Platforms for high-throughput screening of Wnt/Frizzled antagonists

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Signaling cascades initiated by Wnt lipoglycoproteins and their receptors of the Frizzled family regulate many aspects of animal development and physiology. Improper activation of this signaling promotes carcinogenic transformation and metastasis. Development of agents blocking the Wnt-Frizzled signaling is of prime interest for drug discovery. Despite certain progress no such agents are as yet brought to the market or even to clinical trials. One reason for these delays might be the use of suboptimal readout assays. In this article we overview existing and developing assay platforms to screen for Wnt-Frizzled antagonists. Among those, G protein-activating assays built on the emerging GPCR properties of Frizzleds are highlighted.

The Wnt family of evolutionary conserved secreted lipoglycoproteins has important roles in animal development, physiology, and pathology [1]. The very name '**Wnt**' reflects the history of these proteins, because their first representatives were simultaneously discovered as the segment polarity gene **w***ingless* in Drosophila [2] and the integration site *i***nt***-1* of the mouse mammary tumor virus in rodents [3]. The first identified receptor for Wnts, Frizzled (FZD) received its name after a mutation in Drosophila resulted in randomized orientation of hairs and bristles decorating the insect cuticle. Cloning of the mutated gene revealed that FZD had the topology characteristic for G protein-coupled receptors (GPCR) [4], corroborated by similar analysis performed later on mammalian and other animals' FZD proteins [5–7]. In the present post-genomic era, Wnt and FZD families of proteins are known to comprise 19 and 10 homologs in humans, respectively. Their ligand–receptor interactions initiate an intricate signaling network orchestrating complex processes of tissue formation and polarization.

Having crucial functions in animal development, the Wnt-FZD signaling pathways are mostly silent in the adult [1], with physiological and pathological exceptions. The former include the constant activity of the pathway in synaptic remodeling [8] and its reactivation in tissue regeneration after injury [9]. Pathological implications of the Wnt signaling include bone and heart disease [1,10], and cancer [11,12]. Mutational misactivation of the Wnt pathway is the main driving force in more than 90% cases of colon cancer [13], whereas approximately 50% of breast cancer cases are associated with overactivation of this type of signaling [14], including the most aggressive and therapeutically intractable triple-negative breast cancer [15]. Other organs and tissues are also sensitive to carcinogenic transformation upon improper activation of the Wnt-FZD signaling. Furthermore, this type of signaling becomes additionally involved at later stages of cancer progression, such as tumor metastasis [16].

All this underlies an urgent necessity for development of anticancer drugs specifically targeting the Wnt-FZD signaling [11]. In addition to plain antagonists, other molecules interacting with FZD (agonists, inverse agonists, allosteric modulators) might be highly useful; for example FZD agonists are promising in regeneration medicine and stem cell culturing. Development of human and/or humanized monoclonal antibodies against pathway components represents another approach to Wnt-directed drug discovery [11]. Both pharmaceutical industry and academic laboratories actively seek such agents, and several hit compounds have been identified [17,18]. However, none of them has so far been brought to clinical trials (http://clinicaltrials.gov) let alone to registration. We hypothesize that the reason for these delays could be the suboptimal read-out assays which are prone to identify

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molecules acting at rather downstream levels of the pathway. Such limitation reduces the specificity of the resulting hits in respect to the numerous pathways the Wnt-FZD ligand–receptor pairs can activate. We propose that establishment of readout platforms specifically directed at upstream levels of the pathway is crucial for the successful development of anticancer treatments targeting the Wnt-FZD signaling. The following sections will overview the existing and developing platforms, preceded with a brief description of the complex Wnt-FZD pathways relevant for cancer.

Three pathways in Wnt-FZD signaling as different temporal 'floors'

The most important, developmentally and medically, type of Wnt signaling is the β-catenin-dependent pathway [1,13]. It is also the best-characterized: initiated by interaction of a Wnt with a FZD and a single-pass transmembrane co-receptor LRP5/6, it leads to rearrangement of the Axin-based destruction complex of proteins, including adenomatous polyposis coli (APC), glycogen synthase 3β, and casein kinase I. The job of this complex is to bind and phosphorylate cytoplasmic β-catenin, targeting it for ubiquitinylation and proteasomal degradation [1]. Inactivation of the destruction complex in response to the Wnt signal enables accumulation of β-catenin, its translocation to nucleus, and interaction with transcription co-factors such as TCF/LEF. The outcome is expression of a set of growth-promoting target genes, for example, c-myc and cycD (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). The basic ability of the Wnt β-catenin signaling to stimulate cell proliferation underlies its major role in development in addition to carcinogenesis. Mutations in components of the destruction complex APC and to a lesser extent Axin are the major cause of colon cancer [13]. By contrast, in breast cancer this signaling mostly becomes overactivated at the level of Wnt overproduction [14] (as is the case in the historic integration of the mouse mammary tumor virus in the vicinity of int-1, now called Wnt1, forcing overproduction of the latter in the mammary gland [3]).

Another pathway in the family of Wnt-FZD cascades is referred to as planar cell polarity (PCP) signaling [16,19]. Instead of regulating transcription, PCP controls cytoskeleton, mediating cell polarization in response to extracellular cues, providing certain similarities between PCP and chemotaxis [20]. The key role in cytoskeleton reorganization is achieved by FZD signaling to small GTPases of the Rho family [21]. Additionally, a set of proteins (Flamingo/CELSR, Van Gogh/VANGL, Prickle, and FZD itself) accumulate on the plasma membrane in a polarized manner which serves to amplify the initial polarizing information [19]; this process depends on Rab GTPases controlling receptor endocytosis, directed transport along microtubules, and recycling [22]. Developmentally, PCP is manifested in uniform polarization of insect epithelia and mammalian inner ear stereocilia, and in convergent extension cellular movements required for vertebrate gastrulation and neuronal tube closure [19]. In cancer, the PCP pathway is engaged at late stages of tumor progression rendering cancer cells motile and thus promoting metastasis [16].

Finally, the Ca^{2+}-pathway in Wnt-FZD signaling has been identified in several systems [23]. Physiological (and pathophysiological) significance of mobilization of intracellular Ca^{2+} upon Wnt stimulation remains unclear, although this type of response has been shown to modulate the β-catenin and PCP pathways [23].

These three pathways are typically viewed as different, and largely isolated, branches of Wnt-FZD signaling. However, it becomes increasingly clear that the same Wnt-FZD interaction

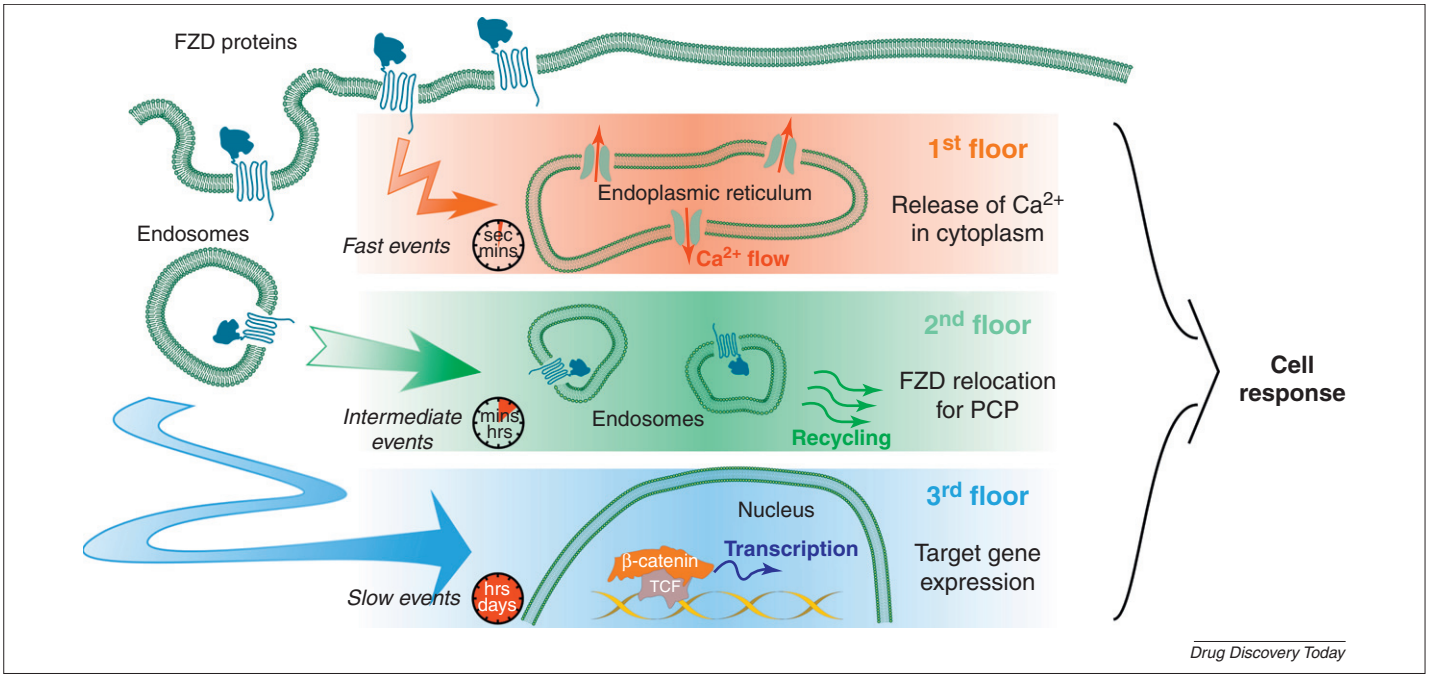

FIGURE 1

'Floors' of the Wnt-FZD signaling. A schematic representation of the temporal hierarchy of Wnt signaling events is given. The red band represents the fastest Ca^{2+}-response; the green highlights the later-occurring endosomal translocation of FZDs to establish the PCP; the blue one is for the slowest β-catenin pathway represented by β-catenin-dependent transcription. Outcome of the signaling at these three 'floors' defines the cell's response to FZD activation. *Abbreviations*: FZD: frizzled; PCP: planar cell polarity.

can induce several 'branches' in the same cell [24]. Furthermore, it is noteworthy that the three pathways operate at very different time scales: the Ca^{2+}-response taking seconds to minutes, cytoskeletal reorganization in PCP – minutes to hours, and β-catenin-dependent transcription – hours to days after cells' stimulation with a Wnt. Thus, we propose that they should not be considered as parallel branches emanating from an activated FZD protein, but rather as temporally hierarchical, sequential 'floors' of the cellular response (Fig. 1). The relative importance of each of the floors of Wnt-FZD signaling is determined by the cellular context (i.e. presence of co-receptors and downstream effectors in addition to extracellular pathway modulating molecules such as sFRP, WIF or Dkk [25]), but also often by the design of the experiment: if it aims at measuring the cells' response to Wnts in the scale of days, faster-happening events such as Ca^{2+} mobilization and cytoskeletal modulations will be omitted from analysis, and vice versa. The hierarchical model of Wnt signaling is supported by the existence of key proteins situated at the top of signaling hierarchy, jointly used by all three pathways: the FZD protein itself, its interaction scaffolding protein Dishevelled (Dvl) [26], and its immediate transducer of the heterotrimeric G protein family [6].

This concept of temporal floors in Wnt signaling has important operational meaning. It implies that, when looking for inhibitors of the β-catenin Wnt signaling, not only the final transcriptional activation can be used as the readout, but also events happening at upper 'floors' and faster time scales, such as rise in intracellular Ca^{2+}. In the following sections we discuss different platforms existing or being developed in Wnt-FZD-directed drug discovery.

Assays based on transcriptional readouts

Currently the leadership among the approaches to the Wnt pathway inhibition belongs to assays based on target gene expression. They can be applied in different cell lines, including those derived from tumors, and typically utilize an artificial reporter [luciferase, green fluorescent protein (GFP)] under the TCF/LEF promoter control whose transcription is induced by accumulation of β-catenin [27]. Analysis at this culminating point of signaling has an advantage of screening for substances affecting the signaling at any level, including even processing and secretion of the Wnt protein itself if the reporter cells are also made to produce the ligand which would activate them in the autocrine manner. Search for the exact target of the hit compounds represents a certain challenge in this approach and may lead to identification of previously unanticipated components of the pathway. A good example is XAV939 identified in a high-throughput screening (HTS) by Novartis (http://www.novartis.com) as a potent suppressor of the Wnt β-catenin signaling. This small molecule turns out to inhibit tankyrase 1 and 2 – poly-ADP-ribosylating enzymes which unexpectedly stabilize Axin [28]. However, tankyrases have many intracellular targets which may restrict the potential of XAV939 in clinical development.

But the fundamental limitation of transcription-based assays for Wnt signaling is that they tend to identify substances acting at downstream steps of the pathway (e.g. Axin stability as in the example above or β-catenin interactions with transcription co-factors [17,18]). This feature was not immediately evident and does not appear in transcription-based assays for a related signaling cascade, the Hedgehog pathway whose high-throughput targeting identified drugs acting at Smoothened, the receptor protein related to FZD [29]; the first of these drugs is already on the market to treat basal cell carcinoma. The underlying reason for this peculiarity of the Wnt transcription-based assays might be the presence of multiple FZD types on the surface of cells used in the assays [30]. Cellular systems where only one FZD isoform would be expressed are currently lacking.

It is clear that substances targeting more downstream steps of the pathway have a higher risk for adverse effects. Indeed, they may affect all the normal cases of engagement of the Wnt signaling such as synaptic plasticity [8] and tissue regeneration [9]. Furthermore, inhibitors of such druggable targets as tankyrase and β-catenin may additionally affect telomere function, mitosis, and cell adhesion [31]. These problems are further aggravated when inhibitors of downstream components (e.g. preventing β-catenin interaction with the TCF transcription co-factor [32]), are explicitly sought. Finally, looking for suppression of Wnt signaling using β-catenin-dependent transcription limits the search to the β-catenin pathway. Nevertheless, cancer cells may also utilize the PCP and Ca^{2+} types of Wnt signaling for their survival and growth [16,33].

Therefore, inhibitors acting on elements in the initiation of the Wnt pathway are required. They can specifically target cancer cell types relying on a particular Wnt or FZD, such as in case of the triple negative breast cancers which are FZD_7-dependent [15]. Such isoform-specific antagonists are in a high demand [11]. Thus, assays focusing on the early components of Wnt signaling are needed. Such assays would be ideal to find hits against breast cancers and metastasizing tumors dependent on overproduction of Wnts [14,33]. At the first glance, these assays may in contrast seem helpless when drugs against cancers induced by mutations in downstream components of the pathway, such as in colon cancer, are to be sought [13]. However, even in these cases, a reinforcement of the signaling by overproduction of Wnts or FZDs, in addition to underproduction of secreted Wnt antagonists, has been reported [13,34,35]. Importantly, blockade of Wnt-FZD interactions by sFRPs (secreted FZD-related proteins) or anti-Wnt antibodies is efficient in suppressing Wnt signaling, slowing down growth of colon cancer cells, and promoting their apoptosis despite the presence of downstream activating mutations [34,35].

Thus, assays focusing on the upstream events in Wnt-FZD signaling may be promising in identifying agents acting against different types of cancer. Such assays and the degree of their development in application to Wnt antagonist screening are summarized in Fig. 2.

Ligand interaction assays

The most upstream event in GPCR activation is the ligand–receptor interaction, and ligand binding assays have been widely used in classical GPCR pharmacology and drug discovery [36]. However, this approach is very problematic in case of Wnt–FZD interactions. Indeed, Wnts are lipoglycoproteins of approximately 40 kDa in size. Their production in bacteria or yeast is impossible despite years of effort, and even in cultured mammalian cell lines production and purification of only some biologically active Wnts out of

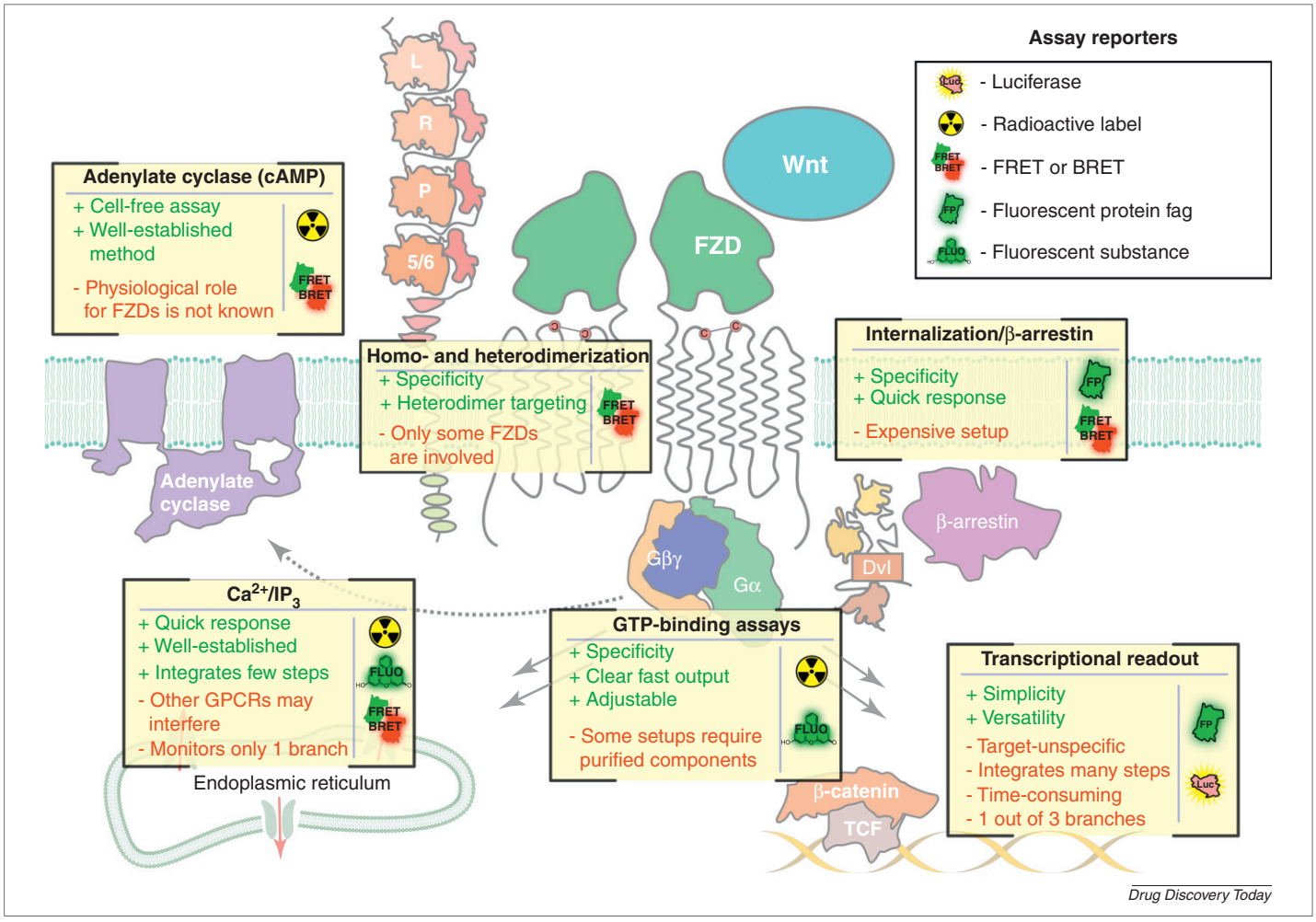

FIGURE 2

Overview of the major features of available Wnt-FZD antagonists screening approaches. The boxes contain brief information about principal advantages and drawbacks of the methods described in the paper, as well as summarize data about reporters available for them. *Abbreviations*: BRET: bioluminescence resonance energy transfer; FRET: fluorescence resonance energy transfer; IP_3: inositol-1,4,5-triphosphate.

the 19 human members of this family has been successful; the resulting proteins are costly. An interesting approach to this problem has been provided by development of Wnt5a- and Wnt1-mimetic peptides [37,38]. Despite their promise, these peptides recapitulate only a subfraction of biological activities of the parental Wnts and can hardly be used in screening for FZD antagonists. In this regard, we consider our attempts to develop, through directed protein evolution approaches, 'super'-Wnts – smaller and easier in production Wnt proteins with the full scope of biological activity [39]. However, these attempts are in their infancy, and at current times establishment of HTS platforms to screen for Wnt-FZD antagonists based on binding assays appears impractical. An additional problem originates from apparent existence of several Wnt binding sites on FZD. Indeed, the highly structured extracellular cysteine-rich domain of FZD is capable of high-affinity interaction with Wnts [40], but is dispensable for the biologic activity of FZD [41]. Thus, ligand binding assays might identify molecules influencing Wnt–FZD interactions, but not necessarily having a functional consequence to the activation of Wnt pathways. Also in this regard the functional readouts of Wnt–FZD binding are required.

Ca^{2+}-related assays

Rise in intracellular [Ca^{2+}] occurs within seconds of GPCR activation and involves fewer intermediates as compared to a transcriptional response. Ca^{2+}-assays are thus more promising for finding small molecules that directly affect the Wnt–FZD interaction. GPCRs best to be screened by Ca^{2+}-methods are coupled to Go or Gq heterotrimeric G proteins [42], and FZDs fall into this category [43–45].

The techniques to follow the Ca^{2+} pathways are well-established and involve monitoring of the second messenger inositol-1,4,5-triphosphate (IP_3, produced by phospholipase C and activating calcium-releasing channels of endoplasmic reticulum) or Ca^{2+} ions themselves. The former measure displacement from the purified IP_3-receptor of radioactively or fluorescently labeled IP_3 by the messenger produced in response to GPCR activation [42,46]. Alternatively, the IP_3 metabolite IP_1 can be measured instead [47].

To directly monitor intracellular [Ca^{2+}], a large variety of fluorescent and luminescent sensors have been developed [48]. Such measurements are somewhat less robust in comparison to IP_3 detection due to a more transient nature of the signal. The choice between IP_3 and the direct Ca^{2+} detection is mainly empirical and

depends on the basal levels of these second messengers in a given cell type. Production of both messengers in response to FZD activation has been explicitly demonstrated [23,49]. However, no high-throughput assay for Wnt-FZD antagonist screening by IP_3 or Ca^{2+} has been formatted yet.

Adenylyl cyclase assay

Many FZDs are known to activate heterotrimeric G proteins of the Gi family [50–53] which are capable of inhibiting cAMP-producing adenylyl cyclase (AC). Furthermore, some FZDs are coupled to the heterotrimeric Gs protein [50,54–56] which instead activates AC. Thus it should be possible to design a Wnt-FZD readout assay monitoring AC activity. While Gs-mediated activation of cAMP production is readily detectable, Gi-induced inhibition of the enzyme is often measured on the background of forced AC activation by forskolin [57].

High-throughput methods to follow AC activity use cAMP analogs and cAMP-specific antibodies, both of which are labeled with fluorescent groups. Concentration of the complex, reduced upon production of 'cold' cAMP upon stimulation of the GPCR-Gs-AC cascade, is quantified by fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) [58]. These methods might be used both for isolated membranes and for cultured cell lines. cAMP measurements in living cells have also been developed utilizing cAMP-sensitive ion channels. In cells expressing these channels a production of the cyclic nucleotide leads to changes in the membrane potential which could be detected by a voltage-sensing fluorescent dye and quantified with a microplate reader. This approach has been successfully employed to screen for GPCR-targeting molecules [59].

High-content screening for Wnt-FZD signaling

The method of high-content screening (HCS) exploits structural rearrangements of the cell induced by receptor activation [60]. HCS requires automatic confocal or epi-fluorescent microscopes used to produce a sequence of images of cells cultured in multiwell plates. FZD-dependent signaling often leads to profound changes in cellular morphology and redistribution of cellular components, and thus is in principle amenable to HCS. However, no such applications have been developed so far.

Among numerous possible HCS assays, the one monitoring FZD endocytosis appears attractive, along the lines established for other GPCRs [61]. Indeed, internalization of activated FZD proteins is utilized in different Wnt pathways and involves early transducers, making it amenable for screening with the focus on the early signaling events [43,62]. In most cases internalization of a GPCR leads to attenuation of the signal. Some GPCRs, and FZDs among them, contradict this rule [22,63] by the use of the endosomal compartment for prolonged and enhanced signaling activity. FZD endocytosis in HCS might be monitored using antibodies specific to extracellular parts of the receptor, or alternatively utilizing FZD fusion with a fluorescent tag not interfering with endocytosis. FZD internalization thus could be readily employed to screen for antagonists or agonists of the pathway in a highly biologically relevant setup. This approach has been applied to screen for compounds inducing FZD_1 endocytosis among FDA-approved drugs, identifying the anthelmintics niclosamide as an indirect inhibitor of Wnt3a signaling [64].

GPCR, and FZD internalization is usually mediated by β-arrestin binding to the intracellular domain of the receptor, which usually requires prior phosphorylation [62,65]. This phenomenon has been applied to increase the robustness of the internalization monitoring by FRET or BRET between tagged GPCRs and β-arrestin. Such a double-labeling method is widely used in the GPCR field and could be observed with any convenient donor–receptor pair [65]. Unusually for GPCRs, the FZD-β-arrestin interaction requires the scaffolding phosphoprotein Dvl as an intermediate [66]. The three proteins, in an immediate proximity to each other, are brought into the endosomal compartment [66,67]. This feature enables application of highly sensitive energy transfer-based detection methods for HCS. Looking for agents blocking interaction of specific FZDs with Dvl is another promising approach [68].

Screenings based on receptor dimerization

Dimer formation by some FZD proteins is necessary for manifestation of their activity [69]. Because dimerization appears as an inherent function and hallmark of correct activation of these FZDs, molecules which affect this phenomenon will be their effective antagonists. Techniques to measure the dimerization ratio, similarly to the β-arrestin monitoring, involve FRET or BRET between complementary fluorescent tags placed on GPCR molecules [70]. This method should also be potent in monitoring receptor heterodimerization which is not a rare feature in GPCR biology and may occur with FZD proteins [70]. As an alternative to the interactions between two FZDs, proximity between FZD and LRP5/6 can also be monitored.

GTP-binding assays

For GPCRs, the most immediate functional outcome of the interaction with ligand is activation of the guanine nucleotide exchange on the cognate heterotrimeric G protein. As with other GPCRs, FZD in some contexts can signal *in vivo* bypassing the G proteins [55]. Despite this, the capacity of FZDs to activate G proteins represents a powerful resource to design Wnt-FZD screening platforms, especially in reconstituted systems ([51,52,54,71,72] http://precedings.nature.com/site/help).

The capability of GPCRs to increase the rate of GTP incorporation is monitored by replacing GTP with its non-hydrolysable labeled analogs, choosing from the main two currently available on the market: the radioactive [^{35}S]GTPγS and the fluorescent GTP-Eu [73]. The former has been applied since the dawn of G protein investigations. It represents a robust and well-established methodology but suffers from the limitations the radioactive label imposes on high-throughput applications. The relatively novel GTP-Eu where GTP is modified with a fluorescent europium chelate has been designed specifically for HTS using GTP-binding GPCR assays [73]. Thanks to its higher affinity to G proteins and dramatically better sensitivity, GTP-Eu is superior to other available fluorescent GTP analogs such as BODIPY-GTPγS [74]. The superb sensitivity of GTP-Eu originates from the physical nature of its fluorophore: unlike in typical fluorescence, it emits light with a delay post-excitation (this phenomenon is known as time-resolved fluorescence or phosphorescence). This feature leads to a drastic improvement of the signal-to-noise ratio, making GTP-Eu an excellent alternative to [^{35}S]GTPγS [75].

FZDs can be provided by different sources for HTS GTP-binding assays. Natural FZD-containing plasma membrane preparations have been used for this purpose, for example, from rat brains which express FZD_1, FZD_3, FZD_6, FZD_7, and FZD_9 [50], or from microglia-derived N13 cell naturally expressing FZD_5 and FZD_9 [51]. Other cell lines also express endogenous FZDs [30]. Alternatively, cultured cells can be transfected to overexpress the FZD of choice to prepare membranes enriched in FZD for the assay [50]. Finally, many functional GPCRs have been expressed in bacteria [76], and FZD proteins followed this route for subsequent utilization in the GTP-Eu-binding assay [71,72]. Clearly, heterotrimeric G proteins (easily purified from porcine brains) have to be added to the assay in this case [50,71,72].

There are additional promising developments of the FZD-measuring GTP-binding assays which have not been fully exploited yet. One is related to the possibility of FZD production in cell-free transcription-translation systems targeting receptors into detergent micelles or liposomes as has been done for several GPCRs [77]. Another relies on FZD-Gα, Wnt-FZD, and even Wnt-FZD-Gα fusion proteins. GPCR-Gα fusions provide the fixed receptor and/or effector ratio and have been successfully used in GPCR assays [78], while creating the active Wnt-FZD fusions omits the need of purifying Wnt proteins [79], the cumbersome and expensive task.

Overall, GTP-binding assays monitor the earliest events in the Wnt pathway and their application for HTS of small-molecule regulators of FZD proteins opens exciting avenues to identify pathway inhibitors acting at the level of the receptor.

Concluding remarks

The Wnt-FZD signaling pathways have key roles in initiation and progression of several types of cancer in human patients. Development of proper readout assays applicable for HTS of small molecules acting on Wnt signaling is of prime importance for anticancer drug discovery. We have overviewed several existing and developing platforms monitoring Wnt pathway activation. Although the current HTS efforts are dominated by the usage of transcription-based assays, it is our conviction that readouts focusing on the more upstream events of the pathway are in urgent demand. These readouts largely exploit the emerging GPCR properties of FZD proteins, the feature for the long time undervalued by Wnt researchers [43]. Embracing FZDs as part of the GPCR superfamily arms the field with the vast inventory of the GPCR-targeting drug discovery approaches [42,61]. We think that development and application of such approaches should boost the Wnt signaling-directed drug discovery. The new assays will hopefully let the Wnt investigators catch up with their peers working on other cancer-related signaling pathways and advance the anti-Wnt drug discovery efforts to clinical trials. Novel specific therapies, primarily against colon and breast cancer, will be the fruit of these efforts.

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