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# APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development

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ABSTRACT

Developmental signaling pathways hold the keys to unlocking the promise of adult tissue regeneration, and to inhibiting carcinogenesis. Patients with mutations in the *Adenomatous Polyposis Coli (APC)* gene are at increased risk of developing hepatoblastoma, an embryonal form of liver cancer, suggesting that Wnt affects hepatic progenitor cells. To elucidate the role of *APC* loss and enhanced Wnt activity in liver development, we examined *APC* mutant and wnt inducible transgenic zebrafish. $APC^{+/-}$ embryos developed enlarged livers through biased induction of hepatic gene programs and increased proliferation. Conversely, $APC^{-/-}$ embryos formed no livers. Blastula transplantations determined that the effects of APC loss were cell autonomous. Induction of wnt modulators confirmed biphasic consequences of wnt activation: endodermal pattern formation and gene expression required suppression of wnt signaling in early somitogenesis; later, increased wnt activity altered endodermal fate by enhancing liver growth at the expense of pancreas formation; these effects persisted into the larval stage. In adult $APC^{+/-}$ zebrafish, increased wnt activity significantly accelerated liver regeneration after partial hepatectomy. Similarly, liver regeneration was significantly enhanced in $APC^{Min/+}$ mice, indicating the conserved effect of Wnt pathway activation in liver regeneration across vertebrate species. These studies reveal an important and time-dependent role for wnt signaling during liver development and regeneration.

## Introduction

Patients with *APC* mutations develop multiple colonic polyps and eventually colon cancer (Kinzler et al., 1991). Approximately 1% of people who carry an *APC* mutation develop an embryonal form of liver cancer, hepatoblastoma (Hirschman et al., 2005), which is thought to originate from hepatic progenitor cells; there is a 1000-fold greater risk in these patients than the general population to develop this type of liver cancer. This suggests that increased β-catenin activity caused by *APC* loss influences the earliest steps of hepatic development and that its dysregulation leads to neoplasia. Furthermore, alterations in the Wnt signaling pathway have been found in a significant fraction of cases of hepatocellular carcinoma (*β-catenin* 20%, *Axin* 10%) and cholangiocarcinomas (*β-catenin* 8%, *APC* 12%, *Axin* 42%) (Taniguchi et al., 2002; Tokumoto et al., 2005), indicating that Wnt acts on several cell types in the liver to induce carcinogenesis. As these data suggest that manipulation of Wnt activity may be therapeutically beneficial to patients with liver disease, we sought to characterize the effects of *APC* loss and increased wnt activity during liver development and regeneration.

Wnt signaling and its main transcriptional mediator, β-catenin, play important roles in controlling tissue patterning, cell fate decisions, and proliferation in many embryonic contexts, including organ development and differentiation (Clevers, 2006). In the absence of Wnt signaling, β-catenin is phosphorylated through the destruction complex consisting of APC, Axin, and Glycogen Synthase Kinase (GSK) 3β, and targeted for degradation. Binding of Wnt ligand to cell surface receptors allows β-catenin to accumulate in the cytoplasm and translocate to the nucleus, where it modulates target gene expression.

The role of wnt signaling in endodermal development was initially described in *Caenorhabditis elegans* (Lin et al., 1995), and is highly evolutionarily conserved (Heasman et al., 2000). Early embryonic

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lethality of mice with homozygous deletion of *β-catenin* initially precluded an analysis of the role of Wnt/β-catenin signaling in vertebrate endodermal development (Haegel et al., 1995). Inducible inactivation of *β-catenin* subsequently revealed a requirement of wnt/β-catenin signaling for intestinal development (Ireland et al., 2004). $APC^{Min}$ homozygous mutant mice, with dysregulated β-catenin activity, fail to form distal visceral endoderm and are embryonic lethal (Chazaud and Rossant, 2006; Moser et al., 1995). Heterozygote mice are viable and develop intestinal neoplasia as adults (Su et al., 1992). The effects of heterozygous *APC* loss on wnt/β-catenin activity and liver development have not been studied in detail.

The liver develops from anterior endodermal progenitor cells. In the zebrafish, endodermal progenitors fated to become liver are identifiable between the 18 somite stage (16 h post fertilization (hpf)) and 24 hpf as a thickening in the anterior endoderm and a restriction of previously pan-endodermal gene expression (Field et al., 2003). As the endoderm develops further, the liver primordium appears as a prominent bud extending to the left from the midline over the yolk sac. At 48 hpf the liver expresses mature markers such as *liver fatty acid binding protein (lfabp)* (Her et al., 2003). Hepatic growth continues through the embryonic and larval stages as the zebrafish liver further differentiates (Wallace and Pack, 2003). The morphogenesis of the endoderm is organized differently in other vertebrate species, such as *Xenopus* and mice (Zorn and Wells, 2007). The molecular mechanisms, identified to date, that initiate and control liver development, however, appear to be well conserved across vertebrate species (Stainier, 2002).

Biochemical analysis of embryonic murine livers and knockdown studies in liver cultures suggested a role for β-catenin in hepatocyte proliferation (Micsenyi et al., 2004; Monga et al., 2003). More recently, the zebrafish *wnt2bb* mutant *prometheus (prt)* demonstrated that mesodermally derived wnt signaling plays a critical role in regulating liver growth during embryogenesis (Ober et al., 2006). Homozygous *prt* mutants showed decreased expression of early liver genes *hhex* and *prox1* at 24 hpf, and have delayed liver formation. In contrast, McLin et al. demonstrated the need for wnt repression in the anterior endoderm for proper liver development in *Xenopus* (McLin et al., 2007). It remains to be determined whether these findings are mutually exclusive, suggesting species-specific variations in signaling requirements, or whether they reflect changing temporal requirements during liver development. Supporting this paradigm, previous studies have revealed alterations in β-catenin levels between pre- and postnatal liver development (Apte et al., 2007; Micsenyi et al., 2004).

During liver regeneration, Wnt signaling is activated shortly after liver resection (Monga et al., 2001). Furthermore, both morpholino knockdown and conditional ablation of β-catenin in mice results in decreased regeneration following partial hepatectomy (Sekine et al., 2007; Sodhi et al., 2005; Tan et al., 2006). To date, however, it has not been shown whether enhanced activation of wnt signaling confers a growth advantage that would accelerate liver regeneration.

To characterize the role of progressive *APC* loss and increased levels of wnt/β-catenin signaling during liver development and generation, we utilized *APC* mutant zebrafish (Hurlstone et al., 2003). The heterozygous mutants are at increased risk of developing intestinal, pancreas and liver tumors in adulthood (Haramis et al., 2006). The liver tumor histology resembles that of hepatoblastomas, suggesting an effect of wnt activation on hepatic progenitor cells. We found that there is a cell autonomous differential response to wnt activation: $APC^{+/-}$ mutants exhibited increased embryonic liver size, while $APC^{-/-}$ status led to an absent liver and embryonic death. Furthermore, we demonstrated biphasic effects of wnt activation during hepatogenesis; early wnt induction led to diminished endoderm formation and failure to specify liver, while wnt activation in mid-somitogenesis influenced endodermal progenitor fate decisions, resulting in increased liver size and decreased pancreas formation. These data suggest that β-catenin signaling affects both endodermal and hepatic progenitor cells. We also demonstrated that enhanced wnt/β-catenin activation accelerates liver regeneration, and that this response is conserved throughout vertebrate species. Our study reveals the WNT signaling pathway as an attractive pharmacological target to manipulate hepatic progenitor cells and to accelerate liver regeneration in humans.

## Materials and methods

### *Zebrafish husbandry*

Zebrafish were maintained according to IACUC protocols. The *lfabp:GFP*, *gut:GFP*, *ptf1α:GFP*, *hs:wnt8-GFP*, *hs:dnTCF-GFP*, and *hs:dkk-GFP* transgenic lines were described previously (Dorsky et al., 2002; Her et al., 2003; Lewis et al., 2004; Ober et al., 2003; Pisharath et al., 2007; Stoick-Cooper et al., 2007; Weidinger et al., 2005). Genotyping for *APC* mutants was performed as described; the wild-type and mutant bands can be distinguished in a single reaction for each sample (Hurlstone et al., 2003).

### *Heat-shock modulation of wnt signaling*

Embryonic heat-shock experiments were conducted at 38 °C for a duration of 20 min. Genotype was determined by the presence of GFP fluorescence at 3 h post heat-induction; sorted non-fluorescent (wild-type) siblings were used as controls.

### *Morpholino knockdown*

Morpholinos (GeneTools) directed against zebrafish *β-catenin* (Lyman Gingerich et al., 2005) or mismatched controls were injected into zebrafish embryos at the one-cell stage at a concentration of 40 μM; injection experiments were replicated ≥3 times.

### *Blastula transplantation*

Embryos were harvested, pronased, and manually dechorionated. 50–100 blastomeres were removed from a donor embryo at the 1000-cell stage and injected at the blastoderm margin of a recipient. Matched donors and recipients were arrayed in multi-well plates until further analysis and genotyped as described for *APC*.

### *In situ hybridization*

Paraformaldehyde-fixed embryos were processed for in situ hybridization using standard zebrafish protocols (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). The following RNA probes were used to detect alterations in endodermal development: *GFP*, *lfabp*, *sterol carrier protein*, *transferrin*, *foxa3*, *sox17*, *hhex*, *prox1*, *pdx1*, *insulin*, *trypsin*, and *ifabp*. Changes in expression compared to wild-type controls are reported as the # altered / # scored per genotype; a minimum of 3 independent experiments of $n \geq 25$ embryos were conducted per analysis.

### *Immunohistochemistry*

Zebrafish embryos, adults and en-bloc abdominal sections as well as resected murine livers were fixed with paraformaldehyde, paraffin embedded and cut in 10 μm serial step-sections for histological analysis. Hematoxylin/eosin staining was performed on alternate sections using standard techniques. Antibodies to β-catenin (1:100, BD 61054), TUNEL (Chemicon International), BrdU (1:2000, Sigma BU-33), and PCNA (1:80, Calbiochem PC10) were visualized by DAB and counterstained with hematoxylin or methylene green. Cell counts were quantified in 5 corresponding sections/genotype for each stain.

### *Caspase assay*

Single embryos ($n=5$ per genotype, two independent experiments) were manually dissociated in lysis buffer and centrifuged. Supernatant (100 μl) was used for the Caspase-Glo 3/7 assay according to manufacturer's protocol (Promega). DNA isolated from the cell pellet was used to confirm *APC* genotype.

### *Confocal microscopy*

GFP transgenic zebrafish embryos were embedded in 1% low melting point agarose containing 0.04 mg/ml Tricaine-S in glass-bottom culture dishes. Microscopy was performed using a Zeiss LSM Meta confocal microscope. A minimum of 10 embryos per genotype were imaged over 3 independent experiments.

### *Flow cytometry analysis*

Whole individual fluorescent embryos were manually dissociated in 0.9% PBS and analyzed for % GFP or dsRed positive fluorescence by flow cytometry; 20,000 cells were analyzed per sample ($n=10$–20/genotype). Genotyping for *APC* was performed by PCR on excess cells following analysis.