Molecular Pathways: Aspirin and Wnt Signaling—A Molecularly Targeted Approach to Cancer Prevention and Treatment

Manish K. Gala and Andrew T. Chan

1Division of Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts
2Channing Division of Network Medicine, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts

Corresponding Authors:
Manish K. Gala, Gastrointestinal Unit, Massachusetts General Hospital, GRJ-711 N, Boston, MA 02114. Phone: 617-724-6007; Fax: 617-724-6832; E-mail: mgala@mgh.harvard.edu
Andrew Chan, Gastrointestinal Unit, Massachusetts General Hospital, GRJ-825C, Boston, MA 02114. Phone: 617-724-0283; Fax: 617 726 3673; E-mail: achan@mgh.harvard.edu

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Abstract

The anti-inflammatory properties of aspirin have resulted in its widespread use as an analgesic, anti-pyretic, and cardioprotective agent. Beyond these applications, multiple observational studies and randomized controlled trials have demonstrated a chemopreventative role for aspirin, particularly in the development of colorectal neoplasia. Given the critical importance of Wnt dysregulation in colorectal carcinogenesis, the interplay between aspirin and canonical Wnt signaling has become a focus of investigation. These studies have illuminated our understanding of the anti-cancer mechanisms of aspirin, yielding the identification of potential biomarkers for which aspirin’s chemopreventative efficacy can be safely optimized into routine clinical practice and providing leads into the discovery of novel preventive and therapeutic targets. In this review, we summarize key experimental and clinical studies of this interaction, as well as highlight future strategies to advance their clinical translation.
Background

Canonical Wnt signaling is an evolutionary conserved pathway involved in embryonic development and tissue regeneration (1). Activation of this pathway through secreted Wnt-protein ligands results in cytosolic stabilization of β-catenin, and its translocation into the nucleus (2). In the nucleus, β-catenin binds to transcription factor 7-like 2 (TCF7L2) to form a transcriptional complex that upregulates the expression of genes involved in cell proliferation and cell migration (Fig. 1A). When this pathway is inactive, cytosolic β-catenin is instead targeted for ubiquination and destruction through a scaffold complex consisting of APC, GSK3β, and Axin. Somatic mutations resulting in enhanced or constituent activation of Wnt signaling occur in many cancer types, notably in colorectal cancer (CRC), where biallelic activation of APC is frequently the incipient event in tumorigenesis (3).

The substantial disease burden of CRC, as the second leading cause of cancer deaths in the United States, has inspired the search for safe and cost-effective chemopreventative measures. Multiple epidemiological studies in diverse populations as well as randomized control trials have shown that aspirin (ASA) use prevents the development of adenomas, lowers the incidence of colorectal cancer, reduces colorectal cancer mortality, and is associated with improved survival among patients with established CRC (4-11). More recently, compelling data support a role for aspirin in reducing the incidence of and death from other cancers, as well as metastatic spread (12-14). Although this human evidence supporting the anti-cancer benefits of aspirin is remarkably strong and consistent, the mechanistic basis for these effects remain poorly understood. Substantial experimental data support a role for aspirin in modulating Wnt
signaling. Based on the fundamental role of Wnt signaling in carcinogenesis, an influence of aspirin mediated through this pathway assumes greater potential relevance when viewed in the context of aspirin’s effects on multiple cancer types. Here we summarize key mechanistic findings from experimental studies, highlight emerging human evidence that highlights aspirin’s role in Wnt signaling, and discuss how further investigation into this interaction may lead to additional translational advances to optimize aspirin treatment for clinical prevention and treatment.

**Role of cyclooxygenases and prostaglandin E$_2$ on Wnt signaling**

As a nonsteroidal anti-inflammatory agent (NSAID), aspirin irreversibly binds and inhibits cyclooxygenase enzymes (PTGS1 and PTGS2, otherwise known as COX-1 and COX-2) responsible for the conversion of arachidonic acid (AA) into intermediates that are processed downstream into pro-inflammatory and homeostatic prostaglandins and related eicosanoids (15, 16). Of these prostaglandins, PGE$_2$, increased by COX-2 upregulation, has been observed to promote colonic adenoma development and progression (17, 18). Increased levels of PGE$_2$ have been found in colonic adenomas and cancers harbored by individuals with Familial Adenomatous Polyposis (FAP) who harbor germline mutations in APC, in addition to a large fraction of sporadic CRC cases (19, 20). PTGS2 (COX-2) deletion, as well as deletion of several PGE2 receptors, decreases intestinal polyp formation in multiple mouse models of FAP (21-25). We have validated the relevance of these laboratory observations to the humans in large prospective cohorts. Analysis of the Nurses’ Health Study (NHS) and Health Professionals Follow-up Study (HPFS) cohorts demonstrated that long-term aspirin use was associated with a relative risk of 0.64 (95% CI, 0.52-0.78) of developing CRCs that
overexpressed COX-2, whereas no effect was seen on tumors with weak or absent COX-2 expression (9). Moreover, we have shown that higher levels of urinary metabolites of PGE$_2$ are associated with an increased risk of adenomas and predict efficacy of aspirin chemoprevention (26).

**Effectors of PGE$_2$/Wnt crosstalk**

Mechanistic investigations of how PGE$_2$ modulates Wnt signaling have identified several key downstream effectors of aspirin (Fig. 1B). The first major target identified was peroxisome proliferator-activated receptor-$\delta$ (PPAR-$\delta$) (27). A member of the nuclear hormone receptor superfamily, PPAR-$\delta$ functions as a transcription factor in the presence of its endogenous ligands, fatty acid metabolites and eicosanoids (28).

Increased expression of PPAR-$\delta$ has been detected in CRCs, and the gene itself is a direct transcriptional target of Wnt signaling via the $\beta$-catenin/TCF7L2 transcriptional complex (29). Activation of PPAR-$\delta$ confers proliferative and anti-apoptotic advantages to CRC lines, and administration of a PPAR-$\delta$ synthetic agonist to $APC^{\text{min}}$ mice increases intestinal polyp size and multiplicity (30). PGE$_2$ additively increases transcription of PPAR-$\delta$ via PI3K/Akt activation (27). Moreover, the protumorigenic effects of PGE2 are dependent on PPAR-$\delta$. Genetic deletion of PPAR-$\delta$ abrogates the increased polyp size and number observed in $APC^{\text{min}}$ mice treated with PGE2. Traditional ASA modified with a covalently-bound nitric oxide moiety administered to $APC^{\text{min}}$ mice diminishes both intestinal tumorigenesis and PPAR-$\delta$ expression measured in the resultant tumors (31).

Investigation of the PGE$_2$/Wnt interaction in development has revealed additional effectors in other tissues. Utilization of transgenic zebrafish models demonstrate that
PGE$_2$ plays a critical role in Wnt activation in hematopoetic stem cells, and administration of the NSAID indomethacin results in reduced PGE$_2$ and Wnt reporter activity (32). This reduction in Wnt transcriptional activity is directly related to decreased $\beta$-catenin availability as opposed to changes in its mRNA levels, thus linking the effects of PGE$_2$ to the inhibition of the $\beta$-catenin destruction complex. Time course experiments revealed that PGE2 treatment resulted in increased phosphorylation of $\beta$-catenin (S675) and GSK3$\beta$ (S9), events that promote $\beta$-catenin stabilization. Interrogation of upstream pathways revealed that these effects were dependent on PGE$_2$ increasing intracellular cyclic AMP (cAMP) levels and protein kinase A (PKA) activation. While higher cAMP levels induced by PGE$_2$ can also activate the PI3K/Akt pathway, the PI3K inhibitor LY294002 failed to significantly blunt Wnt activation by PGE2. Whether the importance of PI3K/Akt pathway in PGE2-mediated Wnt activation observed in intestinal tumors compared to hematopoetic stem cells is explained by tissue specificity, or difference between tumor and normal cells, remains to be elucidated. In vivo, PGE$_2$/Wnt interactions have important roles after injury to liver stem cells and bone marrow progenitor cells. PGE$_2$ is secreted locally after tissue damage, and Wnt activation is noted during the subsequent regenerative response. Treatment with the NSAID indomethacin blunted stem cell proliferation and $\beta$-catenin stabilization required for a regenerative program in these damaged tissues after experimental injury.

Cyclooxygenase-independent effects of aspirin/Wnt interactions

Concordant with the changes in the $\beta$-catenin destruction complex caused by PGE$_2$ inhibition in developmental studies, aspirin treatment of human CRC cell lines also reduces the cytoplasmic pool of $\beta$-catenin available for nuclear translocation (33).
Aspirin treatment resulted in increased ubiquitination of β-catenin, as well as increased phosphorylation of sites (T41 and S45) associated with its targeting for ubiquination and destruction. Interestingly, aspirin did not change the activity of GSK3β. Given the persistent phosphorylation of β-catenin, examination of the phosphatases involved in the β-catenin destruction complex revealed a critical effector role for protein phosphatase 2A (PP2A). Composed of multiple subunits, active PP2A affects Wnt signaling on multiple levels, including dephosphorylation of APC and Axin. Aspirin treatment resulted in the inactivation of PP2A through its own phosphorylation (Tyr 307). A chemical inhibitor of PP2A, okadic acid, replicated the effects of aspirin on β-catenin levels and phosphorylation. In addition, transfection of a phosphorylation resistant construct of PP2A reversed the observed effects on Wnt signaling. No clear link has yet been established between the observed changes of PP2A phosphorylation by aspirin to its inhibitory effects on COX-2/PGE₂ levels.

In addition to PP2A, aspirin may also impact Wnt signaling from other pathways altered in a COX-independent fashion. While epidemiological and in vivo studies highlight a central role for COX-2/PGE₂ in aspirin’s chemopreventative effects, cross-talk with Wnt from other pathways affected by aspirin are also likely. Aspirin administration has been linked to changes in the NF-κβ pathway and angiogenesis, each associated with considerable interactions of their own with active Wnt signaling (34, 35).

Clinical-Translational Advances

Wnt-related biomarkers for targeted aspirin chemoprevention
Concerns about the bleeding risks and other side effects of the long-term use of aspirin have prevented formal recommendations for its widespread use in the primary prevention of CRC (36, 37). The concern over these side effects has prompted investigators to search for biomarkers to identify subgroups of patients in whom the primary chemoprevention of aspirin exceeds possible adverse risks. Genomic biomarkers for aspirin chemoprevention also reinforce the important relationship between aspirin and Wnt signaling. Genome-wide association studies (GWAS) exploring the common-disease, common-variant paradigm have identified numerous loci associated with the development of CRC (38-44). Examining the effect of aspirin chemoprevention with these loci have identified a predictive single nucleotide polymorphism (SNP), rs6983267, on chromosome 8q24 that regulates expression of the oncogene, MYC (45). Aspirin's protective effects were noted only among those with at least one T allele, which was also associated with decreased MYC expression. Interestingly, the T allele plays a functional role in vitro and in vivo, decreasing the affinity of the β-catenin/TCF7L2 transcription complex to a TCF7L2 DNA binding motif that encompasses the SNP (46, 47). These data suggest that susceptibility to the effects of aspirin on the Wnt/CTNNB1 pathway requires a background defined by 8q24 genotype by which β-catenin/TCF7L2 binding is not constitutively active. Fortunately, because the minor allele frequency of the T allele is high (49% among Europeans), the vast majority of the population has a favorable genotype that may benefit from aspirin use.

In addition to SNPs identified by GWAS of CRC development, integrative genomic approaches may further refine the genetic markers of aspirin efficacy relevant
for Wnt signaling. The availability of genotyping data from large well-characterized, longitudinal cohorts permits interrogation of other functional SNPs of Wnt regulators and/or targets determined to be expression quantitative trait loci (eQTL). The NIH-supported the Genotype-Tissue Expression project (GTEx) will calculate eQTLs among broad tissue types using human genotype data and RNA-seq from clinical samples, giving investigators additional candidate SNPs to evaluate (48).

Chemotherapeutic uses of aspirin in Wnt-driven cancers

Extensive molecular phenotyping of CRCs from longitudinal, prospective cohorts have also led to the identification of mutations for which aspirin treatment may serve an important adjuvant role. Regular users of aspirin after the development of CRC from the NHS and HPFS cohorts demonstrated a significant advantage in colon cancer-specific (hazard ratio [HR] for cancer-related death, 0.18; 95% CI, 0.06-0.61), as well as overall (HR for death from any cause, 0.54; 95% CI, 0.31-0.94) survival if their tumors harbored activating PIK3CA mutations (49). No such benefit was observed in those with wildtype PIK3CA. These results were subsequently validated by analysis of participants and their tumors from the Vioxx in Colorectal Cancer Therapy: Definition of Optimal Regime (VICTOR) trial (50). Interestingly, no such benefit was observed with the COX-2 selective inhibitor, rofecoxib (Vioxx), after diagnosis of CRC. These results suggest that aspirin’s effect on PIK3CA-mutant cancers may occur via a COX-2 independent pathway, and additional studies to unravel this mechanism may be fruitful in the development of novel agents against this frequently occurring oncogenic event.

In addition to its application as an adjuvant agent, aspirin has been proposed in combination with newer chemotherapeutics and biologics under development
specifically active in modulating Wnt signaling. Aspirin and other NSAIDs have successfully chemosensitized otherwise resistant \textit{ex vivo} adenomas and human CRC cell lines to recombinant TNF-related apoptosis-inducing ligand (TRAIL), resulting in apoptosis (51). This sensitization was dependent on active Wnt signaling, as induction of dominant negative TCF7L2 in CRC cell lines reversed these effects. Further supporting a dependency on Wnt signaling, no chemosensitization was observed when nondysplastic cell lines and normal colon epithelium were used.

With the extensive interactions between aspirin and Wnt, it will also be exciting to see if aspirin may be used synergistically with newly developed agents that inhibit the activity of porcupine, a Wnt-specific acyltransferase critical for Wnt ligand secretion. Mouse experiments demonstrate porcupine inhibitors to be safe and potent agents in the multiple tumor models driven by excess Wnt ligand (52). Interestingly, porcupine inhibitors have even demonstrated potency against colon cancer cell lines with constitutively active Wnt signaling characterized by mutations in \textit{APC} (53). These results demonstrate that upstream Wnt activation by ligands remains an important modulator of the pathway even in the setting of mutations in the \(\beta\)-catenin destruction complex. Given aspirin’s impact on the \(\beta\)-catenin destruction complex, this combination may prove to be a potent combination given their combined ability to inhibit Wnt signaling at multiple points in the pathway.

\textbf{Aspirin chemoprevention in extracolonic cancers with active Wnt signaling}

Genome-wide analyses of somatic mutational profiles of multiple cancers performed by the Cancer Genome Atlas Network have highlighted a key role for Wnt signaling common to several tumor types (54, 55). In addition, considerable evidence exists that
aspirin may play an important chemopreventative role in other cancers of the gastrointestinal tract (stomach and esophagus), and to a lesser degree cancers of the prostate and lung (14). Studies to verify a potential interaction between aspirin and Wnt signaling may be relevant to these extracolonic cancers where increased Wnt signaling is a frequent event during tumorigenesis. Moreover, as described previously, developmental biology studies of PGE$_2$/Wnt interactions in stem cells support a more generalizable role in many diverse tissue types.

Possible pleiotropic associations may exist among the genomic biomarkers associated with aspirin chemoprevention and cancer-specific survival. In addition to its association with CRC development and aspirin chemoprevention, rs6983267 on 8q24 has been associated with the development of multiple other cancer types, including prostate and stomach, given its functional role as a long-range enhancer for MYC (56, 57). The aspirin chemopreventative effect dependent on rs6983267 may extend to these tumor types as well. Likewise, the cancer-specific survival benefits seen with aspirin use for tumors with PIK3CA mutations may also be more generalizable to extracolonic tumors with concurrently activated Wnt signaling.

**Conclusion**

The interplay between Wnt signaling and aspirin has important implications for chemopreventative and chemotherapeutic strategies. Aspirin appears to modulate Wnt signaling at multiple levels, including effector pathways of COX-2/PGE$_2$, activity of the β-catenin destruction complex, and the expression of key Wnt target genes involved in tumorigenesis. Genomic biomarkers to identify those most likely to benefit from aspirin treatment, including germline common variants for chemoprevention and common
somatic alterations for adjuvant therapy, demonstrate functional effects on Wnt signaling. Future studies focused on rational combination therapies of aspirin with other Wnt-active agents in individuals defined by either specific genotypes or by cancers with similar molecular profiles may yield broad gains in the prevention and treatment of multiple cancers.

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Figure 1. (A) Canonical Wnt Signaling. Without activation of the Wnt ligand, β-catenin interacts with a complex consisting of Axin, APC, and GSK3β and is targeted for destruction. With Wnt ligand stimulation, β-catenin is allowed to accumulate in the cytosol and translocate into nucleus. In the nucleus, it forms a transcriptional activation complex with TCF7L2 to promote transcription of genes involved in proliferation and patterning. (B) The interaction of aspirin with Wnt signaling. Aspirin interacts at numerous levels to inhibit Wnt signaling, including the COX-2/PGE₂ Pathway and the β-catenin destruction complex.
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