Hepatocellular Carcinoma Associated with Liver-Gender Disruption in Male Mice

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Abstract

Hepatocellular carcinoma (HCC) is a male-predominant cancer associated with chronic hepatitis. Like human viral hepatitis, murine Helicobacter hepaticus infection produces inflammation and HCC with a masculine bias. We used this model to identify potential mechanisms of male HCC predisposition. Male weanling A/JCr mice (n = 67) were gavaged with H. hepaticus or vehicle. At 1 year, mice were distributed into four groups: surgical castration, chemical castration, castration followed by dihydrotestosterone supplementation, or sexually intact controls. Responses to infection were compared with IFN-γ challenge alone. At 21 months, there was no significant difference in hepatitis between groups. Neither castration nor androgen receptor agonist altered tumor incidence. Infected mice with severe, but not mild, disease exhibited a mosaic of alterations to sexually dimorphic genes and microsomal long-chain fatty acids. By microarray, tumorigenic hepatitis was strongly associated with liver-gender disruption, defined as the loss of a gender-identifying hepatic molecular signature. IFN-γ alone produced similar changes, demonstrating a role for proinflammatory cytokines in this process. In conclusion, hepatocarcinogenesis in male mice with chronic hepatitis is maturationally impaired and androgen-independent. Proinflammatory cytokines may promote HCC in a male-predominant fashion due to high sensitivity of the masculinized liver to loss of sex-specific transcriptional balance. Liver-gender disruption has pleiotropic implications for hepatic enzyme activity, lipid processing, nuclear receptor activation, apoptosis, and proliferation. We propose a multistep model linking chronic hepatitis to liver cancer through cytokine-mediated derangement of gender-specific cellular metabolism. This model introduces a novel mechanism of inflammation-associated carcinogenesis consistent with male-predominant HCC risk. [Cancer Res 2007;67(24):11536–46]

Introduction

Hepatocellular carcinoma (HCC) is a male-predominant cancer (1). Chronic viral hepatitis is the most common predisposing condition, but other significant risks include alcoholism, aflatoxin B1 ingestion, fatty liver disease, and inborn errors of metabolism (2). Cirrhosis often, but not always, precedes cancer. Even after controlling for known risks, the incidence of HCC in men is more than twice that of women (1). To date, no cause for this gender discrepancy in humans has been identified.

The liver is a sexually dimorphic organ. Gender differences exist with regard to gene expression, mitochondrial function, microsomal enzyme activity, membrane lipid composition, and immune responses (3–6). Liver-gender differentiation commences early in development, but the greatest divergence occurs at puberty (7). In males, testosterone is aromatized within the central nervous system to initiate an endocrine cascade resulting in periodic release of growth hormone from the anterior pituitary (8). Hepatocyte growth hormone receptors transduce pulsed hormone through a tyrosine phosphorylation cascade involving Jak2 and Stat5b. Activated Stat5b migrates to the nucleus and, in conjunction with recognized and unrecognized cofactors, enhances transcription of masculine genes while repressing feminine genes (9). In females, growth hormone is secreted at lower but more continuous levels from the pituitary, Stat5b remains unphosphorylated in the hepatocyte cytoplasm, and the default program of feminine transcription is maintained (7). Pathways other than growth hormone/Stat5b also contribute to liver-gender differentiation, although these are not well defined (10). Sexual dimorphism of the liver has significant metabolic consequences. Xenobiotic clearance of a wide variety of drugs and toxins differs between women and men due, in part, to gender-dimorphic expression of enzymes, such as cytochrome P450 3A4 (3, 11, 12). Rodents also exhibit pronounced liver-gender dimorphism (13, 14).

In spite of the strong influence of gender on liver cancer risk, therapeutic trials targeting sex hormone pathways have proved disappointing. Preliminary clinical results demonstrating a modest benefit with tamoxifen and cyproterone acetate were not replicated in expanded multicenter trials (15). Furthermore, no large placebo-controlled study has shown an improvement in tumor size or patient survival using the competitive androgen receptor antagonist flutamide, leuteinizing hormone–releasing hormone antagonist leuprorelin, or progestin megestrol acetate (16–18). Notably, the above cited trials enrolled only patients with inoperable tumors. Antiandrogen therapy as a chemoprophylactic strategy to delay or prevent HCC in high-risk patients has not been reported.

Like human viral hepatitis, Helicobacter hepaticus infection of A/JCr mice produces chronic inflammation and HCC with a male bias (19, 20). Tumors typically arise after 18 months. At 1 year of age, susceptible males exhibit chronic hepatitis, but not HCC (21). This time point is analogous to the chronic but premalignant stage of human viral hepatitis, when infection frequently is first recognized. We undertook the present study to...
test the hypothesis that castration would slow progression to HCC in mature males with chronic hepatitis and, conversely, that androgen receptor agonism would accelerate tumorigenesis. Unexpectedly, we found that androgens played no direct role in tumor promotion. Instead, we identified a strong association between tumorigenic hepatitis, proinflammatory cytokines, and liver-gender disruption, defined as the loss of a gender-identifying hepatic molecular signature. These observations form the basis of new model of inflammation-associated carcinogenesis that is androgen-independent yet consistent with male-predominant expression of HCC.

Materials and Methods

Study design. Sixty-seven Helicobacter-free male A/JCr weanling mice were purchased from the National Cancer Institute. Mice were gavaged with *H. hepaticus* 3B1 (ATCC 51449; *n* = 43) or broth only (*n* = 24), and infection was confirmed by fecal PCR as described previously (21–23). At 1 year, livers from five intact mice were evaluated by histopathology. The remaining 62 were distributed into four groups: (a) surgical castration (*n* = 14), (b) "chemical castration" with the gonadotropin-releasing hormone antagonist abarelix depot (Plenaxis, 50 µg/g body weight, i.p. once monthly; *n* = 14), (c) surgical castration followed by dihydrotestosterone one supplementation (5 mg of 90-day-release hormone pellets, Innovative Research of America; implanted s.c. over alternating shoulders every 3 months; *n* = 13), or (d) sexually intact controls (*n* = 21). In a separate experiment, *Helicobacter*-free male and female A/J mice (*n* = 10 each) were implanted s.c. with a 7-day Alzet osmotic minipump (Durect Corporation) containing 20 µg recombinant murine IFN-γ (R&D Systems) or vehicle as described elsewhere (24). Mouse husbandry conditions have been described (21). Protocols were compliant with the USPHS Policy on Humane Care and Use of Laboratory Animals and approved by the Massachusetts Institute of Technology Committee on Animal Care.

Histopathology. To minimize the effect of circadian variation, mouse necropsies were performed between 9:00 and 11:30 am. After euthanasia by CO2 inhalation, mouse serum was processed and tissues were collected and evaluated by histopathology as described (21). Liver sections were scored by a comparative pathologist blinded to sample identity. A hepatitis index was generated by combining scores for lobular, portal, and interface hepatitis along with the number of lobes (of four) containing five or more inflammatory lesions (Table 1). Mice with a histologic hepatitis index of ≥4 were defined as having hepatitis. Preneoplastic and neoplastic lesion criteria were adapted from the National Toxicology Program (25). Intralobular foci of cellular alteration were assigned a score of 2, and translobular foci were assigned a score of 2. Foci of cellular alteration are useful as precancerous disease markers (26). Adenomas received a score of 3, and HCC a score of 4. Mice meeting histologic criteria of hepatitis (inflammation index, ≥4) and neoplasia (grade, ≥3) were defined as having tumorigenic hepatitis. Photomicrographs were obtained using a BH-2 microscope (Olympus America) and DXM1200 camera (Nikon Instruments). White color balance and size bars were applied using Photoshop CS2 software (Adobe Systems). Kruskall-Wallis one-way ANOVA and Mann-Whitney U nonparametric statistics were performed using Prism software (GraphPad) with significance set at *P* < 0.05 as described previously (21).

### Quantitative reverse transcription–PCR

Liver RNA was isolated and assessed for quality as described previously (27). Quantitative reverse transcription–PCR (qRT-PCR) was performed with Taqman assay-on-demand or SYBR green reagents (Applied Biosystems). Samples were run in duplicate, and dissociation curves were performed for SYBR green assays to confirm specificity. Genes were selected based on previous disease associations in intact male mice documented by us and others (21, 28). Original primer sequences were designed using MacVector 7.2.3 software (MacVector; Supplementary Table S1). To determine the effect of castration ± dihydrotestosterone supplementation on basal gene expression, fold-change values from uninfected androgen-interrupted groups were compared against uninfected sexually intact controls. Parametric ANOVA and Student’s *t* test were performed similar to the nonparametric assays in the previous section. For the IFN-γ experiment, differential gene expression was determined for gender by comparing vehicle-treated male versus female mice and for cytokine effect by comparing IFN-γ–supplemented versus vehicle-only treated mice of the same sex.

### Table 1. Histopathologic scoring criteria used to determine hepatitis index

<table>
<thead>
<tr>
<th>Criterion</th>
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<tr>
<td>Lobular hepatitis</td>
<td>Small, randomly scattered lobular inflammatory foci</td>
<td>Moderate multifocal inflammation with areas of coagulative hepatocellular necrosis</td>
<td>Moderate-severe coalescing foci of inflammation and coagulative necrosis</td>
<td>Translobular inflammation and coagulative necrosis with significant hepatocyte loss</td>
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<tr>
<td>Portal hepatitis</td>
<td>Small foci of leukocytes (lymphocyte-predominant) in scattered portal triads</td>
<td>Moderately large, portal-restricted, well-circumscribed mononuclear cell-rich aggregates</td>
<td>Large lymphocyte-predominant aggregates forming follicle-like structures</td>
<td>Portal-portal bridging, well-developed follicles</td>
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<tr>
<td>Interface hepatitis</td>
<td>Small foci of indistinctly bounded portal/perportal inflammation extending through limiting plate</td>
<td>Multifocal inflammatory foci extending through limiting plate and beyond first two rows of perportal hepatocytes</td>
<td>Moderate-severe inflammatory foci traversing limiting plates; often accompanied by oval cell hyperplasia</td>
<td>Severe, poorly circumscribed bridging inflammation with hepatocyte loss and widespread oval cell hyperplasia</td>
</tr>
<tr>
<td>Hepatitis index (0–16)</td>
<td>Sum of lobular, portal, and interface hepatitis scores plus number of liver lobes (of four) with five or more inflammatory foci; hepatitis defined by histologic index of ≥4</td>
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Microarray. RNA samples from two sham-inoculated sexually intact mice, surgically castrated mice, and H. hepaticus–infected surgically castrated mice with hepatitis and HCC were submitted for microarray using the GeneChip Mouse Genome 430 2.0 Array (Affymetrix) as described (27). Mice for these analyses were collected in a narrow time window midmorning and were of identical age (21 months) but were not litter-matched or fasted. Mice selected for microarray had no identified extrahepatic lesions (e.g., dermatitis). Nonexpressed transcripts on microarray were filtered with GeneChip Operating Software algorithms (Affymetrix). Gene expression differences of ≥1.5-fold with P < 0.05 were considered significant. For castration and tumor-associated directional analysis, results were normalized by Robust Multichip Average (29). Protein-protein interactions were mapped with Ingenuity Pathways Analysis (Ingenuity Systems). Microarray data were submitted to the Gene Expression Omnibus Database (Series GSE6632).

Immunohistochemistry and ELISA. Immunohistochemistry was performed for β-catenin (1:100; Lab Vision), interleukin-6 (IL-6; 1:500; Abcam), Ki-67 (1:50; BD Biosciences), and nuclear factor-κB (NF-κB; p65 subunit, 1:100; Invitrogen) as described (30). The ARB kit (DAKO) was used for mouse-on-mouse applications. Species, isotype, and concentration-matched irrelevant primary antibody controls were included in all immunohistochemistry assays. Ki-67 (proliferation) labeling indices were determined by counting the number of positive hepatocyte nuclei per 10 high-power (40× ocular objective) fields. Serum dihydrotestosterone (Alpha Diagnostic International) and growth hormone (Diagnostic Systems Laboratories) were measured by ELISA according to manufacturer protocols. Serum from all mice was collected at necropsy between 9:00 and 11:30 am. All mice were evaluated for serum growth hormone along with three Helicobacter-free female control mice from an unrelated study. Intact and surgically castrated dihydrotestosterone-supplemented mice were assayed for dihydrotestosterone-one, along with two uninfected and two infected mice from the surgical and chemical castration groups. Parametric statistics were performed as described above.

Microsomal long-chain fatty acid analysis. Long-chain fatty acid (LCFA) methyl esters from enriched liver microsome fractions isolated with the NE-PER kit (Pierce Biotechnology) were extracted by the Bligh-Dyer method and derivatized with 14% borontrifluoride-methanol (Sigma) based on a published protocol (5). Specific LCFAs were identified and quantified using a commercial gas chromatography/mass spectrometry system composed of a 6890N gas chromatograph and 5973N mass selective detector (Agilent Technologies) operated in positive chemical ionization mode with methane as reagent gas. Samples were derived from five intact infected and five intact uninfected males along with five uninfected adult female mice from an unrelated study. Retention times for the six major microsomal LCFA species (see Results) were determined with commercial standards (Sigma). Data were processed with ChemStation software (Agilent) and analyzed statistically as described above.

Results

Neither castration nor androgen receptor agonism altered HCC incidence. In agreement with prior experience, H. hepaticus–infected male A/JCr mice at 1 year of age exhibited chronic hepatitis but not HCC (21). This confirmed that the H. hepaticus inoculum was virulent and that androgen interventions were instituted before malignancy. At 21 months, we confirmed by histopathology of androgen-sensitive salivary glands, renal glomeruli, and accessory sex glands (31) that the hormonal interventions had the intended physiologic effect. Histopathologic evaluation of liver samples from sham-inoculated mice showed no significant disease, whereas males in all infected cohorts developed characteristic lesions of chronic active hepatitis (Fig. 1A). Most mice with hepatitis also had areas of hepatocellular atypia ranging in progressive severity from intralobular and translobular foci of cellular alteration to adenoma and carcinoma (Fig. 1A; ref. 25). There was no significant difference in hepatitis or tumor incidence between groups (Fig. 1B). Among mice with hepatitis, intact males exhibited a higher mean inflammation index than all castrated groups, but the difference was not statistically significant (Fig. 1B). Because there was no difference in HCC incidence between groups, we sought an androgen-independent mechanism of tumor promotion in mature male mice with preestablished hepatitis.

Castration and disease-associated gene alterations by qRT-PCR. We previously identified genes associated with preneoplastic hepatitis in H. hepaticus–infected mice (27). Here, we extended those evaluations to mice with HCC and determined the effect of androgenic intervention on gene expression. Our qRT-PCR panel included genes known to be expressed in a sexually dimorphic fashion, including the female-predominant genes Cyp2a4 and Cyp4a14 and the male-predominant genes Cyp2b9, Cyp4a12, epidermal growth factor receptor, hydroxysteroid dehydrogenase 3β-V, insulin-like growth factor binding protein-2, and trefoil factor-3 (intestinal; ref. 14). As expected, feminine genes were up-regulated by surgical and chemical castration in uninfected mice, and masculine genes were down-regulated (Fig. 2A). Furthermore, with the exception of Cyp4a14, none of these changes was reversed by the powerful androgen receptor agonist dihydrotestosterone (Fig. 2A). Liver masculinization is regulated predominantly by indirect androgen effects and not by androgen receptor ligand binding, as our findings confirm. H. hepaticus–infected mice with hepatitis regardless of group showed statistically significant up-regulation of H19 and trefoil factor-V (Fig. 1B). All diseased mice except those supplemented with dihydrotestosterone-one also exhibited significantly elevated tumor necrosis factor-α (TNF-α) and ubiquitin D (Fig. 2B). Sexually intact but not castrated groups exhibited disease-associated down-regulation of Cyp2a4, epidermal growth factor receptor, hydroxysteroid dehydrogenase 3β-V, insulin-like growth factor binding protein-2, and up-regulated lipocartin 2 (not shown). Cyp4a14 was down-regulated by disease in both castrated and intact mice, but only reached significance in the latter group. An important confounding variable on hepatic gene expression was ulcerative dermatitis, a common aging lesion of A/JCr and other mice (31). In our panel, ulcerative dermatitis independently down-regulated all four Cyp genes, as well as Igfbp2 (not shown). Therefore, mice with dermatitis were removed from comparative analyses for those genes. Mice with dermatitis were not selected for microarray analysis (below).

Tumor-associated liver-gender disruption revealed by microarray. Because no clear pattern of gene alterations emerged from the qRT-PCR panel, we performed microarray. First, we assessed the effect of castration alone on liver gene expression. Of 45,037 probesets representing ~39,000 unique transcripts, 16,074 were called present in sexually intact mice by GeneChip Operating Software (Supplementary Table S2). Therefore, ~36% of the murine genome were expressed in the adult male liver. In uninfected castrated mice, we identified 2,123 gene alterations (1,413 up-regulated and 710 down-regulated) compared with uninfected sexually intact controls. Thus, ~13% of all liver genes were castration sensitive. Most of these genes have been shown elsewhere to be gender dimorphic and/or associated with failed hepatic masculinization due to impaired growth hormone signaling (e.g., Ames dwarf, lit/lit, and Stat5b−/− mice; refs. 14, 32). Therefore,
Figure 1. Histopathology and immunohistochemistry of H. hepaticus–induced hepatitis and tumorigenesis in mature male androgen-modulated mice.

A, histopathologic features of H. hepaticus–induced chronic active hepatitis and HCC. a, normal liver from sham-inoculated control. b, lobular hepatitis with random mixed inflammation surrounding and traversing central focus of coagulative necrosis. c, marked portal hepatitis with lymphoid follicle-like structures; note sharp delineation along hepatic limiting plate. d, interface hepatitis featuring portocentric inflammation extending into adjacent lobule with disregard for limiting plate boundary. e, normal liver. f, clear cell focus of cellular alteration; note absence distinct border at margin (arrowheads). g, adenoma; note distinct margin with slight compression of overlying hepatocytes (arrowheads). h, interface hepatitis featuring portocentric inflammation extending into adjacent lobule with disregard for limiting plate boundary.

B, hepatitis incidence, inflammation severity (hepatitis index), and tumor incidence summary demonstrating no significant difference in disease outcome between surgically and chemically castrated mice with or without supplementation of the powerful androgen receptor agonist dihydrotestosterone when compared with sexually intact controls.

C, immunohistochemistry. a, no Ki-67+ proliferating hepatocytes in normal mouse liver. b, marked cellular proliferation (intranuclear Ki-67 expression; arrowheads) in HCC. c, in healthy mouse liver, note presence of circulating IL-6 in blood vessels (arrow) and along sinusoidal surfaces; occasional random hepatocytes are also positive (arrowhead). d, increased IL-6+ hepatocytes in regions of fatty degeneration within HCC. e, intranuclear NF-κB (p65) localization in mononuclear inflammatory cells (arrow). f, in healthy mouse liver, β-catenin localized to hepatocyte surface membranes; in contrast, biliary epithelium (arrow) shows β-catenin along the cell surface and diffusely throughout cytoplasm. g, no nuclear translocation of β-catenin in neoplastic hepatocytes. h, proliferating oval cells (arrows) in tumor show the biliary pattern of surface and cytoplasmic β-catenin staining. A, H&E; bar, 80 μmol/L (a, b, d), 160 μmol/L (c), and 40 μmol/L (e–h). C, diaminobenzidine with hematoxylin counterstain; bar, 40 μmol/L (a–d, f–h) and 10 μmol/L (e).
we referred to genes up-regulated by castration as feminine and those down-regulated as masculine. In agreement with the known dimorphism of the murine immune system (6), many of the castration-sensitive genes in our panel were attributable to resident leukocyte populations, including Kupffer cells and natural killer T cells (Supplementary Table S2).

Next, we assessed genes associated with tumorigenic hepatitis in castrated mice. Compared with uninfected surgically castrated controls, H. hepaticus–infected castrated males displayed alterations to 2,848 genes (1,856 up-regulated and 992 down-regulated). Of these, 829 were shown in the previous analysis to be castration sensitive (i.e., gender-dimorphic). Therefore, 29% of disease-associated genes were also castration sensitive ($P < 0.0001$), demonstrating a highly significant bias for gender-dimorphic gene involvement in tumorigenic hepatitis. Many of the castration-sensitive genes altered in diseased mice were associated with inflammation, suggesting that dysregulation of both gender-specific immunity and hepatocellular function contributed to global liver-gender disruption (Table 2). To precisely characterize the direction change of gender-dimorphic genes, we used conservative Robust Multichip Average normalization criteria (29). By this method, 379 genes met the statistical cutoff for castration sensitivity (276 up-regulated and 103 down-regulated; Supplementary Table S3). Diseased mice exhibited 80 up-regulated masculine genes, 163 up-regulated feminine genes, 23 down-regulated masculine genes, and 113 down-regulated feminine genes (Supplementary Fig. S1). Gene ontology analysis and functional network mapping highlighted the central role of proinflammatory cytokine pathways in the altered expression of gender-dimorphic genes. Especially noteworthy were pathways centered on Stat1, TNF-α, IFN-γ, and RelA (NF-κB subunit p65; Supplementary Fig. S1). Because of the insensitivity of the microarray for detecting IFN-γ (33), we performed qRT-PCR for this gene. IFN-γ was up-regulated >8-fold in diseased versus uninfected males ($P < 0.001$, data not shown). Because we used whole liver slices, we were not able to distinguish hepatocyte from nonhepatocyte contributions to total gene expression. The murine immune system also exhibits sexual dimorphism (6), and it is likely that castration-sensitive inflammatory response genes influenced disease outcomes. Taken together, gene expression analyses showed that gender-dimorphic gene alterations were significantly overrepresented in mice with tumorigenic hepatitis and that proinflammatory cytokines were centrally involved in pathogenesis.

**Microsomal LCFA reflected gender and disease status.** LCFA composition in healthy hepatocyte microsomes (endoplasmic reticulum) is gender specific. In agreement with studies in the rat (5), male mice in our study exhibited a higher proportion than age-matched females of palmitic acid (C16:0) as a fraction of the total LCFA pool (Fig. 3A). Females had a higher proportion of stearic (C18:0) and arachidonic acid (C20:4), although only the latter achieved statistical significance. This is consistent with female-predominant expression of LCFA elongase (Elov16), which was increased nearly 2-fold by castration in our study (Supplementary Table S2). Although we had insufficient samples for statistical analysis, castrated male mice exhibited an intermediate phenotype (not shown). In H. hepaticus–infected males with hepatitis, there was significantly decreased stearic (C18:0) and linoleic acid (C18:2) and increased oleic acid (C18:1). Accumulation of the monounsaturated fatty acid oleate correlated with a 5-fold increase in expression of stearoyl-CoA desaturase 2 (Supplementary Table S2).

It is possible that inflammatory cells contributed to altered fatty acid profiles, although we previously showed by morphometric analysis that leukocytes account for a relatively small percentage of liver area in mice with H. hepaticus–induced hepatitis (21). Taken together, assessment of microsomal LCFA confirmed that gender and disease-associated differences in gene expression had functional metabolic consequences. Further work will be required to determine the clinical relevance of fatty acid composition in this and other subcellular compartments.

**Cell proliferation and disease markers in situ.** As expected, nuclear labeling of the cell proliferation marker Ki-67 was much higher in tumors than in normal liver (Fig. 1C). Mice in all androgen-interrupted groups exhibited a modest but statistically significant increase in basal hepatocyte proliferation versus sexually intact uninfected controls (mean 2.1 Ki-67+ hepatocyte nuclei versus 0.2 per 10 high-power fields, respectively). However, there was no difference in HCC incidence or Ki-67 labeling index in neoplasms derived from different mouse groups (not shown), demonstrating that basal hepatocyte replication rate did not correlate with tumor risk. In healthy uninfected mice, IL-6 was identified in circulating form in blood vessels and along sinusoidal linings and intracytoplasmically in randomly scattered hepatocytes. In H. hepaticus–infected mice, IL-6 was expressed in leukocytes and was detected with increased frequency in dysplastic and neoplastic hepatocytes in regions of fatty degeneration (Fig. 1C). As detected by immunohistochemistry, NF-κB (p65 subunit) was expressed primarily by inflammatory cells (Fig. 1C). Because microarray results implicated disease-associated genes in the Wnt/β-catenin signaling family, including Wnt-5a protein, cadherin 1 (E-cadherin), α-catenin, and catenin src (Supplementary Table S2), we performed β-catenin immunohistochemistry on representative tissues. However, unlike the nuclear translocation seen in colon and other cancers (34), in both normal and cancerous liver, β-catenin was localized exclusively to hepatocellular surface membranes. In contrast, bile duct epithelium and proliferating oval cells showed diffuse cytoplasmic, as well surface-anchored, protein (Fig. 1C). From this, we concluded that increased Wnt/β-catenin pathway gene products likely reflected biliary and oval cell hyperplasia. Regardless of cell type, nuclear signal was rare. Therefore, H. hepaticus–induced neoplasms would seem to fall into the β-catenin–negative category of HCC subtypes in agreement with their relatively slow-growing behavior (35).

**Growth hormone correlated poorly with masculinization in aged males.** Most liver masculinization occurs after pulsatile growth hormone induction at puberty (7). We sought to determine whether the panhepatic feminization in mice castrated at 1 year was associated with loss of growth hormone pulsatility. Unexpectedly, all male groups regardless of androgenic intervention showed a widely dispersed serum growth hormone distribution pattern consistent with pulsatile secretion (Fig. 3B). By microarray we observed a 1.5-fold increase in Jak2 mRNA expression in mice with HCC but no difference in Stat5b (Supplementary Table S2). However, for these proteins it is the phosphorylation state that governs in vivo activity (7). We confirmed in a separate assay that circulating dihydrotestosterone was increased in hormone-supplemented mice (Fig. 3C). Because high levels of dihydrotestosterone did not masculinize gene expression (Fig. 2A), testosterone, but not growth hormone or androgen receptor signaling, seemed necessary to maintain male liver phenotype in this cohort of aged mice. Therefore, mechanisms that maintain masculine liver phenotype in...
Liver gene expression changes associated with castration, *H. hepaticus*–induced hepatitis and recombinant murine IFN-γ challenge. A, surgical and chemical castration resulted in up-regulated transcription of feminine liver genes and down-regulation of masculine genes. None of these were reversed by the powerful androgen receptor agonist dihydrotestosterone except Cyp4a14, highlighting the absence of a significant role for androgen receptor signaling in liver masculinization.

B, in our qRT-PCR panel, two genes (H19, Tff3) were significantly up-regulated in all groups of *H. hepaticus*–infected mice with hepatitis F tumors compared with group-matched uninfected controls. Two additional genes (Tnfa, Ubd) were significantly up-regulated in all groups except castrated mice supplemented with dihydrotestosterone.
mature mice may differ from those that induce gender differentiation at puberty. These results highlight the complexity of endocrine regulation of liver-gender determination and show that factors in addition to growth hormone periodicity should be considered when evaluating sex-specific hepatic transcriptional regulation.

**IFN-γ alone recapitulated H. hepaticus–associated transcriptional changes.** To assess the potential role of proinflammatory

<table>
<thead>
<tr>
<th>Table 2. Liver-gender disruption in mice with chronic hepatitis and HCC highlighted by mosaic of disease-associated alterations to castration–up-regulated (feminine) and down-regulated (masculine) genes</th>
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<tbody>
<tr>
<td><strong>Castration up (fem), hepatitis/HCC up</strong></td>
</tr>
<tr>
<td>CD14 antigen</td>
</tr>
<tr>
<td>Caspase 12</td>
</tr>
<tr>
<td>IFN-induced protein 2; Ifit2</td>
</tr>
<tr>
<td>Small inducible cytokine A5 (CCL5)</td>
</tr>
<tr>
<td>Cytochrome b-245, β polypeptide</td>
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<td>Chemokine (C-X-C motif) ligand 10</td>
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<td>Calmodulin-related protein</td>
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<td>T-cell specific GTPase</td>
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<tr>
<td>C-type lectin, superfamily member 12</td>
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<tr>
<td>Tropomyosin 2, β</td>
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<tr>
<td><strong>Castration up (fem), hepatitis/HCC down</strong></td>
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<td>Sphingosine kinase 2</td>
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<tr>
<td>D site albumin promoter binding protein</td>
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<td>Major facilitator superfamily domain containing 2</td>
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<td>Dopachrome tautomerase</td>
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<tr>
<td>Nocturnin</td>
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<tr>
<td>Rigui</td>
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<tr>
<td>Thyroid hormone responsive SPOT14 homologue</td>
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<tr>
<td>Period 2</td>
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<td>Hepcidin antimicrobial peptide</td>
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**NOTE:** Note significant representation of castration-sensitive inflammation-associated genes. Fold-changes shown are mean of H. hepaticus–infected surgically castrated males versus sham-inoculated castrated males. The top 10 characterized genes in each category are listed here. Complete results are presented in Supplementary Table S2.

Abbreviations: fem, feminine; masc, masculine.
cytokines in liver-gender disruption, we determined transcriptional responses in Helicobacter-free mice challenged with recombinant murine IFN-γ. With few exceptions (Cxcl10, Hspa1b, Lpl), genes altered by castration were confirmed as gender dimorphic in this experiment (Supplementary Fig. S2). This validated our assumption that castration sensitivity, largely, was synonymous with sexual dimorphism. Of greater importance, almost all genes up-regulated or down-regulated in mice with tumorigenic hepatitis were altered in the same direction by IFN-γ, suggesting a role for this proinflammatory cytokine in liver-gender disruption and disease pathogenesis (Fig. 2C). Concordant changes were observed for the feminine genes Cd14, Cxcl9 (MIG), H2-aa, Itf2, Ccrn4l, Dbp, Hamp1, and Msfd2, masculine genes Tfi3, Cyp7b1, and Nox4, and gender-neutral genes Ccl2 (MCP-1), Cxcl10 (IP-10), Lpl, Stat1, and Tgtp (Supplementary Fig. S2). However, IFN-γ did not up-regulate in males the exquisitely sensitive feminine gene sulfotransferase hydroxysteroid-prefering 2 and produced a relatively modest reduction in the male-specific transcript HSD3ß-V (Supplementary Fig. S2). Therefore, IFN-γ was not responsible for the hepatic feminization associated with acute H. hepaticus infection. Rather, IFN-γ had a more direct effect on a subset of gender-dimorphic genes resulting in transcriptional alterations in both the masculine and feminine directions.

**Discussion**

Because HCC is a male-predominant cancer, we tested the hypothesis that castration would reduce tumor formation in mice with preestablished hepatitis and, conversely, that androgen receptor agonism would accelerate tumorigenesis. Both arms of this hypothesis were disproved. Neither surgical nor chemical castration of 1-year-old mice with chronic hepatitis reduced HCC at 21 months nor did supplementation of the powerful androgen receptor agonist dihydrotestosterone promote tumors. These findings contrast with the proved efficacy of peripubertal castration for reducing chemical and transgene-promoted liver cancer (36, 37). Therefore, the effectiveness of castration for reducing HCC in male mice is age sensitive. Our results confirm and extend those of Vesselinovitch, who showed that castration at 38 weeks was significantly less effective than castration at 20 weeks or earlier for reducing diethylnitrosamine-initiated HCC (38). Taken together, murine studies show that male susceptibility to hepatocarcinogenesis is maternally imprinted and that the benefit of androgen blockade against HCC is inversely correlated with age. This agrees with the absence of benefit of antiandrogen therapy for HCC in older men and argues against a direct role for male sex hormones in liver carcinogenesis (15–18).

A review of microarray data in the mouse literature shows that the male liver responds to acute injury in part through feminization. We documented this in a prior H. hepaticus study, and similar responses have been reported in widely divergent models of acute hepatic injury (21, 39, 40). Intriguingly, hepatocyte-targeted disruption of β-catenin also results in liver feminization, including decreased expression of the male-predominant gene Egfr (41). Thus, the male liver is predisposed to gender-dimorphic gene alterations in response to a wide variety of perturbations. One potential mechanism for feminization of the male liver after acute injury is inhibition of Stat5b by suppressor of cytokine signaling 3 (Socs3) in response to IL-6 (42). Nevertheless, in the masculinized mature liver, disease-associated gene alterations in the feminine direction do not result in the balanced expression characteristic of the female and do not confer protection against tumor formation.

In contrast to the simple feminization associated with acute injury, chronic tumorigenic hepatitis in our study was associated with a widespread mosaic of sex-dependent gene alterations, resulting in loss of liver-gender identity. Liver-gender disruption was not observed in infected mice without hepatitis, proving that H. hepaticus infection alone was insufficient to incite these changes. In contrast, exogenous IFN-γ did reproduce many of these changes, implicating a role for proinflammatory cytokines in this process. Viewed as a whole, disease expression in this system
was largely a function of the host response. Because we examined whole liver slices, it was not possible to determine which liver cell populations contributed to up-regulated expression of specific genes. We identified castration-sensitive disease-associated genes attributable both to hepatocyte and nonhepatocyte (e.g., leukocyte) cell populations (Table 2). Therefore, loss of gender specificity both in immunity and hepatocyte function likely contributed to disease propagation.

Taken together, results of our study implicate cytokine-driven liver-gender disruption and not androgen signaling as a mechanism of male-predominant HCC promotion. Combining data presented here with results published by others, we have developed a multistep working model of hepatocarcinogenesis with a focus on the male liver (Fig. 4A). In our model, testosterone masculinizes the liver through a well-documented series of events (4). Acute injury invokes a feminizing response that includes alterations to a predictable set of genes, including members of cytochrome P450, hydroxysteroid dehydrogenase, and sulfotransferase enzyme families (27). This may be mediated in part by IL-6–induced Socs3 inhibition of Stat5b (42). A critical role for IL-6 in liver cancer risk was shown in a recent chemical carcinogenesis study wherein IL6−/− male mice displayed a female phenotype of HCC resistance (43). In our working model, if acute hepatic injury does not resolve, the male liver continues down one of two paths: (a) containment through appropriate and balanced physiologic responses or (b) propagation through a self-perpetuating cycle of proinflammatory cytokines, such as IFN-γ, and cell damage, resulting in global liver-gender disruption, metabolic derangement, and HCC. It is likely that molecular crosstalk exists between multiple inflammatory pathways and sex-specific liver gene regulation. An NF-κB response element has been identified in the promoter region of the female-predominant gene CD36 (fatty acid translocase; ref. 44). Whereas some studies have suggested an antitumorigenic role for IFN-γ, these conclusions were based on the behavior of cultured or transplanted tumor cell lines (45, 46). Our in vivo model predicts a protumorigenic effect of chronic proinflammatory cytokine exposure. In all likelihood, the effect of IFN-γ and other cytokines on liver carcinogenesis is context specific.

We postulate that deranged hepatocellular metabolism is the physiologic connection between liver-gender disruption and HCC. For example, murine enzymes involved in cholesterol and bile acid metabolism, Cyp7a1, Cyp7b1, and Cyp8b1, are gender dimorphic. Cyp7a1, the rate-limiting enzyme in classic bile acid synthesis, is female predominant, whereas Cyp7b1 and Cyp8b1 in the mouse are
masculine genes (10). Animals in our study with tumorigenic hepatitis showed suppression of Cyp7b1 and Cyp8b1 but no change in Cyp7a1 mRNA levels. Elsewhere, mice with gene knockout of the bile acid sensor farnesoid X receptor were shown to develop spontaneous liver neoplasms, and alterations to the above Cyp genes were associated with tumorigenesis (47). Based on the wide range of transcriptional alterations, the diseased male liver likely accumulates altered pools of cholesterol, bile acids, and LCFA’s, as shown in our study for microsomal LCFA. This could result in shunting toward alternative pathways, oxidative damage, and activation of proinflammatory second messengers, as well as disrupted signaling through lipid-sensitive nuclear receptors, such as peroxisome proliferator–activated receptors and retinoid X and liver X receptors (48).

The model we have developed regards gender-specific hepatocellular function not merely as a product of individual genes but a balanced and interdependent system. As such, hepatic feminization associated with acute responses could leave the male liver in precarious metabolic balance, amplifying the subsequent effect of type I cytokines, such as IFN-γ. Together, these forces would result in global liver-gender disruption, derangement of hepatocellular metabolism, and HCC with a male bias. Nevertheless, the similar transcriptional profiles observed in male and female mice challenged with IFN-γ suggest that proinflammatory cytokines are important in tumor promotion in both sexes. Regardless of gender, tumor risk in our model is determined primarily at the level of the hepatocyte, in agreement with the cell autonomous origin of HCC in chimeric mouse models (49, 50). Further study will be needed to determine at the molecular level how the loss of gender-specific metabolic balance contributes to the oxidative damage, epigenetic alterations, and dysregulated proliferation and apoptosis kinetics that are the hallmarks of hepatocellular transformation.

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