Conditional Disruption of *Axin1* Leads to Development of Liver Tumors in Mice

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BACKGROUND & AIMS: Mutations in components of the Wnt signaling pathway, including β-catenin and AXIN1, are found in more than 50% of human hepatocellular carcinomas (HCCs). Disruption of *Axin1* causes embryonic lethality in mice. We generated mice with conditional disruption of *Axin1* to study its function specifically in adult liver. **METHODS:** Mice with a *LoxP*-flanked allele of *Axin1* were generated by homologous recombination. Mice homozygous for the *Axin1fl/fl* allele were crossed with AhCre mice; in offspring, *Axin1* was disrupted in liver following injection of β-naphthoflavone (*Axin1fl/fl/Cre* mice). Liver tissues were collected and analyzed by quantitative real-time polymerase chain reaction and immunoprecipitation, histology, and immunoblot assays. **RESULTS:** Deletion of Axin1 from livers of adult mice resulted in an acute and persistent increase in hepatocyte cell volume, proliferation, and transcription of genes that induce the G_2/M transition in the cell cycle and cytokinesis. A subset of Wnt target genes was activated, including *Axin2*, *c-Myc*, and *cyclin D1*. However, loss of Axin1 did not increase nuclear levels of β-catenin or cause changes in liver zonation that have been associated with loss of the adenomatous polyposis coli (APC) or constitutive activation of β-catenin. After 1 year, 5 of 9 *Axin1fl/fl/Cre* mice developed liver tumors with histologic features of HCC. **CONCLUSIONS: Hepatocytes from adult mice with conditional disruption of Axin1 in liver have a transcriptional profile that differs from that associated with loss of APC or constitutive activation of β-catenin. It might be similar to a proliferation profile observed in a subset of human HCCs with mutations in AXIN1. *Axin1fl/fl* mice could be a useful model of AXIN1-associated tumorigenesis and HCC.**

Keywords: Mouse Model; Liver Cancer; Carcinogenesis; BNF.

BASIC AND TRANSLATIONAL LIVER

Hepatocellular carcinoma (HCC) is the most common liver cancer in humans and is strongly associated with deregulation of Wnt signaling components including β-catenin, AXIN1, AXIN2 (conductin), and TCF1, which together are mutated in more than 50% of cancers.[1,2] β-catenin (CTNNB1) mutations are found in 20% to 40% of HCCs,[2] adenomatous polyposis coli (APC) germline mutations predispose to hepatoblastomas, and AXIN1 and AXIN2 mutations have been found in 16% and 3% of cases of HCC, respectively.[3,4] Mutations of AXIN1 in HCC are associated with loss of heterozygosity, supporting the idea that AXIN1 is a tumor suppressor. As with other solid tumors, a large number of genetic and epigenetic changes accumulate during oncogenic progression. Specific mutations have been identified in genes including p53, pRB, PTEN, PIK3CA, CDKN2A, and RAS family members.[5] Recent studies have integrated transcriptomic, genetic, chromosomal, and methylation profiling to define subsets of HCCs that map onto molecular pathways and clinical phenotypes (reviewed in Zucman-Rossi[2]). Of particular note, mutations to CTNNB1 and AXIN1 were associated with distinct tumor subsets. Expression of mutant β-catenin was linked to well-differentiated, chromosomally stable tumors, whereas AXIN1 mutations were more closely with poorly differentiated, chromosomally unstable tumors that had a distinct spectrum of associated mutations.[5–8]

Nuclear β-catenin regulates the transcription of Wnt target genes by associating with TCF transcription factors.[9] Levels of β-catenin are kept low through the action of the multiprotein β-catenin turnover complex, comprising Axin, CK1, GSK-3, and APC, which targets β-catenin for degradation.[4] The activity of the β-catenin turnover complex is down-regulated by Wnt signaling in multiple contexts and is strongly associated with the regulation of stem cells.[10] In cancer, mutations to Wnt/β-catenin signaling components inappropriately activate TCF-dependent transcription.[11–13]

In addition to its role in regulating β-catenin, Axin associates with and regulates the activity of a number of other proteins, including p53, Myc, and Smad3.[14–18] Furthermore, Axin, APC, GSK-3, and β-catenin bind and regulate components of the mitotic spindle and centrosome.[19–24] Axin2 (but not Axin1) has been shown to play a positive role in centrosome splitting before mitosis,[19] and in colorectal tumors, the up-regulation of AXIN2 transcription following APC loss has been suggested to drive chromosomal instability.[25] By contrast, Axin1 (but

Abbreviations used in this paper: AFP, α-fetoprotein; APC, adenomatous polyposis coli; BNF, β-naphthoflavone; CPS, carbamoyl phosphate synthetase; GS, glutamine synthetase; qRT-PCR, quantitative real-time polymerase chain reaction.

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not Axin2) binds the γ-tubulin ring complex and regulates microtubule nucleation through a β-catenin–independent process.[21]

Mouse models have been used to study the loss of APC and mutation of β-catenin in the liver. Acute, widespread, liver-specific loss of APC caused high mortality and was associated with induction of nuclear β-catenin and hepatomegaly. Lower-level deletion of APC led to the induction of well-defined tumors after 8 to 9 months.[26,27] By contrast, none of the models in which β-catenin was mutated or overexpressed gave rise to tumors unless they were combined with other oncogenic drivers.[1]

Axin1 loss-of-function mice die between 8 and 10 days of gestation with axial defects.[28] Mice lacking the APC-binding RGS domain of Axin1 ($Axin^{\Delta RGS}$) or lacking exon 2 of Axin1 (containing the translation start and RGS domain) phenocopied the embryonic Axin1 null phenotype, suggesting that APC binding is essential for Axin1 function.[29,30] To allow the analysis of Axin1 function postembryonically, we generated a conditional *loxP*-flanked allele and have examined the consequences of gene deletion in the liver. Acute loss of Axin1 induced hepatocyte proliferation and a "proliferation" transcriptional program. A subset of Wnt target genes was activated that was distinct from that regulated by activation of β-catenin or loss of APC. Primary HCCs developed after a latency of 1 year. This model should provide a powerful tool to investigate Axin1 function in adult tissues.

Materials and Methods

Gene Targeting and Recombination

Details of the gene-targeting vector are described in Supplementary Materials and Methods. All animal experiments were performed according to UK Home Office regulations. *Ah-Cre* mice were intercrossed with mice carrying *LoxP*-flanked *Axin1* ($Axin1^{fl/fl}$) and *Rosa 26 LacZ* reporter alleles (*LacZ*) as indicated.[31,32] Cre activity was induced by 4 intraperitoneal injections of β-naphthoflavone (BNF; 80 mg/kg body wt) over 4 days. Littermates were used as controls wherever possible and as indicated.

Quantitative Real-Time Polymerase Chain Reaction Analyses

Total RNA was extracted using an RNeasy Kit (Qiagen, West Sussex, UK). Microarray analysis was performed as described in Supplementary Materials and Methods. Reverse transcriptions were performed using the ImProm-II System (Promega, Southampton, UK). Expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) in triplicate and was normalized to levels of beta 2-microglobulin (B2M) and β-actin in a DNA Engine (MJ Research PTC-200 Thermal Cycler PCR; Bio-Rad, Hercules, CA) using SYBR Green (Bioline, London, UK). qRT-PCR primer sequences are listed in Supplementary Materials and Methods (Supplementary Table 1).

Protein Extraction, Immunoprecipitation, and Western Blots

Liver samples were snap frozen in liquid nitrogen and stored at −80°C. For Western blots, frozen tissue samples were homogenized and lysed with protein lysis buffer (Mammalian Cell Lysis Kit; Sigma, Dorset, UK). For immunoprecipitation, tissue samples were homogenized and lysed as previously described.[33]

Histology and Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 5-μm sections as described.[34] The list of antibodies used in the immunostaining can be found in Supplementary Materials and Methods. β-galactosidase staining was performed as described previously.[31,32]

β-catenin Suspension Bead Array–Based Assay

Analysis of biological function, localization, and post-translational modification of the different forms of β-catenin was performed as previously described[35] and also can be found in Supplementary Materials and Methods.

Results

Generation of Conditional Axin1 Mutant Mice

Mice bearing a conditional *Axin1* allele in which exon2 was flanked by loxP sites were generated by homologous recombination in ES cells (Figure 1*A–D*). The numbers of $Axin1^{fl/fl}$, $Axin^{wt/fl}$, and $Axin1^{wt}$ generated by heterozygous breeding were 20, 50, and 31, respectively, suggesting that the *loxP*-flanked allele did not interfere with embryogenesis ($\chi^2 = 2.45$). Homozygous mice bearing *LoxP*-flanked *Axin1* alleles were fertile and showed no visible abnormality, suggesting that the integrated *LoxP* sites did not interfere with function. $Axin1^{fl/fl}$ mice were intercrossed with *AhCre* mice to allow inducible liver gene deletion following induction of the Cre expression with BNF[31,32] (Supplementary Figure 1*A*).

Repeated intraperitoneal exposure of cohorts of $Axin1^{fl/fl}$/*Cre* or control mice to BNF for 4 days resulted in high-level *Axin1* deletion in the liver (>90%), as determined by quantitative PCR (Supplementary Figure 1*C*) and whole-mount β-galactosidase staining of $Axin1^{fl/fl}$*/Cre; Rosa26R* mice that contained the inducible Rosa26R reporter allele (Supplementary Figure 1*A* and *B*). Gene deletion led to the loss of Axin1 protein, as assessed by immunoblotting and immunoprecipitation with antibodies against different regions of the Axin1 molecule (Figure 1*E* and *F* and Supplementary Figure 2). Recent studies have shown that embryonic deletion of exon 2 of *Axin1* using a similar *loxP* targeting strategy resulted in a lethal embryonic phenotype identical to that of the $Axin1^{Tg1}$-null allele.[30] Taken together, these data suggest that Axin1 protein was lost from the livers of BNF-treated $Axin1^{fl/fl}$*/Cre* mice.

Acute Deletion of Axin1

$Axin1^{fl/fl}$*/Cre* mice displayed no obvious signs of physical illness after 4 days of treatment with BNF. However, livers were enlarged 1.2-fold when compared with BNF-treated, age-matched controls as assessed by the liver/body weight ratio ($Axin1^{fl/fl}$*/Cre*, 0.067 ± 0.001; $Axin1^{wt}$*/Cre*, 0.056 ± 0.004; $P = .028$). Interestingly, hep-