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Modulating and measuring Wingless signalling

Jean-Paul Vincent

MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW71AA, United Kingdom

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ABSTRACT

The main Wnt ligand of *Drosophila* activates a conserved canonical signalling pathway to regulate a plethora of cellular activities during development, regeneration and nervous system function. Here I first describe experimental means of measuring and modulating Wingless signalling in *Drosophila* cell culture. Various reporters have been devised by placing TCF-binding sites or DNA fragments from known target genes upstream of luciferase-coding sequences. Signalling can be activated in cells by addition of Wingless conditioned medium, treatment with a chemical inhibitor of Shaggy/GSK3 or transfection with a plasmid encoding activated Armadillo (*Drosophila* β-catenin). Measuring Wingless signalling in intact tissue is somewhat more challenging than in cell culture. Synthetic transgenic reporters have been devised but further improvements are needed to achieve sensitive responsiveness to Wingless at all times and places. As an alternative, gene traps in *frizzled3* and *notum*/*wingful*, two context-independent endogenous targets, can be used as reporters. It is hoped that further modification of these loci could lead to more versatile and sensitive means of detecting signalling. Many genetic tools are available to trigger ectopic signalling or prevent endogenous signalling. These mostly rely on RNAi-producing transgenes or the generation of mutant patches by mitotic recombination. New developments in genome engineering are opening further means of manipulating the components of Wingless signalling with exquisite temporal and spatial precision.

1. Introduction

Wnts form a family of secreted proteins that act as signalling molecules in a variety of situations [1,2]. Although distinct transduction pathways have been identified, signalling mediated by β-catenin is seen as the core Wnt pathway. It is triggered by engagement of a Wnt with a member of the Frizzled family of 7-pass transmembrane receptors and the co-receptor LRP5/6, which leads to inhibition of the so-called β-catenin destruction complex, comprising Axin, APC and GSK3β. As a result, β-catenin accumulates in the nucleus and, along with TCF, an HMG box DNA binding protein, assembles a transcriptional regulatory complex. The *Drosophila* genome encodes 7 Wnts, 4 Frizzled receptors and one LRP5/6 homolog, called Arrow. Wingless is considered the main Drosophila Wnt, as it controls cellular activities at multiple times and places during development as well as in the adult [3]. Transduction of the Wingless signal absolutely requires Arrow, while Frizzled and Frizzled2 act as redundant receptors. Frizzled3 has been associated with Wingless signalling as a non-essential attenuator while so far, there is no evidence that Frizzled4 contributes to Wingless signalling. In summary, canonical Wnt signalling in *Drosophila* involves one main ligand, Wingless, two redundant receptors Frizzled and Frizzled2 and one co-receptor, Arrow. Like vertebrate canonical Wnt signalling, the Wingless pathway involves inhibition of the destruction complex and stabilisation of β-catenin, which is called Armadillo in *Drosophila*. Stabilised Armadillo interacts with Pangolin (*Drosophila* TCF), and recruits the transcriptional coactivators Legless (homologue of Bcl9) and Pygopus. The best understood form of transcriptional regulation is activation, whereby Armadillo displaces the transcriptional co-repressor Groucho and recruits co-activators at target loci. Direct repression by Armadillo has also been demonstrated although the underlying mechanism is less well understood. The Wingless pathway has been implicated in a large number of processes in *Drosophila* including cell fate determination in the embryonic epidermis, specialisation of the gut, heart patterning, appendage growth and patterning, adult gut homeostasis and memory formation [3–8]. This wide range of function parallels the importance of Wnt signalling in mammals. Because of the relatively small number of Wnt signalling components in *Drosophila* and the tractability of this model system, there is great interest in using *Drosophila* to investigate the basic cell biology underlying the numerous ways that Wnt signalling regulate cell behaviour. One key aspect of such research requires the ability to modulate and measure signalling activity

E-mail address: jvincen@nimr.mrc.ac.uk

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both in cultured cells and in intact tissues. Here I review the most commonly used tools and reagents used to achieve this, emphasizing those that are publicly available.

2. Wingless signalling in cultured cells

2.1. Drosophila cell lines

Cultured *Drosophila* cells have several features that make them particularly amenable to high throughput screening for modulators of Wingless signalling. RNAi is easily achieved by addition of double stranded RNA to the culture medium and unlike mammalian cells, *Drosophila* cells do not mount an innate immune response to long RNA duplexes (http://www.flyrnai.org). Moreover, the *Drosophila* genome is relatively non-redundant, allowing gene function to be readily uncovered. Three *Drosophila* cell lines have been used to study Wingless signalling [9,10]. Clone8 cells were derived from imaginal discs and are therefore thought to be of epithelial origin. Kc167 cells were derived from dissociated embryos and are thought to have characteristics of haemocytes, the main blood cells of *Drosophila*. Another cell line called S2 also resemble embryonic blood cells. They express all the components of Wingless signalling except for the two Frizzled receptors, Frizzled and Frizzled2 and therefore do not respond to exogenous Wingless. However, these receptors are present in a separately maintained S2 line (called S2R+), thus conferring responsiveness. Although all three cell lines (Clone8, Kc167, S2R+) respond to exogenous Wingless, their differ in the extent of the response and in their ease of handling [11]. S2R+ cells are the most commonly used, perhaps because they are relatively robust and adhere somewhat to the substrate, facilitating media changes. Details on how to maintain these cell lines can be found at http://www.flyrnai.org/DRSC-CEL.html.

2.2. Reporters of Wingless signalling

Upon addition of exogenous Wingless or other treatments that activate Wingless signalling (see below), all three cells lines above have been shown to activate the TOPFlash reporter, a synthetic construct initially developed to measure Wnt signalling in vertebrate cells [12]. This reporter includes 7 TCF binding sites (AGATCAAAGGgggta; where lower case indicate spacer sequence) upstream of a minimal promoter comprising the TATA box of the thymidine kinase promoter (M50 Super 8× TOPFlash; see https://www.addgene.org/12456/). Vertebrate researchers typically use as a negative control a plasmid comprising mutated TCF sites (AGGCCAAAGG) upstream of Renilla luciferase (FOPFlash) [12]. The TOPFlash assay has been adapted to *Drosophila* cells in various guises. The first reported *Drosophila* TOPFlash assay involved transfection of Clone8 cells with dTF12 (12 TCF sites upstream of a minimal heat shock promoter driving firefly luciferase) [13,14]. The cells were co-transfected with PolIII-RL (a construct expressing Renilla luciferase under the control of the constitutive PolIII promoter) to provide information about transfection efficiency, cell number and cell viability. In a similar subsequent screen [11], S2R+ cells were transfected with Super TOPFlash, the reporter used in vertebrate cells. For normalisation, a plasmid expressing Renilla luciferase under the control of the constitutive *copia* promoter was used. Although this study suggests that the minimal thymidine kinase promoter is acceptable in *Drosophila*, it is likely that a *Drosophila* promoter (e.g. the minimal *heat shock* promoter) would be more effective. It is also worth noting that the *Drosophila* consensus TCF site differs slightly from the vertebrate sequence [15] and it is conceivable therefore that the TOPFlash reporter could be further optimised for *Drosophila*. Moreover, helper sites identified in Wingless-regulated genes have been shown to boost the activity of synthetic Wingless reporters. For example, in Kc167 cells, a plasmid comprising 6 copies of a combined site (TCF + helper; called TH) upstream of the minimal heat shock promoter and luciferase (6TH-hs-luc) was 10-fold more responsive to transfected actin-Arm* (*actin* promoter driving expression of activated Armadillo; see below) than a similar construct containing 6 copies of only the TCF sites [16]. It appears therefore that 6TH-hs-luc could possibly be the best synthetic transcriptional reporter yet available for cultured *Drosophila* cells. Another efficient reporter was created by inserting 2.2 kb of regulatory sequence from *notum* (AKA *wingful*), a known target gene, upstream of the minimal *heat shock* promoter and the luciferase ORF (wf-hs-luc; [17]). Side-by-side performance comparison of wf-hs-luc and 6TH-hs-luc in clone8, Kc and S2R+ cells will be required to determine which should be the reporter of choice.

Because of their quantitative nature and dynamic range, luciferase-based assays are well suited to measuring Wingless signalling in cultured *Drosophila* cells. However, the TOPFlash signal takes several hours to develop, the time needed for transcription and translation of luciferase to a sufficient level, and there are times when a faster assay is required. One of the first measurable sign of Wingless signalling is an increase in the level of Armadillo protein. This can be measured by Western blot (with Mab N27 A1; see Table) within 2 h of pathway activation, at least in clone8 cells and S2R+ cells [9,10,18]. Like TOPFlash assays, western blots provide an average measure of signalling activity within a cell population. However, it is conceivable that with improved detection methods, the level of Armadillo could be assessed in individual cells. Yet another method to measure Wingless signalling activity in cultured cells is to assess the expression of known Wingless target genes by quantitative RT-PCR (qRT-PCR). This has been demonstrated for two target genes, *notum/wingful* and *naked* [19]. It is hoped that new methods of genomic engineering [20] will enable the creation of more practical reporters e.g. by inserting GFP downstream of the endogenous promoter within the cell's genome. Such reporters are expected to provide a more physiological measure of signalling than a transfected construct.

2.3. Transcriptional repression by Wingless

By design, the TOPFlash assay detects transcriptional activation by Wingless. However, direct negative targets are known to exist both in intact tissues (see below) and in cell culture, e.g. *Ugt36Bc*, *pxn and tig* [19]. Characterisation of these negative targets has led to the identification of a novel TCF repressor site. For example, such a site is present in three copies upstream of Ugt36Bc, making up a 178 bp repressor element. This was used to devise a reporter of repression comprising this element upstream of the metalothionine (MT) promoter driving luciferase expression (Ugt178-MT-Luc; [19]). Addition of copper sulphate activates the MT promoter and this is prevented, in an Armadillo-dependent manner, by activation of Wingless signalling.

2.4. Activating and preventing Wingless signalling in cell culture

The most 'natural' way to activate Wingless signalling in cultured cells is to add exogenous Wingless. There is no commercial source of Wingless but conditioned medium is easily produced from publicly available cells carrying *tubulin-wingless* (S2-tub-Wingless). These cells are typically grown for 3 days in Schneider's medium supplemented with fetal calf serum before conditioned medium is harvested and used neat to activate signalling (note that serum significantly improves the stability of Wingless in the conditioned medium). Alternatively, Wingless can be produced by transfecting a vector expressing Wingless under the control of the