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The effect of oncoprotein v-erbA on thyroid hormone-regulated genes in hepatocytes and their potential role in hepatocellular carcinoma

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Abstract Mutant forms of thyroid hormone receptor (TR) with dominant negative activity are frequently found in human hepatocellular carcinoma (HCC). Interestingly, the v-erbA oncogene, known to exert a dominant-negative effect on the expression of thyroid hormone (T3)-responsive genes, led to the development of HCC in a transgenic mouse model. Thus it is possible that the oncogenic activity of v-erbA in hepatocytes may be mediated by its dominant negative activity on T3-responsive genes. Microarray analysis was used to identify genes differentially expressed in murine hepatocytes in culture (AML12 cells) stably transfected with v-erbA and exposed to T3. The Affymetrix GeneChip Mouse Genome 430 2.0 array consisted of over 39,000 transcripts representing well-known genes. We have identified twenty T3-responsive genes that are negatively regulated by v-erbA at 3 h, and eighteen genes at 24 h, such as follistatin, activin βC, thrombomodulin, Six1, Rasgrp3 and Ndrg2, as well as genes that are regulated by v-erbA only, such as angiopoietin 1 and Igfr2. We have identified T3 responsive genes that are dysregulated by v-erbA. These genes are known to be involved in carcinogenesis. Our studies may provide insight into the potential role of mutant forms of TR in the pathogenesis of HCC.

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Abbreviations

dCHIP	DNA-Chip analyzer
HCC	Hepatocellular carcinoma
Igf2r	Insulin-like growth factor 2 receptor
NASH	Non-alcoholic steatohepatitis
Ndrg2	N-myc downstream regulated gene 2
Pparγ	Peroxisome proliferator-activated receptor
Rasgrp3	Ras-GTP-releasing protein 3
Six1	Sine oculis-related homeobox-1 homolog
Thbd	Thrombomodulin
T3	Thyroid hormone
TR	Thyroid hormone receptor

Introduction

Thyroid hormone (T3) regulates expression of genes that play important roles in development, differentiation, growth and other aspects of metabolism. T3 binds to the thyroid hormone receptors (TRs) α and β, which in turn act as transcription factors of T3-responsive genes. Interestingly, naturally occurring mutant forms of the thyroid hormone receptors (TRα1 and TRβ1) which act as dominant negative repressors of T3-dependent gene expression are found in about 70% of human hepatocellular carcinoma (HCC) cases analyzed [1]. In addition, mutant TRs have also been implicated in renal clear cell carcinoma and

certain thyroid and gastric neoplasias [2–4]. Taken together, these observations are suggestive of a role for mutated thyroid hormone receptors in tumorigenesis, perhaps by repressing expression of T3-responsive genes, and indicate that T3 may play a protective role in that process.

The oncoprotein v-erbA, a mutated version of avian TRα, can silence T3-responsive genes by antagonizing ligand-dependent gene regulation by T3. v-erbA also acts as dominant negative repressor of retinoic acid (RA)-responsive genes. The repressive activity of v-erbA is linked to oncogenesis in avian erythroleukemia [5]. In addition, transgenic mice over-expressing v-erbA develop HCC [6]. It is not known if v-erbA's purported role in carcinogenesis is the result of deregulation of T3 and/or RA-responsive genes, or rather a direct effect on gene expression. We have previously shown that v-erbA not only plays a dominant-negative role in the expression of RA-responsive genes but is also responsible for regulation of a large number of genes known to be involved in carcinogenesis [7].

Since liver is a major target of T3-dependent gene regulation, we conducted the present study to determine by microarray analysis the effect of v-erbA on T3-responsive genes in v-erbA transfected hepatocytes in culture (AML12 cells). The non-transformed adult hepatocyte cell line AML12 is a good model to study hormonal regulation in hepatocytes. We have previously shown that these cells express endogenous TRα and TRβ at the mRNA and protein level [7]. Furthermore, these cells maintain the expression of liver-specific genes and are a good model to study T3 regulation of apoptosis, sharing the same control mechanisms and regulation of apoptosis as primary hepatocyte cell culture and in vivo studies [8–11].

Thus in the current studies, we identified T3-responsive genes that are negatively regulated by v-erbA, and researched their potential role in tumor development.

Materials and methods

Cell culture

The non-transformed murine hepatocyte AML 12 cells were grown in modified Eagles's/Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 nM dexamethasone and ITS (insulin, transferrin and selenium, Invitrogen, Carlsbad, CA) at 37°C in an atmosphere with 5% CO_2.

In order to study the effect of v-erbA on T3-responsive genes, untransfected AML12 cells and cells stably transfected with v-erbA were exposed to 10 nM T3 for 3 h and 24 h in the presence of 10% stripped fetal bovine serum (Hyclone, Logan, UT). Controls for both groups were AML12 cells that were not exposed to T3.

Stable transfections

The gag-v-erbA oncogene was cloned into a KpnI/NotI-digested vector containing the hygromycin-resistance gene (pcDNA3.1/H^+). This plasmid was linearized wih SspI and transfected with Lipofectamine Plus (Invitrogen) into AML12 cells. For the purpose of selection of transfected cells, the culture medium was supplemented with hygromycin at 100 µg/ml. Isolated colonies were selected 2 weeks after transfection.

V-erbA expression at the mRNA and protein levels were confirmed by Northern and Western blot analyses, respectively, as described previously [12]. Based on the high levels of v-erbA expression, the V6 clone was selected for present studies [12].

Microarrays

RNA for gene expression analysis was obtained from untreated AML12 cells (AML12 ctrl), AML12 cells exposed to 10 nM T3 (AML12 + T3), untreated v-erbA-transfected cells (V6), and v-erbA-transfected cells exposed to 10 nM T3 (V6 + T3). RNA isolation and preparation for microarray analysis have been previously described [12]. The Affymetrix GeneChip Mouse Genome 430 2.0 array consisting of over 39,000 transcripts representing well-known genes was used for these studies. Assays of triplicate samples were performed at the core facility at the University of Missouri at Columbia. Expression data thus obtained were analyzed by the software DNA-Chip Analyzer (dCHIP) available at www.dchip.org. Microarray expression data were deposited in the Gene Expression Omnibus Database (GEO, National Center for Biotechnology Information, accession number series GSE 15458).

Quantitative, real-time PCR (qPCR)

First-strand cDNA for real-time quantitative PCR analysis was synthesized from five micrograms of total RNA (prepared as described above) using random primers and SuperScript™ III Reverse Transcriptase kit (Invitrogen) according to manufacturer's instructions.

Quantitative PCR reactions were performed in a DNA Engine Opticon 2 System (Bio-Rad, formerly MJ Research Inc.). Samples were prepared using the DyNAmo™ SYBR® Green qPCR kit from Finnzymes (New England Biolabs, Beverly, MA). Reaction mixes consisted of 10 µl of SYBR green-containing master mix, 1 µl of 1:5 dilution of cDNA prepared as described above, and 1 µl of 10 µM amplification primers for selected genes (20 µl total reaction volume). The sequences of gene-specific primers designed for qPCR are presented in Table 1. Thermocycling conditions have

Table. 1 Sequence of forward (F) and reverse (R) primers used to determine relative gene expression by qPCR

Gene	Primer sequence
Fst	(F) 5′-ACGTGTGAGAACGTGGACTG-3′
	(R) 5′-CATTCGTTGCGGTAGGTTTT-3′
Igf2r	(F) 5′-CTTCCCTTTTCTGGCCTTCT-3′
	(R) 5′-GAAGGTGGAAAGCTCACAGC-3′
Ndrg2	(F) 5′-ACACCTTATGGCTCGGTCAC-3′
	(R) 5′-TCTCTTGCATATCCCCGAAC-3′
Rasgrp3	(F) 5′-CCGACTCAAAGACACCCATT-3′
	(R) 5′-GAAGCCATCACAGTCAGCAA-3′
Six1	(F) 5′-ATTCTCTTCCAGGCCTCACA-3′
	(R) 5′-ACCAAACTGGAGGTGAGTGG-3′
Tgfb2	(F) 5′-CCTTTTCTGCGTCAGTGTGA-3′
	(R) 5′-ACATTTGTGCGCATCTTCTG-3′
Thbd	(F) 5′-TACTGGCGATAACCACACCA-3′
	(R) 5′-CAGCTTCAGTTGCTGTCGAG-3′

been previously described [7]. Calculations of relative gene expression in treatment samples versus controls (normalized to Gapdh as reference control gene) were performed using Genex Macro™ version 1.1 software (Bio-Rad Laboratories). Each gene was tested in multiple PCR reactions, and the mean of at least three reactions was used to calculate expression levels.

Western blot analysis

Cell lysates were obtained from AML12 cells 3 h and 24 h after incubation with 10 nM T3 (or vehicle). Proteins were submitted to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following separation, proteins were transferred to Immunoblot PVDF Membrane (Bio-Rad, Hercules, CA). The membranes were probed with goat polyclonal antibodies that recognize Ndrg2 and Six1 (Sc-19467 and Sc-9709, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200. Secondary antibody was bovine anti-goat IgG-HRP (Sc-2350, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:5,000. The bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce-Thermo Fisher Scientific, Rockford, IL).

Results and discussion

Effect of T3 and v-erbA on global gene expression in AML12 cells

We have identified 231 genes that are up-regulated by T3 $\geq$ 2-fold at 3 h and 274 genes that are similarly down-regulated during the same time period. Of these, 136 T3-responsive genes were modulated by v-erbA $\geq$ 2-fold. At 24 h, 223 genes were up-regulated and 219 genes were down-regulated $\geq$ 2-fold by T3. One hundred and seventy-six of these genes were regulated by v-erbA (complete list of genes regulated by T3 and v-erbA can be found at Gene Expression Omnibus Database, accession number series GSE 15458).

While the majority of T3 and v-erbA responsive genes are either directly or indirectly up- or down-regulated, the microarray identified 20 T3-responsive genes that are negatively regulated by v-erbA at 3 h. At 24 h, we found 18 T3-responsive genes negatively regulated by v-erbA (Table 2). Among these T3-responsive genes dysregulated by v-erbA there are genes known to be involved in cellular processes associated with tumorigenesis, such as cell proliferation and differentiation, apoptosis, cell migration, vascularization and resistance to cancer drugs. In contrast, a recent study found that v-erbA target genes expressed in immature erythroid progenitors during differentiation (the natural target cells of v-erbA) were not regulated by T3 [13]. It appears that the dominant-negative effect of v-erbA on T3-responsive genes may be cell-type specific. In addition, v-erbA may also play a role in tumorigenesis that is independent of T3-mediated gene regulation. Our analysis also identified over 1,000 v-erbA-responsive genes that are not regulated by T3 and as described below some of these are involved in carcinogenesis.

Microarray results were verified by real time PCR (qPCR) of a representative group of genes. Any discrepancies between microarray results and qPCR data could be accounted for by differences in the specificity of each technique: whereas the microarray often employs sequences from the 3'UTR of the gene, we consistently used highly specific primers made from the coding region of the gene. Overall, the results obtained by qPCR concurred with the microarray analysis (Table 3).

Effect of v-erbA on T3-responsive genes

T3-responsive genes that are also modulated by v-erbA can be categorized in three groups: genes that are up- or down-regulated by both T3 and v-erbA with responses of roughly the same magnitude (81 genes at 3 h, 105 genes at 24 h); genes that are up-regulated or down-regulated by both T3 and v-erbA but where the responses are equal or more than 2-fold different (25 genes at 3 h, 38 genes at 24 h); and genes that are up- or down-regulated by T3 and v-erbA in opposite directions (20 genes at 3 h, 18 genes at 24 h).

T3-responsive genes that are dysregulated by v-erbA will likely offer the most insight into the oncogenic effects of v-erbA in AML12 cells (Tables 2, 3). A number of these T3-responsive genes dominantly regulated by v-erbA are known to be involved in control of cell proliferation and/or

Table. 2 Dominant-negative effect of v-erbA (V6 clone) on T3-responsive genes

Gene	Accession	AML + T3 (vs. AML12ctrl) fold change	V6 + T3 (vs. AML12 + T3) fold change	Time
5-Hydroxytryptamine (serotonin) receptor 2C	NM_008312	–2.61	2.49	3 h
Ataxin 2 binding protein 1	NM_021477	2.16	–2.31	3 h
Calcitonin receptor-like	AB015595	2.22	–6.11	24 h
Casein kappa	NM_007786	2.67	–2.76	24 h
Contactin 1	BB318787	–6.55	2.23	24 h
Cytochrome P450, family 17, subfamily a, polypeptide 1	NM_007809	2.72	–2.07	3 h
Cytochrome P450, family 17, subfamily a, polypeptide 1	NM_007809	6.54	–2.12	24 h
D site albumin promoter binding protein	BB550183	2.35	–2.37	24 h
Elongation factor RNA polymerase II	BB139475	–2.41	2.17	3 h
Follistatin (Fst), mRNA	BB444134	–2.72	4.21	3 h
Forkhead box D1	BB662927	–2.11	2.54	24 h
Gap junction membrane channel protein alpha 1	M63801	–11.49	2.67	24 h
Glucosaminyl (N-acetyl) transferase 1, core 2	AK017462	–2.52	2.06	3 h
Activin beta-C	U95962	2.33	–2.19	3 h
Neuroendocrine secretory protein antisense	AI561892	2.65	–6.14	24 h
Nims mRNA for NF-E2 inducible megakaryocyte specific protein	BG076300	–2.05	2.02	24 h
N-myc downstream regulated gene 2	NM_013864	3.05	–6.94	24 h
Oxysterol binding protein-like 6	BG070848	–2.24	3.73	3 h
PHD finger protein 19	AK014030	–30.69	2.46	3 h
Procollagen, type V, alpha 3	NM_016919	–3.68	2.1	3 h
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	BQ176864	–2.73	2.96	3 h
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	BQ176864	–3.96	2.11	24 h
RAS, guanyl releasing protein 3	BB042252	–2.45	4.3	3 h
RNA binding motif protein 14	BC010294	–2.05	2.29	24 h
Serum/glucocorticoid regulated kinase 2	NM_013731	3.2	–5.59	3 h
Sideroflexin 2, mRNA (cDNA clone MGC:30418 IMAGE:5043343)	BB377927	2.57	–2.16	3 h
Sine oculis-related homeobox 1 homolog (Drosophila)	BB137929	–2.03	2.72	3 h
Small EDRK-rich factor 1	AA709993	–2.12	2.64	24 h
Sodium channel, voltage-gated, type IV, alpha polypeptide	NM_133199	2.35	–4.11	3 h
Solute carrier family 20, member 1	BB126659	6.32	–2.88	24 h
SRY-box containing gene 5	AI528773	–2.67	2.14	3 h
Tetraspanin 13	BB807707	–8.82	2.07	24 h
Thioesterase superfamily member 5	AK004155	3.01	–3.36	3 h
Thioesterase superfamily member 5	AK004155	2.21	–3.82	24 h
Thrombomodulin	NM_009378	2.12	–3.62	24 h
TSC22 domain family, member 1	AV009804	2.04	–3.71	3 h
Zinc finger protein 467	BE628275	2.32	–6.52	24 h
Zinc finger protein 521	BC021376	–2.46	3.02	3 h

induction of apoptosis. Dysregulation of activin signaling is involved in pathological conditions such as hepatic inflammation, fibrosis, acute liver failure and liver cancer [14]. Specifically, activin A inhibits replication and promotes apoptosis in hepatocytes. It has been proposed that liver tumors may escape activin growth control by over-expressing follistatin, an activin A antagonist [15]. Increased follistatin expression is found in about 60% of liver tumors in humans as well as in animal models [16] and increased follistatin levels can be detected in the blood of patients with alcoholic cirrhosis and HCC [17]. We found that while T3 down-regulated follistatin, v-erbA increased

Table. 3 Fold-difference in gene expression determined by qPCR and microarray

Gene	AML12 + T3 vs. AML12 ctrl		V6 + T3 vs. AML12 + T3	
	qPCR	Microarray	qPCR	Microarray
Fst	−1.4	−2.7	5.7	4.2
Igf2r	N/F	N/F	−14.2	−12.7
Ndrg2	2.7	3.1	−4.4	−6.9
Rasgrp3	−1.7	−2.5	4.2	4.3
Six 1	−1.3	−2.0	5.0	2.7
Tgfb2	N/F	N/F	2.9	4.8
Thbd	1.2	2.1	−51.6	–3.6

its expression both at 3 h and 24 h. We also found that activin β C subunit was down-regulated by v-erbA (−2.2 and −4.5 fold by microarray and qPCR, respectively). The role of activin β C in liver biology and disease is controversial. However, over-expression of activin β C in the mouse liver has been shown to inhibit DNA regeneration of hepatic cells and to induce apoptosis in human and rat hepatoma cell [18, 19]. Taken together, our observations are consistent with a scenario where v-erbA might induce hepatocyte proliferation and inhibit apoptosis by simultaneously repressing activin β C and over-expressing follistatin.

We also found that Six1 (sine oculis-related homeobox-1 homolog), a homeodomain-containing transcription factor whose enhanced expression is associated with metastasis was up-regulated by v-erbA (2.7-fold at 3 h) and down-regulated by T3 (−2.0-fold at 3 h). Rhabdomyosarcoma cell lines over-expressing Six1 show enhanced in vitro proliferation as well as increased cellular invasiveness [20]. Six1 is also reported to be upregulated in primary and metastatic breast cancer [21]. In addition, we found that thrombomodulin (Thbd) expression was down-regulated by v-erbA (−3.6-fold) and up-regulated by T3 (2.1-fold). In agreement with our findings, increased serum levels of Thbd have been previously described in hyperthyroid patients [22]. Low expression of Thbd has been associated with high incidence of metastasis in pancreatic islet cell-derived tumors [23]. Deregulation by v-erbA of Six1 and Thbd is consistent with v-erbA-mediated acquisition of metastatic properties important in tumor progression.

We found that v-erbA up-regulates Ras-GTP-releasing protein 3 (Rasgrp3) at 3 h (4.3-fold), while T3 down-regulates its expression (−2.5-fold). The role of GTP-releasing proteins is to facilitate GTP binding and RAS protein activation. In addition, Rasgrp3 expression is known to be up-regulated in vessels that form in response to tumor angiogenic signals [24]. Up-regulation of Rasgrp3 by v-erbA could be an indication of deregulation of Ras signaling, which is associated with oncogenic transformation.

Furthermore, v-erbA down-regulated N-myc downstream regulated gene 2 (Ndrg2) expression (−6.9-fold at 24 h) while T3 up-regulated it (3.0-fold at 24 h). Ndrg2 is a Myc-repressed gene with potential as tumor suppressor [25] which has been reported to be significantly reduced in several types of cancer, including liver cancer [26]. In addition, over-expression of Ndrg2 suppressed invasion and migration of a highly invasive cell line [27], suggesting that Ndrg2 may play a role in suppressing tumor metastasis in HCC. V-erbA may play a role in tumor invasion by down-regulating Ndrg2.

Moreover, the dominant negative activity of v-erbA on several T3 responsive genes, such as Ndrg2 and Six1, was confirmed at the protein level by western blot analysis (see Fig. 1). The dominant negative activity of v-erbA on Ndrg2 was confirmed at the protein level. However, the western blot did not show T3-mediated down-regulation of Six-1 convincingly. Since the down-regulation of Six-1 was only 1.3 to 2 folds by qPCR and microarray, respectively, such modest difference may not be prominently reflected at the protein expression level, particularly when the baseline expression (−T3) was low. Importantly, expression of Six-1 was clearly up-regulated at the mRNA and protein levels by the oncoprotein v-erbA (Tables 2 and 3, Fig. 1).

Taken together, these observations of the dominant negative effect of v-erbA on the expression of T3-responsive genes that appear to confer protection against a variety of functions associated with tumorigenesis are consistent with a role for this oncogene in the development of HCC.

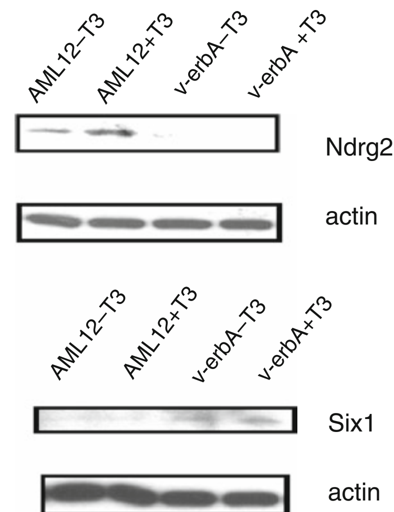

Fig. 1 Dominant negative activity of v-erbA on T3 responsive genes by western blot analysis. Analysis of Ndrg2 and Six1 expression in AML 12 cells control and AML12 cells stably transfected with v-erbA by Western blot analysis, in the absence and presence of 10 nM T3 for 24 h. Antibodies used for immunodetection were polyclonal anti-Ndrg2 or anti-Six1 antibodies. Equal loading in each lane was verified by assessing their actin concentration

Table. 4 v-erbA-responsive genes not regulated by T3 in AML12 cells

	Gene	Accession number	Fold-difference (3 h)	Fold-difference (24 h)	Function
Abi1	abl-interactor 1	AW912678	16.52	N/F	Promotes cell adhesion and migration
Acvr1c	Activin A receptor, type IC	BB396526	4.11	5.41	TGF-β signaling pathway
Angpt1	Angiopoietin 1	BB453314	2.14	2.37	Promotes angiogenesis, enhances tumor growth
Angptl3	Angiopoietin-like 3	BC019491	4.5	−9.66	Lipid metabolism, angiogenesis, cell adhesion and migration
Apoa1	Apolipoprotein A-I	NM_009692	−15.68	N/F	Lipid metabolism
Apoa2	Apolipoprotein A-II	NM_013474	N/F	−3.56	Lipid metabolism
Apoc1	Apolipoprotein C-I	NM_007469	−6.95	−6.65	Lipid metabolism
Apoc2	Apolipoprotein C-II	NM_009695	−2.95	−4.4	Lipid metabolism
Apoc3	Apolipoprotein C-III	BC021776	−3.19	−8.29	Lipid metabolism
Apoc4	Apolipoprotein C-IV	BC024657	−2.2	−2.87	Lipid metabolism
Apoh	Apolipoprotein H	NM_013475	−4.05	−4.7	Recognition of dying cells
L-Fabp	Fatty acid binding protein 1, liver	NM_017399	−2.82	N/F	Lipid metabolism
Fosb	FBJ osteosarcoma oncogene B	NM_008036	2.47	N/F	Negative regulation of cell matrix adhesion
Fgg	Fibrinogen, gamma polypeptide	NM_133862	−6.72	N/F	Fibrosis
Fbf12	Fibroblast growth factor 12	BQ175704	3.5	N/F	Cell proliferation
Fbf7	Fibroblast growth factor 7	NM_008008	−2.34	−5.14	Cell proliferation
Fbfr3	Fibroblast growth factor receptor 3	NM_008010	2.54	2.8	Cell proliferation
Fmo5	Flavin containing monooxygenase 5	NM_010232	−3.19	−2.68	Oxidation reduction
Hsd3b4	Hydroxysteroid dehydrogenase-4, delta<5>-3-beta	NM_008294	−6.78	N/F	Oxidation reduction
Inhbb	Inhibin beta-B	BB253137	N/F	−2.28	TGF-β signaling pathway
Igf1	Insulin-like growth factor 1	AF440694	2.3	N/F	Lipid metabolism, anti-apoptosis
Igf2r	Insulin-like growth factor 2 receptor	BG092290	−12.74	−17.65	Lipid metabolism, growth regulation
Notch4	Notch gene homolog 4 (Drosophila)	NM_010929	2.03	N/F	Cell differentiation
Nr0b2	Nuclear receptor subfamily 0, group B, member 2	BC019540	−4.6	−5.26	Regulation of transcription
Ppargca	Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha	AV337619	9.89	9.2	Regulation of transcription
Pparg	Peroxisome proliferator activated receptor gamma	NM_011146	2.15	2.31	Regulation of transcription
Tll1	Tolloid-like	NM_009390	3.38	5.11	Cell differentiation
Tgfb2	Transforming growth factor, beta 2	BF144658	4.81	3.55	Cell growth and proliferation
Tgfbr3	Transforming growth factor, beta receptor III	BM122301	6.84	N/F	Cell growth and proliferation

v-erbA-dependent genes not regulated by T3

Approximately 1,000 genes were regulated by v-erbA (but not by T3) at 3 h in AML12 cells. At 24 h, that number was close to 1,500. We have highlighted a number of these genes whose role in carcinogenesis is well established (Table 4). Some of the v-erbA responsive genes not regulated by T3 were regulated by RA. Specifically, v-erbA effected dominant negative activity on the RA responsive genes Fmo5, Nr0b2, and Ppargca (Table 4) [12]. Other v-erbA responsive genes not regulated by T3 or RA, may be directly targeted by v-erbA.

Some of the genes shown in Table 4 have been specifically implicated in liver damage leading to tumor development. Angiopoietin 1, for example, is secreted by activated hepatic stellate cells in primary cultures and increased in human fibrotic livers [28]. Insulin-like growth factor 2 receptor (Igf2r) is believed to be a tumor

suppressor gene, and altered expression of this gene has been implicated in liver carcinogenesis [29]. Our results indicate that v-erbA down-regulates Igf2r dramatically, which is consistent with a role in tumor development. We have also found peroxisome proliferator-activated receptor γ (Pparγ) to be considerably up-regulated by v-erbA. Interestingly, mice deficient in liver-specific Pparγ are protected from hepatic steatosis, a condition that may progress to non-alcoholic steatohepatitis (NASH) [30]. It has been reported that patients with NASH are at risk for the development of hepatocellular carcinoma [31]. In addition to Pparγ, a number of v-erbA-responsive genes are involved in lipid metabolism. Dysregulation of fatty acid metabolism leads to accumulation of fatty acids and triglycerides in the liver, resulting in NASH which can lead to fibrosis and cirrhosis, the latter being a common feature of HCC independent of etiology.

Conclusion

We have identified T3-responsive genes dysregulated by v-erbA that play a role in cellular processes involved in tumorigenesis, either specifically in HCC or in cancers in general. Our observations are in agreement with a role for these genes in apoptosis, metastasis, and cell proliferation. In our studies v-erbA-induced over-expression of follistatin and down-regulation of Ndrg2 and Thbd are particularly interesting since these genes have been implicated in hepatic malignancies. Since TR mutants with dominant negative activity are frequently found in patients with HCC, and v-erbA exerts dominant negative effect on the expression of T3-responsive genes, we speculate that our findings may provide insight into the role of these genes in the pathogenesis of human HCC. In addition, we have found that v-erbA also modulates genes, such as Igf2r, that are not regulated by T3. Some of these genes are directly involved in tumor development, while others contribute to hepatic injury believed to be the basis of hepatocellular carcinoma. We propose that v-erbA transfected AML12 cells may be a useful cell culture system in which questions about the early stages of development of hepatocellular carcinoma can be studied.

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