Development of a high-content imaging-based technique to measure Dickkopf-1 binding to low-density lipoprotein receptor-related protein 6

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Introduction

Dickkopf-related protein 1 (Dkk1) is a member a family of secreted proteins which inhibits β-catenin-dependent Wnt signalling by binding to low-density lipoprotein receptor-related protein 6 (LRP6), a component of the LRP6/wnt/Frizzled receptor ternary signalling complex (Ahn et al., 2011; Bourhis et al.,2011; Matoba et al., 2017).

Dkk1 dysregulation in the brain has been linked to Alzheimer's disease (AD). Dkk1 levels are elevated in human AD brain and transgenic mouse AD models (Caricasole et al., 2004; Rosi et al., 2010), while acute amyloid-β (Aβ) oligomers exposure to mouse brain slices induced Dkk1 expression and subsequent Dkk1-mediated synaptic loss (Purro, Dickins, & Salinas, 2012). Rat primary cortical neurons treated with Aβ also induced Dkk1 expression, via a clusterin-dependent pathway, with Dkk1-dependent neurotoxicity linked to signalling mediators and gene expression changes associated with the non-canonical/planar cell polarity (PCP)-wnt pathway (Killick et al., 2014). Interestingly, blockade of Dkk1 and the associated signalling pathways with blocking antibodies or siRNA, has shown to be protective in preclinical models of AD (Killick et al., 2014; Purro et al., 2012).

As part of our efforts to identify novel small molecule Dkk1-LRP6 inhibitors, we require assays with which to screen and triage test compounds. Cell-based Dkk1-LRP6 assays reported in the literature use either modified Dkk1 protein and/or do not possess suitable throughput for drug screening.

Aim: To develop a novel cell-based immunocytochemical (ICC) technique utilising high-content imaging (HCI) and data analysis (HCDA) suitable for the screening of protein and small molecule inhibitors of Dkk1-LRP6 binding.

Methods - Assay Principle

HEK293 cells transiently transfected with human LRP6 were incubated with recombinant Dkk1 protein at 4°C in a clear bottom 96-well plate. The assay was terminated with washing with ice cold PBS followed by cell fixation by PFA (4 % w/v). Dkk1 and LRP6 were stained for via Immunocytochemistry. Images were generated using the PerkinElmer Operetta High-content imaging system. LRP6 and Dkk1 staining are visualised in yellow and red, respectively.

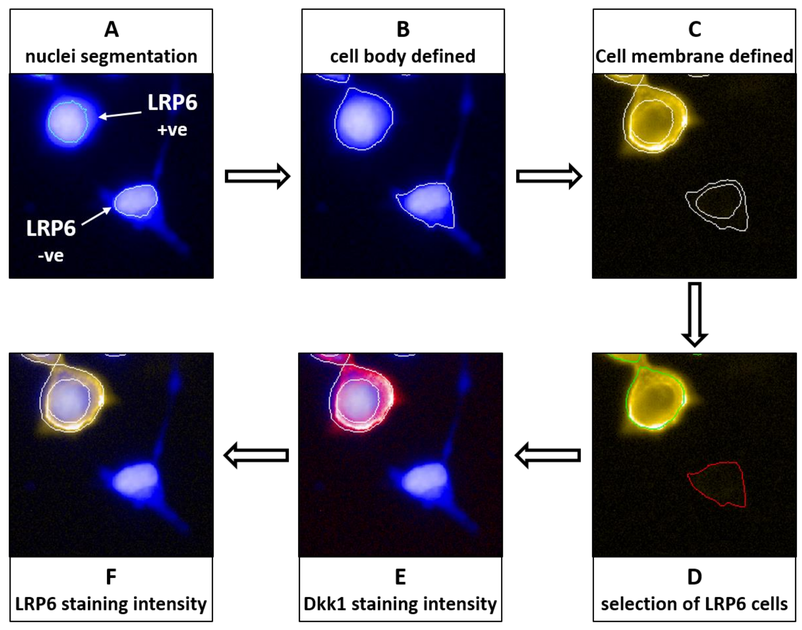

Fig 1: Image analysis using PerkinElmer Columbus software. Stained cells were segmented according to their nuclei (A), followed by definition of the cell body (B) and then cell membrane region (C). A threshold for successful LRP6 transfection was used to select the LRP6 cell population for further analysis (D, green-circled vs red-circled cells). Within this LRP6 positive population, the intensity of Dkk1 staining, visualised in red (E) and the intensity of LRP6 staining visualised in yellow (F), both in the membrane region, was determined. Intensity values were not calculated for LRP6 negative cells. These values were then used to calculate the Dkk1/LRP6 ratio. Cells in these images were transfected with hLRP6 and treated with Dkk1 (800 ng/mL) for 2 h. Images were captured in the DAPI, TRITC and AF647 channels using a 40x long WD objective.

Optimised assay reveals saturable Dkk1-LRP6 binding with low NSB

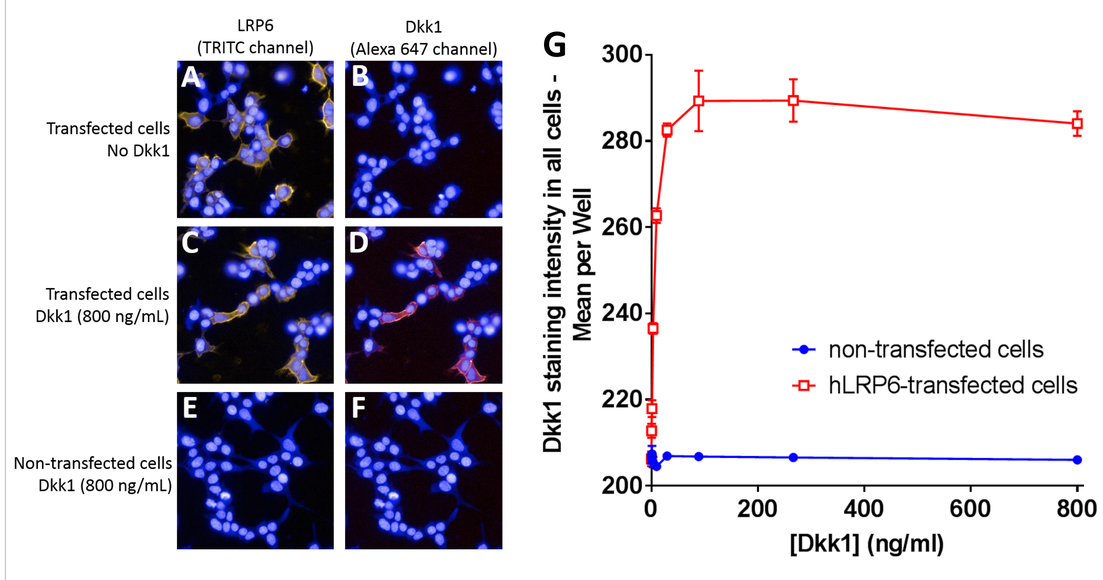

Fig. 2: Immunocytochemical staining for human LRP6 produced staining in LRP6 transfected cells only, visualised in yellow (A and C), which was absent in non-transfected cells (E). Co-staining for human Dkk1 produced overlapping staining in transfected cells treated with exogenous recombinant Dkk1 protein, visualised in red (D). This Dkk1 staining was absent in non-treated transfected cells (B) and in non-transfected cells treated with Dkk1 (F). Saturation analysis revealed saturable, concentration-dependent binding of Dkk1 to transfected cells (G – red squares), which was wholly absent in non-transfected cells (G – blue circles). Data represents mean ± s.e.m. *n*=3.

Characterisation of Dkk binding kinetics

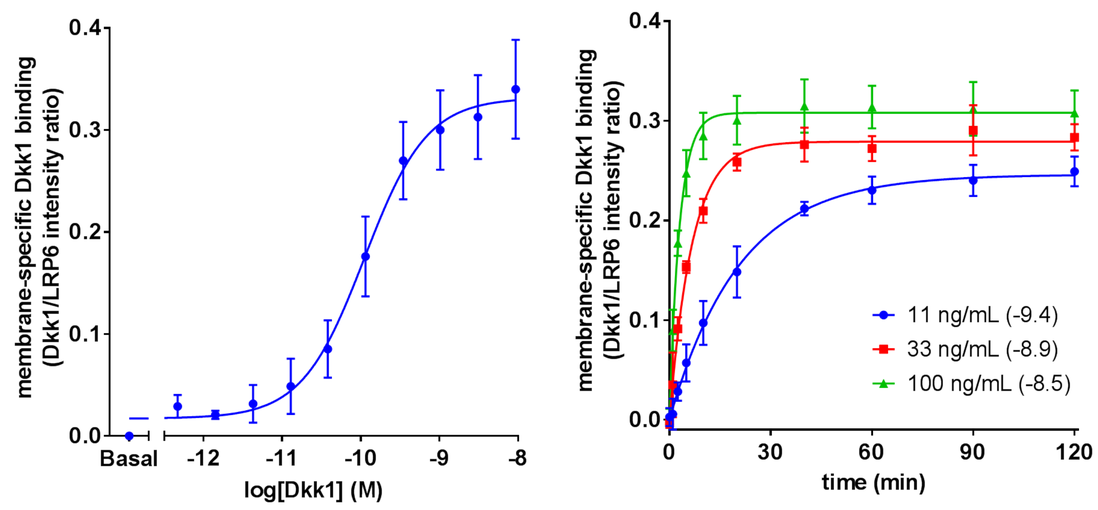

Fig. 3: Incubation of HEK-hLRP6 cells with increasing concentrations of exogenous recombinant hDkk1 for 2 h revealed LRP6-specific, concentration-dependent binding of Dkk1 with an IC_{50} = 0.11 ± 0.02 nM, while association kinetic experiments demonstrated the utility of the technique to investigate Dkk1 binding kinetics (Dkk1 k_{obs} - 11 ng/mL = 4.8 ± 0.5; 33 ng/mL = 15.1 ± 1.1; 100 ng/mL = 32.9 ± 2.8 min-1). Data shown represent mean ± s.e.m, *n*=3.

Dkk1 protein selectivity and competition displacement by other Dkk isoforms

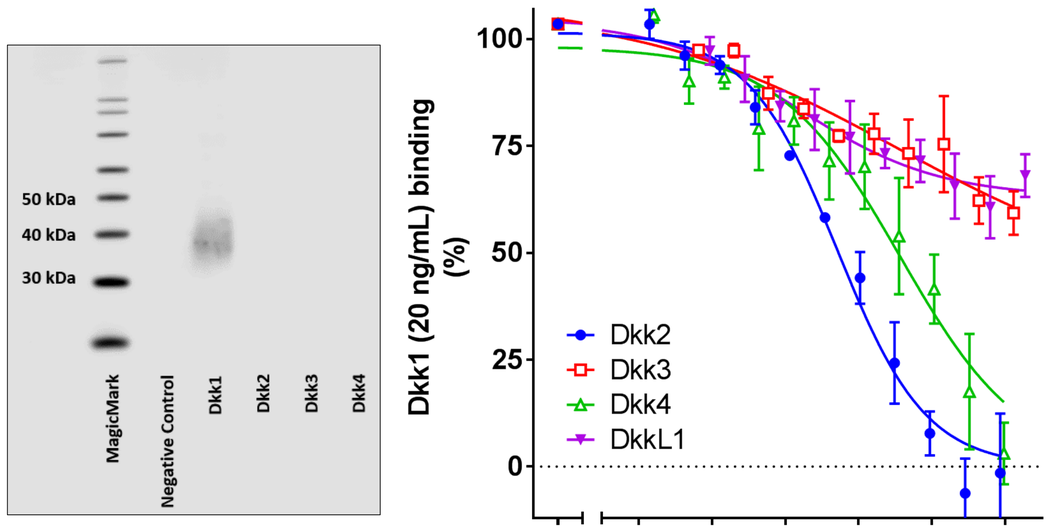

Fig 4: The selectivity of the anti-human Dkk1 antibody for recombinant hDkk1 versus recombinant human Dkk2, -3, and -4 was confirmed by western blotting. Incubation of hLRP6-transfected cells with increasing concentrations of exogenous recombinant proteins for 2 h, in the presence of hDkk1 (20 ng/mL Dkk1 pre-incubated for 1 h) revealed concentration-dependent displacement of Dkk1 binding by Dkk2 and 4 (pIC50 Dkk2 = 0.54 ± 0.06; Dkk4 = 4.95 ± 2.60 nM). Data shown is expressed as percentage of Dkk1 binding and represent mean ± *n*=3.

Utility to identify small molecule Dkk1-LRP6 binding inhibitors

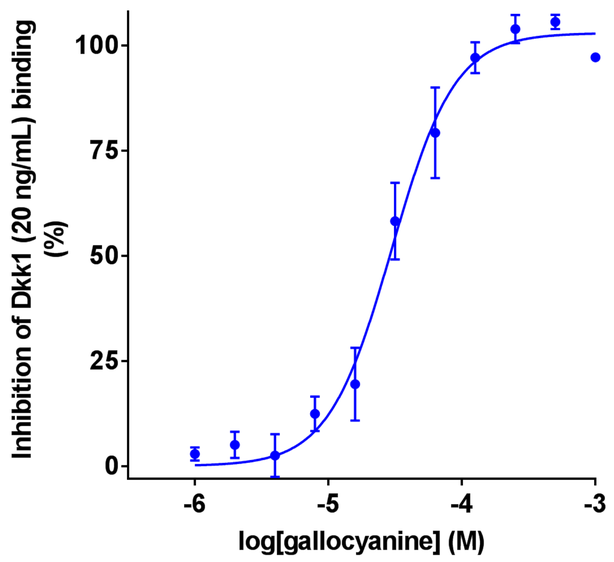

Fig 5: The previously reported small molecule **Gallocyanine** is confirmed as an inhibitor of Dkk1-LRP6 binding (Iozzi *et al*., 2012).

Incubation of HEK-hLRP6 cells with increasing concentrations of gallocyanine for 2 h in the presence of hDkk1 (20 ng/mL Dkk1 preincubated for 1 h) revealed concentration-dependent displacement of Dkk1 binding. Data shown is expressed as percentage inhibition of Dkk1 binding and represent mean ± s.e.m., *n*=3.

Z factor 100 μM Gallocyanine = 0.83 ± 0.04.

Conclusion

We have developed a validated cell-based assay, suitable for the screening of inhibitors of Dkk1-LRP6 binding, and provide the basis for additional assay development, investigating Dkk1-LRP6 pharmacology. We aim in future to use this technique to assess novel small molecule inhibitors of Dkk1-LRP6 binding as potential therapeutics in the treatment of Alzheimer's Disease.

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Reference details available on request from lead author.