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Short communication

Number and brightness analysis of sFRP4 domains in live cells demonstrates vesicle association signal of the NLD domain and dynamic intracellular responses to Wnt3a

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ABSTRACT

The Wnts are secreted, lipidated glycoproteins that play a role in cellular processes of differentiation, proliferation, migration, survival, polarity and stem cell self-renewal. The majority of Wnts biological effects are through binding to specific frizzled (Fzd) receptor complexes leading to activation of downstream pathways. Secreted frizzled-related proteins (sFRPs) were first identified as antagonists of Wnt signalling by binding directly to Wnts. They comprise two domains, a Fzd-like cysteine rich domain (CRD) and a netrin-like domain (NLD). Subsequently sFRPs have been shown to also interact with Fzd receptors and more diverse functions have been identified, including potentiation of Wnt signalling. Many aspects of the biology of this family remain to be elucidated. We used the number and brightness (*N&B*) method, a technique based on fluorescence fluctuation analysis, to characterise the intracellular aggregation and trafficking of sFRP4 domains. We expressed sFRP4 and its' domains as EGFP fusions and then characterised the effect of endogenous Wnt3a by fluorescence confocal imaging. We observed vesicular trafficking of sFRP4 and that the NLD domain has a vesicular association signal. We found that sFRP4 and the CRD formed oligomeric aggregates in the perinuclear region while the NLD was distributed evenly throughout the cell with a larger proportion of aggregates. Most significantly we observed intracellular redistribution of sFRP4 in response to Wnt3a suggesting that Wnt3a can modulate intracellular localisation and secretion of sFRP4. Our results reveal a number of novel findings regarding sFRP4 which are likely to have relevance to this wider family.

1. Introduction

The Wnts are secreted, lipidated glycoproteins (at least 19 in humans) that transduce signals by binding to specific frizzled (Fzd) receptor complexes (reviewed in Schulte, 2010) leading to activation of canonical or non-canonical pathways depending upon molecular context. Wnts are functionally integral to many processes during embryonic development and play an important role in homeostasis in adult tissues. The downstream pathways activated by Wnts play a role in a diverse array of cellular processes including differentiation, proliferation, migration, survival, polarity and stem cell self-renewal (Clevers and Nusse, 2012; Wang et al., 2012). Aberrant Wnt signalling is associated with several disorders, especially cancer (Anastas and Moon, 2013).

The mammalian secreted frizzled-related proteins (sFRPs) are a family of five proteins which were first identified on the basis of their antagonistic effect upon Wnt signalling. Subsequent research has indicated more diverse functions for sFRPs, including potentiation of Wnt signalling in certain contexts (Uren et al., 2000; Kress et al., 2009; Von Marschall and Fisher, 2010; Xavier et al., 2014), spacial diffusion of Wnts (Mii and Taira, 2009) or even Wnt independent effects (Martin-Manso et al., 2011). They contain two domains, a Fzd-like cysteine rich domain (CRD) and a netrin-like domain (NLD). The CRD of sFRPs was initially believed to play a role analogous to the CRD of Fzds as the principal mediators of Wnt binding (Lin et al., 1997). Although some studies have suggested that sFRPs interact with Wnts via their CRD domain (Lin et al., 1997) subsequent experiments have indicated that the NLD may

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play a more prominent role in this interaction (Bhat et al., 2007; Lopez-Rios et al., 2008). In addition it has been demonstrated that SFRPs and Fzds can also interact via their CRD domains to form heterodimers and homodimers suggesting an alternative mechanistic basis for sFRPs to influence Wnt signalling (Bafico et al., 1999; Rodriguez et al., 2005).

The biological significance of sFRPs is evinced by studies in developmental models and the frequent observation of aberrant expression in many cancers. Despite this evidence many aspects of the biology of this family remains to be elucidated. In malignant mesothelioma (MM) sFRP4 has been shown to be downregulated by promoter methylation and to function as a tumour suppressor (Lee et al., 2004; Kohno et al., 2010). Nearly all studies of sFRPs to date have focussed upon their extracellular interactions and very little is known about the intracellular trafficking, localisation and behaviour of sFRP4 and its domains. Using a mesothelioma cell model which showed little or no sFRP4 expression (Fox et al., 2013) we undertook to characterise the intracellular trafficking of sFRP4 domains, particularly in cells exposed to endogenous Wnt3a.

We used a recently described fluorescence microscopy technique (Ossato et al., 2010), Number and Brightness (*N*&*B*) fluctuation spectroscopy analysis, to study intracellular localisation and aggregation of sFRP4, sFRP4 CRD and sFRP4 NLD. The basis for the *N*&*B* method is the analysis of fluorescence intensity distributions and permits measurement of both the number (*N*) and brightness (*B*) at each pixel in a stack of images. Since the brightness is directly related to the number of molecules aggregated, the method has been previously applied to live cell image analysis of intracellular protein aggregation (Ossato et al., 2010; Vetri et al., 2011). Our imaging and image analysis studies suggest that sFRP4 is localised in the perinuclear region and this localisation is likely to be a property of the CRD domain. We found that the NLD was likely to have a vesicle association signal involved in secretion of sFRP4. Notably we found that intracellular localisation and trafficking of sFRP4 domains could be modified in response to Wnt3a.

2. Materials and methods

2.1. Cell culture

The malignant mesothelioma cell line JU77 was used in this study (Manning et al., 1991). JU77 cells were cultured in RPMI 1640 supplemented with 5% foetal bovine serum, 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100ug/ml) (all from Thermofisher Hyclone, Vic., Aust).

2.2. Plasmid constructs

Expression vectors for the human sFRP4 gene and the CRD and NLD domains were a kind gift from Prof. Roberts Friis, University of Bern, Switzerland. The sFRP4 constructs were prepared by PCR and cloning into the pEGFP-N1 vector (Clontech, CA, USA) so that the full length sFRP4, the CRD domain or the netrin C terminal domain were expressed in frame as amino terminal fusions to the GFP. The sFRP4, CRD and Netrin constructs retained the Kozak and signal sequences of sFRP4. The parental pEGFP-N1 vector expressing GFP was used as a control. Plasmid DNA for transfection was prepared using a HiSpeed Plasmid Midi Kit (Qiagen, Vic., Australia).

2.3. Transfection

Transient transfections were performed on JU77 cells using FUGENE® HD reagent and the pEGFP-N1 plasmid vector constructs. Cells were seeded at a density of 80% confluency in 250ul RPMI growth medium in 8 well Lab Tek chambered #1.0 Borosilicate cover glass system w/cvr (ThermoScientific) on the day of transfection. Following transfection with Reagent:DNA in a ratio of 3:1 for 24 h, a further 48 h of incubation was allowed for the protein to be expressed. The transfection reagent was removed from the cells and replaced with standard complete RPMI medium after 48 h. For the Wnt3a treatment group, 48 h post transfected cells in chambers were replenished with recombinant human Wnt3a (R&D systems) 250 pg/mL in RPMI complete medium for 6 h and imaged under confocal microscope. The GFP-only vector was also expressed to act as a control. Transfected JU77 cells expressing GFP-fusion protein fluorescence were measured using a Nikon-A1+ confocal microscope (Nikon, Tokyo, Japan with 488 nm laser point scanning).

2.4. Confocal live cell imaging and number and brightness analysis

Number and brightness (NB) analysis was performed on the transfected samples and measured by the time series of 100 frames of 256 × 256 pixel, obtained with a HV 96, offset-8 and laser 1.0 setting was used to obtain barely bright enough, best focus, and visible images to see the same GFP fluorescence for all the groups using a scan speed of 1/2. The pixel size was 50 nm with a pixel dwell time of 23.5 ìs, and the pinhole was set at 33.0 ìm. These image stacks were analysed using SimFCS software (Laboratory for Fluorescence Dynamics, University of California, and Irvine CA). The detector calibration was obtained from 100 frames of background image taken before and after the experimental image stacks using the exact same settings but with the laser turned off as described previously (Dalal et al., 2008). Brightness (*B*) of fluorescence particle, $B = 1$ values represent the immobile fraction of the image, and $B > 1$ values represent the mobile fraction (Dalal et al., 2008). To obtain the molecular brightness in photons/molecule/s, the $B = 1$ value was divided by the pixel dwell time. The *B* vs intensity plot was analysed using two cursors. A red cursor selects all the pixels of the image that have *B* values between 1 and 1.5 and paints the pixels of the image in red. A second green cursor was used to select these pixels that have *B* values between 1.5 and 4 which were painted in green. There are essentially no pixels with *B* values above 4. Therefore the *B* images show in green the regions of the cell where larger aggregates form (larger *B* values). For each image the fraction of green and red pixels was also calculated. Changes upon stimulation of Wnt3a were also calculated by normalising the difference of green pixels in the image before and after Wnt3a activation by the total number of pixels above a minimum of intensity threshold.

3. Results

3.1. Establishment of B value for monomeric EGFP

In order to determine the brightness (*B*) of monomeric EGFP we transfected JU77 cells with the parental pEGFP-N1 vector and acquired images as described above. This enabled us to establish background correction and subsequently determine the *B* value due to monomeric EGFP expressed in JU77 cells essentially as previously described (Plotegher et al., 2014). As a result of this analysis we were able to determine that the brightess of monomeric EGFP was distributed from 1 to 1.5 with a mean value for *B* 1.25.

3.2. Peri-nuclear localisation and vesicular trafficking of sFRP4

The nature of the intracellular localisation of sFRP4 (or indeed sFRPs in general) and whether it forms oligomeric aggregates is not known. Therefore JU77 cells were transiently transfected with sFRP4–EGFP and observed by live cell confocal imaging 72 h after transfection. The sFRP4–EGFP was localised predominantly in the