Abstract

The Wnt/ß-Catenin signaling pathway is a key regulator of proliferation, differentiation, and programmed cell death in stem and progenitor cells. Misregulated or aberrant signaling events in the pathway contributes to the pathology of diseases such as cancer and neurodegenertion. It stands to reason that if we can monitor and influence Wnt/ß-Catenin signaling in stem and progenitor cells with sufficient resolution, it will be possible to design rationally targeted therapies. The body of work presented in this thesis contributes to the realization of this goal through discovery of molecular agents for controlling Wnt/ß-catenin signaling, examination of the biological mechanisms linking this pathway to disease, and identification of cases where pharmacological perturbation of Wnt/ß-Catenin signaling may have therapeutic utility.

Table of Contents

List of Abbreviations

hESC - human embryonic stem cell

Preface

I conducted the research presented in this thesis dissertation with the goal of elucidating how Wnt/ß-catenin signaling directs behavior in stem and progenitor cells. Within this realm, I chose to investigate contexts in which findings may inform novel therapeutics. To accomplish this, I worked within a framework

Projects began with a high-throughput screen to discover molecules that regulate Wnt/ß-catenin signaling in decidedly interesting contexts. The molecules with the greatest activity in the screen are chosen as hits, and are fed into a pipeline of filtering and validation steps. Finally, I characterized the activity of the most promising molecules in a series of cell culture, animal, and disease models.

From project to project, which was iteratively improved upon.

I looked to maximize the amount of usable information gathered, and to optimize relevance to disease using experimental models.

In efforts to drive discovery, I tested and honed a set of methodologies that can be used to perform automated and high-throughput experiments in stem and progenitor cells. To direct discovery towards clinical relevance, I designed and iterated upon a pipeline to perform primary data filtering, secondary validation, and ultimately to test discoveries in clinically relevant disease models.

By conducting high throughput screens of arrayed small molecule libraries, using stem and progenitor cell lines that have been engineered to encapsulate genetic reporter systems, it has been possible to identify clinically-approved compounds that were not previously known to affect Wnt, as well as entirely novel Wnt regulators whose biological mechanisms were later determined.

1 - Introduction

1.1 - Background

1.1.1 - Stem Cell targeted Therapies

Properties of Stem Cells We know in large part where stem and progenitor cell niches reside within adult organisms. We have identified cases where these niches contribute to homeostasis or disease depending on their environment. We have identified sets of markers that reliably indicate where a cell lies within the spectrum between pluripotency and terminal differentiation, and this information can be used to determine the fate decisions that a cell has made in its lifetime. These advances have greatly impacted our understanding of the mechanisms by which dysregulation of signaling in stem cells contributes to disease.

Additionally, the field possesses an increasingly rich understanding of how stem cells may manipulated to generate desired cell types, and conversely how to induce differentiated cells to gain pluripotent capability.

Requirement of Adult Stem Cell Niches for Homeostasis

Cancer Stem Cells The 'cancer stem cell' hypothesis holds that the same pathways regulate self-renewal in stem cells and cancer cells, and that there are stem cells within the tumor that posess indefinite potential for self-renewal. [Tannishtha Reya. Nature 2001]

Ex-Vivo Directed Differentiation

Adult Neurogenesis

1.1.2 - Wnt/ß-Catenin Signaling in Stem Cells

Wnt Regulation of Devloping Tissues Wnts are a family of roughly 20 secreted glycoproteins, that are ligands for Fzd receptors. Wnts were first discovered based on their role in cell polarity in Drosophila and axis induction in Xenopus during organism development. Wnt/ß-catenin signaling, also referred to as canonical Wnt signaling, involves the acumulation of ß-catenin in the cytosol subsequent to membrane receptor activation. ß-catenin is then translocated to the nucleus where it binds a set of transcription factors, ultimately driving expression of various target genes. In contrast, ß-catenin independent, or non-canonical, Wnt signaling is thought not to direct gene expression.

In the ensuing decades since the discovery of this signaling pathway, Wnt has been determined to play a central part in most aspects of embryonic development as well as in the homeostasis of some adult tissues (Clevers, 2006). A commonality between these adult tissues, which include the colon crypts and germinal brain regions, is the presence of stem cell niches. Involvement of Wnt signaling is also implicated in certain tissues during disease, a notable example is cancer tumorogenesis.

Wnt in Cancer Tumorogenesis Misregulation of the Wnt pathway has been linked to various types of tumors. The 'cancer stem cell' hypothesis holds that the same pathways regulate self-renewal in stem cells and cancer cells, and that there are stem cells within the tumor that posess indefinite potential for self-renewal. [Tannishtha Reya. Nature 2001]

Wnt in Adult Neurogenesis

Wnt in hESCs

1.1.3 - Pharmacologic regulators of Wnt/ß-Catenin signaling

We have relatively few tools to modify Wnt signaling both experimentally and therapeutically. Zach Zimmermans paper notes this.

Lithium is in clinical use and part of its mechanism may be due to Wnt signaling. This is talked about in my Simva paper.

Many are under investigation FIGURE OUT WHICH ONES ARE UNDER INVESTIGATION frizzled?

1.2 Approach

1.2.1 Discovering Small Molecule Regulators of Cell Signaling

In the research presented, I looked to discover small molecule regulators of the Wnt/ß-catenin pathway. I was particularly interested in small molecules because of their applicability to both basic research and clinical drug development. As opposed to genetic approaches such as siRNAs and shRNAs to induce a gain or loss of function in cell signaling, small molecules can be included in an experimental paradigm with relatively little overhead and higher relative predictability of effect [CITATION]. This effect carries over when administering small molecules to animals, which tends to be more efficacious than genetic materials. This can be due to cell permeability and bioavailability [CITATION]. Finally, small molecules have a long proven track record in treating human diseases.

Consistent with my overall goals, small molecules are available in the form of arrayed libraries. This is important in that it allows for high-throughput screens to be performed by automated equipment, rather than being tested individually by hand. These contents of the libraries can be produced from curated lists, such as the library of human experienced compounds in the riluzole in simvastatin papers. Libraries can also be produced combinatorially around a scaffold in order to target a specific motif such as the ATP binding pocket. This type of library is seen in the Wiki paper and also in the JW screen.

Finally, I prioritized the discovery of small molecule regulators of Wnt/ß-Catenin signaling because the small molecules that I reported on served as deliverables in and of themselves alongside the journal articles. This means that future projects have a reliable physical starting place from which to conduct experiments. The molecules I report on are commercially available, and in some cases are already approved for human use. This means that the time it takes for another group to begin experiments based off of my findings will be minimized, and ongoing studies of these molecules such as in clinical trials will be able to draw inference from my projects.

1.2.2 Understanding Biological Mechanisms through Cell Signaling Events

The projects that I participated in attempted to further our understanding of cell behavior. In each case I used Wnt/ß-Catenin signaling as an entry point from which to address biological mechanisms that govern behavior. This entailed selectively monitoring and perturbing Wnt signling. I monitored Wnt signaling while using established methods to perturb other pathways, in order to find where multiple pathways may cross-talk with one another. To determine the role of Wnt in homeostatic and disease processes, I perturbed Wnt signal-

ing while monitoring behaviors such as cell death, proliferation, induction of differentiation, and lineage specification.

I chose to focus on Wnt/ß-catenin signaling in part because of its established role in diseases including melanoma and various neurodegenerative disorders. This helped to guide the direction in which I proceeded through projects, as I was able to place my experimental results within context of the body of knowledge about the specific disease.

In spite of what is known about how misregulated Wnt/ß-catenin signaling can precipitate disease states, there are relatively few therapies targetting this signaling pathway. This served as another reason that I focused on this pathway.

1.2.3 Exploiting Cell Signaling to Inform Novel Therapeutics

The idea of targeting pathways in disease has been around for a while

There were certain successes, particularly in cancer, see Gleevec

However cancer is fast moving and changing, see Vemurafinib

A new idea is to target cancer stem cells with Wnt

Why? Wnt has less functional redundancy than ERK/MAPK

An aim of my work was to identify opportunites where perturbation of cell signaling in stem and progenitor cells may inform the development of a rational therapeutic.

This approach worked in my projects where:

with ril and simva we already knew something about targets

with WIKI we were able to connect to known mechanism

later on with BRAF, Wnt was able to synergize w vemurafinib

To date, the field of translational life sciences has made major headway towards development of therapies that utilize pharmacologic manipulation of stem and progenitor cells.

1.3 Contribution to Published Works

1.3.1 - Biechele et. al, 2010

- developed plan to connect and process data
- aimed at maximizing our effectiveness in prioritizing hits
- Convert raw data to human interpretable readout
- sort across various drug agencies (FDA, euro, world)
- perform statistical analysis in HTS (Z Score)
- manually curate results, resolve replicates from multiple libraries

1.3.2 - James et. al, 2012

I helped design the high throughput screen for WIKI

I helped run followup assays in H1s

Finally I took the followup assays from H1s and miniaturized it so that it could be done in HTS (see patent in appendix)

1.3.3 - Robin et. al, 2014

I did this entire project myself, basically.

- I identified that statins would be an interesting hit to follow up on from the small molecule screen
- I generated a reporter cell line from primary aNPCs, and validated simvastatin in this line
- I ran RT-PCR as a secondary confirmation that simvastatin enhances WNt
- I treated differentiatin aNPCs with simvastatin and stained for cell type specific markers, revealing that simvastain drives cell fate towards neuronal lineage
- I treated mice with simvastatin and observed an increased number of batgal positive cells in the DG
- I looked at cell type specific markers in the DG and saw that simvastatin treatment enhances adult neurogenesis
- I treated reporter cells with various metabolites from the HMGCR pathway and determined that simvastatin enhances WNT signaling by depleting isoprenoids

2 - Chemical-genetic screen identifies riluzole as an enhancer of Wnt/ -catenin signaling in melanoma

2.1 - Introduction

I was an author on a paper about riluzole.

2.2 - Journal Article

2.2.1 - Summary

To identify new protein and pharmacological regulators of Wnt/ -catenin signaling, we used a cell-based reporter assay to screen a collection of 1857 human-experienced compounds for their ability to enhance activation of the -catenin reporter by a low concentration of WNT3A. This identified 44 unique compounds, including the FDA-approved drug riluzole, which is presently in clinical trials for treating melanoma. We found that treating melanoma cells with riluzole in vitro enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease cell proliferation. Furthermore riluzole, like WNT3A, decreases metastases in a mouse melanoma model. Interestingly, siRNAs targeting the metabotropic glutamate receptor, GRM1, a reported indirect target of riluzole, enhance -catenin signaling. The unexpected regulation of -catenin signaling by both riluzole and GRM1 has implications for the future uses of this drug.

2.2.2 - Highlights

- A cell-based screen identified 44 compounds that enhance WNT/ -catenin signaling
- Riluzole, approved for treating ALS, was found to enhance WNT/ -catenin signaling
- Riluzole, in clinical trials for melanoma, decreases melanoma metastases in mice
- GRM1 negatively regulated WNT/ -catenin signaling in melanoma cells

2.2.3 - Introduction

Wnt proteins act as ligands for members of the Frizzled family of serpentine receptors and for the LRP5/6 coreceptors. Activation of the pathway results in -catenin (CTNNB1) stabilization and nuclear accumulation. Aberrant Wnt/ -catenin signaling arising from either hyper- or hypoactivation has been linked to numerous clinical conditions, most notably cancers (Moon et al., 2004). Several types of cancer have been linked to mutations in core Wnt/ -catenin pathway genes that result in constitutive ligand-independent activation of the pathway (Moon et al., 2004). However, as described below for melanoma, elevation of Wnt/ -catenin signaling correlates with or may promote favorable biological or clinical outcomes.

Wnt/ -catenin signaling is a major regulator of melanocyte differentiation (Thomas and Erickson, 2008), so it is not surprising that this pathway is also involved in the pathogenesis of malignant melanoma. In a mouse model of malignant melanoma, Wnt/ -catenin signaling itself is insufficient for melanocyte

transformation, but activation of this pathway can work synergistically with active Ras signaling to promote tumor formation (Delmas et al., 2007). By contrast, several studies using patient-derived tumor samples have reported that melanoma progression is associated with the loss of nuclear -catenin, a clinical marker of Wnt/ -catenin activation. Furthermore, improved survival is seen in patients with higher levels of cytosolic or nuclear -catenin (Bachmann et al., 2005, Chien et al., 2009, Kageshita et al., 2001 and Maelandsmo et al., 2003), suggesting that active Wnt/ -catenin signaling in patients predicts improved prognosis. Interestingly, activation of Wnt/ -catenin signaling by Wnt3a in a mouse melanoma model results in decreased proliferation in vitro and in vivo, along with increased expression of melanocyte differentiation markers usually lost with melanoma progression, pointing to potential mechanisms that might explain the improved prognosis of patients with elevated -catenin (Chien et al., 2009). Collectively, these observations underscore the potential importance of determining whether any existing approved drugs enhance Wnt/ -catenin signaling and whether they may have therapeutic benefit in treating melanoma.

In the present study, we used a cell-based screen to identify several small molecule enhancers of Wnt/ -catenin signaling. We focused on one enhancer compound, riluzole, an FDA approved therapeutic for amyotrophic lateral sclerosis that is under investigation in clinical trials as a melanoma therapy (Yip et al., 2009).

2.2.4 - Results and Discussion

Using the -catenin-activated luciferase reporter (BAR) (Biechele and Moon, 2008) (see Figure S1A available online) in a cell-based assay, we screened for small molecules that enhance the activation of the reporter by exogenous WNT3A (Figure 1 inset). We optimized the WNT3A stimulus to obtain an EC20 (Figure S1B) in HT22 neuronal cells and screened a library of 1857 human experienced chemicals (1500 unique). We identified 47 (44 unique) chemicals that enhanced the WNT3A stimulus greater than 1.5-fold (Figure 1A; Supplemental Database), several of which displayed a dose-dependent enhancement of Wnt/ -catenin signaling upon rescreening (Figures S1C and S1E).

To rule out the formal possibility that some of the primary hits from the screen act directly on luciferase activity rather than on the Wnt/ -catenin pathway, we used a second cell-based Wnt/ -catenin reporter system in which Venus fluorescent protein is the reporter gene (Rekas et al., 2002). WNT3A stimulation of cells stably expressing this reporter promoted an increase in both the number of Venus positive cells and their mean fluorescent intensity (MFI) by FACS analysis (Figure 1B). To validate that Venus expression is tightly linked to -catenin signaling, the expression of endogenous -catenin target genes in Venus-positive and -negative cells was compared. Consistent with a positive correlation, the Venus-positive population of untreated cells, or the Venus-positive population

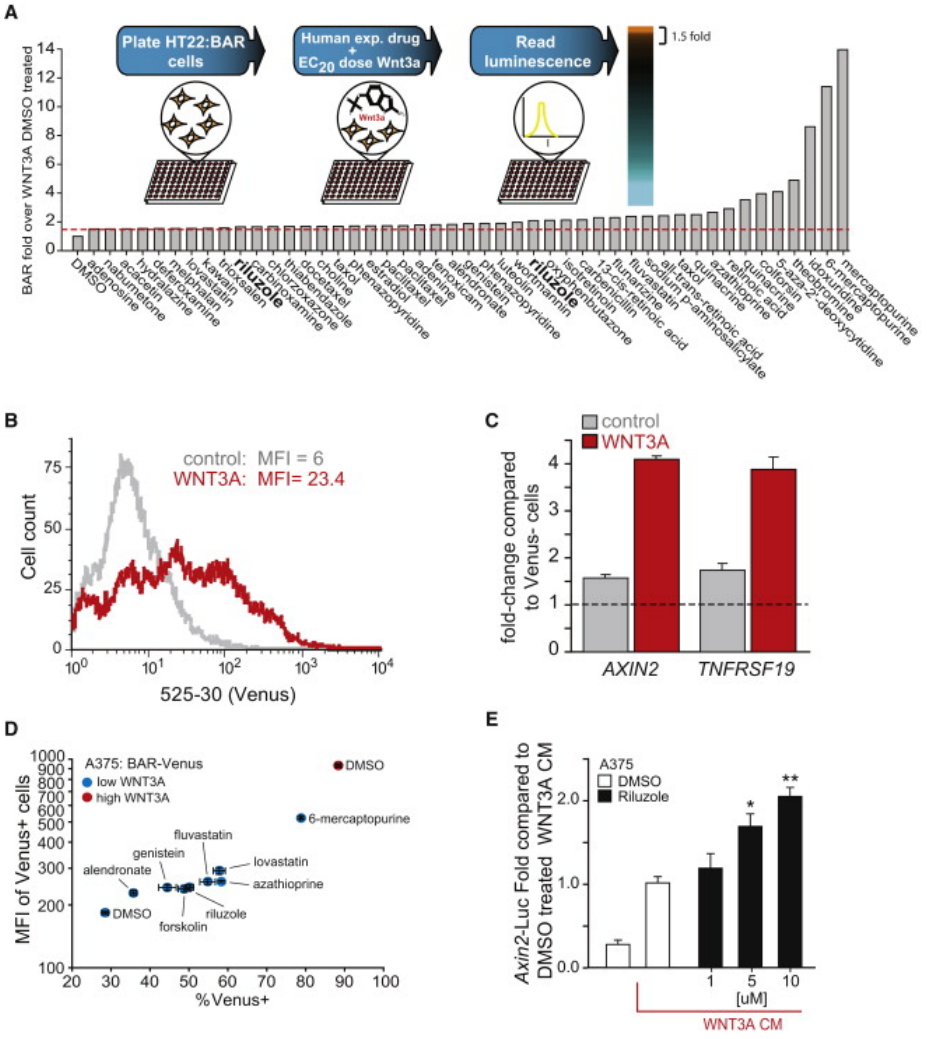

Figure 1: Figure 1

of cells that had been treated with WNT3A-conditioned media prior to FACS sorting, showed elevated expression of the -catenin target genes AXIN2 and TNFRSF19 when compared with Venus-negative cells (Figure 1C). In this assay, several primary screen hits enhanced the WNT3A-mediated increase in the percentage of Venus-positive cells and their MFI (Figure 1D).

Candidate hits from the primary screen were analyzed with respect to their being mentioned in the literature and in clinical trials databases, enabling us to generate a list of diseases where these bioactive molecules were being studied. We compared this list to the diseases being treated with lithium chloride, a classic inhibitor of GSK3 which is the only available patient-experienced drug that is known to activate Wnt/ -catenin signaling. As a result of this analysis, we focused on the drug riluzole (brand name Rilutek) (Figure S1D), an FDA-approved drug used to slow the progression of amyotrophic lateral sclerosis (ALS) (Aggarwal and Cudkowicz, 2008). Riluzole is under intensive evaluation as a treatment for bipolar disorder, a condition where lithium chloride is a first-line treatment (Pittenger et al., 2008). Lithium is also being evaluated as a treatment for ALS (Fornai et al., 2008), which is also consistent with the possibility that these two unrelated compounds may regulate the same signaling pathway(s).

Riluzole enhanced Wnt/ -catenin signaling in both the primary screen in HT22 neuronal cells (Figure S1E) and in adult hippocampal progenitor cells (Figure S1F). Furthermore, riluzole enhanced the stabilization and nuclear localization of -catenin in U2OS cells (Figures S2A and S2B). As riluzole is in clinical trials for treatment of metastatic melanoma (Yip et al., 2009) and decreased Wnt/ -catenin in clinical samples has been associated with melanoma progression (Chien et al., 2009), we focused on validating this hit and investigating its mechanism of action in the context of melanoma. We first demonstrated that riluzole enhances Wnt/ -catenin signaling in a dose-dependent manner in A375 melanoma cells stably expressing BAR confirming the observations in neuronal cells (Figure S2C). As a control, we showed that riluzole does not activate a control reporter construct (fuBAR) in which the TCF/LEF sites required for responding to -catenin have been mutated (Figure S2C). We next demonstrated that riluzole enhances the WNT3A-dependent activation of the promoter of a known direct target gene of -catenin, Axin2 (Figure 1E). Moreover, riluzole, like WNT3A, upregulates several genes involved in melanocyte development (Trpm1, Met, Mitf, Sox9, Kit, and Si) alone (Figure 2A) and in synergy with exogenous WNT3A (Figure 2B).

Consistent with the reported ability of WNT3A to elevate levels of transcripts involved in melanocyte differentiation (Chien et al., 2009), treating B16 melanoma cells with WNT3A leads to a dose- and -catenin-dependent increase in cellular pigmentation (Figure 2C; Figure S2D). Treatment of these cells with riluzole also increases pigmentation, and does so in a -catenin-dependent manner that is synergistically enhanced by cotreatment with WNT3A (Figure 2C; Figure S2D). Analysis of riluzole-treated B16 cells in which siRNAs have been used

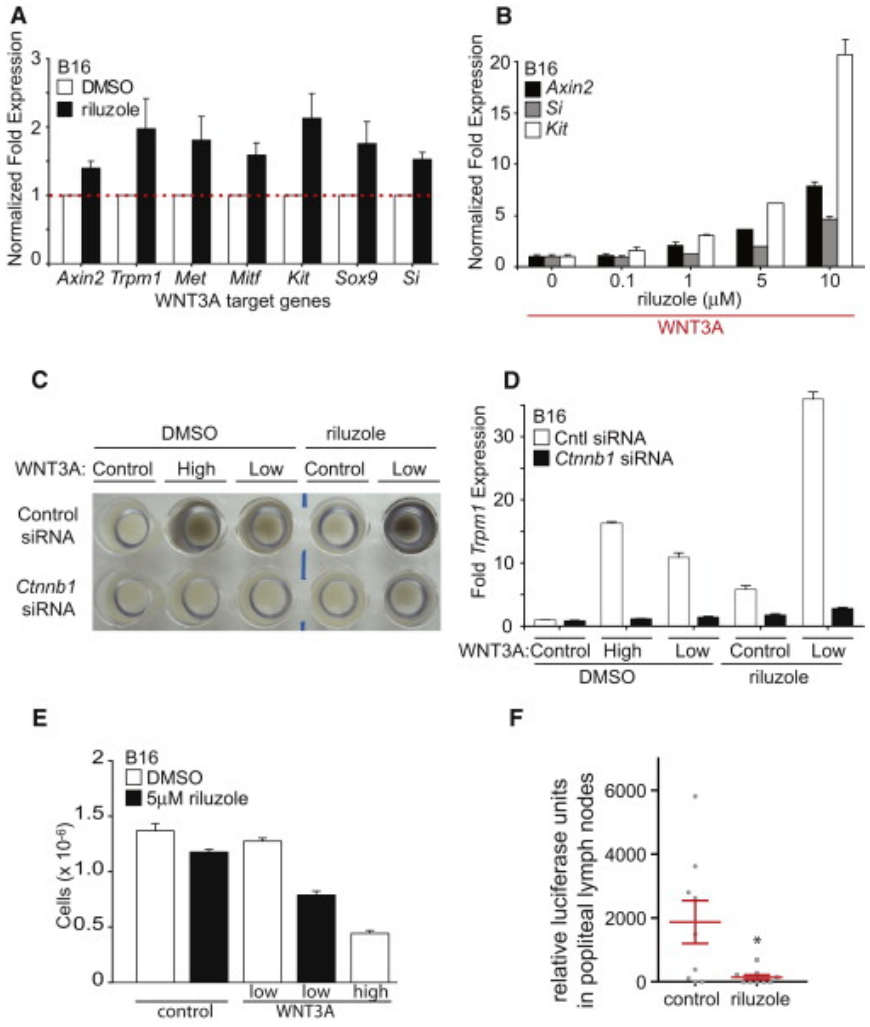

Figure 2: Figure 2

to deplete endogenous -catenin showed that Wnt-3a and riluzole synergize in a -catenin-dependent manner to elevate the expression of Trpm1 (Figure 2D).

We have previously demonstrated that elevated Wnt/ -catenin signaling in melanoma cell lines cultured in vitro, and in melanoma patients, correlates with a reduction in cell proliferation (Chien et al., 2009), raising the question as to whether riluzole would also affect proliferation. Synergy between riluzole and WNT3A was observed in the reduction of proliferation in B16 cells (Figure 2E). We also observed a decrease in the proliferation of several human melanoma cell lines in the presence of riluzole (Figure S2E).

We then tested whether riluzole inhibits melanoma progression in vivo using B16 melanoma cells implanted into the footpads of mice, followed by treating the mice with riluzole. There was a striking reduction in metastases compared with vehicle-treated mice, as measured by the detection of cells in the sentinel popliteal lymph node (Figure 2F). Collectively, the data show that riluzole both mimics and enhances the ability of WNT3A to promote differentiation of melanoma cells toward a more melanocyte-like state, that riluzole decreases the proliferation of melanoma cells in vitro, and that riluzole reduces the metastasis of melanoma cells in vivo.

To better understand the mechanism by which riluzole enhances Wnt/ -catenin signaling in melanoma cells, we explored known signaling targets and pathways previously linked to riluzole. In a phase 0 trial of riluzole for treatment of melanoma, it was shown that tumors from patients treated with riluzole exhibited decreased phosphorylation of ERK (Yip et al., 2009). However, treatment of A375 human melanoma cells with riluzole resulted in no change in ERK phosphorylation (Figure S3A). Another possible mechanism for riluzole to enhance Wnt/ -catenin signaling involves the activation of PKA through a riluzole-mediated increase in cAMP levels (Duprat et al., 2000 and Taurin et al., 2006). To test this mechanism, A375 cells were treated with riluzole or with forskolin, a small molecule known to activate adenylyl cyclase and thus PKA (Seamon et al., 1981). Cell lysates from forskolin, but not riluzole-treated cells, showed a significant enhancement in PKA-phosphorylated CTNNB1 as well as other protein substrates relative to controls (Figure S3B).

Previously, a mouse with a high incidence of spontaneous melanoma-like lesions was found to carry a mutation that led to elevated expression of metabotropic glutamate receptor 1 (GRM1) (Pollock et al., 2003). Riluzole has been reported to inhibit glutamate release and reuptake and thereby inhibit activation of metabotropic glutamate receptors (Doble, 1996). If riluzole enhances Wnt/ -catenin signaling through a mechanism involving GRM1 then reducing GRM1 expression might enhance Wnt/ -catenin signaling. To test this hypothesis, we transfected A375:BAR melanoma cells with two independent siRNAs targeting GRM1, or with siRNAs targeting CTNNB and AXIN1/2 as controls. Both GRM1 siRNAs depleted GRM1 transcript levels to approximately 10% of control siRNA transfected cells (Figure S3C). Strikingly, reduction of GRM1 transcripts by siRNAs synergized with WNT3A in the BAR luciferase reporter assay,

demonstrating that endogenous GRM1 functionally represses Wnt/ -catenin signaling (Figure 3A). GRM1-directed siRNAs also enhanced WNT3A-mediated upregulation of endogenous AXIN2 transcripts (Figure S3D). Consistent with these findings, three structurally unrelated GRM1-selective small molecule antagonists enhanced WNT3A-stimulated BAR activity in A375:BAR cells in a dose-dependent manner (Figure 3B). Furthermore, like riluzole, all three GRM1-selective antagonists enhance the pigmentation of B16 melanoma cells alone and synergize with WNT3A (Figures 3C and 3D; Table S1), while antagonists of the related glutamate receptor, GRM5, have no effect on BAR activity or B16 melanoma cell pigmentation (Figure S3E and Table S1). Collectively, these data identify GRM1 and not GRM5 as a novel regulator of Wnt/ -catenin signaling and the likely target of riluzole-mediated enhancement of Wnt/ -catenin signaling.

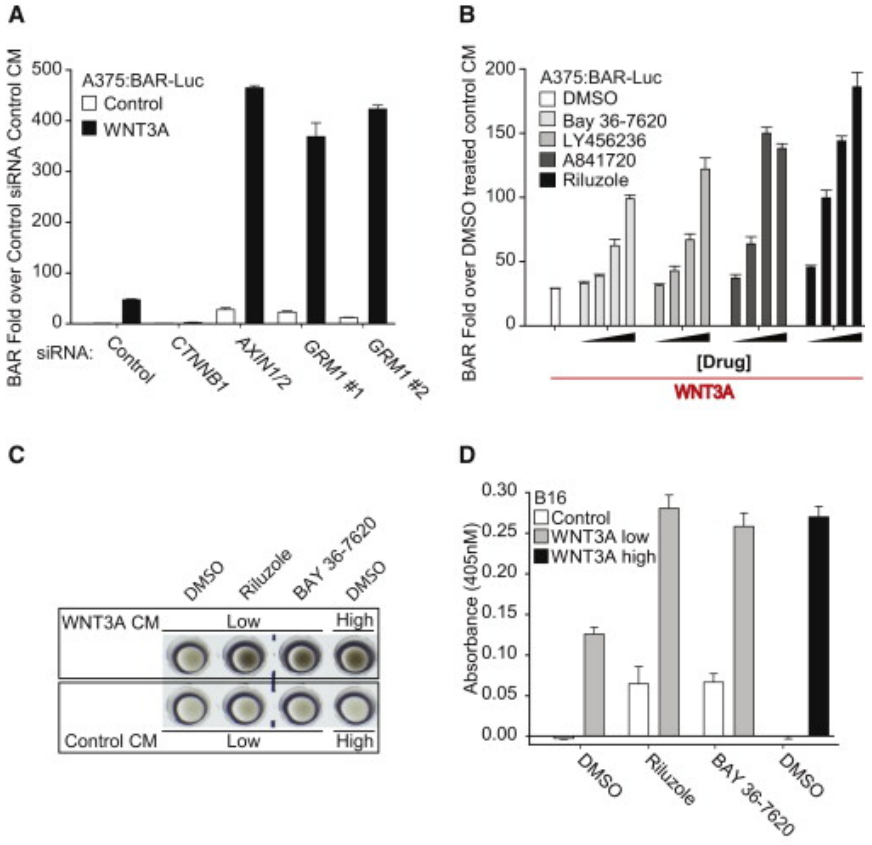

Figure 3: Figure 3

2.2.5 - Significance

This article describes the identification and characterization of the FDA-approved drug riluzole as an enhancer of Wnt/ -catenin signaling in melanoma cells. In melanoma cells, riluzole enhances the ability of WNT3A to regulate gene expression, to promote pigmentation, and to decrease proliferation. Furthermore, riluzole, like WNT3A, decreased metastases in vivo in a mouse melanoma model. Investigating the mechanisms of action of riluzole revealed that endogenous metabotropic glutamate receptor GRM1 but not GMR5 represses Wnt-mediated activation of -catenin signaling in melanoma cells. Given that riluzole is in clinical trials for treating melanoma, our data that riluzole modulates -catenin signaling, combined with prior data that -catenin signaling has complex effects in different cancers, should stimulate further investigation into the use of riluzole in cancer therapies.

2.2.6 - Experimental Procedures

Full details of materials and experimental procedures used in this study can be found in the Supplemental Experimental Procedures that accompany this Brief Communication (available online).

2.2.7 - Acknowledgments

A.J.C. was supported by the National Cancer Institute (K08CA128565) and R.T.M. by the HHMI. This project was supported by the National Cancer Institute's Initiative for Chemical Genetics under Contract No. N01-CO-12400 and by the Department of Defense (W81XWH-07-1-0367). We thank Stephanie Norton for technical assistance.

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2.3 - Conclusion

We learned some interesting things about riluzole.

3 - WIKI4, a novel inhibitor of tankyrase and Wnt/ß-catenin signaling

3.1 - Introduction

I was an author on a paper about wiki.

3.2 - Journal Article

3.2.1 - Abstract

The Wnt/ß-catenin signaling pathway controls important cellular events during development and often contributes to disease when dysregulated. Using high throughput screening we have identified a new small molecule inhibitor of Wnt/ß-catenin signaling, WIKI4. WIKI4 inhibits expression of ß-catenin target genes and cellular responses to Wnt/ß-catenin signaling in cancer cell lines as well as in human embryonic stem cells. Furthermore, we demonstrate that WIKI4 mediates its effects on Wnt/ß-catenin signaling by inhibiting the enzymatic activity of TNKS2, a regulator of AXIN ubiquitylation and degradation. While TNKS has previously been shown to be the target of small molecule inhibitors of Wnt/ß-catenin signaling, WIKI4 is structurally distinct from previously identified TNKS inhibitors.

3.2.2 - Introduction

Wnt family genes encode highly conserved secreted glycoproteins, which activate downstream signal transduction pathways important in development and tissue homeostasis. Wnts can signal through one of several pathways, including the conserved Wnt/ß-catenin pathway. The Wnt/ß-catenin pathway is activated by Wnt ligands binding to Frizzled serpentine receptors and to LRP5/6 co-receptors, leading to the post-translational regulation of the stability of ß-catenin (encoded by CTNNB1) (reviewed in [1]). In the absence of a Wnt signal, cytosolic CTNNB1 is bound by the scaffolding proteins Adenomatous Polyposis Coli (APC) and AXIN1, and the kinases Casein Kinase 1 (CSNK1A1) and Glycogen Synthase Kinase (GSK). Sequential phosphorylation of CTNNB1 by CSNK1A1 and GSK3 leads to its recognition by a ubiquitin ligase protein complex and its subsequent degradation by the proteasome. Upon activation of Wnt/ß-catenin signaling, this "destruction complex" is inhibited, resulting in accumulation of newly translated CTNNB1, which then translocates to the nucleus where it acts as a co-activator during transcription of target genes that ultimately lead to context-dependent changes in cell proliferation, specification, or differentiation.

Wnt/ß-catenin-dependent transcription plays critical roles in both embryonic development and in adults [2], [3]. Examination of mice and zebrafish that are transgenic for ß-catenin-dependent reporters has revealed that ß-catenin signaling is spatially and temporally regulated [4]–[9]. Not surprisingly, Wnt/ß-catenin signaling plays many roles in development, including patterning of all three germ layers [10]–[15]. In addition, we and others have shown that ectopic activation of the Wnt/ß-catenin pathway can drive differentiation of human embryonic stem cells (hESCs) towards mesodermal and endodermal lineages [16], [17]. Lastly, Wnt/ß-catenin signaling is activated by acute injury and

functions in regenerative responses [18], as well as in diverse chronic diseases including cancers (colorectal cancer [19], liver cancer [20], [21], Wilms tumor [22], [23], lymphoma [24], [25], myeloma [26], [27], [28], leukemias [29], [30]) and neuropsychiatric diseases [31].

There have been a growing number of small molecule inhibitors of Wnt/ß-catenin signaling (reviewed in [32]), which at a minimum should provide tools for modulating the pathway in vitro. For example, Huang and colleagues have described a small molecule inhibitor of Wnt/ß-catenin signaling that works by inhibiting the adenosine di-phosphate (ADP) ribosylase protein, Tankyrase (TNKS) [33], [34], [35]. Inhibiting the activity of TNKS leads to elevation of levels of AXIN, thereby promoting the degradation of CTNNB1 and inhibiting Wnt/ß-catenin signaling [33], [34], [35].

In an effort to identify additional small molecule inhibitors of Wnt/ß-catenin signaling, we screened A375 melanoma cells stably transduced with a ß-catenin-activated reporter (BAR). To ensure Wnt pathway-specificity, we cross-screened A375 cells containing luciferase reporters activated by different signaling pathways and eliminated those compounds that inhibited multiple pathways. Using this approach we identified a novel Wnt inhibitor, Wnt Inhibitor Kinase Inhibitor 4 (WIKI4), which effectively blocks Wnt/ß-catenin reporter activity in diverse cell types, including cancer cells that display elevated ß-catenin signaling due to activating APC mutations. WIKI4 inhibits the expression of Wnt target genes as well as the functional effects of Wnt/ß-catenin signaling in colorectal carcinoma cells and hESCs. We subsequently established that WIKI4 antagonizes Wnt/ß-catenin signaling via inhibition of TNKS activity.

3.2.3 - Materials and Methods

Reagents The reporters described in this manuscript are lentiviral plasmids containing 12 binding sites for transcription factors downstream of the Wnt/ß-catenin (5 -AGATCAAAGG-3) (previously described in [36]), Nuclear Factor Kappa B (NF-kB, 5 -GGGAATTTCC-3), Transforming Growth Factor Beta (TFGß, 5 -AGCCAGACA-3), and Retinoic Acid (RA, 5 -GGTTCACCGAAAGTTCA-3) signaling pathways which are each separated by distinct 5-base pair linkers. The transcriptional binding cassettes are located upstream of a minimal thymidine kinase promoter and the firefly open reading frame. Each reporter also contains a separate phosphoglycerate kinase promoter that constitutively drives the expression of a puromycin resistance gene. To engineer stable cell lines that express the reporters, cells were infected with un-concentrated virus, and selected with puromycin (2 µg/mL).

H1 (WiCell) and H1-BAR hESC lines were maintained on irradiated MEF feeders in 20% Knockout Serum Replacement medium +8 ng/ml FGF2 (KSR medium) and passaged weekly using dispase as previously described [17]. NALM6 human pre-B cells (DSMZ) were grown in RPMI 1640 with 10% fetal bovine serum (FBS) and 55 µM ß-mercaptoethanol, A375 malignant melanoma

cells (ATCC) were grown in RPMI 1640 with 5% FBS. DLD1 colorectal carcinoma (ATCC), SW480 colorectal carcinoma (ATCC), U2OS osteosarcoma (ATCC) cells were grown in DMEM/F12 with 10% FBS. Wnt3A conditioned medium (CM) and control L CM were generated as previously described [17] from L cells and L-Wnt3A cells (ATCC).

The following antibodies were used: AXIN1 (2087; Cell Signaling Technology), AXIN2 (2151; Cell Signaling Technology), CTNNB1 (9562; Cell Signaling Technology), S33/37T41P-CTNNB1 (9561; Cell Signaling Technology), S45P-CTNNB1 (9564; Cell Signaling Technology), GCTM2 (kind gift from Martin Pera, University of Melbourne, Australia; GCTM-2 antibody previously described in [37], [38], [39]), CD9 (mAB4427; Millipore), TUBB1 (T7816; Sigma-Aldrich), UBIQUITIN (SC-8017, Santa Cruz Biotechnology). The following compounds were used in this study: MG132 (474790, EMD Millipore), XAV-939 (S1180, Selleck Chemical), U0126 (U-6770, LC Labs), and WIKI4 (7990417, Chembridge). All sequences used for real-time PCR or siRNA transfection are listed in Table S1 or previously published [17]. All molecules used in the structure activity relationship analysis are detailed in Table S2.

High Throughput Small Molecule Screen Screening was performed using the facilities of the Quellos High Throughput Screening Facility at the Institute for Stem Cell and Regenerative Medicine in Seattle, WA. Compounds dissolved in DMSO were obtained from Chembridge (a custom selection of 6,492 entities from Chembridge's KINASet library). For the primary screen, performed in duplicate, A375 malignant melanoma cells stably expressing BAR were cultured in growth medium (DMEM/5%FBS/1%antibiotic). 4000 cells per well were transferred to clear bottom 384-well plates (BD Falcon; Fisher Scientific 08-772-004) in 30 µL of growth media using a Matrix WellMate (ThermoScientific). The following day 50 nL of each compound (final concentrations of 370 nM and 10 µM) and 10 µL of Wnt3A-conditioned media (EC20 dose) was transferred to the cells. On the third day, 10 µL of resazurin (final concentration 0.1 mg/mL) was added to the cells, and after a three hour incubation viability was assessed by quantifying the fluorescent reduction product of resazurin using an Envision Multilabel plate reader (PerkinElmer). Finally, 5 µL of Steady-Glo (Promega) was added to each well, and luciferase was quantified using the Envision Multilabel plate reader. The fold-increase over the background of DMSO controls for viability and luciferase was calculated. Inhibitors were chosen for further analysis if they inhibited Wnt3A-dependent luciferase production at the 370 nM concentration (normalized Steady-Glo fold change <0.5) but did not decrease resazurin reduction at the 10 µM concentration (viability fold change >0.9). For the Z-factor calculation (Z), we used the following equation [40]:

In this equation µ equals the mean calculated from the positive (c+) and negative (c-) control replicates, and equals the standard deviation calculated from the positive (c+) and negative control replicates (c-). For the secondary screening, compounds that satisfied our hit criteria were re-screened for their ability

to inhibit stimulation of reporters for the Wnt/ß-catenin, NF-kB, TGFß, and RA pathways stably expressed in A375 melanoma cells as described above.

Functional Cell Assays For the DLD1 colony forming assays, single DLD1 cells were plated at 1000 cells per well (6-well) and cultured overnight in DMEM containing 0.5% FBS. The next day, compounds were added to the media, and the cells were subsequently cultured for ten days with refreshment of the media and compound occurring every two days. At the end of the culture period, the colonies in each well were counted.

For the flow cytometric analysis of cell surface markers in hESCs, H1-BAR-VENUS hESCs were seeded as small clusters on MEFs in KSR media at ~35,000 cells per square cm. The following day, the medium was replaced with 50% (vol/vol) conditioned medium (control L or Wnt3A CM) in KSR media with or without inhibitors, XAV-939 or WIKI4. DMSO served as a vehicle control for the compounds. The medium was replenished daily. After 6 days of treatment, hESCs were isolated as single cells with TrypLE Express (Invitrogen) and counted. 500,000 cells were immunolabeled with 100 ul primary antibodies: GCTM2 (hybridoma supernatant, 1:2, a kind gift from Martin Pera, University of Melbourne, Australia; GCTM-2 antibody previously described in [37], [38], [39]) and CD9 (TG30 clone, 1:100, Millipore). Cells were then incubated with isotype-specific secondary antibodies (Invitrogen): goat anti-mouse IgM-Alexa 647 (1:100) and goat anti-mouse IgG2a-biotin (1:5000), followed by PE-Cy7-streptavidin (1:250). hESCs were resuspended in 140 ng/ml DAPI in KSR media, then passed through a cell strainer prior to analysis on a BD FACSCanto II flow cytometer. Results were quantified using FlowJo software. The percentage of GCTM2 and CD9 double-positive hESCs was determined from the DAPI-negative (viable), DsRED-positive gated population. DsRED is constitutively expressed in H1-BAR-VENUS cells, thus this gating strategy serves to exclude any MEFs.

For the gene expression analysis in hESCs, total RNA was isolated using TRIZOL according to the manufacturer's protocol (Invitrogen). 2.5 µg RNA was used for cDNA synthesis with RervertAid First Strand cDNA Synthesis kit (Fermentas). cDNA was diluted 100-fold, then used as template for quantitative PCR (2 µl cDNA per 10 µl reaction) using Applied Biosystems SYBR Green-based detection according to the manufacturer's protocol on a Roche Lightcycler 480 instrument. Duplicate reactions were performed for each sample. Transcript copy numbers were normalized to GAPDH for each sample, and fold expression over the untreated control was calculated for each gene of interest. Primer sequences are previously published [17] or listed in Table S1.

For all low-throughput siRNA experiments, siRNAs were reverse-transfected at a final concentration of 10 nM using RNAiMAX (#13778-075, Invitrogen) according to the manufacturer's instructions.

Biochemistry For the compound wash-off experiments, ten million SW480 or DLD1 colorectal carcinoma cells were treated overnight with DMSO (D9170; Sigma-Aldrich), WIKI4, or XAV-939 at the concentrations indicated. Cells were then washed off and treated for one hour with the indicated treatments, and then lysed in RIPA buffer (50 mM Tris-cl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 5 mM ADP-HPD and 5 mM N-ethyl maleimide). Lysates were immunoprecipitated overnight with the indicated antibodies and analyzed by western blot. The in vitro TNKS2 assay was acquired from commercial sources (80565; BPS Bioscience).

3.2.4 - Results and Discussion

Identification of WIKI as a Small Molecule Inhibitor of Wnt/ß-catenin Signaling To make an assay for Wnt/ß-catenin signaling suitable for high throughput screening, we generated A375 melanoma cells stably infected with a ß-catenin-activated luciferase reporter (BAR) [23], [36] and selected populations in which luciferase activity is increased at least 4,000-fold by WNT3A. We tested the robustness of our assay by calculating the Z-factor (Z) values [40] using probes that are known to enhance (U0126 [41], Riluzole [42], and GSK3B inhibitor IX [43]) or inhibit (XAV-939 [33]) Wnt/ß-catenin signaling (Figure S1A). For all control probes, we found the Z values to be greater than .45 (Figure S1A), a value considered robust in high throughput screening assays [40]. Following validation of our assay, we then screened A375 melanoma cells at two concentrations of a small molecule library in the presence of a twenty percent effective concentration (EC20) dose of WNT3A. We focused on small molecules that reduced expression of the luciferase reporter at a low dose (330 nM) and that did not kill cells at a high dose (10 µM) relative to controls treated with dimethyl sulfoxide (DMSO), with the expectation that these criteria would filter out compounds that inhibited BAR due to cellular toxicity. Five compounds met our criteria for further study by significantly decreasing Wnt/ß-catenin signaling without causing toxicity at either dose (Fig. 1A).

We next asked whether any of the five compounds preferentially modulated Wnt/ß-catenin signaling by comparing the repression of BAR in A375 cells relative to luciferase reporters for the Nuclear Factor Kappa B (NF-kB), Transforming Growth Factor Beta (TGFß), and Retinoic Acid (RA) signaling pathways (Fig. 1B). Of the five candidate Wnt/ß-catenin inhibitors that we tested, WIKI4 (left panel, Fig. 1C) was the only inhibitor of BAR that did not also inhibit the reporters for NF-kB, TGFß, and RA (Fig. 1B). Furthermore, WIKI4 has demonstrated activity in one of nine published assays (http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?cid=2984337), supporting our contention that WIKI4 is not a general inhibitor of activity in high throughput screening assays. We then demonstrated that WIKI4 inhibits Wnt/ß-catenin signaling in several other cell lines, including DLD1 colorectal cancer cells (Fig. 1D), NALM6 B cells (Figure S1B), U2OS osteosarcoma cells (Figure S1B) and hESCs (Figure S1C). In all cell types tested, we observed that

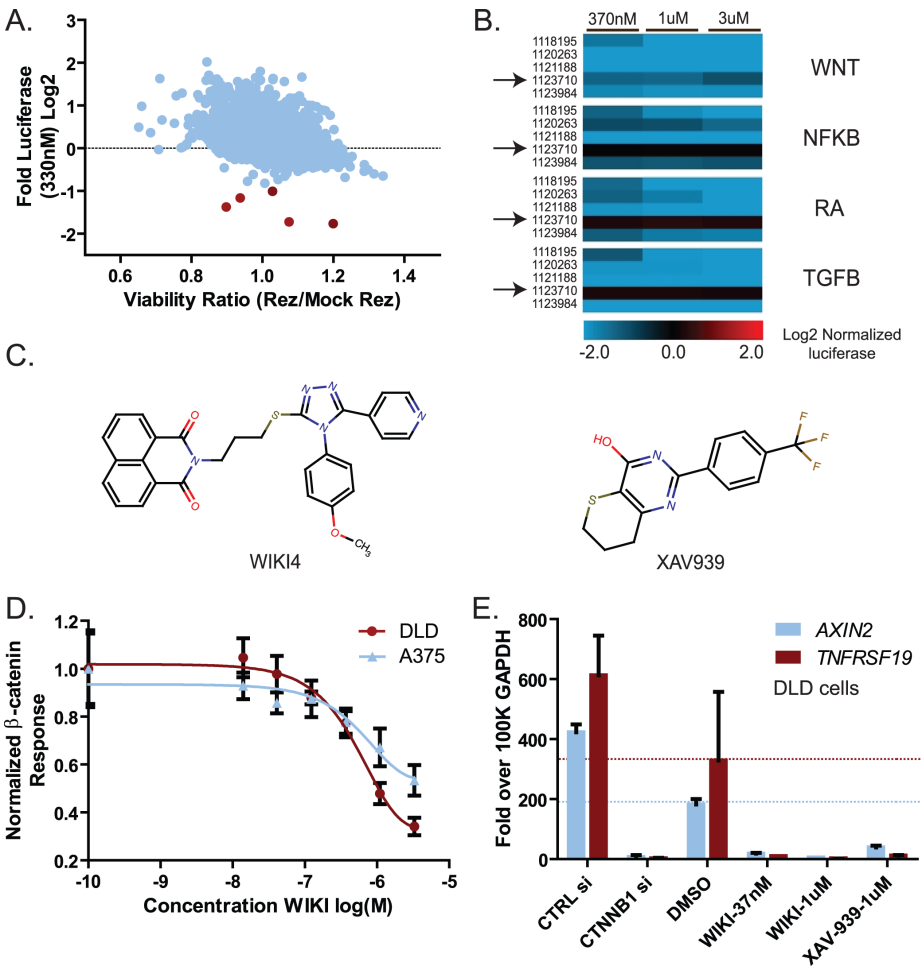

Figure 4: Figure 1

WIKI4 potently inhibited Wnt/ß-catenin signaling and that its half-maximal response dose was ~75 nM.

We next investigated whether WIKI4 is sufficient to inhibit expression of Wnt/ß-catenin target genes in DLD1 colorectal carcinoma cells, which express a truncated form of the Wnt/ß-catenin inhibitor APC [44]. We found that incubation of DLD1 cells overnight with either WIKI4 or the structurally distinct TNKS inhibitor, XAV-939 (right panel, Fig. 1C) [33], resulted in decreased steady-state abundance of AXIN2, and TNFRSF19 (Fig. 1E), which is consistent with WIKI4 acting as an inhibitor of Wnt/ß-catenin signaling. Furthermore, we observed that WIKI4 is sufficient to inhibit WNT3A-dependent increases in the expression of AXIN2 and TNFRSF19 in hESCs (Figure S1D, S1E). Thus we have identified WIKI4 as a new inhibitor of Wnt/ß-catenin signaling that regulates the pathway in several cell types.

To determine which chemical groups in WIKI4 are required for its ability to inhibit Wnt/ß-catenin signaling, we next performed a structure activity relationship analysis (Figure S2). WIKI4 has a molecular weight of 522 and a calculated partition coefficient of 4.8, putting it near the limits of "druglikeness" by Lipinski's Rule of Five [45]. WIKI4's mass and complexity is greater than XAV-939 (Fig. 1C), and identification of small active WIKI4 analogs could provide more opportunities for modification while maintaining its druglike properties. To identify less complex WIKI4 analogs and to determine which portions of WIKI4 are required for activity, we searched for commercially available analogs. We queried the ZINC [46] and eMolecule (www.emolecules.com) databases and identified 62 WIKI4 analogs for further testing (Table S3). We assayed the Wnt/ß-catenin inhibitory activity of a subset of these compounds (Figure S2). Our results indicate that the traizole's 4-pyridyl and 4-methoxyphenyl groups tolerate some modification, but the latter group could not be removed (Figure S2A). Additionally, substitution of the 1,8-naphthalimide group with a phthalimade group eliminated activity as did replacement of the 1,8-naphthalimide group with a methyl or phenyl group (Figure S2B).

WIKI4 Inhibits the Cellular Responses to Wnt/ß-catenin Signaling
We next asked whether cells treated with an effective dose of WIKI4 would show a reduction in Wnt/ß-catenin-mediated responses at the cellular level. As DLD1 colorectal cancer cells require ß-catenin signaling for growth in limiting culture experiments [47], these cells provide an excellent functional model of the pathway in which to test small molecules. We found that WIKI4 inhibits growth of DLD1 cells relative to DMSO controls in media containing low serum (Fig. 2A). This result demonstrates that WIKI4 inhibits a known cellular response to Wnt/ß-catenin signaling.

Given that cellular responses to Wnt/ß-catenin signaling are diverse and context-dependent, we next examined the effects of WIKI4 on hESCs. Activation of Wnt/ß-catenin signaling in hESCs alters their cell fate and causes them to differentiate into early mesoderm and endoderm lineage cells [16], [17]. Upon stim-

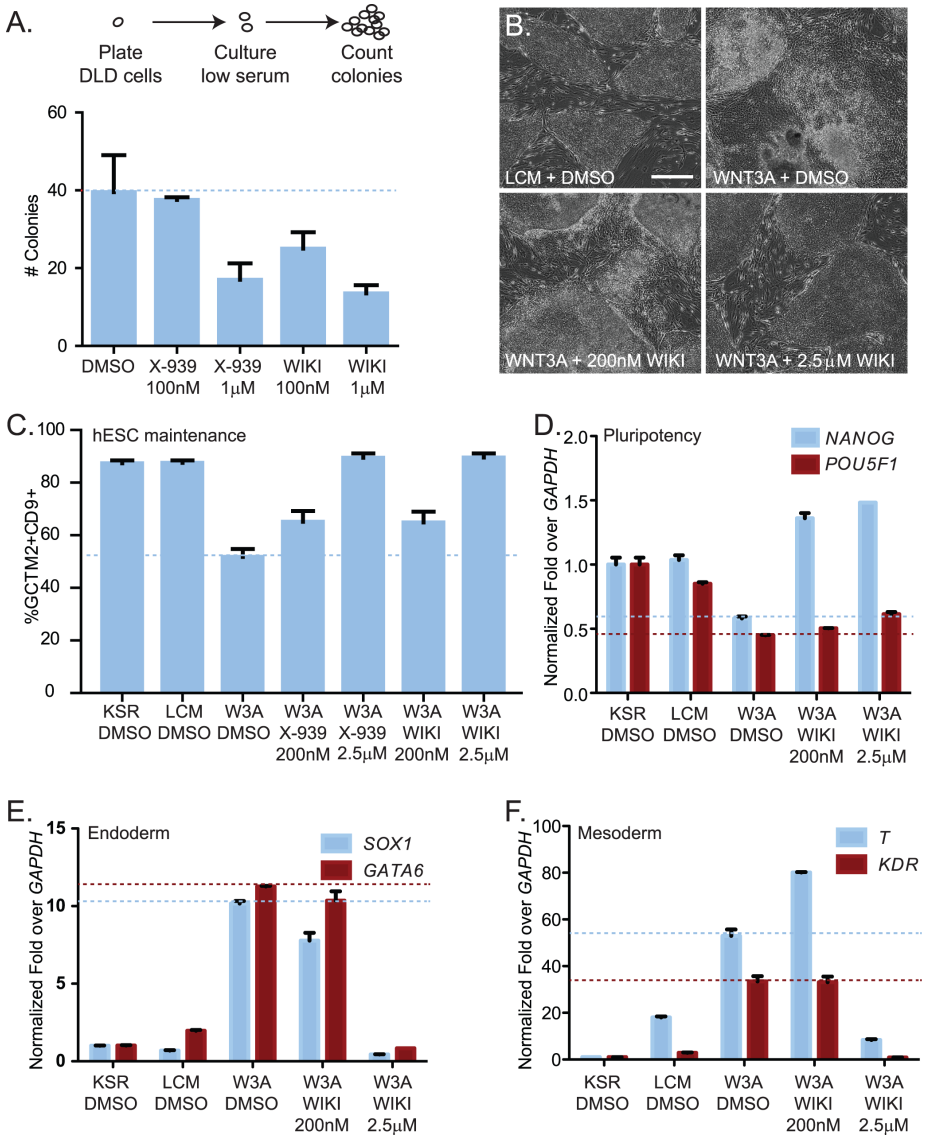

Figure 5: Figure 2

ulation with Wnt3A for 6 days, hESC colonies exhibit overt phenotypic changes that include loss of compact colony structure (top panels, Fig. 2B), decreased co-expression of cell surface markers of undifferentiated hESCs (GCTM2 and CD9, Fig. 2C) and decreased steady-state RNA abundance of pluripotency genes (NANOG and POU5F1, Fig. 2D). Additionally, treatment of hESCs with WNT3A leads to increased expression of genes associated with endoderm (SOX17 and GATA6, Fig. 2E) and mesoderm (T and KDR, Fig. 2F) differentiation. We found that in hESCs treated with both Wnt3A and WIKI4, the WNT3A-dependent effects that we typically observe on colony morphology (bottom panels, Fig. 2B), expression of cell surface markers (Fig. 2C) and expression of markers of pluripotency and differentiation (Fig. 2D, 2E, 2F) were eliminated. We conclude that WIKI4 inhibits Wnt/ß-catenin-mediated processes in hESCs, as well as in DLD1 cells, suggesting that WIKI4 acts on a conserved component of the Wnt/ß-catenin signaling pathway.

WIKI4 Increases Steady-state Abundance of AXIN1 After stimulation of A375 melanoma cells with Wnt3A, we observed that the steady-state abundance of the scaffold protein AXIN1 is reduced (left time course, Fig. 3A) and conversely, abundance of cytosolic CTNNB1 increases (left time course, Fig. 3A). Additionally, we observed that the abundance of CTNNB1 that is phosphorylated at sites that are regulated by the destruction complex components CSNK1A1 (S45, left time course Fig. 3A) and GSK3B (S33, left time course, Fig. 3A) is decreased following Wnt3A stimulation. We next investigated whether WIKI4 regulates the biochemical changes associated with Wnt/ß-catenin signaling. We found that WIKI4 inhibits WNT3A-dependent increases in the steady-state abundance of cytosolic CTNNB1, inhibits Wnt3A-dependent decreases in steady-state abundance of AXIN1, and inhibits Wnt3A-dependent decreases in abundance of phosphorylated of ß-catenin (S33 and S45) (right time course, Fig. 3A). Taken together, our findings indicate that WIKI4 modulates Wnt-dependent changes in the abundance and phosphorylation of known core components of the Wnt/ß-catenin signaling pathway.

We next examined whether WIKI4 alters steady-state abundance of AXIN1 and the related AXIN2 in another cell type. Increases in the steady-state abundance of the AXIN scaffolding proteins have been shown to correlate with decreases in the steady-state abundance of cytosolic CTNNB1, even in APC-mutant colon cancer cells [33], [34]. To test the effects of WIKI4 on AXIN levels in APC-mutant cells, DLD1 colorectal cancer cells were treated with WIKI4 for two, four, six or 24 hours and processed for western blotting. We observed that WIKI4 significantly increased the steady-state abundance of AXIN1 and AXIN2 (Fig. 3B) to levels similar to those seen with treatment with the TNKS inhibitor XAV-939.

To further investigate how WIKI4 regulates AXIN protein abundance, we queried whether WIKI4 treatment promotes expression of AXIN mRNA or whether it prevents the degradation of AXIN by the proteasome. Using

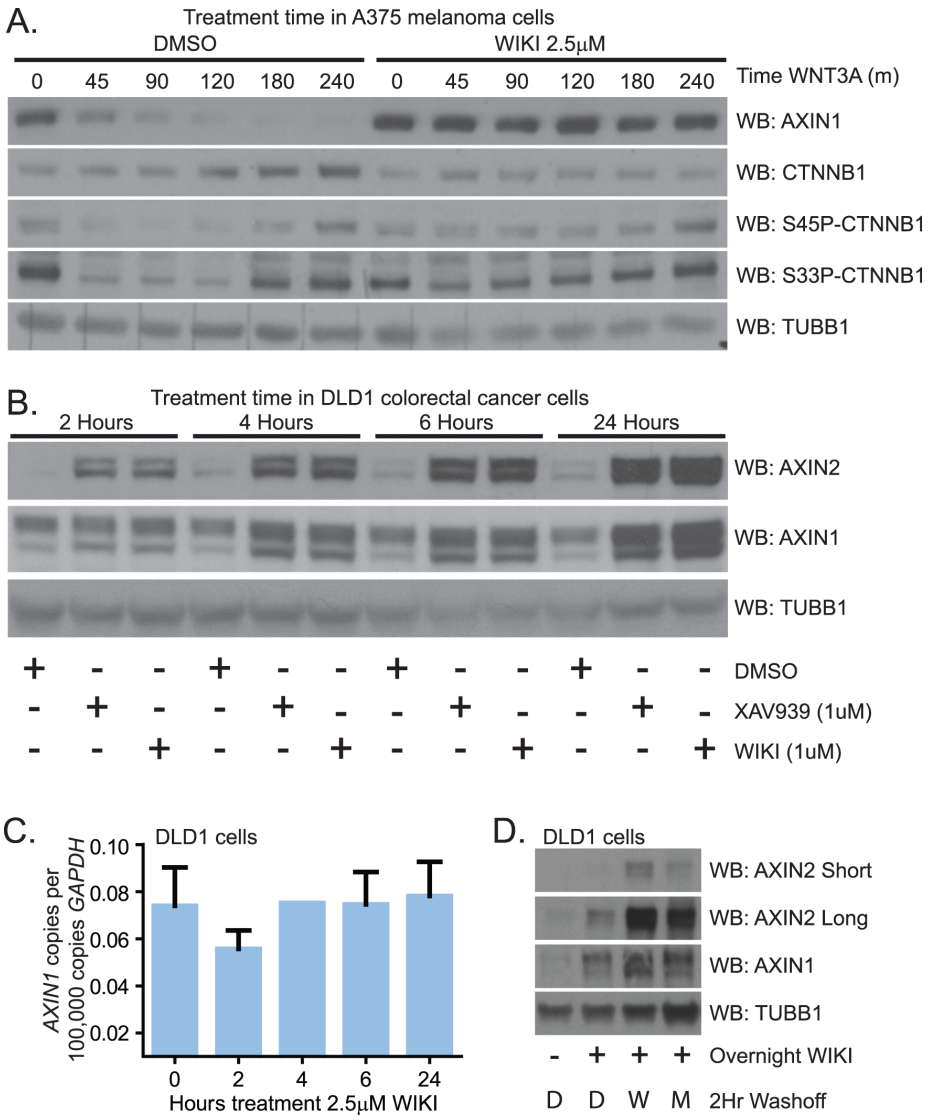

Figure 6: Figure 3

quantitative PCR (qPCR) analyses of DLD1 colorectal carcinoma cells, we found that steady state levels of AXIN1 (Fig. 3C) and AXIN2 (Fig. 1D) transcripts were not increased upon treatment with WIKI4. To test whether WIKI4 inhibits AXIN protein turnover, we treated DLD1 cells overnight with WIKI4, and then released them from treatment the next day for two hours (wash-off). We found that cells continuously treated with WIKI4 during the wash-off period exhibited increased abundance of AXIN1 and AXIN2 relative to cells treated with DMSO (Fig. 3D, compare lanes two and three), suggesting that WIKI4 prevents turnover of the AXIN proteins. When DLD1 cells were treated with the proteasome inhibitor MG132 during the wash-off period, AXIN1 and AXIN2 protein abundance remained elevated (Fig. 3D, compare lanes 3 and 4). Taken together, the qPCR and wash-off experiments suggest that WIKI4 increases the steady-state abundance of AXIN proteins by preventing their degradation by the proteasome.

WIKI4 Blocks the Activity of TNKS2 and Prevents AXIN Ubiquitylation AXIN1 is modified sequentially by two enzymes in order for it to be recognized by the proteasome for degradation. First, AXIN1 is ADP-ribosylated by the TNKS1 and TNKS2 enzymes [33]. Subsequently, ADP-ribosylated AXIN1 is bound by the E3 ubiquitin ligase RNF146, which specifically catalyzes its ubiquitylation (Fig. 4A, [48], [49]). To test whether WIKI4 prevents ubiquitylation of AXIN protein, we treated SW480 (Fig. 4B) and DLD1 (Figure S3A, S3B) colorectal carcinoma cells overnight with WIKI4, and subsequently incubated for two hours with either MG132 alone or MG132 and WIKI4 (wash-off). We found that inhibition of the proteasome during the wash-off period with MG132 led to an increase in the abundance proteins bound to ubiquitin (Fig. 4B, Fig. S3A, S3B left panels). We further observed that WIKI4 treatment during the wash-off period reduced the detection of ubiquitin in immunoprecipitated AXIN2 (Fig. 4B, Figure S3A) and AXIN1 (Figure S3B), suggesting that WIKI4 indeed inhibits AXIN ubiquitylation.

One possible explanation for WIKI4-dependent inhibition of AXIN ubiquitylation is that WIKI4 directly inhibits TNKS-mediated ADP-ribosylation of AXIN. The ADP-ribosylation activity of TNKS proteins can be assayed in vitro by quantifying their ability to catalyze auto ADP-ribosylation. To investigate the hypothesis that WIKI4 inhibits the catalytic activity of TNKS proteins, we performed in vitro auto-ADP-ribosylation assays using recombinant TNKS2. Similar to what is observed for the known TNKS inhibitor XAV-939, we found that WIKI4 prevents auto-ADP-ribosylation of TNKS2 at an IC50 of ~15 nM (Fig. 4C). In contrast to the effects of XAV-939 and WIKI4, a second ATP analog, U0126, failed to inhibit auto-ADP-ribosylation of TNKS2, demonstrating that our assay is specific (Figure 4C). Taken together, our data suggest that WIKI4 inhibits Wnt/ß-catenin signaling by inhibiting tankyrase activity, and thus preventing the ubiquitylation and degradation of AXIN proteins.

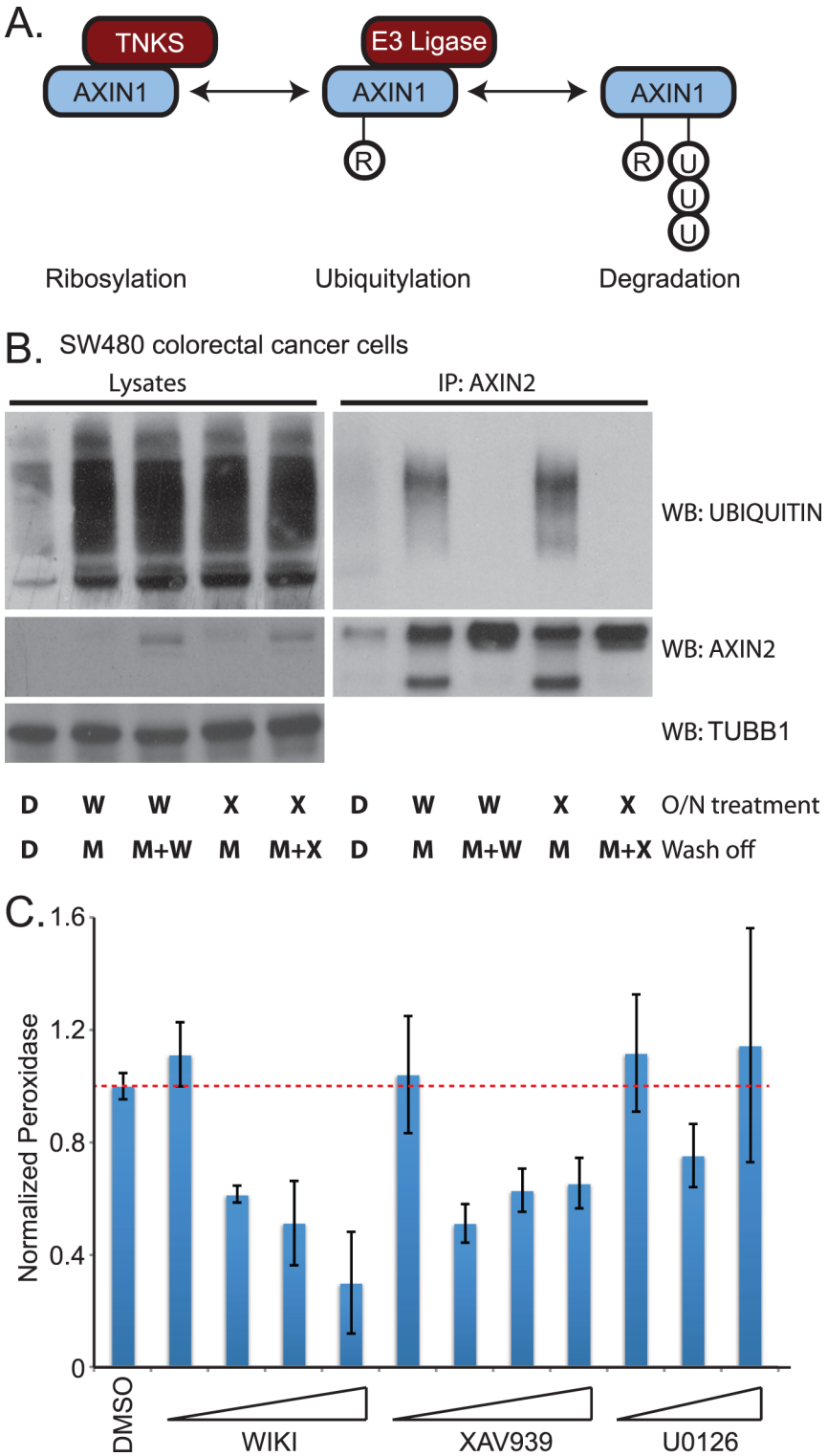

Figure 7: Figure 4

3.2.5 - Conclusions

In summary, we have identified and characterized WIKI4, a novel small molecule inhibitor of Tankyrase that leads to inhibition of Wnt/ß-catenin signaling in multiple cell lines and in hESCs. As the structure of WIKI4 is distinct from the other published Tankyrase inhibitors [33], [34], [35], it is unlikely to share off-target effects with those molecules. Therefore, WIKI4 will be useful as a complementary biological probe for researchers who wish to inhibit the Wnt/ß-catenin pathway by inhibiting Tankyrase.

3.3 - Conclusion

We learned some interesting things about wiki.

4 - Simvastatin Promotes Adult Hippocampal Neurogenesis by Enhancing Wnt/ -Catenin Signaling

4.1 - Introduction

I was an author on a paper about simvastatin.

4.2 - Journal Article

4.2.1 - Summary

Statins improve recovery from traumatic brain injury and show promise in preventing Alzheimer disease. However, the mechanisms by which statins may be therapeutic for neurological conditions are not fully understood. In this study, we present the initial evidence that oral administration of simvastatin in mice enhances Wnt signaling in vivo. Concomitantly, simvastatin enhances neurogenesis in cultured adult neural progenitor cells as well as in the dentate gyrus of adult mice. Finally, we find that statins enhance Wnt signaling through regulation of isoprenoid synthesis and not through cholesterol. These findings provide direct evidence that Wnt signaling is enhanced in vivo by simvastatin and that this elevation of Wnt signaling is required for the neurogenic effects of simvastatin. Collectively, these data add to the growing body of evidence that statins may have therapeutic value for treating certain neurological disorders.

4.2.2 - Introduction

A large body of evidence indicates that statins, a class of drugs typically used to treat hyperlipidemia, are therapeutically beneficial for neurological disorders. Statins have been shown to improve outcome following traumatic brain injury and stroke (Chen et al., 2003, Karki et al., 2009, Lu et al., 2007, Mahmood et al., 2009 and Wu et al., 2008). Simvastatin rescues cerebrovascular and memory-related deficits in mouse models of Alzheimer disease (AD) (Li et al., 2006, Tong et al., 2009 and Tong et al., 2012), and recent meta-analysis of clinical studies concluded that statins provide a slight benefit in the prevention of AD and all-type dementia (Wong et al., 2013). While these effects have been attributed to reduction of inflammation, reduced oxidative stress, upregulated PI3K/AKT signaling, and enhanced neurogenesis, the mechanisms by which statins are beneficial in neurological disorders are not fully understood.

Previously, we reported a chemical genetic screen that revealed that several statins activate a -catenin-responsive luciferase reporter (BAR) in a cell-based assay (Biechele et al., 2010). This result supports prior in vitro studies that have shown that statins modulate Wnt/ -catenin signaling (henceforth referred to as Wnt signaling) in human neuronal cells (Salins et al., 2007), in rat mesangial cells (Lin et al., 2008), and in mouse embryonic stem cells (Qiao et al., 2011). Given that Wnt signaling is a key regulator of adult hippocampal neurogenesis (Jang et al., 2013, Kuwabara et al., 2009, Lie et al., 2005, Luo et al., 2010, Mao et al., 2009 and Seib et al., 2013), we sought to determine whether statin-mediated enhancement of the Wnt pathway can occur in this region of the brain and to characterize any downstream effects on neurogenesis. We chose to focus on simvastatin (simva), as it is a lipophilic statin capable of crossing the blood-brain barrier (Tamai and Tsuji, 2000) and is commonly studied in neural contexts.

We find that simva enhances Wnt signaling in the adult hippocampus and that Wnt signaling is required for statins to enhance neuronal specification in differentiating adult neural progenitor cells (aNPCs). Through examination of various stage-specific markers in vivo, we determine that simva treatment increases the number of newborn neurons in the dentate gyrus (DG) by enhancing proliferation of intermediate precursor cells (IPCs) in the subgranular zone (SGZ). Finally, we determine that the effect of simva on the Wnt pathway is independent of cholesterol and is mediated by inhibition of isoprenoid biosynthesis.

4.2.3 - Results

Simva Enhances Wnt Signaling In Vitro and In Vivo We recently reported that lovastatin and fluvastatin enhance Wnt signaling (Biechele et al., 2010). In the present study, we extended our analysis to simva, a statin of clinical relevance to neurological disease. To monitor Wnt activity, we transduced cultured aNPCs with BAR driving expression of Venus fluorophore. Consistent with prior studies (Wexler et al., 2009), we saw no reporter activity under basal culture conditions, and addition of simva by itself did not induce reporter expression (not shown). However, in combination with a low dose (20 ng/ml) of recombinant WNT3A ligand (rcWNT3A), simva promoted a dose-dependent increase in the percentage of Venus+ cells following 4 days of treatment (Figures 1A and 1B).

If, as suggested above, simva were able to synergize with Wnt signaling, then one would predict that such treatment should increase the expression of endogenous Wnt target genes. To test this, we treated aNPCs with simva or DMSO, in combination with either rcWNT3A or vehicle control (CHAPS), and used quantitative real-time PCR to monitor the levels of two genes that are known to be directly regulated by Wnt signaling in adult neural cells, Axin2 and CyclinD1 (Mao et al., 2009). As expected, rcWNT3A promoted increases in Axin2 and CyclinD1 expression compared to CHAPS, while simva alone had negligible effects. However, the expression of these genes was greatly enhanced by the combination of simva and rcWNT3A, similar to the synergy seen with the BAR reporter. We observed a 7.7-fold increase in Axin2 and a 5.2-fold increase in CyclinD1 expression (Figure 1C). Thus, both BAR reporter and expression levels of -catenin target genes show that simva enhances Wnt signaling in cells where the pathway is activated at a low level.

We then directly investigated whether dosing mice with simva via oral gavage enhances Wnt signaling in the brain. Based on our in vitro data, we focused on the DG of the hippocampus, one of the germinal brain regions where aNPCs reside and where WNT3A is secreted throughout adulthood (Garbe and Ring, 2012 and Lie et al., 2005). We treated adult C57BL/6J mice harboring a reporter transgene (BAT-GAL) that drives expression of nuclear-targeted -galactosidase (-gal) in response to Wnt signaling (Maretto et al., 2003), and which has been used to measure Wnt signaling in the DG (Garbe and Ring, 2012 and Mazumdar

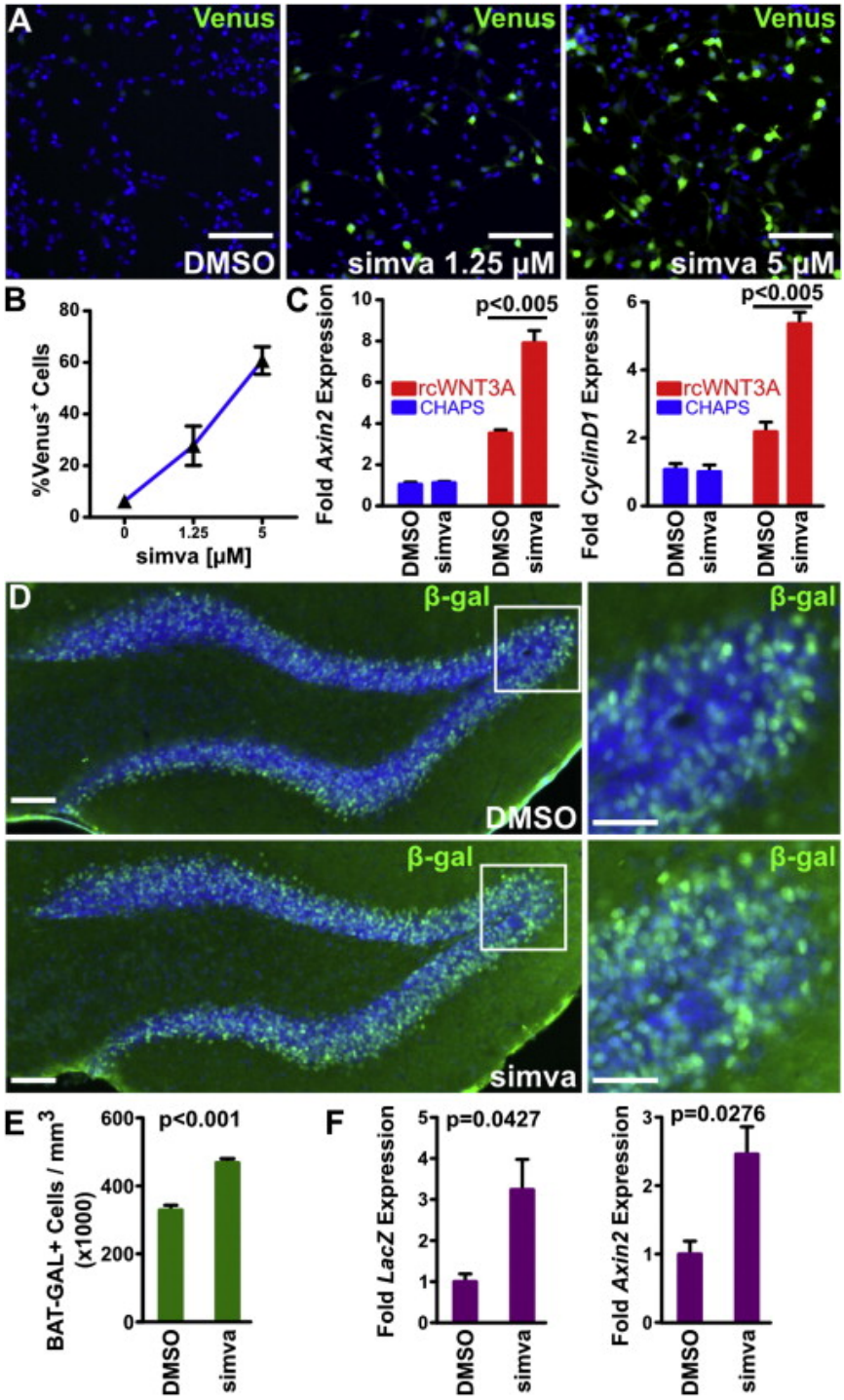

Figure 8: Figure 1

et al., 2010). Based on previous studies, we treated BAT-GAL mice with either 10 mg/kg simva or DMSO by oral gavage daily for 7 days (Chen et al., 2003 and Karki et al., 2009). Mice were sacrificed 4 hr after the final drug treatment. We observed a 1.4-fold increase in the density of nuclei containing -gal in the DG of mice treated with simva (Figures 1D and 1E), while the volume of the DG was unchanged between treatment groups (not shown). To test whether these results reflect changes in expression levels of Wnt targets, we collected total RNA from the hippocampus and performed real-time PCR analysis. We measured a significant increase in the average expression of both reporter gene LacZ and endogenous target gene Axin2 in mice treated with simva compared with vehicle-treated mice (Figure 1F). Together with the BAT-GAL immunostaining, these results demonstrate that systemic administration of simva enhances Wnt signaling in the DG of adult mice.

Simva Enhances Neuronal Specification via Wnt Signaling We then investigated the effect of simva on aNPCs during differentiation. We induced differentiation in aNPCs as described previously (Luo et al., 2010, Palmer et al., 1999 and Smrt et al., 2007), in the presence of drug treatment for 4 days and stained for lineage-specific markers (Figure 2A). While simva slightly reduced the total number of cells detected following differentiation (Figure 2B), simva treatment lead to a 3.6-fold increase in the number of TUJ1+ neurons and a significant decrease in the number of GFAP+ astroglial cells (Figures 2C and 2D). To further assess the effect of simva on aNPC differentiation, we measured expression of linage-specific genes using real-time PCR. mRNA levels of the neuron-specific genes Tuj1 and NeuroD1 were significantly increased following differentiation in aNPCs treated with simva (Figure 2E). Levels of the astroglial genes Gfap and Aqp4 were lower in simva-treated aNPCs, but the difference was not statistically significant (Figure 2F). These results, consistent with previous reports of Wnt enhancement during aNPC differentiation (Luo et al., 2010), indicate that simva influences lineage specification in aNPCs toward increased production of neurons and decreased production of astroglia.

In order to test whether the effect of simva on aNPC differentiation is due to enhanced Wnt signaling, we also tested simva in combination with a Wnt pathway antagonist. For this, we employed the small molecule XAV939. Importantly, XAV939 blocks simva-mediated enhancement of Wnt signaling in aNPCs (Figure S1 available online). We reasoned that if simva enhances neuronal differentiation via enhanced Wnt signaling, then a combination of XAV939 and simva should not elicit this effect.

As expected, XAV939 treatment significantly decreased the number of neurons formed following aNPC differentiation. We found that in aNPCs treated concurrently with XAV939 and simva, the percentage of TUJ1+ cells was equivalent to DMSO-treated cells and significantly reduced as compared to cells treated with simva alone (Figures 2A and 2C). Additionally, levels of the neuronal genes Tuj1 and NeuroD1 were decreased with combined simva and XAV939 treatment

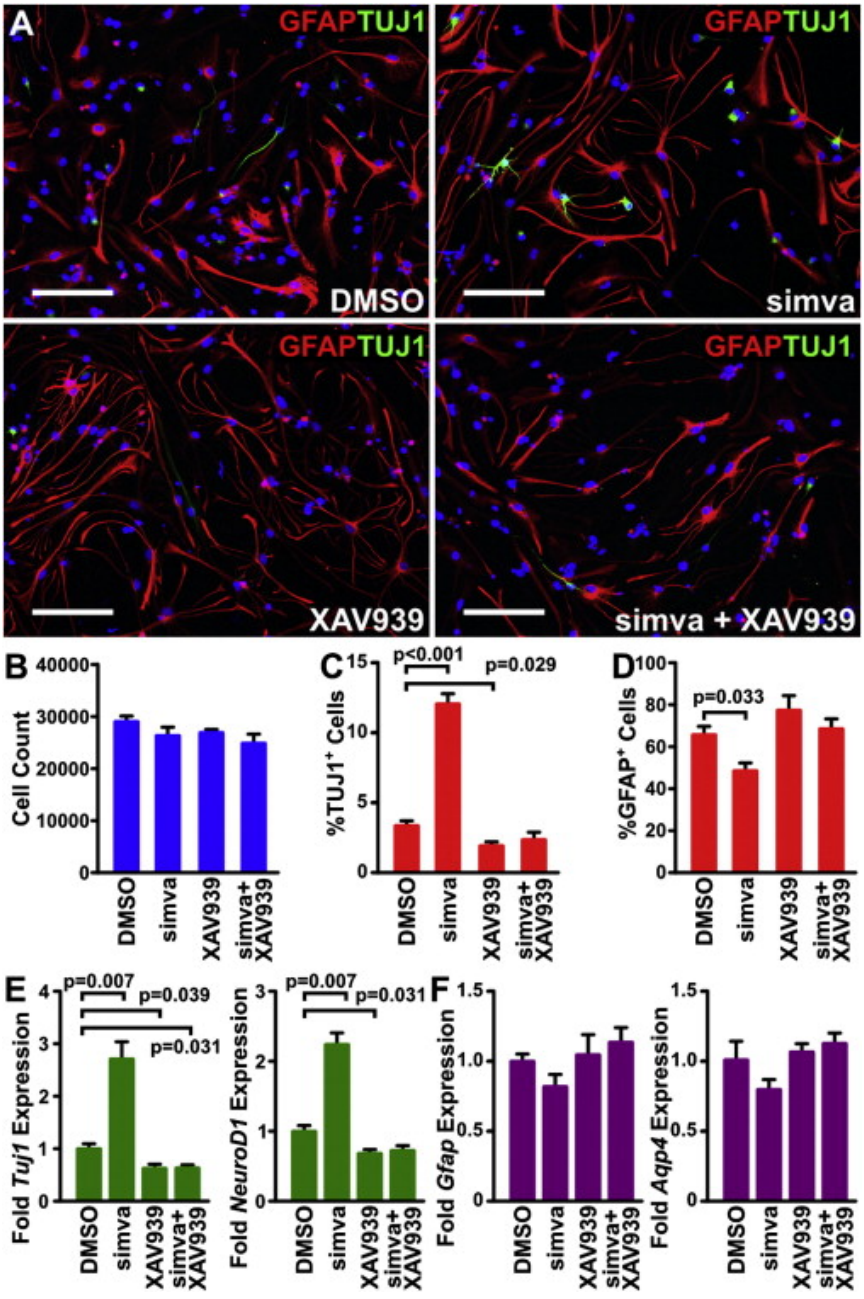

Figure 9: Figure 2

compared with DMSO (Figure 2D), while levels of the astrocytic genes Gfap and Aqp4 were not significantly changed (Figure 2E). These data demonstrate that blocking Wnt signaling abolishes the ability of simva to enhance neuronal differentiation and suggest that this effect of simva is Wnt signaling-dependent.

Simva Enhances Adult Hippocampal Neurogenesis Having observed that simva enhances Wnt signaling in the DG of adult mice and increases neuronal specification in cultured aNPCs, we next assessed the effects of the simva treatment described above on in vivo hippocampal neurogenesis. To assess overall cell proliferation in the SGZ, we first examined the DNA replication marker MCM2 (Figure 3A). Quantification of cell numbers using confocal z stack images revealed that mice treated with simva had 1.7-fold more MCM2+ cells per DG compared to control (Figure 3B). To test whether the simva-mediated increase in DG cell proliferation affects the formation of new neurons, we examined the immature neuron marker DCX. We did not find a significant difference in the number of DCX+ cells per DG for simva versus control (not shown). However, when we injected mice with a single dose of EdU (50 mg/kg) 24 hr prior to perfusion to specifically label cells in S phase toward the end of treatment, we saw that the number of DCX+/EdU+ cells per DG was increased 1.4-fold with simva treatment (Figures 3C and 3D). To test whether simva treatment affects cell survival, we counted the apoptotic nuclei within the same area of the DG. Cleaved caspase-3 (cCASP3) staining revealed no significant difference in the number of cCASP3+ cells per DG in simva-treated mice (Figure S2).

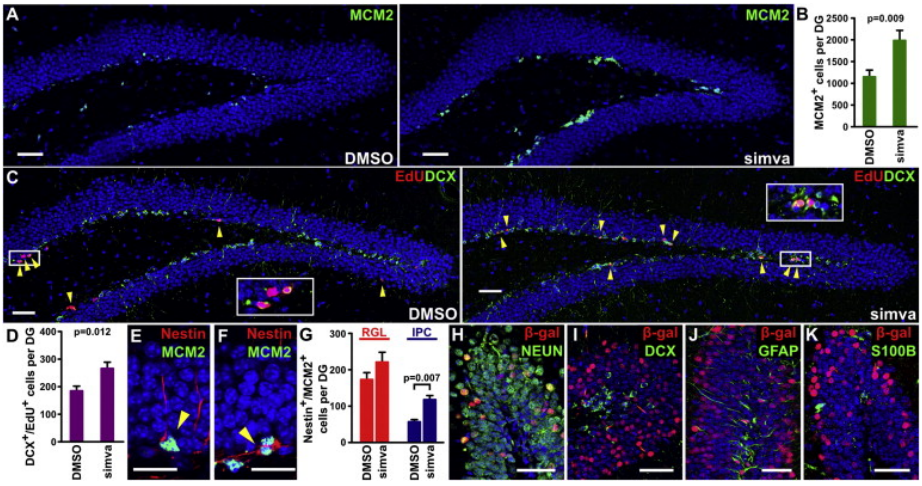

Figure 10: Figure 3

The increase in the number of proliferating cells within the SGZ that we observed as a result of simva treatment could be due to increased IPC proliferation or increased production of IPCs from radial-glia like early precursors (RGLs)

(Bonaguidi et al., 2011). We looked to see what effect simva has on these populations by labeling for progenitor marker Nestin alongside MCM2. Activated RGLs, which asymmetrically divide to form new IPCs, were identified as Nestin+/MCM2+ with radial morphology (Figure 3E). Proliferating IPCs were identified as Nestin+/MCM2+ cells with horizontal morphology (Figure 3F). We observed a modest, but not statistically significant, increase the number of activated RGLs per DG in simva-treated mice. Meanwhile, the number of proliferating IPCs per DG was increased 2-fold in simva-treated mice (Figure 3G).

BAT-GAL staining revealed that Wnt signaling is active in some, but not all, of the cells in the DG of both simva- and control-treated mice (Figure 1D). To identify the cell types that exhibit active Wnt signaling following simva treatment, we costained tissue for -gal and a panel of cell-type-specific markers. We observed that a majority of nuclear -gal expression occurred in cells labeled with the mature neuron-specific protein NEUN in the granule cell layer (GCL) (Figure 3H), with very little -gal in immature neurons (DCX+) (Figure 3I). We saw significant -gal expression in GFAP+ cells in both the GCL and SGZ (Figure 3J). While GFAP can label both astrocytes and immature precursors, cells with nuclear -gal expression were rarely labeled with the astrocyte marker S100B (Figure 3K).

We found that simva increases overall cell proliferation in the SGZ, leading to an increase in the number of newly formed neurons. When we looked to delineate between different progenitor pools, we found that IPC proliferation was significantly increased. Additionally, we observed Wnt reporter expression in both GFAP+ and NEUN+ cells following simva treatment. Taken together, these data suggest that oral simva treatment enhances adult neurogenesis in the mammalian hippocampus.

Simva Enhances Wnt Signaling via Depletion of Isoprenoids Finally, we investigated the mechanism by which simva can enhance Wnt signaling. Statins antagonize HMG-CoA-reductase (HMGCR), the rate-limiting enzyme in the sterol biosynthetic pathway. This pathway is responsible for de novo synthesis of cholesterol as well as isoprenoids (Endo, 1992). To test whether inhibition of sterol biosynthesis enhances Wnt signaling, we used small interfering RNAs (siRNAs) to knock down HMGCR in human neuroblastoma (SH-SY5Y) cells harboring BAR driving luciferase. Similar to our aNPC data, we found that simva enhances Wnt signaling in these cells with a low dose of WNT3A conditioned media (CM), but not with control CM (Figure S3). We tested three unique siRNAs to knock down HMGCR (Figure S4) and a control nontargeting siRNA. Cells transfected with HMGCR siRNAs and treated with WNT3A CM showed significantly increased reporter induction (Figure 4A).

Downstream of HMGCR, sterol biosynthesis bifurcates to produce either the cholesterol-precursor squalene or the isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) (Fears, 1981) (Figure 4B). Statins,

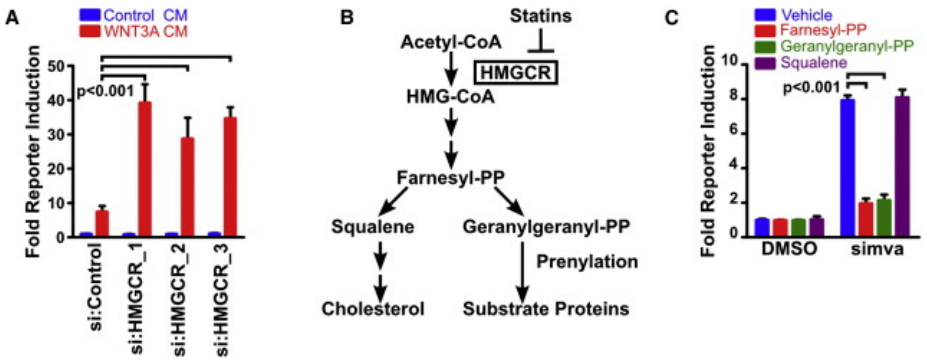

Figure 11: Figure 4

via inhibition of the sterol biosynthetic pathway, deplete cellular pools of cholesterol, FPP, and GGPP (Hughes, 1996). To test whether any of these products are involved in simva enhancement of Wnt signaling, we treated SH-SY5Y BAR cells with 5 M simva, WNT3A CM, and exogenous FPP, GGPP, or squalene. We reasoned that if statin-mediated depletion of any of these metabolites caused the enhancement of the Wnt pathway, then adding the responsible metabolite back to the cells would inhibit the ability of simva to enhance BAR activity. Notably, addition of 10 M GGPP or 10 M FPP to simva-treated cells significantly reduced simva-mediated Wnt enhancement, while 10 M squalene did not have an effect (Figure 4C). Since FPP is used to synthesize GGPP, the effect may be due to depletion of GGPP alone or a combination of FPP and GGPP depletion. Therefore, we conclude that simva enhances Wnt signaling via depletion of isoprenoids and not cholesterol.

4.2.4 - Discussion

Simva is under investigation for its potential therapeutic effects outside of hyperlipidemia treatment. While statins have been reported to enhance Wnt signaling in vitro, it was heretofore not known whether statins can enhance this pathway in vivo and in the context of neurogenesis. Here we provide evidence that oral simva treatment enhances Wnt signaling in the mammalian adult hippocampus. This is significant in that aside from lithium, no other clinically approved compound has been demonstrated to enhance Wnt signaling in the brain (Zimmerman et al., 2012).

The observations in this study are consistent with reports of increased hippocampal neurogenesis due to both simva treatment (Chen et al., 2003, Lu et al., 2007 and Wu et al., 2008) and increased Wnt signaling (as cited in introduction). Importantly, we demonstrate a link between these phenomena by probing Wnt's role in simva enhancement of neurogenesis in vitro, and subsequently investigating the effect of enhanced Wnt signaling during multiple stages of in vivo adult

hippocampal neurogenesis.

While we showed a requirement for Wnt signaling in increased neuronal differentiation among simva-treated aNPCs, it remains possible that additional signaling pathways play a role in simva's effect on overall neurogenesis. Further, while others have demonstrated beneficial neurological effects of simva in disease models (as cited in introduction), the present study was performed using healthy animals and did not monitor later stages of neuronal development or behavioral outcomes.

To help map the biological connection between enhanced Wnt signaling and enhanced neurogenesis, we examined costaining of BAT-GAL with various cell-type-specific markers following simva treatment. However, a comparison showing differences in temporal expression patterns between different in vivo Wnt reporters presents a potential caveat to this approach (Garbe and Ring, 2012).

The mechanism underlying statin enhancement of Wnt signaling had not been previously reported. Providing initial insight, we show that HMGCR loss of function is sufficient to enhance the Wnt pathway. Furthermore, we demonstrate that simva acts on Wnt signaling by depleting isoprenoids, rather than through a cholesterol-dependent mechanism. Prenylation guides membrane localization of small GTPases such as RAS and RHO-associated kinases and other signaling proteins (Zhang and Casey, 1996), and serves as a regulatory mechanism for these enzymes that can be targeted therapeutically (Gelb et al., 2006). To this point, recent studies have measured an age-dependent increase of isoprenoid levels in brains of mice (Hooff et al., 2012) and have identified an overabundance of isoprenoids in the brains of AD patients (Eckert et al., 2009). The identity of the specific prenylated protein or proteins responsible for the effect of simva on the Wnt pathway remains elusive. However, there are a number of prenylated proteins known to regulate Wnt signaling (e.g., RAC1 and RHOA) that may serve as candidates for future studies (Schlessinger et al., 2009).

4.2.5 - Experimental Procedures

BAR Venus Experiment with aNPCs aNPCs were plated on polyornithine- (Sigma-Aldrich) and laminin-coated (Life Technologies) optical imaging plates (Corning) in proliferation media containing FGF (Life Technologies) and epidermal growth factor (EGF; PeproTech) (see Supplemental Experimental Procedures). Drugs were added as indicated 24 hr after plating. Following 4-day drug treatment, Hoechst 33342 dye (Sigma-Aldrich) was added at 1 g/ml.

Mouse Experiments Eight- to 10-week-old C57BL/6J WT and BAT-GAL mice were used in this study. All animal-related procedures were approved by the Institutional Animal Care and Use Committee of the University of Washington and were conducted in accordance with the guidelines of the National

Institutes of Health. For oral gavage, simva was dissolved in DMSO and diluted in 1% carboxymethylcellulose (Sigma-Aldrich) in water. For EdU experiments, mice were injected intraperitoneally with 50 mg/kg EdU dissolved in DMSO. For details on tissue collection, see the Supplemental Experimental Procedures.

aNPC Differentiation To induce differentiation, aNPCs were plated on polyornithine- and laminin-coated optical imaging plates with differentiation media containing 5 M forskolin (Sigma-Aldrich) and 1 M retinoic acid (Sigma-Aldrich) and lacking EGF and fibroblast growth factor (see Supplemental Experimental Procedures).

Antibody Staining, Imaging, and Quantification BAR Venus aNPCs were imaged with a fluorescence microscope (Nikon), and antibody-stained aNPCs were imaged with a Nikon A1 confocal microscope. TUJ1+ and GFAP+ aNPCs were manually counted using ImageJ, and percentage was determined by dividing by total number of nuclei. BAT-GAL+ cell density and DG volume was determined using stereology software and semiautomated counting with a fluorescence microscope. To quantify MCM2+, DCX+, EdU+/DCX+, and Nestin+/MCM2+ cells we collected z stacks and counted manually using ImageJ (see Supplemental Experimental Procedures).

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4.2.6 - References

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4.3 - Conclusion

We learned some interesting things about simvastatin.

5 - Conclusion

The concluding chapter should show clearly how the preceding chapters form a coherent substantial body of work and how significantly this body of work advances our knowledge.

Summarize overall results I chose to frame my work on cell signaling pathways based on recent work.

My contribution of original knowledge in this area is the discovery of various molecules that can be used to manipulate Wnt/ß-Catenin signaling. This includes repurposed clinically-approved compounds that were not previously known to affect Wnt, and novel compounds whose biological mechanisms were later determined.

Successes There was a lot to be gained by focusing on Wnt signaling We had a great reporter system that could reliably be used for high throughput screening, also other reagents like antibodies, and most importantly expertise of peers in academic lab environment

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We were able to cover a lot of ground in terms of interesting disease contexts, as this became ou
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We delivered a number of molecules to the community (Robin et al., 2014) These can be used immediately by clinicians body of knowledge that can be applied to interpretation of patient data

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and also can be used by biologists as research molecules
can be purchased on tocris
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We prototyped a system for HTS that worked in diverse cell types

Shortcomings Possible shortcoming: block one pathway and cancer will find a way around it NATURE REVIEWS CLINICAL ONCOLOGY | EDITORIAL Predicting cancer's next move

While at times the self imposed filter of small molecules was helpful, it also restricted the possible solutions that could be found for a problem

Likewise with Wnt signaling, aside from the gains of tying a research project to a particular pathway, its likely that disease pathologies span indescriminately across signaling pathways. It may be more beneficial in some cases to consider all signaling pathways simultaneously, as is the case in systems biology, as opposed to 'monolithic' focus on a known pathway.

A shortcoming of my work with small molecules was my relative inability to determine novel targets. With Riluzole, we were fairly certain that the effect was due to metabotropic glutamate receptor, but there was conflicting data. In the case of the WIKI paper, Ricker worked for a long time and barely found it – and it was already known. In the case of the simvatin, I was able to confirm that the effect on Wnt was due to simvas known MOA, but when I attempted to trace the steps back to Wnt signaling I could not succesfully connect the dots with small molecules alone.

Although I gave some attempt at using genetic and proteomic approaches, these did not yield an answer. I believe that if I had focused more strongly on possessing the correct genetic and proteomic tools to monitor and manipulate Wnt signaling I could have learned the answer – and would be more suited to answer future questions.

Despite the effort put forth to do HTS in stem cells, we did not identify anything unique to stem cells when we did these screens. Wiki works in other cell types, as do the peptide mimetics that I identified. We hoped to learn about a possible interaction between CTNNB1 and OCT4, but only in traditional low-throughput experiments was our lab able to address these questions. The mistake here was to perform a wholistic discovery experiment with an anticipated result in mind – these two approaches are not compatible.

Suggestions for future work Determine whether wnt enhancement can truly overcome vemurafinib resistance

Closely follow clinical paradigms – if something aside from small molecules comes along, then quickly adopt it to research

Prioritize performing experiments from different domains in tandem. In order to build a strong case that a certain signaling event occurs, I found that it is not only necessary to use small molecule perturbation but also through genetic means such as siRNA.

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