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.

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Human 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),...
constitutively active SRF causes liver expansion in SRF-VP16<sup>iHep</sup> mice. Mice carrying the conditional Rosa<sup>26</sup>(SRF-VP16) allele<sup>20</sup> were bred with animals expressing tamoxifen-inducible hepatocyte-specific Cre<sup>ERT<sub>2</sub></sup> (<sup>Alfp-Cre<sup>ERT<sub>2</sub></sup></sup> mice) (Supporting Fig. 1A,B) to get SRF-VP16<sup>iHep</sup> mice. Treatment of SRF-VP16<sup>iHep</sup> 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 Cre<sup>ERT<sub>2</sub></sup> 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 Cre<sup>ERT<sub>2</sub></sup> activation in SRF-VP16<sup>iHep</sup> 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 Cre<sup>ERT<sub>2</sub></sup> alleles, or both, never developed increased LBWR or HCC during this time (n > 143). In livers of SRF-VP16<sup>iHep</sup> 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<sup>iHep</sup> livers with LBWR greater than 15% displayed many macroscopically visible premalignant proliferation, antiproliferative, and senescent effects on HCC xenografts were obtained upon down-regulation of MRTFs/MKLs. Collectively, this implies SRF contributions to hHCC formation. We provide here the first in vivo evidence supporting this concept. We generated the SRF-VP16<sup>iHep</sup> mouse line, permitting conditional expression of the SRF-VP16 protein in hepatocytes upon Cre-mediated deletion of a STOP-flox cassette.<sup>20</sup> SRF-VP16 carries the VP16 transcriptional activation domain of <i>Herpes simplex</i> virus, thereby eliciting constitutive SRF activity.<sup>21</sup>

In SRF-VP16<sup>iHep</sup> 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<sup>iHep</sup> HCCs share molecular features with distinct subgroups of hHCCs, including overlapping gene expression signatures,<sup>2,22</sup> activating <i>Ctnnb1</i> mutations,<sup>23</sup> and hypomethylation of <i>lgf2/H19</i> oncofetal genes.<sup>22</sup> Thus, SRF-VP16<sup>iHep</sup> mice identify the SRF-mediated convergence of sustained MAPK and Rho/actin signaling as an oncogenic driver of HCC.

**Materials and Methods**

**Stochastic Hepatocyte-Specific Expression of SRF-VP16.** Stop-floxed SRF-VP16 mice (Gt(Rosa)26-Srfflex1/wt::SRF-VP16<sup>Alfp-CreERT2</sup> mice)<sup>20</sup> were bred with Srfflex1<sup>Alfp-CreERT2</sup> (floxed <i>Srf</i> exon 1)<sup>24</sup> and Alfp-Cre<sup>ERT2</sup> animals (Supporting Fig. 1A) to obtain triple transgenic mice, Srfflex1<sup>Alfp-CreERT2</sup>::SRF-VP16<sup>iHep</sup> (termed SRF-VP16<sup>iHep</sup>; for polymerase chain reaction [PCR] genotyping: Supporting Materials and Methods). Liver specificity of Cre<sup>ERT2</sup> activity (Supporting Fig. 1B), its tamoxifen-inducible activation (Supporting Fig. 1B), and its spontaneous activity (Supporting Fig. 1B,C) are evidenced. Animal housing and handling was in accordance with the Federation 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; <i>CTNNB1</i> mutational analysis of hHCCs; expression profiling of murine samples; quantitative real-time PCR; isolation and analysis of murine intrahepatic immune cells (IHICs); statistical analysis.
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-CreERT2 control animals lacking the SRF-VP16 allele, displayed multiple individual green cells rather than cell colonies (Fig. 1F, upper). Thus, spontaneous CreERT2-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<sup>Hep</sup> 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<sup>Hep</sup> 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. 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<sup>Hep</sup> 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<sup>Hep</sup> 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<sup>Hep</sup> livers displayed elevated numbers of β-galactosidase/p21-positive senescent cells (Fig. 4A, upper and middle), accompanied 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<sup>high</sup>Gr-1<sup>+</sup>), macrophages (CD11b<sup>+</sup>F4/80<sup>+</sup>), and CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> 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. We sequenced the Ctnnb1 gene of 26 separately dissected SRF-VP16<sup>Hep</sup> 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. 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<sup>Hep</sup> nodular
and five SRF-VP16<sup>Ind</sup> 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 (Cmnb1, 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-VP16<sup>Ind</sup> 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 Cmnb1 (mHCC<sub>A</sub> tumors) (n = 3) or mutated Cmnb1 (mHCC<sub>B</sub> 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., Bel-2, Ctgf, Egr1, Egr2, Flna, c-fos, Myh9, Tagln2, Tgb2, Thbs1, Tpm1, Tuft1, Vcll, Vim, Vill, and Zymx).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). Of these, Igf2 and H19 were the most strongly up-regulated in both Categories I and II. The 25 most strongly down-regulated transcripts 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 and hHCCs, particularly the G1/G2-enriched SC10 subset. The commonality included dysregulation of oncofetal gene expression.
elevated H19 gene expression. Regarding the highly expressed Igf2 gene in murine HCCs, no differences to constitutive low control levels of CpG methylation were seen upstream of the mP1 promoter (site *, Fig. 7B) nor around the mP2 promoter (sites 1-3, Fig. S3), similar to the highly expressed Cd63 gene (Fig. 7B). Promoter-associated CpG sites of the imprinted Airn gene, encoding an antisense regulator of Igf2r, showed a trend towards demethylation, which might be hyperproliferation-associated, but this failed to reach statistical significance. Also, the Meg3 and Peg3 genes, usually subject to imprinting control, were strongly overexpressed in SRF-VP16-triggered mHCC (Table S3). Other highly expressed genes, Igfbp6 and 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 hIGF2 expression is frequent in human G1-type HCCs,2 we investigated the cohort of 40 human HCCs regarding hIGF2 expression and CpG methylation. We focused on three CpG dinucleotides around the hP3 promoter (sites 1-3; Fig. 7C, lower), previously implicated in tumor-associated hP3 promoter activation.29 25% of hHCC specimens, including the majority of G1/G2 tumors and the SC10 tumors, showed both high hIGF2 gene expression and promoter hP3 hypomethylation (Fig. 7C, upper). Thus, the SC10 subtype of hHCC display hIGF2 promoter hypomethylation congruent with SRF-VP16-triggered mHCC.

Discussion

SRF-VP16<sup>Hep</sup> mice provide the first 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),13,14 pathways frequently activated in cancer cells.6,12 A comparable scenario was revealed for oncogenic human Ets proteins in mimicking Ras/MAPK signaling.30

In SRF-VP16iHep mice, within 10 weeks, spontaneous hepatocellular activation of CreERT2 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-VP16iHep 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 Cmmbl gene, mapping to codons equivalent to those mutated in hHCCs.

The profile of dysregulated genes in SRF-VP16iHep mHCCs encompasses a subgroup of 182 entries shared with the 960-membered set of direct SRF target genes mediating the serum response of transformed fibroblasts27 (Table S8). This strong overlap attests to SRF-mediated the serum response of transformed fibroblasts27 (Table S8). 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Author names in bold designate shared co-first authorship.

Supporting Information

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