Dysregulated Serum Response Factor Triggers Formation of Hepatocellular Carcinoma

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The ubiquitously expressed transcriptional regulator serum response factor (SRF) is controlled by both Ras/MAPK (mitogen-activated protein kinase) and Rho/actin signaling pathways, which are frequently activated in hepatocellular carcinoma (HCC). We generated SRF-VP16iHep mice, which conditionally express constitutively active SRF-VP16 in hepatocytes, thereby controlling subsets of both Ras/MAPK- and Rho/actin-stimulated target genes. All SRF-VP16iHep mice develop hyperproliferative liver nodules that progresses to lethal HCC. Some murine (m)HCCs acquire Ctnnb1 mutations equivalent to those in human (h)HCC. The resulting transcript signatures mirror those of a distinct subgroup of hHCCs, with shared activation of oncofetal genes including Igf2, correlating with CpG hypomethylation at the imprinted Igf2/H19 locus. Conclusion: SRF-VP16iHep mHCC reveal convergent Ras/MAPK and Rho/actin signaling as a highly oncogenic driver mechanism for hepatocarcinogenesis. This suggests simultaneous inhibition of Ras/MAPK and Rho/actin signaling as a treatment strategy in hHCC therapy. (HEPATOLOGY 2015;61:979-989)

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**H**uman hepatocellular carcinoma (hHCC) belongs to the five most lethal cancers worldwide.1 Liver cirrhosis, alcohol abuse, or chronic hepatitis virus infection, and type 2 diabetes-associated nonalcoholic steatohepatitis predispose to HCC. Despite their extreme heterogeneity, hHCCs could be classified (G1-G6, or S1-S3) based on gene expression signatures, genomic and epigenetic alterations.2-4 Aberrant activation of WNT/β-catenin, Jak/STAT, PI-3K/Akt signaling pathways, and activation of the Ras/MAPK (mitogen-activated protein kinase) and Rho/actin cascades cause HCC formation.3,5-7 Both Ras/MAPK and Rho/actin cascades regulate cell proliferation and differentiation.6 Rho/actin signaling additionally determines polarity, adhesion, and mechanosensory and migratory activities of normal and cancerous cells.8,9 Activation of Rho/actin signaling in hHCC is frequently elicited by deletion of Rho/Rac/Cdc42-inhibiting tumor suppressors, e.g., DLC1.7,8,10 DLC1 encodes a Rho inhibitor with Rho-GAP function and is deleted in up to 50% of liver cancers.7,10 Synergistic oncogenic crosstalk of Ras/MAPK and Rho/actin signaling has been described,11,12 but their joint impact on target gene expression remains unclear.

The transcription factor SRF (serum response factor) is activated by both Ras/MAPK and Rho/actin signaling, engaging distinct target gene profiles and involving alternative cofactors (ternary complex factors [TCFs], myocardin related transcription factors [MRTFs])13,14 (Fig. 1A). Elevated expression of SRF was reported in high-grade hHCCs.15,16 SRF was activated by the X and core proteins of hepatitis B virus (HBV) and C virus (HCV),...
respectively.\textsuperscript{17} 

dLC1-deleted HCC cells revealed activation of Rho/actin signaling and associated nuclear localization of SRF cofactors MRTF-A/B (MKL1/2).\textsuperscript{18} Furthermore, antiproliferative, prosenescence effects on HCC xenografts were obtained upon down-regulation of MRTFs/MKLs.\textsuperscript{19} Collectively, this implies SRF contributions to hHCC formation. We provide here the first in vivo evidence supporting this concept. We generated the SRF-VP16\textsuperscript{Hep} mouse line, permitting conditional expression of the SRF-VP16 protein in hepatocytes upon Cre-mediated deletion of a STOP-flox cassette.\textsuperscript{20} SRF-VP16 carries the VP16 transcriptional activation domain of \textit{Herpes simplex} virus, thereby eliciting constitutive SRF activity.\textsuperscript{21}

In SRF-VP16\textsuperscript{Hep} mice, conditional activation of SRF-VP16 elicited broad changes in hepatocellular gene expression resulting in hyperproliferative nodules, followed by rapid progression to HCC. Importantly, SRF-VP16\textsuperscript{Hep} HCCs share molecular features with distinct subgroups of hHCCs, including overlapping gene expression signatures,\textsuperscript{2,22} activating \textit{Ctnnb1} mutations,\textsuperscript{23} and hypomethylation of \textit{Igf2/H19} oncofetal genes.\textsuperscript{22} Thus, SRF-VP16\textsuperscript{Hep} mice identify the SRF-mediated convergence of sustained MAPK and Rho/actin signaling as an oncogenic driver of HCC.

**Materials and Methods**

\textit{Stochastic Hepatocyte-Specific Expression of SRF-VP16.} Stop-floxed SRF-VP16 mice (\textit{Gt(Rosa)26-Sor\textsuperscript{tm1(SRF-VP16)Antu} mice)}\textsuperscript{20} were bred with \textit{Srfflex1/wt::SRF-VP16} (floxed \textit{Srfflex1})\textsuperscript{24} and \textit{Alfp-CreERT2} animals (Supporting Fig. 1A) to obtain triple transgenic mice, \textit{Srfflex1/wt::SRF-VP16\textsuperscript{Hep}/::Alfp-CreERT2\textsuperscript{Hep}} (termed SRF-VP16\textsuperscript{Hep}; for polymerase chain reaction [PCR] genotyping: Supporting Materials and Methods). Liver specificity of CreERT2 activity (Supporting Fig. 1B), its tamoxifen-inducible activation (Supporting Fig. 1B), and its spontaneous activation (Supporting Fig. 1B,C) are evidenced. Animal housing and handling was in accordance with the Federal of European Laboratory Animal Science Associations and approved by local ethics committees (Regierungspräsidium Tübingen).

The Supporting Materials and Methods describe experimental details for the following: histological analysis, immunoblot analyses, and antibodies for immunoblotting and immunohistochemistry; analysis of genomic mutations of mHCCs; quantitative high-resolution DNA methylation analysis of murine samples; methylation profiling of hHCCs; expression profiling of hHCCs; genomic DLC1 status of hHCCs; \textit{CTNNB1} mutational analysis of hHCCs; expression profiling of murine samples; quantitative real-time PCR; isolation and analysis of murine intrahepatic immune cells (IHICs); statistical analysis.

**Results**

Constitutively Active SRF Causes Liver Expansion in SRF-VP16\textsuperscript{Hep} Mice. Mice carrying the conditional \textit{Rosa26(SRF-VP16)} allele\textsuperscript{20} were bred with animals expressing tamoxifen-inducible hepatocyte-specific CreERT2 (\textit{Alfp-CreERT2} mice) (Supporting 1A,B) to get SRF-VP16\textsuperscript{Hep} mice. Treatment of SRF-VP16\textsuperscript{Hep} mice with tamoxifen caused efficient induction of SRF-VP16 expression. However, marginal spontaneous activity of Cre-recombinase was observed in the absence of tamoxifen, leading to SRF-VP16 expression in a few hepatocytes. Employing the Cre-responsive mT/mG reporter allele (Supporting Materials and Methods), we quantified this spontaneous CreERT2\textsuperscript{Hep} activation to generate per liver, within the first 10 weeks of age, an accumulated total of 0.38\% ± 0.08\% hepatocytes (n = 9) (Supporting Fig. 1C,D).

Spontaneous CreERT2\textsuperscript{Hep} activity in SRF-VP16\textsuperscript{Hep} mice caused hyperproliferation of effected hepatocytes, leading to multiple premalignant nodules throughout the livers, accompanied by age-dependent increases in liver mass reaching a liver weight-to-body weight ratio (LBWR) of up to 33\% (Fig. 1C, Supporting Fig. 2). 80\% of all animals developed HCC within 25-40 weeks of age (n > 93) (Fig. 1B,C; Supporting Fig. 2). Mice lacking either SRF-VP16 or CreERT2 alleles, or both, never developed increased LBWR or HCC during this time (n > 143). In livers of SRF-VP16\textsuperscript{Hep} mice, but not of control animals, recombination and expression of SRF-VP16 was observed at DNA, RNA, and protein levels (Fig. 1D,E).

SRF-VP16\textsuperscript{Hep} livers with LBWR greater than 15\% displayed many macroscopically visible premalignant...
nodules (Fig. 1C), each likely derived from clonal expansion of an individual SRF-VP16-expressing hepatocyte. In support, we crossed the mT/mG Cre reporter allele into SRF-VP16iHep mice and identified, at the age of 10 weeks, multiple green nodules representing colonies of hepatocytes with CreER T2 activity (Fig. 1F, lower). Alfp-CreER T2 control animals lacking the SRF-VP16 allele, displayed multiple individual green cells rather than cell colonies (Fig. 1F, upper). Thus, spontaneous CreER T2-mediated activation of SRF-VP16 expression in a subset of hepatocytes caused their hyperproliferation, leading...
to premalignant nodules followed by progression to HCC.

Liver Expansion Upon Hyperproliferation of SRF-VP16-Expressing Hepatocytes. Liver histology of SRF-VP16\textsuperscript{Hep} mice revealed foci of small hepatocytes in the perivenular parenchyma indicating hepatocellular proliferation. These foci rapidly expanded to hyperproliferative nodules composed of small basophilic hepatocytes with slightly enlarged nuclei, all strongly expressing the proliferation-associated SRF target gene Egr1 (Fig. 2A, upper). Increases in nodule size correlated with increasing LBWR (Fig. 2A, upper, 2B). All Egr1-positive nodules showed proliferative activity, displaying an average of 15% Ki67-positive cells (Fig. 2A, lower, 2C). While these nodules initially showed a clear demarcation to surrounding nonneoplastic parenchyma (Fig. 2D, left), in some lesions atypia developed and individual cells infiltrated into the surrounding parenchyma (Fig. 2D, right).

Progression From Hyperproliferative Nodules to HCC. All SRF-VP16\textsuperscript{Hep} livers containing multiple hyperproliferative nodules displayed solid microtrabecular growth of small basophilic hepatocytes (Fig. 3A,A',D), expressing the polarity marker DPP IV of nontransformed hepatocytes.\textsuperscript{25} Above 20 weeks of age, the majority of animals (n = 51) harbored one to several macroscopically visible tumors (Fig. 3A-C) with pseudo-glandular and irregular trabecular growth patterns (Fig. 3E,F) as unequivocal characteristics of HCC. Tumor cells lost expression of DPP IV (Fig. 3E, right). Together, SRF-VP16\textsuperscript{Hep} livers revealed progression from premalignant, hyperproliferative nodules to HCC, as initiated by sporadic hepatocyte-specific expression of SRF-VP16.

Senescent Hepatocytes and Infiltrating Lymphocytes in SRF-VP16\textsuperscript{Hep} Livers. We estimated up to 100,000 hyperproliferative nodules per liver (Figs. 1C, 2A), a high number contrasted with the lower number (less than 5) of HCCs within one liver (Fig. 3A-C). Thus, progression from premalignant nodules to HCC was rare, possibly impaired by cellular tumor-suppressive mechanisms. SRF-VP16\textsuperscript{Hep} livers displayed elevated numbers of β-galactosidase/p21-positive senescent cells (Fig. 4A, upper and middle),\textsuperscript{26} unaccompanied by activated caspase 3-mediated apoptotic activity (Fig. 4B). Further, tumor tissue displayed nests of infiltrating immune cells (Fig. 4A), with elevated levels of neutrophils (CD11b\textsuperscript{high}Gr-1\textsuperscript{1}), macrophages (CD11b\textsuperscript{low}F4/80\textsuperscript{1}), and CD8\textsuperscript{1} T cells, but not CD4\textsuperscript{1} T cells (Fig. 4C,D).

SRF-VP16-Triggered HCC Progression May Associate With β-Catenin Mutations. In hHCC, activating mutations of the CTNNB1 are frequently observed.\textsuperscript{3} We sequenced the Ctnnb1 gene of 26 separately dissected SRF-VP16\textsuperscript{Hep} mHCCs. Twelve samples (46%) carried Ctnnb1 missense mutations affecting codons 32, 34, 37, or 41 (Supporting Table S1), representing frequently mutated codons of CTNNB1 in human cancers.\textsuperscript{23} Ha-Ras (codon 61) and B-Raf (codon 600) mutations, were not found (not shown).

Expression of Candidate SRF Target Genes. Quantitative real time PCR (qRT-PCR) of candidate gene transcripts from nine control, 17 SRF-VP16\textsuperscript{Hep} nodular
(LBWR, ranging from 6.6 to 26.9%), and five SRF-VP16I Hep mHCC tissues (Fig. 5) revealed dramatic up-regulation of the proliferation-associated, Ras/MAPK-stimulated immediate-early genes (IEGs) Egr1, Egr2, and c-fos in both nodular and HCC tissue (column (i)). The β-actin and Vinculin genes, normally regulated by Rho/actin signaling, were also up-regulated in nodular and mHCC tissues (column (ii)). Tumor proliferation genes (Cnmnb1, c-Myc) were prominently up-regulated in mHCCs (column (iii)). Carcinoma progression genes (Cdh1, Mmp14, and Vim) showed significant up-regulation in nodules and mHCC (column (iv)). Collectively, in SRF-VP16I Hep mice, liver tissues display up-regulation of direct SRF target genes normally stimulated by either MAPK or Rho/actin signaling.

Genome-Wide Gene Expression Profiling of Murine Tumor Tissues. In genome-wide RNA expression profiling we compared control livers (n = 3) with premalignant nodular liver tissue (n = 3) and HCC tissues carrying either wild-type Cnmnb1 (mHCCA tumors) (n = 3) or mutated Cnmnb1 (mHCCB tumors) (n = 3). Altogether, about 1,330
transcripts were found differentially expressed in nodular and HCC tissues (Table S2). RT-PCR validation was obtained for all genes investigated (including those studied in Fig. 5, plus eight others). Many genes carrying identified CArG-boxes were up-regulated (e.g., Bcl-2, Ctgf, Egr1, Egr2, Flna, c-fos, Myh9, Tagln2, Tgfb2, Tfeb1, Tpm1, Tuf1, Vcl1, Vim, Vill, and Zyx).27 The Venn diagram (Fig. 6a) revealed 224 dysregulated transcripts shared by all three types of liver tissue (Category I, Table S3) and a distinct set of 358
transcripts dysregulated in HCCs but not nodules (Category II, Table S4). In all, 68 (Category III, Table S5), 226 (Category IV, Table S6) and 317 transcripts (Category V, Table S7) were dysregulated exclusively in nodular tissue, mHCCA, and mHCC B, respectively. For each category, the 10 most strongly up- and down-regulated genes are displayed (Fig. 6B).

The 25 most strongly up-regulated transcripts of Categories I and II represented oncofetal genes (Igf2, H19, Bex1), and genes involved in proliferation/survival (Cpe, Gldn, Fstl3, Psat1, Igf2, Cd63, Lcn2, Plat1, Tspan8, Tspan13, Timp1), cytoskeletal activities (Actn3, Krt20, Vim, Vil1), immune-regulation (Ly6d, Klrb1a) and lipid metabolism (Scd2, Ly6d, Akr1c18, Lpl2). The Cnmnb1-mutated HCC B tumors (Category V) selectively showed up-regulation of proto-oncogenes c-fos, and c-Jun, as well as Wnt signaling components (Fzd3, Tcf7, Axin2). Down-regulated transcripts of Categories I and II included the tumor suppressor genes Sdha, Ndrg2 and Igfals.22,28

**Shared Gene Expression Signatures of Murine and hHCCs.** Cross-species comparison of our murine samples was performed with a cohort of 40 human HCCs,22 which was analyzed for genomic DLC1 deletions, SRF mRNA expression, and CTNNB1 mutation status (Fig. 6C). 60% hHCCs displayed genomic DLC1 loss and 50% displayed SRF mRNA overexpression (Fig. 6C). Tumors overexpressing SRF either displayed elevated IGF2 expression or clustered with CTNNB1 mutations (Fig. 6C,D, upper). Further, hHCC subclasses (G1 to G6) were assigned according to Boyault classification.2 Combined unsupervised hierarchical clustering of gene expression profiles from murine and hHCCs was performed, applying a gene set of the SRF-VP16-derived "58 most strongly up-regulated transcripts." A strong murine/human expression overlap with a subgroup of 10 hHCCs, henceforth called "subcluster of 10" (SC10), was observed (Fig. 6D). SC10 hHCCs displayed a stronger relatedness to the murine specimen than to any of the other 30
hHCCs and were enriched for IGF2-overexpressing tumors. 70% of the G1 or G2 tumors belonged to SC10, while none of the G6 tumors did (Fig. 6D). In close agreement, upon applying the murine gene set of “50 most dysregulated (up or down) transcripts in the unsupervised hierarchical clustering,” a subcluster of eight human tumors (SC8) was identified (not shown). All tumors of SC8 are contained in SC10. Individual genes specifying the mHCC/hHCC overlap included the imprinted or developmentally expressed genes Igf2, H19, Bex1, Peg3, and Cd133/Prom1. Additional development-regulated transcripts dysregulated in SRF-VP16 tissues included oncofetal genes Afp, Epcam, Gpc3, Igf2bp3, Plaur, Sox4, Sox9, Vil1, and Vim. In summary, comparative expression profiling identified high relatedness between SRF-VP16-derived mHCCs and hHCCs, particularly the G1/G2-enriched SC10 subset. The commonality included dysregulation of oncofetal gene expression.

Epigenetic Dysregulation of Igf2/H19 in Both Murine and Human HCCs. Overexpression of the normally imprinted Igf2/H19 genes in both SRF-VP16-derived mHCCs (Fig. 7A) and G1 subclass hHCCs suggested common epigenetic alterations. CpG methylation of the Igf2/H19 imprinting control region (DMR), investigated in 40 independent SRF-VP16-triggered mHCCs, indeed revealed hypomethylation in both nodules and tumors (Fig. 7B), correlating with
elevated \textit{H19} gene expression. Regarding the highly expressed \textit{Igf2} gene in murine HCCs, no differences to constitutive low control levels of CpG methylation were seen upstream of the \textit{mP1} promoter (site *, Fig. 7B) nor around the \textit{mP2} promoter (sites 1-3, Fig. S3), similar to the highly expressed \textit{Cd63} gene (Fig. 7B). Promoter-associated CpG sites of the imprinted \textit{Airn} gene, encoding an antisense regulator of \textit{Igf2r}, showed a trend towards demethylation, which might be hyperproliferation-associated, but this failed to reach statistical significance. Also, the \textit{Meg3} and \textit{Peg3} genes, usually subject to imprinting control, were strongly overexpressed in SRF-VP16-triggered mHCC (Table S3). Other highly expressed genes, \textit{Igfbp6} and \textit{Ly6d}, displayed significant CpG hypomethylation (Fig. 7B). Collectively, this indicates SRF-VP16-triggered mHCC formation being linked to epigenetic alterations of both imprinted and nonimprinted genes.

Since elevated \textit{hIGF2} expression is frequent in human G1-type HCCs,\textsuperscript{2} we investigated the cohort of 40 human HCCs regarding \textit{hIGF2} expression and CpG methylation. We focused on three CpG dinucleotides around the \textit{hP3} promoter (sites 1-3; Fig. 7C, lower), previously implicated in tumor-associated \textit{hP3} promoter activation.\textsuperscript{29} 25% of hHCC specimens, including the majority of G1/G2 tumors and the SC10 tumors, showed both high \textit{hIGF2} gene expression and promoter \textit{hP3} hypomethylation (Fig. 7C, upper). Thus, the SC10 subtype of hHCC display \textit{hIGF2} promoter hypomethylation congruent with SRF-VP16-triggered mHCC.

\section*{Discussion}

\textit{SRF-VP16}\textsubscript{\textit{Hep}} mice provide the first \textit{in vivo} evidence for dysregulated, constitutive activity of the transcription factor SRF to trigger cancer. Constitutive SRF-VP16
activity elicited gene expression profiles, which mirrored, in part, SRF activity stimulated by combined Ras/MAPK and Rho/actin signaling (Fig. 1A), pathways frequently activated in cancer cells. A comparable scenario was revealed for oncogenic human Ets proteins in mimicking Ras/MAPK signaling.

In SRF-VP16<sup>iHep</sup> mice, within 10 weeks, spontaneous hepatocellular activation of CreERT<sup>2</sup> generated an SRF-VP16-overexpressing cell population constituting ~0.4% of all hepatocytes. The single molecular event of induced SRF-VP16 expression elicited high proliferative activity, leading to rapid hepatocyte expansion and formation of premalignant dysplastic lesions (nodules). Subsequently, from these numerous nodules progression to a small number of malignant HCC occurred. The SRF-VP16<sup>iHep</sup> mouse model therefore permits the study of molecular events associated with both initiation and progression of cancer. Close to 50% of murine SRF-VP16-triggered tumors displayed activating point mutations in the Ctnnb1 gene, mapping to codons equivalent to those mutated in hHCCs.

The profile of dysregulated genes in SRF-VP16<sup>iHep</sup> mHCCs encompasses a subgroup of 182 entries shared with the 960-membered set of direct SRF target genes mediating the serum response of transformed fibroblasts (Table S8). This strong overlap attests to SRF-mediated serum response of transformed fibroblasts as similarly reported for G1 subtype hHCCs. Furthermore, the hHCC cohort studied here revealed CpG hypomethylation at the human IGF2 hP3 promoter in the majority of SC10/G1/G2 hHCCs. This correlated with elevated IGF2 expression (Fig. 7C) and reflected the constitutively low CpG methylation at mIgf2 promoters in murine tissues (Fig. S3). Yet the precise mechanism leading to the massive deregulation of the Igf2/H19 locus in the SRF-VP16-triggered mHCCs remains to be elucidated.

Within an individual SRF-VP16<sup>iHep</sup> liver, only few isolated mHCC lesions develop from thousands of premalignant nodules (Fig. 1), indicating tumor suppressor mechanisms preventing malignant transformation to occur more frequently. The presence of senescent hepatocytes within and surrounding the premalignant nodules, plus the presence of infiltrating leukocytes (Fig. 4), suggested tumor suppression by senescence-associated immune surveillance to govern the mouse model.

In conclusion, the SRF-VP16<sup>iHep</sup> mouse model, which is shown to display, in part, gene expression profiles elicited upon combined oncogenic Ras/MAPK and Rho/actin signaling, identifies SRF target genes to fulfill oncogenic driver functions in HCC. SRF activation by virally expressed HBV and HCV proteins adds a clinically relevant component to SRF’s suggested role in liver carcinogenesis. The molecular relationship between SRF-VP16-triggered mHCCs and G1/G2 hHCCs strengthens our suggestion of SRF playing a crucial role in liver carcinogenesis. Simultaneous molecular targeting of both Ras/MAPK and Rho/actin signaling pathways, including direct inhibition of SRF, is suggested as a therapeutic strategy for treatment of human HCC.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.27539/suppinfo.