonic development and can lead to tumor formation when aberrantly activated [1–4]. The Wnt receptor complex that activates the canonical pathway contains two components: a member of the Frizzled (Fz)[2] family (there are 10 of these seven-transmembrane-span proteins in humans) and either one of two single-span transmembrane proteins, low-density lipoprotein-receptor-related proteins 5 (LRP5) and LRP6 [5–10]. LRP5/6 activation is inhibited by secreted proteins belonging to the Dickkopf (Dkk) family (for reviews see [11,12]). LRP6-deficient mice are prenatal lethal, whereas LRP5 deficient mice have normal embryogenesis, grow to adulthood and are fertile, but show defects in bone accrual, eye development, lipoprotein and glucose metabolism [13–15]. Moreover, a high bone mass phenotype has been associated with the single point mutation G171V in LRP5 [16,17], outlying its critical importance in bone homeostasis. LRP5G171V variant reduces Dkk1 inhibition of the Wnt signaling and therefore suggests that interfering with LRP5/Dkk1 interaction could be a viable approach for therapeutic intervention to increase bone mass.

The Dkk family comprises four members (Dkk1 to Dkk4) and a unique Dkk3-related protein named Soggy (Sgy). Dkks contain two characteristic cysteine-rich domains (Cys-1 and Cys-2) separated by a linker region of variable length [18,19]. Cys-2, in particular, is highly conserved among all members of the family and contains 10 conserved cysteine residues; this is similar to the proteins of the colipase family, with which Dkk proteins share weak sequence similarity [19,20].

The identification of the Wnt canonical signaling pathway as a key actor in skeletal biology and disease provided new avenues to find new bone anabolic drugs for the treatment of osteoporosis. Disrupting Dkk1 inhibition of the Wnt signaling pathway may provide the means to achieve this goal. In this paper we describe the expression, purification and functional testing of key components of this signaling pathway, Dkk1, Dkk2, LRP5 and LRP6. The availability of these functionally active proteins provides us the tools to further study this pathway and screen for novel drug candidate for the management of bone diseases.

Materials and methods

Reagents

Proteins were expressed using commercially available mammalian cell lines: CHO (*ATCC CCL-61*), L-cells (*ATCC CRL-2648*) and

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[2] *Abbreviations used:* Fz, Frizzled; LRP5, lipoprotein-receptor-related proteins 5; Dkk, Dickkopf; Sgy, Soggy; CM, conditioned medium; HRP, Horse Radish Peroxydase; CV, Column Volumn; LIA, luminescent interaction assay; IMAC, Immobilized Metal Affinity Chromatography.

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FreeStyle 293-F cells (Invitrogen #R790-07). L-cells cells were grown in DMEM (Gibco #31053-028), CHO cells in DMEM/F12 (HAM) (Gibco #21765-029). Media were supplemented with 5% FBS (Sigma #F2442), 1% sodium pyruvate (BioWhittaker #BE13-115E), 1% L-glutamine (BioWhittaker #BE17-605E), 100 U/ml penicillin and 100 μg/ml streptomycin (BioWhittaker #DE17-602E). FreeStyle 293-F cells were grown in FreeStyle 293 expression medium (Gibco #12338-018) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Blasticidine at 0.5 μg/ml (Invitrogen #R210-01) was added for amplification of the stably transfected cells. Heparine (Sigma #H3149) was added at 0.1 g/L for production.

Cloning and expression of EC-LRP5 and EC-LRP6

Secreted ectodomains of human LRP5 and LRP6 (here after named EC-LRP5 and EC-LRP6) were constructed as follows. hLRP5 and hLRP6 coding sequences (kindly provided by DeLi Shi) were first mutated to introduce StuI and HindIII restriction sites after aa1389/aa1365 of, respectively, hLRP5 and hLRP6 using the QuickChange site-directed mutagenesis kit (Stratagene). Mutated cDNAs were then subcloned, respectively, into pcDNA3 and pCS2 expression vectors using appropriate restriction sites to generate cMyc-6His-tag fusion.

FreeStyle 293-F cells were co-transfected as described by the manufacturer (Invitrogen) with EC-LRP coding vectors and vectors containing coding sequences for Mesd (pcDNA3.1-Flag-MesdC2) and Rap (pRKh-Rap) [21–23] at a ratio of 1:0.5:0.3, for a total of 1.2 μg DNA/10^6 cells. Conditioned medium (CM) was harvested 3–4 days after the transfection by centrifugation at 2000*g* for 10 min. Cells were then diluted at 10^6 cells/ml in fresh medium and grown for another 2–4 day period. The same harvest procedure was used two more times. EC-LRP5/6 conditioned media were subjected to western blotting using tetra-His antibody (Quiagen) and expression levels were estimated by comparison with the known amounts of a reference protein.

Cloning and expression of mDkk1-HRP and mDkk2-HRP

To allow detection of Dkk1 and Dkk2 proteins, they were fused to Horse Radish Peroxydase (HRP). These proteins were also tagged with 6His at their C-terminus and an IgK signal peptide was added at their N-terminus, allowing efficient secretion of the proteins in the cell culture medium. A vector expressing IgK/mDkk1(aa31-end)/HRP/6His was obtained as follows. A PCR product corresponding to the HRP open reading frame was obtained using EcoRI-HRP/sense and HRP-6His-Stop codon/antisense primers and pCS2-xmDkk1-HRP (kindly provided by Christof Niehrs) as template. The PCR product was digested by EcoRI and cloned into EcoRI/PmeI digested pSK-GW-032 plasmid, a derivative of pEF-Dest51 expressing mDkk1 [24].

A vector expressing IgK/mDkk2/HRP/6His was obtained as follows. A PCR product corresponding to IgK/mDkk2 (aa28-259) was obtained using BsrGI-IgK/sense and Dkk2-EcoRI/antisense primers and pSec-Tag2C-mDkk2 (kindly provided by Chi Faucheu) as template. The PCR product was digested by BsrGI/EcoRI and cloned into BsrGI/EcoRI digested pSK-GW032-HRP-6His (described above).

CHO cells were transfected with mDkk1-HRP and mDkk2-HRP coding constructs using Fugene (Roche, #1814443) as described by the manufacturer. Stably expressing clones were obtained for each cell line after selection with 5 μg/ml blasticidine (Invitrogen, #46-1120), followed by limited dilution in 96-wells plates. mDkk1-HRP and mDkk2-HRP expression was assayed for all clones by luminescence assay. The best expressing clones were then amplified for production using 2125 cm^2 roller bottles and about 100 L of conditioned cell culture supernatant were prepared for each protein.

Protein purification

All purification steps were performed either on ice or in a refrigerated closet, operating at 4 °C using an Äkta purifier system (GE Healthcare Life sciences) and monitored by on-line UV detection at 280 nm and 254 nm. Bellow we describe a typical purification process for each of the proteins used in this study.

Purification of EC-LRP6 from culture medium

Two liters of culture medium were thawed in cold water and supplemented with 100 mM Pipes pH 6.5 buffer and 3 M NaCl solutions to obtain 10 mM Pipes pH 6.5 and 300 mM NaCl final concentrations. The sample was centrifuged (Beckman Avanti XP, rotor JLA 16.250) at 29,416*g* for 1 h at 4 °C before loading at 2.5 ml/min on a 50 ml Ni^{2+} chelating sepharose fast flow column (GE Healthcare Life sciences) equilibrated in 10 mM Pipes pH 6.5, 500 mM NaCl. The flow rate was set to 5 ml/min when the UV detector displayed an A_{280nm} value of half the maximum value observed during the load phase and conserved until the end of this purification step. When A_{280nm} returned to the baseline level, the column was washed with a 20 mM Pipes pH 6.5, 500 mM NaCl and 10 mM imidazole buffer to remove non-specifically bound material. A wash with a 10 mM Pipes pH 6.5, 100 mM NaCl buffer reduced the ionic strength of the buffer. Then EC-LRP6 protein was eluted from the column by a linear gradient of imidazole up to 250 mM with a buffer containing 10 mM Pipes pH 6.5, 100 mM NaCl, 500 mM imidazole. The presence of EC-LRP6 in the elution fractions was assayed by Coomassie blue stained 4–12% SDS–PAGE. The fractions containing the EC-LRP6 protein were pooled and diluted (1/3) with a 10 mM Pipes pH 6.5 to further reduce the ionic strength before application on a 5 ml HiTrapQ column (GE Healthcare LifeSciences, equilibrated in 10 mM Pipes pH 6.5, 100 mM NaCl) at 2.5 ml/min. Elution was performed stepwise at 5 ml/min, with steps at 200, 250 and 300 mM NaCl followed by a 25 ml linear gradient to 500 mM NaCl. The presence of EC-LRP6 in the elution fractions was assayed by Coomassie blue stained 4–12% SDS–PAGE. The fractions containing the EC-LRP6 protein were pooled.

Purification of EC-LRP5 from culture medium

Four hundred and fifty milliliters of culture medium were thawed in cold water and supplemented with 100 mM Tris pH 8 buffer, 2 M imidazole solution and NaCl to obtain 50 mM Tris pH 8, 300 mM NaCl and 10 mM imidazole final concentrations. The sample was filtered through a GE Express Millipore filter (0.22 μm) at 4 °C before application at 0.6 ml/min on a Ni^{2+} affinity prepacked column (His Trap, 5 ml, GE Healthcare Life sciences) equilibrated in 50 mM Tris pH 8, 300 mM NaCl. The flow rate was set to 5 ml/min when the UV detector displayed a A_{280nm} value of half the maximum value observed during the load phase and conserved until the end of this purification step. When A_{280nm} returned to the baseline level, the column was washed with a 50 mM Tris pH 8, 300 mM NaCl, 20 mM imidazole buffer to remove non-specifically bound material. The ionic strength is reduced by two successive washes with 10 mM Pipes pH 6.5, 300 mM NaCl buffer followed by a 10 mM Pipes pH 6.5, 100 mM NaCl buffer. Then EC-LRP5 protein was eluted from the column by a 25 ml linear gradient to 250 mM imidazole with a buffer containing 10 mM Pipes pH 6.5, 100 mM NaCl, 250 mM imidazole. The presence of EC-LRP5 in the elution fractions was assayed by Coomassie blue stained 4–12% SDS–PAGE and western blotting. The fractions containing the EC-LRP5 protein were pooled.

mDkk1-HRP purification

Four thousand and five hundred milliliters of culture medium were thawed and supplemented with 1 M Tris pH 8, 1 M NaCl and 2 M imidazole solutions to obtain 100 mM Tris pH 8, 250 mM NaCl and 10 mM imidazole as final concentrations. The sample was filtered through a GE Express Millipore filter (0.22 μm) at 4 °C before application at 4.5 ml/min on a 50 ml Ni^{2+} sepharose fast flow (GE Healthcare Life sciences) equilibrated in 100 mM Tris pH 8, 250 mM NaCl and 10 mM imidazole. After extensive washing with the equilibration buffer, the UV_{A280nm} returned almost to zero and the flow rate was raised to 5 ml/min. Elution was performed as a two step procedure, with a linear gradient to 38.5 mM imidazole in 3 Column Volumn (CV) and hold at this point for one more CV, followed by a 250 ml linear gradient to 200 mM imidazole. The luminescence of the elution fractions was measured and the positive fractions were pooled, diluted 10 times with 50 mM Tris pH 9 and loaded on a 5 ml HiTrapQ column (GE Healthcare Life sciences) at a 5 ml/min flow rate. Elution was performed by a linear gradient to 1 M NaCl in 10 CV at 2.5 ml/min. The luminescence of the elution fractions was measured and the mDkk1-HRP corresponding fractions were pooled.

mDkk2-HRP purification

Three thousand and four hundred milliliters culture medium were thawed in the water bath and supplemented with 1 M Tris pH 8, 1 M NaCl and 2 M imidazole solutions to obtain 50 mM Tris pH8, 350 mM NaCl and 10 mM imidazole final concentrations. The sample was centrifuged in a Beckman Avanti XP centrifuge with the JLA 16.250 rotor, at 14,000 rpm, 1 h, 4 °C, before application at 3.5 ml/min on a 50 ml Ni^{2+} sepharose fast flow (GE Healthcare Life sciences) equilibrated in 50 mM Tris pH 8, 350 mM NaCl and 10 mM imidazole. The flow rate was set to 5 ml/min then 10 ml/min when the UV detector displayed a A_{280nm} value of half the maximum value observed during the load phase and conserved until the end of this purification step. Elution was performed with a step at 30 mM imidazole (in 50 mM Tris pH 8, 350 mM NaCl) followed by a linear gradient to 400 mM imidazole in 1 CV. The luminescence of the elution fractions was measured and the positive fractions were pooled, diluted with 50 mM Tris pH 7.8 to achieve a NaCl concentration of 100 mM NaCl and loaded on a 5 ml HiTrap SP column (GE Healthcare Life sciences) at a 5 ml/min flow rate. Elution was performed by a step at 250 mM NaCl followed by a 25 ml linear gradient to 1 M NaCl at 5 ml/min. The luminescence of the elution fractions was measured and the mDkk2-HRP corresponding fractions were pooled.

Measurement of luminescence for the purification samples

All dilutions were performed using DMEM medium supplemented with 5% (v/v) SVF (both from Gibco). A standard luminescence curve was created with serial dilutions of peroxydase (POD, Roche applied science, #108090) from 10^{-3} to 1.56×10^{-5} POD units. The assays were performed in 96-well plate by adding to the analyzed samples (diluted or not) 200 μl of SuperSignal WestPico (Pierce). The luminescence was measured immediately with a Fluoroskan-Ascent FL (labsystems) and used to determine the quantity of protein in the sample assuming that the luminescence specific activity in the Dkk-HRP constructs equals that of POD (250 U/mg).

Binding assays

Luminescent interaction assay (LIA)

A luminescent interaction assay was developed using EC-LRP5/6 and mDkk1/2-HRP proteins. The LIA was prepared by coating the bottom of a 96-1/2-well high protein binding plate (Greiner bio-one #675074) with 5 μg/ml EC-LRP5 or EC-LRP6 solutions, diluted in assay buffer (10 mM Pipes pH 6.5–200 mM NaCl-10% glycerol). After an overnight incubation, plates were washed 3 times with assay buffer + 0.05% (v/v) Tween20 and were blocked with assay buffer + 5% (w/v) skim milk (Difco) for 2 h. Then mDkk1-HRP or mDkk2-HRP diluted in assay buffer + 1% (w/v) skim milk was added for 3 h at 4 °C. Plates were washed before adding SuperSignal WestPico Chemiluminescent substrate (Pierce) and luminescence was read immediately on a Fluoroskan-Ascent FL.

Competition assay with mDkk1-V5-6His

With the system described above, we also set up a competitive assay with mDkk1-V5-6His [24] as the competitor to the binding of mDkk1-HRP to EC-LRP5/6. The protocol is the same until the overnight incubation. Then plates were washed 3 times with wash buffer (assay buffer + 0.05% Tween20). At this point, mDkk1-HRP (150 nM) was added and increasing amounts of mDkk1-V5-6His (from 300 pM to 900 nM) diluted in assay buffer + skim milk 1% final, 50 μl/well. Plates were incubated 3 h at room temperature, and then washed before adding SuperSignal WestPico Chemiluminescent substrate (Pierce) and luminescence was read immediately on a Fluoroskan-Ascent FL.

Wnt signaling cell-based assay

Activation or inhibition of Wnt signaling were assessed with a cell-based Wnt-dependent luciferase reporter assay [25]. Mouse fibroblast L-cells plated in 96-well plates (DMEM, 10% FCS) at 2×10^4 cells/cm^2, were transiently co-transfected with TCF 1 expression construct, the Wnt-luciferase reporter construct TOP-flash (Upstate Biotechnology) (200 ng total DNA; using Fugene 6 (Life Biotechnology). Co-transfection with 4 ng of pRL-TK (Promega, Madison, WI), which encodes a Renilla luciferase gene downstream of a minimal HSV-TK promoter, was systematically performed to normalize for transfection efficiency. Sixteen hours after transfection, cells were washed, cultured for 24 h in media containing 2% FBS and 15% Wnt3a-CM in the presence or absence of various recombinant proteins. Controls were performed in the absence of Wnt3a-CM. Cells were then lysed and luciferase assays were performed with the Dual Luciferase Assay Kit (Promega) according to the manufacturer's instructions. 10 μl of cell lysates were assayed for firefly luciferase and for Renilla luciferase activity. Firefly luciferase activity was normalized to Renilla luciferase activity. Wnt3a-CM was prepared from Wnt3a-producing L-cells, as described by [26].

Analysis on SDS–PAGE and western blot

The deposits were prepared by mixing 40 μl of sample with 10 μl of sample buffer 5× [27]. The SDS–PAGE were performed using 4–12% BisTris (MOPS running buffer) pre-casted gels (Invitrogen) and were stained with Coomassie blue (Imperial protein stain, ThermoScientific, #24615). For western blot, the gels were transferred onto nitrocellulose membranes (Invitrogen). SeeBlue molecular weight markers were used in both analysis and Magic XP markers were used for western blot (both from Invitrogen). For detection of the His-tagged protein, the membranes were incubated with the anti tetra-His (Qiagen #34670) followed by an incubation with anti-Mouse IgG peroxydase conjugated (Amersham #NA931 V) for ECL + revelation on a Biorad FX imager.

Results

Expression of EC-LRP5 and 6

FreeStyle cells were transiently transfected with vectors encoding EC-LRP5 and EC-LRP6 proteins. An IgK peptide signal was added to the N-terminus part of the protein for their efficient secretion into the culture medium. Cells were also co-transfected with vectors coding for Mesd and Rap proteins and, as shown previously [21–23,28], an increase in the EC-LRP5/6 protein expression level was observed when co-transfection was carried-out (data not shown).

Under same experimental conditions, the expression level of EC-LRP5 is consistently 3–5 times lower than the EC-LRP6 (Fig. 1A). The expression levels are good for large (>155 kDa) secreted proteins in mammalian cells, ranging from 2.5 to 3 mg/L for EC-LRP5 to 4–10 mg/L for EC-LRP6. In both cases the expression levels increase with time, with a maximum reached at 5 days post-transfection (Fig. 1B). This lag time to reach the highest expression level could be due to a limiting rate in the folding/secretion processes of such large proteins. Sequential harvest of the cell culture supernatant was possible every 3–4 days for a total of 13 days.

EC-LRP6 purification

The CM was clarified by high-speed centrifugation then loaded to Nickel chelate resin. Almost no EC-LPR6 was lost in the flow-through of this affinity column (data not shown). The imidazole elution was performed in a low salt buffer, allowing to reduce the dilution of the sample before loading on an ionic exchange column. After extensive washing, most of the protein was eluted in the fractions that correspond to the 200 mM NaCl step (data not shown). 2.9 mg of 85% pure EC-LRP6 were obtained (200 μg/ml, estimation based on Coomassie blue-stained gel, Fig. 2A), which corresponds to a final yield of 1.45 mg/L of CM.

EC-LRP5 purification

For EC-LRP5, the clarification of the buffered CM was performed by filtration through a 0.22 μm membrane. Some EC-LRP5 protein was released after loading on the Ni^{2+} affinity column during the wash step to remove non-specifically bound material with a 20 mM imidazole buffer (data not shown). However, most of the protein was found in the gradient elution fractions, resulting in a 225 μg batch of an 80% pure protein (concentration of 25 μg/ml, as estimated by Coomassie blue-stained gel (Fig. 2B)), corresponding to a yield of 500 μg/L of CM.

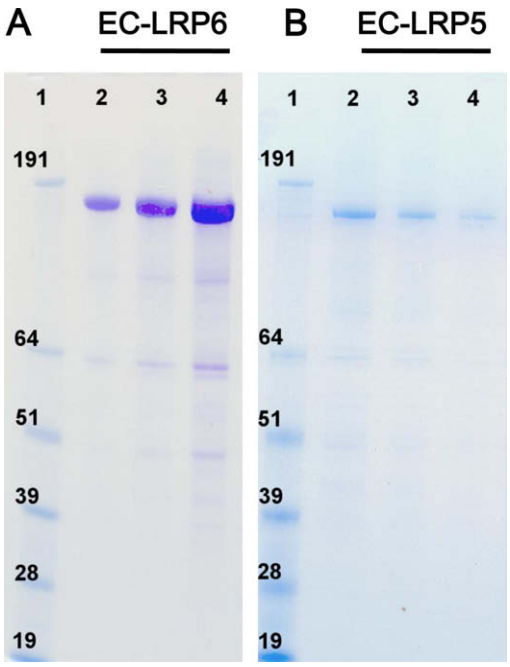

Fig. 2. Purification of EC-LRP5 and EC-LRP6. The final protein batches were analyzed for their purity using Coomassie blue stained 4–12% BisTris SDS–PAGE. (A) Analysis of various amount of the purified EC-LRP6 protein. Loading: 4, 8 and 16 μl for lanes 2, 3 and 4, respectively. Lane 1: MW markers (kDa). (B) Analysis of various amount of the purified EC-LRP5 protein. Loading: 16, 8 and 4 μl for lanes 2, 3 and 4, respectively. Lane 1: MW markers (kDa). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Expression of mDkk1 and mDkk2-HRP

All attempts to obtain mDkk1 and mDkk2-HRP expression by transient transfection in CHO-S, DG44 or FreeStyle 293-F failed. Only few luminescences could be detected in supernatants from CHO transfected cells. Considering these very low expression levels, stably-expressing CHO clones were isolated for each Dkk-HRP

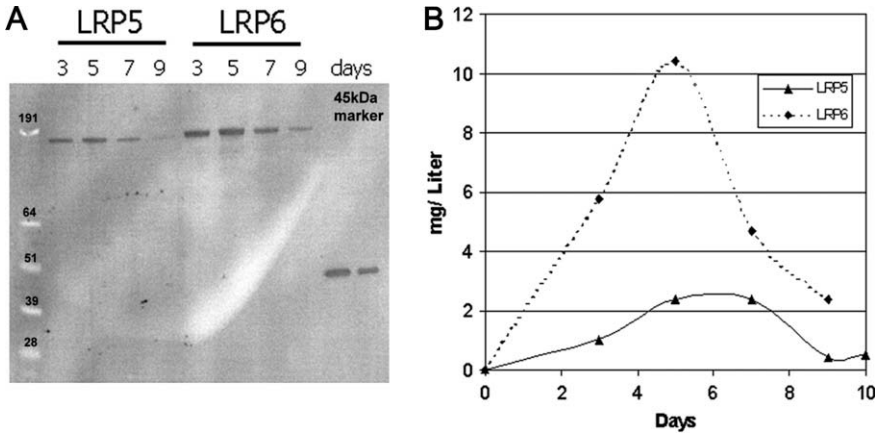

Fig. 1. Analysis of the expression of EC-LRP5 and EC-LRP6. (A) Analysis of EC-LRP5 and EC-LRP6 expression by western blot. Conditioned media harvested at different time point post-transfection were analyzed by western blot with anti-tetra-His antibody (Qiagen). The amount of EC-LRP5 and EC-LRP6 proteins was estimated by comparing the intensity of the bands with those of 50 and 100 ng of a 45 kDa 6His-tagged protein. (B) Time course of the expression level of EC-LRP5 and EC-LRP6 proteins in transiently transfected FreeStyle cells. Plot of the average protein expression levels at different time point post-transfection.

protein. Moreover, as previously described for another Dkk protein [24], the addition of heparine at 0.1 g/L during the production process increases 5- to 10-fold the recovery of Dkk-HRP proteins from the conditioned medium (CM). The use of roller bottles and sequential harvests allowed rapid production up to 100 L of CM for each of those proteins.

mDkk1-HRP purification

Due to the low expression levels, the luminescent properties of the HRP moiety were used to detect the presence of the protein in the CM. We made the assumption that the luminescence in the sample was uniquely due to the Dkks-HRP protein.

The buffered CM was clarified and loaded on Ni^{2+} affinity column. Less than 10% of the protein was found in the flow-through (by luminescence measurement). The mDkk1-HRP protein eluted as two peaks, one peak at 38.5 mM imidazole and the second during the linear gradient to 200 mM imidazole. This behavior may be explained by a heterogeneous glycosylation of the protein (fuzzy bands). All these fractions were pooled, diluted and loaded on ion exchange column. Almost all the protein was retained on the ion exchange chromatography since about 86% of the protein was recovered in the pooled fractions from this step (Fig. 3A). Using the luminescence standard curve, the amount of purified mDkk1-HRP protein from 4.5 L of CM was estimated to 660 μg (final concentration of 40 μg/ml) corresponding to a final yield of 146.6 μg/L of CM).

mDkk2-HRP purification

CM was clarified by high-speed centrifugation then loaded onto Nickel chelate resin. Almost no luminescence was detected in the flow-through and wash fractions (data not shown). Elution was performed with a step at 30 mM imidazole to remove most of the non-specifically bound material, followed by a linear gradient to 400 mM imidazole in 1 CV. The fractions containing the luminescence were pooled, diluted with a suitable buffer to achieve a 100 mM NaCl concentration, and loaded on a sulfo-propyl ion exchanger. As shown in Fig. 3B, the step at 250 mM NaCl removed contaminants, whereas the mDkk2-HRP protein eluted in the NaCl gradient. This process allowed to obtain 850 μg of 65% pure mDkk2-HRP (concentration of 75 μg/ml, an estimation based on Coomassie blue-stained gel, western blotting and luminescence, see Fig. 3C), corresponding to a final yield of 250 μg/L of CM.

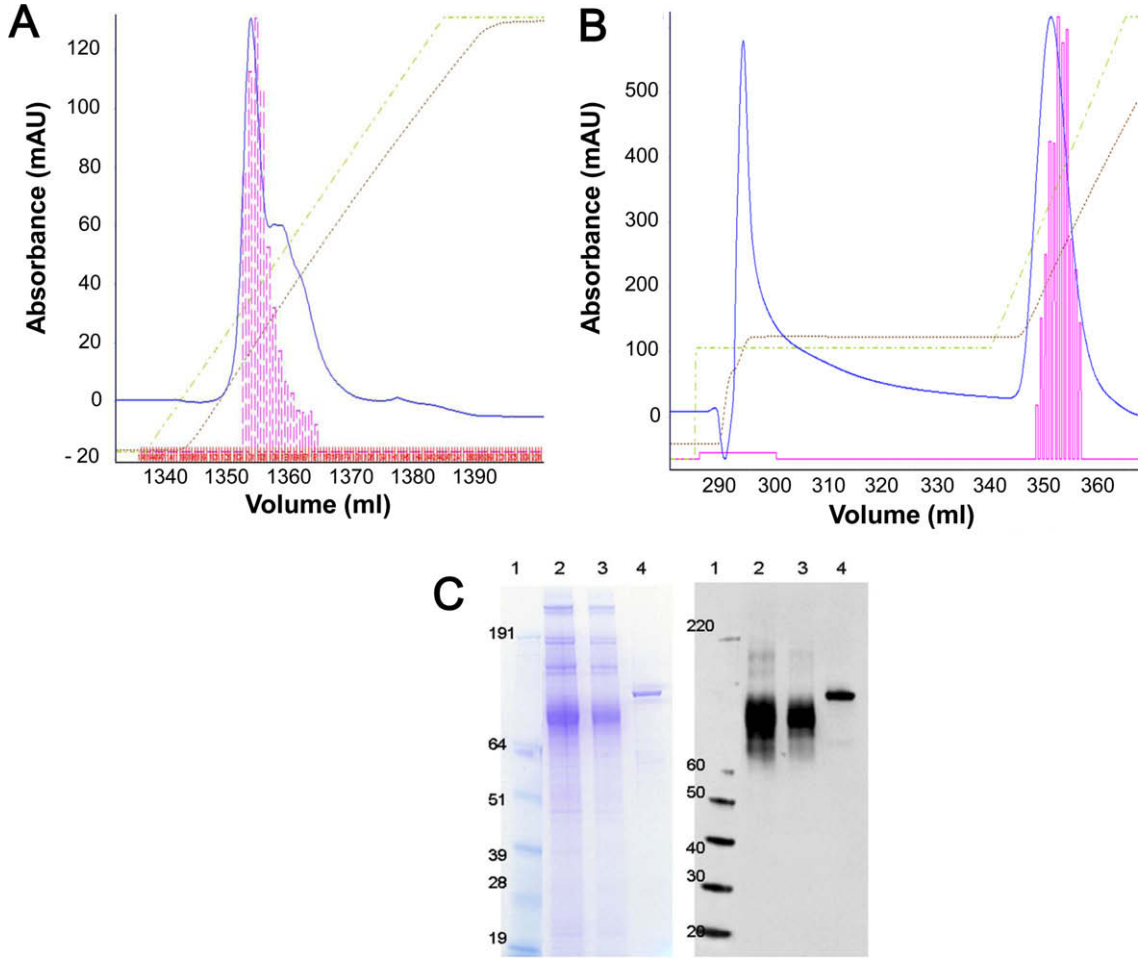

Fig. 3. Dkk-HRP proteins purification and analysis of the final batches. (A) Elution chromatogram of the mDkk1-HRP HiTrapQ column. The UV_{280nm}, NaCL gradient and conductivity are represented as solid, semi-dotted and dashed lines, respectively and luminescence of elutions fractions as histograms. (B) Elution chromatogram of the mDkk2-HRP HiTrapSP column. The UV_{280nm}, NaCL gradient and conductivity are represented as solid, semi-dotted and dashed lines, respectively, and luminescence of elution fractions as histograms. (C) Coomassie blue-stained SDS–PAGE and western blot of the final mDkk2-HRP batch. 4–12% BisTris NuPAGE were ran with MOPS buffer and proteins were either revealed by Coomassie blue staining or transferred to a nitrocellulose membrane and revealed with anti tetra-His antibody (Qiagen). Coomassie blue-stained gel loading: lane 1: MW markers (kDa), lane 2 and 3: 8 and 4 μl purified mDKK2-HRP, lane 4: 400 ng of a 110 kDa 6His-tagged protein. Western blot loading: lane 1: MW markers (kDa), lane 2 and 3: 4 and 2 μl purified mDKK2-HRP, lane 4: 200 ng of a 110 kDa 6His-tagged protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

All the purified proteins were aliquoted and stored at −80 °C with 10% (v/v) glycerol as a cryoprotectant.

Functional activity of purified proteins

The biological activity of mDkk1-HRP and mDkk2-HRP proteins were first tested by measuring their ability to inhibit the Wnt pathway triggered by Wnt3a containing medium in the Wnt signaling luciferase reporter cellular assays. These assays were performed in different cell types including MC3T3, fibroblast L-cells and C3H10T1/2 cells. As shown in Fig. 4A, mDkk1/2-HRP proteins dose dependently inhibit Wnt signaling in fibroblast L-cells. Comparable results were obtained for the other used cell lines (data not shown). IC_{50} values of 2nM were measured for both mDkk-HRP proteins.

The same cell-based assay was used to investigate the biological activity of EC-LRP6. As shown in Fig. 4B, Wnt3a increases luciferase signal when added to cells transfected with the Wnt-dependent luciferase reporter. Furthermore, as expected, addition of mDkk1 protein to Wnt3a-stimulated cells inhibits the luciferase response (Fig. 4B). Soluble and active EC-LRP5 or 6 proteins are expected to act as dominant negative of the Wnt pathway. Accordingly, our data show that addition of increasing amount of purified EC-LRP6 leads to dose-dependant inhibition of the Wnt signaling, antagonizing the action of Wnt3a (Fig. 4B). On the other hand, when EC-LRP6 was added in the presence of both Wnt3a and mDkk1 (LRP6 binds to both Wnt and Dkk1), it was able to release the mDkk1 inhibition of the Wnt pathway, restoring the activation of the Wnt pathway to almost the level observed in absence of mDkk1 (Fig. 4B). These data demonstrate the biological relevance of the purified EC-LRP6 produced with this approach and confirm that this protein has a dominant negative activity on the Wnt pathway.

Binding assays (LIA)

Based on the luminescent properties of the HRP moieties, a luminescent interaction assay was developed to assess and evaluate the binding of mDkk1/2-HRP proteins to EC-LRP5/6 proteins, respectively. The EC-LRP proteins were directly coated on the 96-well plate at 4 °C and to avoid non-specific binding, a blocking step was added before adding the mDkk-HRP proteins. To further reduce background, those proteins were also diluted in a blocking buffer containing 1% w/v skim milk. Binding curves were obtained (Fig. 5A and B) and show a difference between EC-LRP5 and EC-LRP6 in their binding selectivity for Dkk proteins. Indeed, EC-LRP6 does not discriminate between Dkk1 and Dkk2, although the binding on Dkk1 is slightly higher than on Dkk2 (Fig. 5A). Conversely, EC-LRP5 displayed a clear selectivity for Dkk2 versus Dkk1 (Fig. 5B). Finally, the interaction of Dkk1 with EC-LRP5 is the weakest interaction monitored in this assay (Fig. 5A and B).

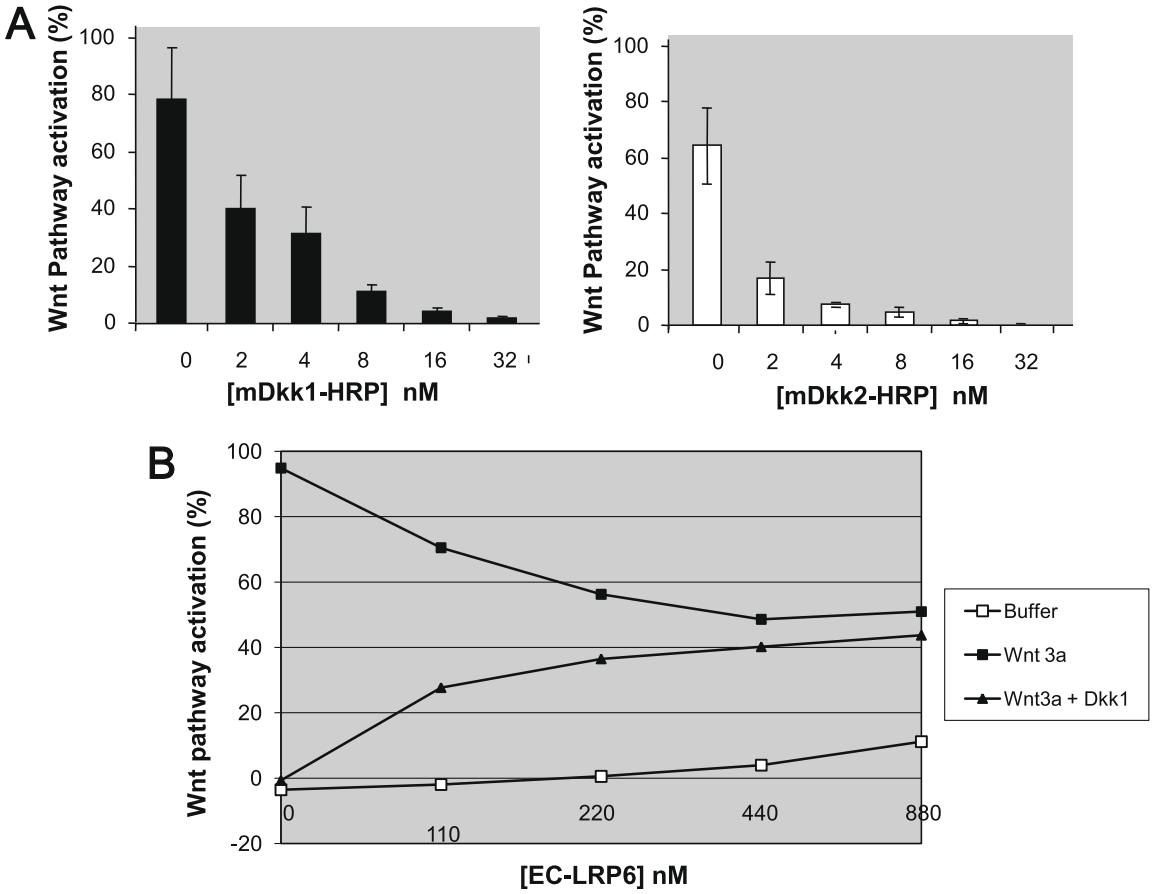

Fig. 4. Biological activity of produced proteins in a luciferase reporter assay. (A) Wnt pathway inhibition by mDkk-HRP proteins. Fibroblast L-cells were transiently transfected with Wnt-luciferase reporter construct TOPflash in the presence of 15% Wnt3a-CM and increasing amounts of mDkk-HRP proteins. Luminescence was recorded and presented as a normalized signal (Firely/Renilla) versus mDkk-HRP proteins concentration. (B) Effect of purified EC-LRP6 protein on the Wnt pathway activation. Fibroblast L-cells were transfected as described in (A) and increasing amounts of EC-LRP6 protein was added with various combinations of Wnt3a-CM and 150 ng/ml mDkk1-V5-6His. References for the measurement of the Wnt pathway activation: the 0% corresponds to treatment with buffer only or 15% Wnt3a-CM + 150 ng/ml mDkk1-V5-6His; the 100% activation corresponds to treatment with 15% Wnt3a-CM only.

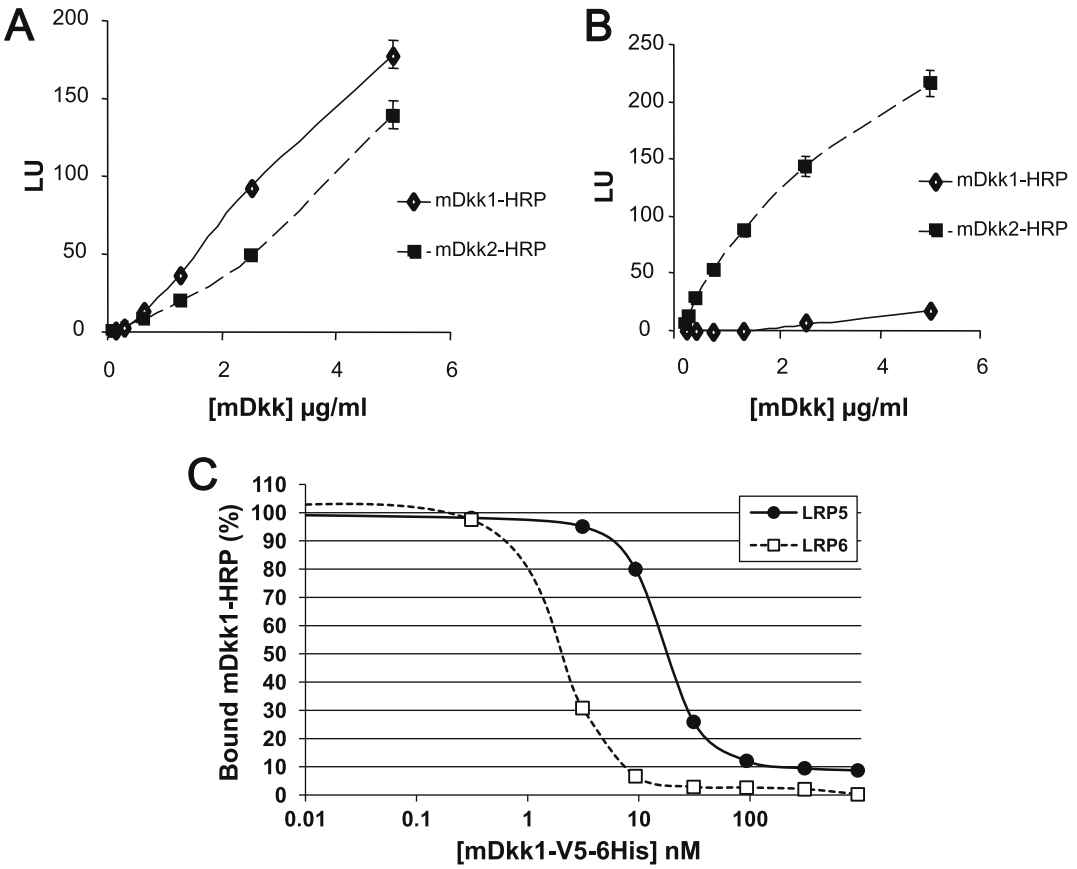

Fig. 5. Binding of mDkk-HRP to EC-LRP proteins measured by LIA. (A) EC-LRP6 binding selectivity curves versus mDkk1-HRP and mDkk2-HRP. EC-LRP6 protein was directly coated on the 96-well plate overnight at 4 °C then incubated for 3 h at 4 °C with increasing amounts of either mDkk1 or mDkk2-HRP proteins. Bound mDKK-HRP proteins were quantified by adding SuperSignal WestPico Chemiluminescent substrate (Pierce) and luminescence reading. (B) EC-LRP5 binding selectivity curves versus mDkk1-HRP and mDkk2-HRP. Same as (A) but coating was performed with EC-LRP5 protein. (C) Competitive binding of mDkk1-V5-6His versus mDkk1-HRP to EC-LRP6. Same as in (A) but mDkk1-V5-6His was added as the competitor to the binding of mDkk1-HRP to EC-LRP5/6.

To further characterize those interactions, a competitive binding assay was performed using mDkk1-V5-6His, without the HRP moiety, as a competitor. The results presented in Fig. 5C clearly show that mDkk1-V5-6His is able, as expected, to compete the binding of mDkk1-HRP to both EC-LRP6 and EC-LRP5 proteins. Furthermore, there is a clear difference in the curves obtained with either EC-LRP6 or EC-LRP5, the latter curve being shifted to higher mDkk1-V5-6His concentrations. If we assume that the HRP moiety does not modify the affinity of mDkk1 for LRPs, this confirms that the binding of Dkk1 to EC-LRP6 is stronger than to EC-LRP5. From the curves we can derive IC_{50} values of approximately 2 nM for the Dkk1/EC-LRP6 interaction and 18 nM for the Dkk1/EC-LRP5 interaction. This result confirms the trend observed above with the LIA, where mDkk1-HRP/EC-LRP5 showed the weakest interaction of the tested panel.

Discussion

The Wnt signaling pathway provides new avenues to identify novel drug candidate for the treatment and management of distinct diseases [12,29–31]. Therefore the availability of tools relevant to this pathway, such as recombinant proteins, is a prerequisite for developing appropriate screening and selection assays. In addition, the availability of such tools will contribute to investigate and increase our knowledge about the Wnt signaling cascade. In the present report, we describe for the first time the expression and purification of the soluble form of the Wnt co-receptors LRP5 and LRP6. Furthermore we also describe the purification of HRP-Dkk1 and Dkk2 fusion proteins and demonstrate its use to accurately and easily measure binding and interaction with LRP5/6. All recombinant proteins were tested, found to be biologically active and their binding properties characterized.

For expressing EC-LRP proteins, it proved critical to co-express them with Mesd and Rap to obtain sufficient expression level. It has been shown previously that Mesd and Rap, through their respective chaperoning and trafficking activities, enhanced the expression level of secreted LRP6 [21–23,28]. This was clearly the case for the EC-LRP constructs, with a slight increase of the expression levels upon co-transfection with vectors containing Mesd and Rap proteins. Despite this, the expression levels of EC-LRP5 and EC-LRP6 were different, with EC-LRP6 being expressed between 3 and 5 times more than EC-LRP5 (4–10 mg/L versus 2.5–3 mg/L of culture medium). For both proteins, the best expression levels were obtained 5–6 days post-transfection, as shown in Fig. 1B. This is surprising since a more classical approach would have led us to harvest only 2–3 days post-transfection only. The lower expression level of EC-LRP5 combined with the loss of protein during the removal of contaminants did not permit us to achieve the same good purification yield as for EC-LRP6. In order to minimize the loss of protein, we decided to perform only one chromatographic step with the Ni^{2+} chelating column. Even with this precaution, the overall yield for EC-LRP5 is only 500 µg/L of culture medium where it is 1.45 mg/L for EC-LRP6 corresponding to 35–40% purification efficiency. Those medium figures reflect the challenge inherent to the purification of poorly expressed proteins.

The expression levels for mDkk1-HRP and mDkk2-HRP were very low and we did not detect those proteins by western blot in the culture medium. Therefore we used the HRP activity measurement to isolate the best productive producer and used roller bottles for production, which permitted to increase the productive surface but also the volume of the medium for each harvest. Moreover, as previously described [24], the adjunction of heparine at 0.1 g/L during the production increased 5- to 10-fold the recovery of Dkk-HRP proteins in the conditioned medium. Using the roller bottles, we were able to rapidly produce up to 100 L of culture supernatant for each of those two proteins. We then took the advantage of the HRP moiety to follow-up the production and the purification processes. We assumed that the measured luminescence was directly due to the recombinant proteins and that the specific activity (U/mg) of the HRP moieties is the same as for the commercially available peroxydase used to create the standards curve. The reliability of this quantification method was checked by analyzing by western blot an amount of mDkk-HRP proteins determined by this method and comparing it to known amounts of reference proteins. The purification schemes are roughly the same for the two proteins, with an Immobilized Metal Affinity Chromatography (IMAC) as first purification step followed by an ion exchange chromatography. But there was a difference in the ionic exchange step, with opposite strategies for mDkk1-HRP and mDkk2-HRP (anion exchanger versus cation exchanger, respectively). Nevertheless, this step was efficient in both cases and led to final yields close to 145 and 250 μg/L of culture for mDkk1-HRP and mDkk2-HRP, respectively.

Since our goal was to develop tools to study the Wnt pathway inhibition, we then checked the biological relevance of the produced proteins. As the presence of the HRP moiety can modify the binding of Dkks to the EC-LRPs, we first tested that the mDkks-HRP proteins were able to disrupt the Wnt signaling pathway. We clearly showed that mDkk1-HRP and mDkk2-HRP inhibit Wnt signaling with almost comparable IC_{50} values, in a nano-molar concentration range. Those values correlate well to previously published data [32–34] that described Kd data in the nano-molar range for Dkk1 with LRP5/6. We also investigated how EC-LRP6 interfered with the Wnt pathway on a luciferase reporter assay. The results (Fig. 4B) clearly showed the biological activity of the EC-LRP6 in this system. Increasing amounts of EC-LRP6 led to the dose-dependant inhibition of the Wnt3a induced activation of the Wnt pathway whereas, in the experiment where both Wnt3a and Dkk1 were added, EC-LRP6 permitted to release the Dkk1 inhibition. Indeed, EC-LRP6 serves as a decoy to membrane bound LRP6, thus either trapping Wnt3a or making an inefficient ternary complex with Wnt3a and Frizzled for signal transduction and finally, releasing Dkk1 inhibition of the Wnt pathway by direct binding to Dkk1. In this later mechanism, EC-LRP6 also serves as a decoy receptor for Dkk1.

This is in agreement with previously published results, showing that EC-LRP6 behaved as a negative dominant protein in Wnt pathway activation studies [9,35]. This also illustrates the pivotal role of LRP6, which is involved in either the LRP6/Wnt/Frizzled complex and in the LRP6/Dkk1/Kremen complex [11,12].

However, despite the fact that many data have been produced over the last years [33,34], the binding properties of the isolated molecular partners have never been studied so far. We investigated the binding properties of mDkk1-HRP and mDkk2-HRP on the EC-LRPs. The data obtained in the LIA experiments clearly showed that EC-LRP5 discriminates mDkk1 from mDkk2, with a better binding to mDkk2 than to mDkk1. Conversely, EC-LRP6 does not display such selectivity with only a slightly better binding to mDkk1 as compared to mDkk2.

Moreover, in a competition assay with mDkk1-V5-6His, we found that, whereas the IC_{50} of Dkk1 for EC-LRP6 can be estimated to 2nM, which is in the range of previously published constants [32–34], the IC_{50} for binding of Dkk1 to EC-LRP5 is close to 20 nM, one order of magnitude more than for LRP6. The discrepancy with previously reported constants may be due to the cellular contexts that were used in previous studies and where it is difficult to define the exact contributions of both LRP5 and LRP6 to the measurements. Altogether, these data indicate a selectivity of mDkk1 for LRP6 with respect to LRP5 and further investigations may deliver more accurate interaction constants.

In summary, we described for the first time the production and partial characterization of components of the Wnt signaling pathway, in amounts sufficient to test them in *in vitro* and cell-based assays to study their binding properties. Two proteins of the LDL-receptor-related protein family were produced as soluble ectodomains, EC-LRP5 and EC-LRP6, as well as mDkk1-HRP and mDkk2-HRP. All of those proteins were found to be active in Wnt pathway inhibition assays. Altogether, these results provide us with powerful tools to study the inhibition of the Wnt signaling pathway.

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