DRUG DISCOVERY TODAY TARGETS

Investigating Wnt signaling: a chemogenomic safari

Michael McMillan and Michael Kahn

Chemical genomics is a powerful method to complement more traditional genetic techniques (i.e. knockout mice, siRNA) for the dissection of complex signaling networks. Wnt signaling in mammals is a complex and crucial regulator of diverse functions. The Wnt–β-catenin pathway initiates a signaling cascade that is crucial in both normal development and the initiation and progression of cancer. A key step in Wnt activation of target genes is the nuclear translocation of β-catenin and the formation of a complex between β-catenin and members of the T-cell factor (TCF) family of transcription factors. Using a forward chemical genomics strategy, we identified ICG-001, a selective inhibitor of a subset of Wnt–β-catenin-driven gene expression. This chemogenomic tool enables us to dissect this complex signaling network and to better understand the role of Wnt signaling in both normal and pathophysiological settings.

Chemical genomics: definition

The term 'chemical genomics' (or chemical genetics; here we will use the terms interchangeably) is a relatively recent addition to the research lexicon and has been used to describe a variety of endeavors. Although the name implies a mix of elements of classical medicinal chemistry and genetics, it is also used to describe a wide range of new technologies related to aspects of these disciplines, such as HTS [1,2] and novel methods of target identification [3–6]. Chemical genetics involves the study of living systems and its goal is to aid our understanding of the molecular basis of complex phenotypes. It assumes that small organic molecules can be found that selectively modulate the cellular network of complex interactions that control phenotypes. Several excellent recent reviews have appeared that cover most of the literature to date [7–13].

By analogy to genetics, chemical genomics is divided into forward and reverse chemical genomics. Reverse chemical genomics refers to the modification of a particular gene/protein and the examination of the phenotype produced by the modification. The majority of modern drug discovery uses this basic approach, by screening small molecules *in vitro* against a defined target, selecting a bioactive small molecule and finally observing the phenotypic consequences of inhibiting this target in living systems. This is an excellent approach to understand the role of a specified target within the overall living system.

The focus of this review will be exclusively forward chemical genomics (FCG), which by our definition requires the successful integration of all of the following steps: (i) cell- or organism-based screening and discovery of a small molecule that elicits a specific phenotypic response [14,15]; (ii) identification of a specific molecular target, which could be responsible for the activity of the small molecule: (iii) validation of the proposed target, including a demonstration of specificity of the small molecule; and (iv) upon

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successful completion of steps i–iii, use of the small molecule to conduct further studies regarding the basic biology of the molecular target or interaction, including the pathway(s) in which it participates and its role under both physiological and pathophysiological settings. Without properly characterizing and validating the target and mode-of-action, it is extremely challenging to gain a better understanding of basic biology.

FCG does not bias the choice of molecular target, as is the case with reverse chemical genomics; rather it seeks to find any relevant target(s) in a pathway and, as such, is well adapted to study complex signaling networks, where all the components and interactions might not be known. A FCG 'knockout' or 'knockdown' interacts with only a small part of the total surface of its protein target and can, therefore, remove a specific subset of protein–protein interactions for a target at a specific time during development (cellular or organismal); however, by definition a genetic 'knockout' or 'knockdown' will remove all of the interactions of that protein. This is an important distinction and of particular relevance for the analysis of networks because the orchestration of complex signaling networks generally involves multidomain scaffolding or 'master organizing' proteins that interact with a large number of partners to affect the proper cellular response. Ultimately, the validation of a therapeutic target rests on the cumulative weight of all experimental evidence and chemical genomics can provide additional valuable information that is currently not readily provided by genetic methods. Here, we will demonstrate how FCG investigations can augment more traditional genetic tools to dissect the Wnt signaling network and enrich our understanding of the processes it controls.

Library design and the intersection of chemical and biological space

With the completion of the human genome sequence, it is now possible to deduce the number and sequences of most of the genes and hence the proteins they encode. However, having a 'parts list' does not tell us how the 'parts interact to operate the 'machine' as a whole. The function of many proteins, which share crucial homology domains, can be grouped into 'superfamilies' (i.e. kinases, GPCRs and zinc fingers) and the number of members of a particular superfamily can be estimated from sequence homologies. Thus, the approximate number of classical pharmaceutical targets (i.e. GPCRs, peptidases, kinases, ion channels, and so on) can be estimated.

Historically, medicinal chemistry has shown that certain 'privileged scaffolds' [16] (i.e. particular chemical classes, benzodiazepines, β-lactams) target specific superfamilies quite effectively. For example, efforts to develop inhibitors for protein kinases have demonstrated that the preponderance of these structures fall within the pi-heterocycle category that mimic ATP [17]. Indeed, for each class or superfamily of targets, there is a defined subset of 'chemical space' or motif, which best overlaps with the 'biological space' that the superfamily recognizes.

Therefore, the design of the small-molecule library and what 'chemical space' it occupies is of great importance to interact with a molecular target successfully. Merely populating random 'chemical space', no matter how large a library is constructed, will not be productive if there is no overlap with the 'biological space' of interest (Figure 1). If the molecules in the library are too 'simple', it is doubtful that they will be selective enough to be useful to further our understanding about a particular target or pathway. By definition, libraries of biologically active compounds, which have been selected due to their activity towards other targets, will be nonselective and again of questionable use in probing novel complex signaling pathways [18]. However, their core structures could serve as useful scaffolds on which to develop focused libraries for related superfamily targets. 'Directed libraries' towards one class of targets offer a 'semi-rational' approach to probe members of a superfamily.

Investigations using peptidomimetic templates

To investigate complex signaling networks, we have chosen to direct our libraries towards potential protein–protein interactions because these are the basic components from which these networks are constructed. Our design of small-molecule chemical libraries for targeting protein–protein interactions has been guided by the simplistic elegance of nature. Nature has used three basic secondary structure building blocks for the construction of all proteins: α-helices, β-strands and reverse turns [19,20]. Through combinatorial derivatization of these three architectural elements, and hundreds of millions of years of optimization, specific protein–protein recognition events have been developed to control complex genetic programs that are essential to the survival of cells and multicellular organisms.

Over the years, our laboratory has developed small-molecule peptidomimetic templates, which mimic these secondary structure elements [21–23] and have proven activity against a wide range of protein targets, including GPCRs and peptidases. These peptidomimetic templates can be synthesized in a combinatorial fashion using solid phase synthetic protocols. Libraries (of up to tens of thousands) of small molecules can be synthesized based on these templates and used for high-throughput screens. The 'chemical space' of these libraries should overlap well with the 'biological space' for protein–protein interactions because they were designed to mimic recognition patterns that nature has optimized for a billion years. Although targeting a specific protein–protein interaction with a small molecule has generally proven problematic [24,25], screening a directed library of small molecules against a network of protein–protein interactions within a signal transduction pathway increases the odds of finding active compounds directed at one of the potential targets involved in the regulation of these pathways.

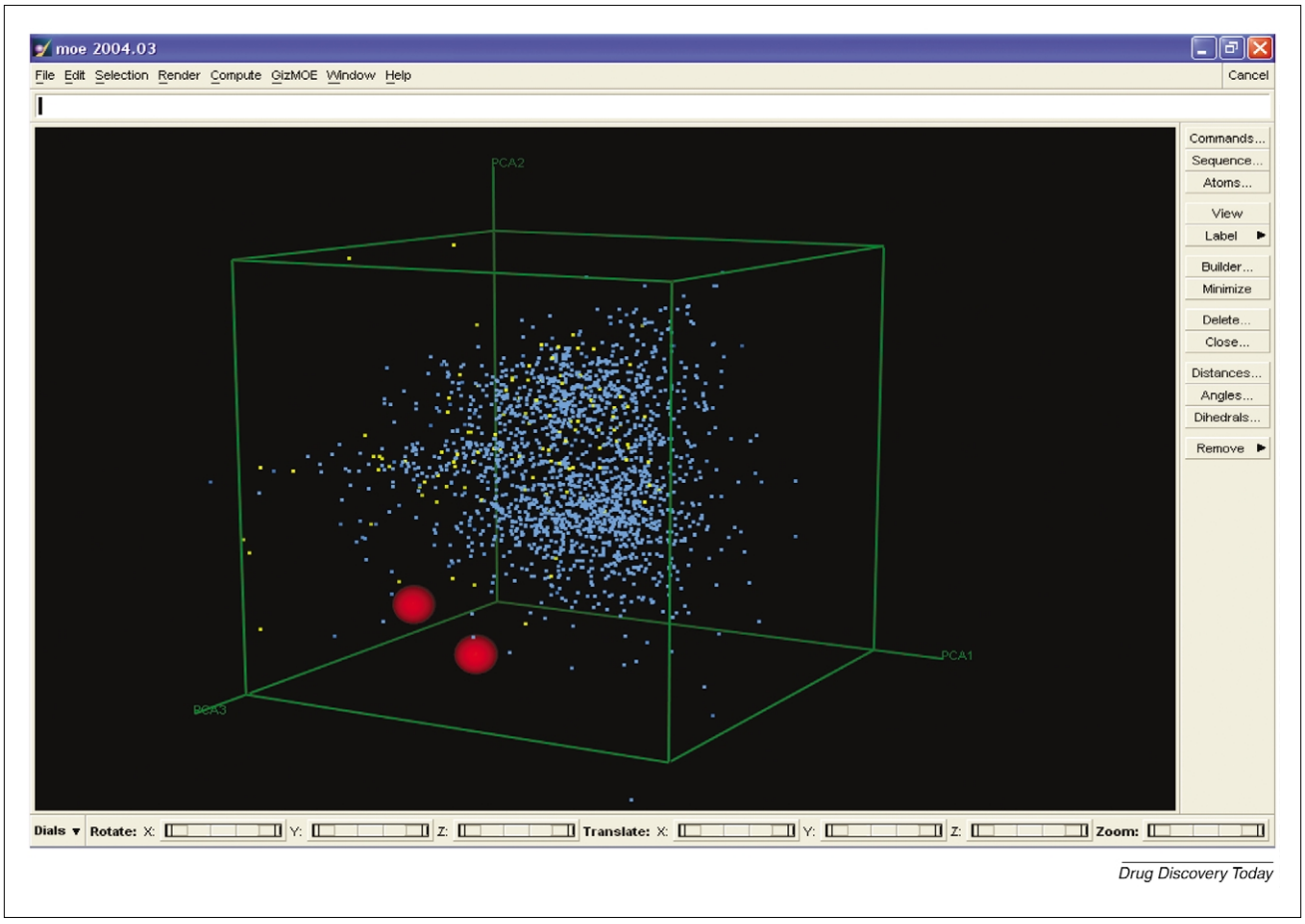

FIGURE 1

A 3D representation showing the first three components of principal component analysis (PCA). Twenty descriptors are selected to allow 75% of the variance to be retained with a database containing the following: ~1600 'clean' NCI diverse representative set (blue) (diversity set derived from ~140,000 NCI compounds; http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html); ~100 'clean' anti-cancer compounds (yellow) [61,62]; ICG-001 and ICG-427 (red).

Chemogenomic investigation of Wnt signaling: reflections on a safari

Wnt signaling in mammals is a complex and crucial regulator of diverse functions (Figure 2). In the mammalian genome, 19 Wnt genes bind to two receptor families, the Frizzled proteins (10 members) and lipoprotein receptor related proteins 5 and 6 (LRP-5/6). The Wnt genes are powerful regulators of both cell proliferation and differentiation and the Wnt–β-catenin pathway initiates a signaling cascade that is crucial in both normal development and the initiation and progression of cancer. The hallmark of this pathway is that it activates the transcriptional role of the multifunctional protein β-catenin [26–30].

Under physiological conditions, the Wnt–β-catenin pathway regulates the expression of a range of genes involved in promoting proliferation and differentiation. In >85% of colon cancers, one of two components [either APC (adenomatous polyposis coli) or β-catenin itself] of the 'β-catenin destruction complex', which normally regulates the degradation of cytoplasmic β-catenin by the proteasome, is mutated leading to an accumulation of nuclear β-catenin and constitutive expression of its target genes [31]. Many of these genes, including *cyclin D1* [32,33] and *c-myc* [34] have crucial roles in cell growth, proliferation and differentiation, and are inappropriately activated in colon cancer. For this reason, we initially decided to look for antagonists of this pathway in colon cancer cells. Although our initial goal was quite well defined, it initiated a 'safari' that has provided, and continues to provide, a great deal of new and exciting information about Wnt signaling. Furthermore, this 'safari' has provided us with new insights into one of the most fundamental control points in cell biology; that is, a cell's switch from proliferation to differentiation, which is crucial not only in cancer and other diseases but also in stem cell biology and normal development.

Step one: screening of a small-molecule library

The beginning of the 'safari' starts with the establishment of a high throughput phenotypic screen. Due to mutations in APC, SW480 colon carcinoma cells exhibit constitutive translocation of β-catenin to the nucleus, and, thus, high basal Wnt–β-catenin transcription as assessed by the consensus TCF–β-catenin luciferase reporter construct

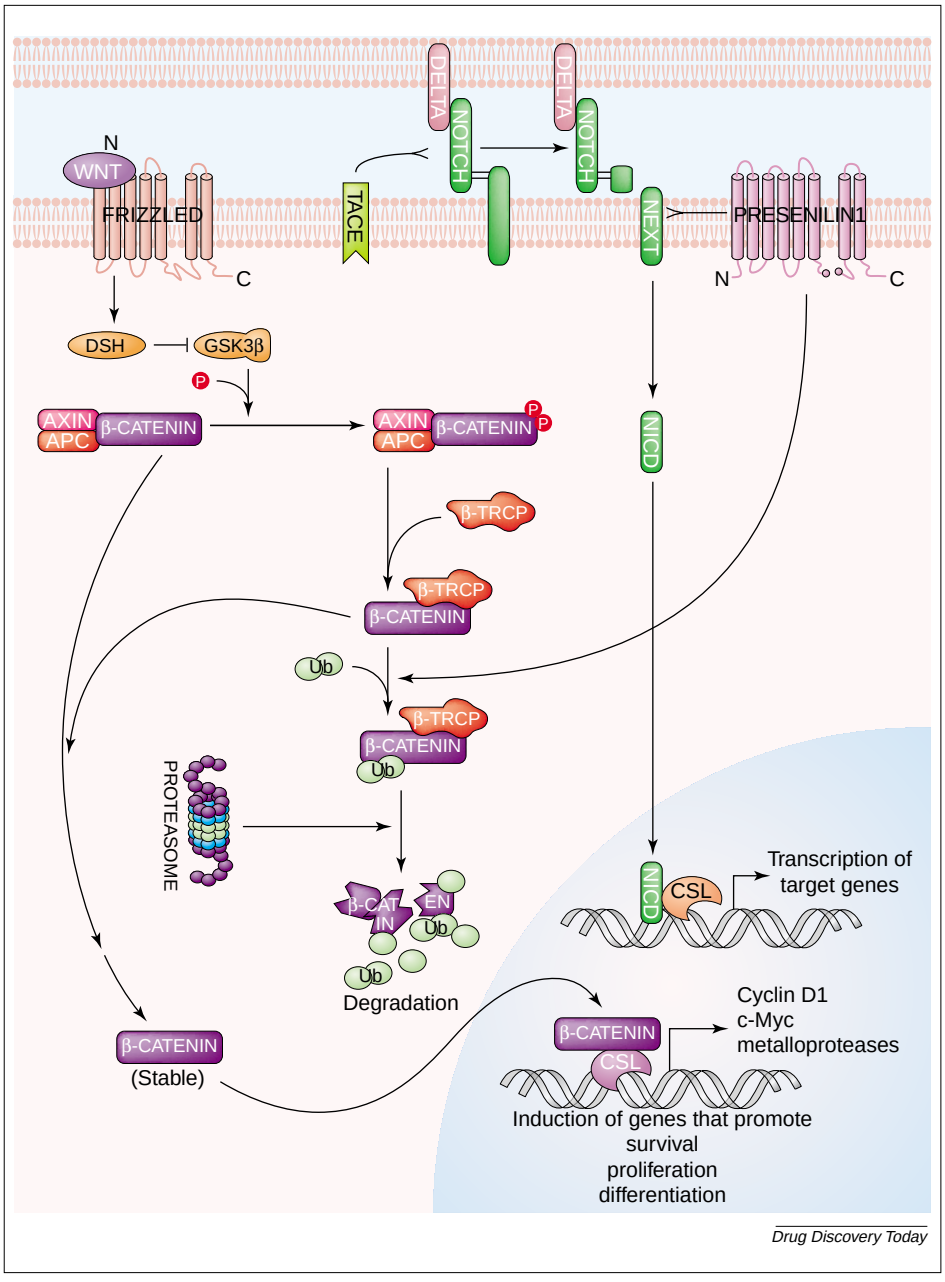

FIGURE 2

Presenilin action in Notch and Wnt signaling.

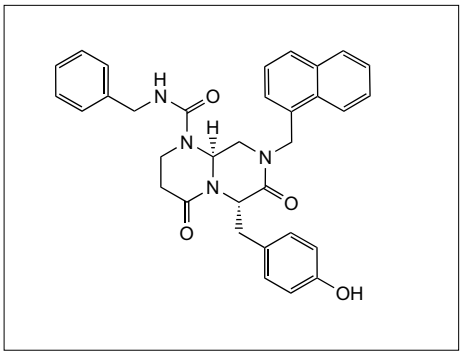

FIGURE 3

Chemical structure of ICG-001.

TOPFLASH [34]. Using this reporter assay, we screened a secondary structure-templated small-molecule library of 5000 compounds [22,23] for inhibitors of β-catenin–TCF-mediated transcription. Three closely related compounds were identified in the initial screen, from which we selected the most potent one, ICG-001, for further investigation (Figure 3) [35].

Step two: target identification

Having identified a 'selective' hit molecule, the next step in the process was to identify its molecular target(s). Although several procedures have been described for this step, including affinity chromatography and phage 'panning', this is often a troublesome and disconcerting part of the FCG process [4–6] and in great measure why the FCG strategy has seen limited use in drug discovery in recent years.

To find the molecular target of IGC-001, we synthesized a biotinylated derivative, ICG-002, for use as an affinity reagent to probe a mixture of solubilized proteins. The only major band retained on the ICG-002 affinity column that was eluted specifically by ICG-001 had an apparent molecular weight of 225 kDa and was identified by immunoblotting as the transcriptional coactivator Creb-binding protein (CBP). This was interesting, in that to generate a transcriptionally active complex, β-catenin recruits the transcriptional coactivators, CBP or its closely related homolog p300 [36,37], as well as other components of the basal transcription machinery. However, CBP/p300 are extremely large and highly homologous proteins that interact with perhaps hundreds of proteins to coordinate transcription [38]. Hence, CBP could be just a 'sticky protein' and the binding observed might have nothing to do with the activity of ICG-001

Specificity of ICG-001

The power of a chemical genomic tool lies in its ability to affect a biological interaction selectively. To explore the selectivity of ICG-001, we initially used reporter gene assays. ICG-001 did not inhibit other CBP/p300-dependent reporters, including AP-1 and CRE [35], and showed no significant activity in a general pharmacological screen against ~50 well-characterized receptors, ion channels and enzymes. ICG-001 is selectively cytotoxic to a wide range of cancer cells, while displaying almost no toxicity to normal differentiated cells and inducing differentiation in stem/progenitor cells (i.e. C2C12 myoblasts). cDNA microarray analysis demonstrated that ICG-001 had a very selective effect on global gene transcription. Interestingly,

two of the genes downregulated by the compound are *S100A4* and *survivin*, the number one and four mRNAs upregulated in cancer cells, respectively [39].

Step three: validation of the molecular target

The next step in the process is to confirm that the biological activity of the hit molecule ICG-001, identified in step one, is due to its interaction with the target identified in step two (i.e. CBP). It is important to confirm that at the stringency used, the hit molecule interacts with the identified target and that this interaction is responsible for the biological activity observed. This validation requires the integration of a variety of techniques (biochemical and genetic) and is a crucial but often overlooked step, which will enable us to continue confidently on our safari.

ICG-001 selectively disrupts the CBP–β-catenin but not the p300–β-catenin interaction

To demonstrate that ICG-001 bound to the proposed molecular target (CBP) in cells, we performed the following experiment. Nuclear lysates prepared from cells transfected with CBP had a ~4–6 fold increased incorporation of ^{14}C-labeled ICG-001 compared to controls (β-catenin or empty vector transfected). Immunoprecipitation of β-catenin with CBP was inhibited by ICG-001 in a concentration-dependent manner. The binding of ICG-001 to CBP is specific; the compound did not interfere with the binding of β-catenin to p300 [35]. To exclude the possibility of an indirect association between CBP and ICG-001 mediated via another cellular component, we used recombinant CBP, p300 and β-catenin proteins to map the binding domains. We found that ICG-001 interacted with amino acids 1–111 of CBP. The C-terminal region of β-catenin (647–781) interacted with the same 1–111 amino acids of both CBP and p300 [35]. However, only the CBP–β-catenin interaction and not that between p300 and β-catenin was disrupted by ICG-001. This data confirmed the direct association between CBP and ICG-001, and the ability of ICG-001 to selectively disrupt the CBP–β-catenin interaction, without affecting the p300–β-catenin interaction, despite the extremely high degree of homology between the two co-activators.

The biological activity of ICG-001 is CBP dependent

To further confirm that the biological activity of ICG-001 was due to its ability to bind CBP and block the CBP–β-catenin interaction, we performed a series of experiments akin to classical 'gain of function' and 'loss of function' genetic experiments. We anticipated that transfection of additional CBP should raise the IC_{50} of the inhibitor. Indeed, transfection of increasing amounts of CBP (but not p300 or β-catenin) raised the IC_{50} of ICG-001 in a dose-dependent manner. Specific downregulation of CBP (but not p300) protein levels lowered the IC_{50} values dramatically and hence increased the inhibitory effects of ICG-001. Thus, the specific interaction of ICG-001 with CBP and its ability to block the CBP–β-catenin interaction is directly correlated with its observed bioactivity.

Step four: the real excitement begins: exploring the biology

Having characterized the molecular target of the compound screened in step one and demonstrated the selectivity of the compound for the molecular target, we can now safely enter the most exciting part of the safari – exploring the unknown aspects of the pathway. In the case of ICG-001, we chose to first explore the effects of differential coactivator usage (i.e. CBP versus p300) on TCF–β-catenin-mediated transcription. This would be difficult to do using classic 'knockout' or 'knockdown' techniques because CBP and p300 interact with a large number of partners other than β-catenin [38]. Therefore, genetic deletion of CBP has complex consequences affecting a multitude of different transcription factors. However, using ICG-100 we were able to specifically and selectively block only the CBP–catenin interaction. Now we are in a position to explore uncharted areas of Wnt signaling.

Chemogenomic investigation of differential coactivator usage

In our cDNA microarray analysis, we observed a marked reduction in the transcription of *survivin* (~70%), along with markers of cell proliferation, such as *PCNA* and *Ki67*. Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins that is prominently upregulated in many human cancers [40–42], yet is virtually undetectable in normal adult tissues. The mechanisms controlling *survivin* gene transcription in cancer cells remain elusive. Recent studies in colon cancer cells suggest that regulation of *survivin* expression is at least partially TCF–β-catenin dependent [43,44]. Using ICG-001 to block the interaction between CBP and β-catenin, we found a dose-dependent inhibition of *survivin* gene transcription and expression in colon carcinoma cells lines, as well as in breast and prostate cancer cells [35,45]. This suggested that in a variety of cancer cells *survivin* gene transcription is TCF–β-catenin regulated in a CBP-dependent manner [45].

Chromatin immunoprecipitation assay

To further analyze the effects of ICG-001 on *survivin* transcription, we used the chromatin immunoprecipitation assay to investigate the events occurring at the *survivin* promoter. ICG-001 treatment reduced the occupancy of CBP and increased the recruitment of p300 to the *survivin* promoter with concomitant increased recruitment of the transcriptional repressors SUMO-1, HDAC6 and PML [45–47]. Interestingly, we observed three promoter-specific differential outcomes in colorectal cancer cells at TCF–β-catenin-regulated gene promoters upon disrupting the β-catenin–CBP interaction with ICG-001. At the *cyclin D1* promoter, we observed a decrease in message and no recruitment of p300 [35]; at the *c-myc* promoter, we

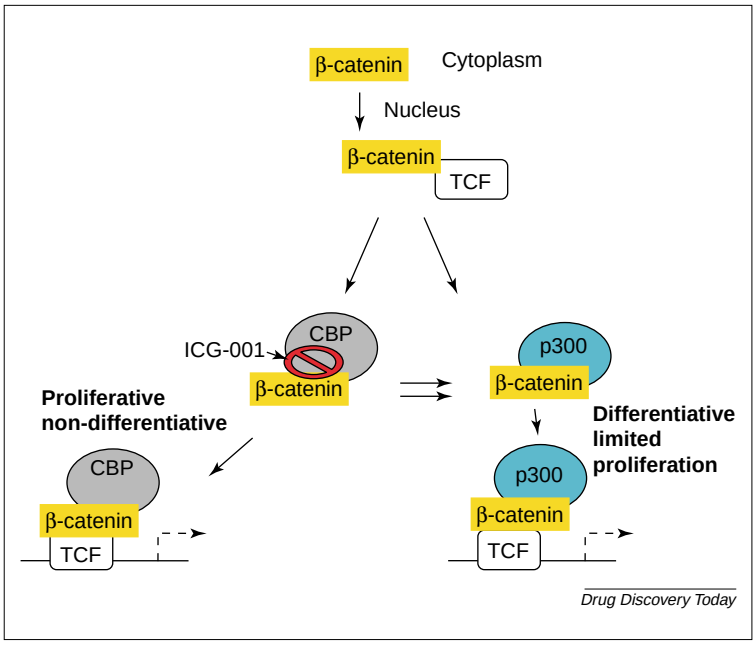

FIGURE 4

Model for differential coactivator usage in Wnt–β-catenin transcription and its role in regulating proliferation versus differentiation.

observed an increase in message with increased p300 occupancy; whereas at the *survivin* promoter, we observed increased recruitment of p300 along with additional proteins associated with transcriptional repression and a corresponding decrease in *survivin* message [45].

Wnt–β-catenin signaling in neuronal differentiation and Alzheimer's disease

Canonical Wnt signaling has been shown to promote self-renewal in a variety of tissue stem cells, including neuronal stem cells and hematopoeitic stem cells [29]. However, in neuronal precursor cells, activation of the canonical Wnt pathway can promote or inhibit differentiation depending on the experimental circumstances [48–50]. A clear explanation for the differential effects of Wnt activation on neuronal precursors is not available at present.

Alzheimer's disease (AD) is characterized by the formation of amyloid plaques and neurofibrillary tangles and extensive synapse loss, which is strongly correlated with the decline of cognitive function. Although significant effort has been devoted to link either amyloid deposits or neurofibrillary degeneration with the progression of AD, it is possible that these two markers are independent manifestations of a common underlying defect in neuronal homeostasis and plasticity [51]. Missense mutations in presenilin-1 (PS-1) are associated with early-onset familial AD (FAD) [52]. PS-1 is a crucial component of the γ-secretase complex and mutant PS-1 increases the production of amyloid plaque [53], as well as the proteolysis of Notch intracellular domain (NICD) [54]. PS-1 has also been shown to interact with β-catenin and other members of the armadillo family of proteins [55,56].

BOX 1

Key points

- ICG-001 represents the first selective small-molecule chemogenomic tool to modulate Wnt/catenin signaling.
- ICG-001 selectively blocks the CBP–β-catenin interaction without affecting the highly homologous p300–β-catenin interaction.
- ICG-001 has provided a unique tool to explore differential coactivator usage in the Wnt/catenin signaling pathway.
- The switch from β-catenin–CBP to β-catenin–p300 controls one of the most fundamental cell switch points (i.e. the switch from proliferation without differentiation – in other words, in stem cells/progenitors and cancer) to the initiation of a differentiative program with limited proliferative capacity.

FAD mutant PS-1 affects Wnt signaling

To investigate the role of Wnt signaling in a cell-based model of FAD, we performed a series of experiments. PC-12 stably expressing the FAD mutation PS-1/L286V showed defective NGF-induced neurite outgrowth and reporter assays demonstrated increased TCF–β-catenin-mediated signaling compared to controls. Moreover, we demonstrated that in these same cells, increased TCF–β-catenin-mediated signaling is correlated with an increase in cyclin D1 expression. This is particularly interesting because repression of cyclin D1 transcription and cell cycle arrest are highly coordinated with neurogenesis [57,58].

This led us to hypothesize that misregulated TCF–β-catenin–CBP dependent signaling in the mutant PC-12/L286V cells was responsible for defective differentiation and neurite outgrowth. To test this hypothesis, we used ICG-001. Treatment of the mutant cells with ICG-001 decreased *cyclin D1* reporter gene transcription and message levels. Treatment of mutant cells with NGF and ICG-001 led to morphologically normal neurite outgrowth and differentiation [59].

A switch from proliferation to differentiation

Despite their high degree of homology and similar patterns of expression, CBP and p300 have unique and distinct roles in gene regulation [38]. Our chemogenomic studies with ICG-001 present strong evidence that CBP and p300 have distinct functions in the regulation of *survivin,* and *cyclin D1* transcription [35,45]. We have developed a model, which proposes that TCF–β-catenin–CBP driven transcription is crucial for proliferation without differentiation (e.g. in cancer and stem cells), whereas a switch to TCF–β-catenin–p300 mediated gene expression is an essential first step in normal cellular differentiation (Figure 4). Aberrant regulation of the balance between these two related transcriptional programs is associated with a wide array of diseases, including cancer and FAD [35,45,59]. Further studies to understand endogenous mechanisms controlling this coactivator switching and its implications are in progress.

Therapeutic potential

Although ICG-001 is a powerful tool for dissecting Wnt signaling (Box 1), it is not a drug and we will have to wait for the development of its cogeners to fully determine its utility in the clinic for the treatment of cancer [35], FAD [59] and potentially other hyperproliferative diseases. However, we are encouraged by the fact that we seem to be targeting 'cancer stem cells' [60] using CBP–β-catenin antagonists (e.g. ICG-001).

The safari continues

ICG-001 and related analogs (e.g. ICG-427 that selectively inhibits the β-catenin–p300 interaction) are powerful tools, which, in conjunction with more traditional genetic and molecular biological approaches, help to explore the Wnt pathway, the multitude of genes that it regulates and the genetic programs, both normal and aberrant, that it controls. The ability to selectively block a subset of protein–protein interactions provides the unique ability to modulate signaling networks without completely ablating them. Further studies to dissect Wnt signaling, as well as its interactions with other signal transduction pathways (e.g. growth factors, nuclear receptors, and so on), are in progress.

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