Overexpression of fatty acid synthase in chemically and hormonally induced hepatocarcinogenesis of the rat

Matthias Evert, Regine Schneider-Stock and Frank Dombrowski

Institut für Pathologie, Otto-von-Guericke-Universität Magdeburg, Magdeburg, Germany

Fatty acid synthase (FAS) is the key enzyme of *de novo* fatty acid synthesis and has been shown to be involved in carcinogenesis of numerous human malignancies, including breast, colorectal, and prostate carcinomas, often associated with a worse prognosis. Although FAS is mainly expressed in the liver, an implication of FAS in hepatocarcinogenesis, has not yet been investigated. FAS expression is stimulated by insulin and glucose, and insulin is also the primary trigger of hepatocarcinogenesis in an endocrine experimental model, which is induced by low-number transplantation of islets of Langerhans into the livers of diabetic rats. We therefore investigated, whether FAS is implicated in hepatocarcinogenesis in this model and in comparison to chemically induced hepatocarcinogenesis after *N*-nitrosomorpholine (NNM) treatment in diabetic and normoglycemic rats. Preneoplastic clear-cell foci of altered hepatocytes (FAH), harvested after laser-microdissection of kryostat sections, showed an overexpression of FAS messenger RNA in gene expression profiles, done by array-hybridization, and in quantitative RT-PCR (Light-Cycler). Virtually, all (96–98%) of the subsequently investigated FAH and the glycogenotic hepatocellular adenomas and carcinomas showed an additional strong FAS protein overexpression. In the NNM-model, FAS protein was also overexpressed in the vast majority (87%) of glycogenotic FAH and neoplasms, in particular in the diabetic animals. Also two spontaneous glycogenotic FAH in control animals displayed strong FAS overexpression. Basophilic lesions and neoplasms, which occasionally develop out of the primary clear-cell FAH at later stages of carcinogenesis, however, lost FAS overexpression. In conclusion, FAS overexpression is an early phenomenon in spontaneous, hormonally and chemically induced rat hepatocarcinogenesis, demonstrable in early clear-cell (glycogenotic) FAH and hepatocellular neoplasms. FAS overexpression can be attributed to the local hyperinsulinemia in the transplantation model and belongs to cellular and metabolic alterations in the chemical model, which are induced by an insulinomimetic but yet unknown mechanism.

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After low-number transplantation of islets of Langerhans into the livers of diabetic rats, the liver acini draining the blood from the islet grafts show cellular alterations, the foci of altered hepatocytes (FAH).¹ FAH resemble the glycogen-storing or clear-cell phenotype of preneoplastic foci known from experimental and human hepatocarcinogenesis.^{2,3} In the transplantation model, these preneoplastic FAH may develop into hepatocellular adenomas (HCA) and carcinomas (HCC) within 6–24 months after

islet transplantation.⁴ Previous results indicate that insulin is the primary trigger for the development of FAH and hepatocarcinogenesis in this model.^{1,4–7} In the beginning of the carcinogenic process, however, these FAH can be regarded as purely adaptive in nature. At this stage, insulin effects manifested in several aspects, that is, an increase in glycogen storage and proliferative activity, lipid accumulation, and altered expression of several insulin-related proteins, including IGF-I and its binding proteins, such as IGFBP-1 and IGFBP-4.^{1,4,6} Moreover, we recently demonstrated increased insulin signalling within the FAH, reflected in a translocation of the insulin receptor and overexpression of several insulin signal transduction proteins, such as insulin receptor substrate-1, Raf-1 and Mek-1.⁷ The carbohydrate metabolism in FAH has also been

Correspondence: Professor Dr med. F Dombrowski, Institut für Pathologie, Otto-von-Guericke Universität Magdeburg, Leipziger Strasse 44, D-39120 Magdeburg, Germany.
E-mail: frank.dombrowski@medizin.uni-magdeburg.de
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thoroughly investigated and was altered in an insulin-typical manner,⁵ showing downregulation of glucose-6-phosphatase or upregulation of hexokinase and glucose-6-phosphate dehydrogenase among other altered enzyme activities. Based on the lipid accumulation, one might presume that insulin also altered the lipid metabolism in these preneoplastic hepatocytes.

Part of this study is a tentative evaluation of the gene expression profile in very early FAH, performed by array-hybridization, which identified an overexpression of fatty acid synthase (FAS) in the FAH as one of the major alterations. FAS is a multifunctional enzyme complex catalyzing different reactions in the biosynthesis of long-chain fatty acids, starting from acetyl-CoA and malonyl-CoA.^{8–10} Thus, it plays a key role in the *de novo* lipid synthesis in rodents and humans. It is usually expressed at low levels in most adult tissues, as nutritional fatty acids downregulate *de novo* fatty acid synthesis. However, it is expressed more strongly in the liver, in the lactating breast, or during embryonic development.^{11,12} Insulin is known to transcriptionally regulate FAS expression.^{13–15}

Several human neoplasms overexpress FAS, including colorectal,^{16,17} endometrial,¹⁸ gastric,¹⁹ mammary,^{20,21} ovary,²² and prostate²³ adenocarcinomas, as well as oral²⁴ and pulmonary²⁵ squamous cell carcinomas, often associated with poor prognosis.^{22,26–30} Moreover, FAS inhibition triggered apoptosis in the MCF7 human breast cancer cell line.³¹ Regarding its low expression in most tissues, FAS inhibition is thus an interesting target for cancer therapy.^{32–34} Although FAS is physiologically mainly expressed in the liver, the implication of FAS in hepatocarcinogenesis has not yet been investigated.

In addition to the gene expression profile mentioned above and quantitative RT-PCR, we examined FAS expression in the transplantation model immunohistochemically, using streptozotocin- and autoimmune-diabetic rats. Furthermore, we evaluated FAS expression in chemically induced preneoplastic foci after *N*-nitrosomorpholine (NNM)-treatment and in spontaneous FAH in old control animals, which resemble hepatocarcinogenesis in the transplantation model in several aspects.³⁵

Materials and methods

Animal Treatment

Animals were subdivided into four experimental groups (EG) and one control group (CG). In EG I ($n=46$), the main group, diabetes was induced in adult 3-month-old inbred male Lewis rats by treatment with a single subcutaneous dose of streptozotocin (80 mg/kg body weight). Then, the animals received only 250–450 islet grafts as reported in detail previously,¹ so that mild hyper-

glycemia (250–300 mg/dl) persisted for at least 10 months. Rats were killed under anesthesia between 2 weeks and 24 months after transplantation.

EG II consisted of BB/Pfd rats of the same age. These animals have become diabetic by an auto-immunological mechanism, similar to human type I diabetes at an age of 3–5 months. Approximately 70% of these BB/Pfd rats showed a previously described spontaneous re-establishment of self-tolerance to the islet cells, caused by an unknown mechanism.³⁶ Only these rats were used to prevent immunological destruction of the transplanted islets and only animals showing vital transplants at the time of killing were included in this study ($n=12$). Transplantation was performed as in EG I. Animals were killed between 2 weeks and 12 months after transplantation.

Hepatocarcinogenesis in Lewis rats of EG III and EG IV was chemically induced. In EG III ($n=7$), 4-month-old animals received a daily administration of NNM via oral application for 6 weeks (16 mg/kg body weight). Thereafter, they were kept for another 26 weeks before they were killed.

EG IV-rats ($n=7$) were initially made diabetic by streptozotocin injection as described for the main group (EG I). Then, they also received NNM and were killed 26 weeks after cessation of the NNM treatment as EG III.

The CG was made up of 10 nondiabetic rats that were not transplanted. Animals of this group served for observing spontaneous hepatocarcinogenesis and were killed at the age of 2 years.

All animals were investigated immunohistochemically, except for two and seven animals of EG I, which were instead examined by atlas-array hybridization and quantitative RT-PCR, respectively. Housing and treatment of the animals were in line with the guidelines of the Society for Laboratory Animal Service (GV-SOLAS) and the strict German animal protection law.

Tissue Sampling and Processing

For array analysis and quantitative RT-PCR, liver tissue obtained from the middle lobe 2 weeks after transplantation and six additional HCC from five animals 12–24 months after transplantation were snap-frozen in isopentane (at -120°C) and immediately stored at -80°C . Thereafter, and in the remaining animals, the livers were fixed by perfusion with a fixation cocktail, embedded in paraffin, and stained with hematoxylin and eosin and with the periodic acid–Schiff reaction as described previously.¹

Laser Microdissection

For array analysis and quantitative RT-PCR, several FAH were microdissected using the Laser Microbeam Microdissection Microscope (Palm, Bernried,

Germany) as shown previously.³⁷ For quantitative RT-PCR, 20–30 serial kryostat sections (8 μ m thickness) were microdissected. As we performed only two array analyses, we wanted to exclude any interference in validity owing to amplification errors caused by utilization of PCR-based methods. Thus, 250 serial cryostat sections containing FAHs were laser-microdissected for one array analysis; the corresponding control was also harvested by laser microdissection from unaltered tissue of 50 cryostat sections. Total RNA was extracted with the RNAeasy-Kit (Qiagen, Hilden, Germany).

Array Analysis

Array analysis was performed in two animals 14 days after islet transplantation using nylon arrays containing 1176 gene-specific primers for cDNA labeling (BD Atlas™ Nylon cDNA Expression Array, Rat 1.2, Cat.# 7854-1, BD Biosciences/Clontech, Heidelberg, Germany). The quantity of the samples and integrity of the messenger RNA (mRNA) were evaluated by photometric measurement and agarose gel electrophoresis. Equal amounts (4.5–7 μ g) of total RNA were then labelled with ³²P (Amersham, Braunschweig, Germany) and transcribed into complementary DNA (cDNA). Hybridization was performed according to the manufacturer's instructions. The membranes were finally visualized on X-ray-films (2–3 days processing). Array analysis was carried out in order to obtain a tentative overview of the gene expression in very early FAH after transplantation and not to define a characteristic pattern of alterations. Thus, we did not make a computerized evaluation of the membranes to detect each slight difference. Only differences that were clearly visible to the naked eye were considered for further investigation.

Quantitative RT-PCR

Quantitative RT-PCR was performed in laser-microdissected FAH and unaltered liver tissue of two animals. After RNA preparation (RNAeasy kit, Qiagen, Hilden, Germany) and reverse transcription (Reverse transcription system, Promega, Madison, WI, USA), cloning and PCR-amplification were conducted as described elsewhere.³⁷ Briefly, FAS and β -actin (internal control) were amplified using the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche, Mannheim, Germany). Sequences of primers, hybridization probes, annealing temperatures, and length of PCR-products are given in Table 1. Relative expression levels of different samples of FAHs were calculated by quantification of FAS levels, normalized to the endogeneously expressed house keeping gene β -actin according to the formula: $2^{(Rt-Et)}/2^{(Rn-En)}$. Rt and Et are the threshold cycle numbers for the reference gene β -actin and the FAS gene in FAHs, respectively,

Table 1 Sequences of primers, hybridization probes, annealing temperatures and lengths of PCR-products

Rat-fatty acid synthase: NM 017332^a, 281-bp^b, annealing temperature: 65°C

FAS-F	5'-Agg TgC TA g Agg CCC TgC TA-3'
FAS-R	5'-gTg CAC AgA CAC CTT CCC AT-3'
hyb FAS-X	5'-TAG TgA TgA ggT CCA CAG CAG CAG C-X-3'
hyb FAS-LC	5'-LC-CCC ggT CCT CCA ggC TCT TCA gT-p-3'

Rat- β -actin: NM 031144^a, 232-bp^b, annealing temperature: 71°C

β -actin-F	5'-CAC ggC ATT gTA ACC AAC Tgg gAC-3'
β -actin-R	5'-CAg Tgg TAC gAC CAg Agg CAT ACA gg-3'
hyb β -actin-X	5'-ACA ggg TgC TCC TCA ggg gCC AC-X-3'
hyb β -actin-LC	5'-LC-CgC AgC TCA TTg TAG AAA gTg Tgg TgC CA-p-3'

^aGenBank accession number.

^bLength of PCR product (base pairs), -F and -R = forward and reverse primer, hyb = hybridization probe, X = Fluorescein, LC = Light Cycler Red 640, p = phosphate.

whereas Rn and En fulfill the same purpose for the unaltered tissue.

Immunohistochemistry

Sections of 5 μ m thickness were deparaffinized and boiled for 20 min in Dako Target Retrieval Solution High pH (Dako, Denmark, No. S3307) in a microwave oven (450 W). Incubation with the primary antibody (monoclonal mouse-Ig-G-anti-FAS, BD Transduction Laboratories, Germany, No. 610963) was performed overnight at 4°C. Biotinylated horse-anti-mouse/rabbit-IgG (H + L, Alexis Corp., Germany No. VC-BA-1400) was used as the second antibody, and visualization was done automatically by the StreptABC method (Vectastain ABC-AP Kit AK 500, Alexis Corp.) using alkaline phosphatase as enzyme and neufuchsin as chromogen. Slides were finally counterstained with hemalaun. Polyclonal antiinsulin, antiglucagon and antisomatostatin antibodies (Dako; dilution 1:200, 4°C incubation for 20 h) were used as described previously to prove the existence of the transplanted islets or smaller remnants within the foci or tumors.⁴

Results

Atlas-Array-Hybridization

As a whole, the gene expression profile in both animals was relatively congruent and showed a pattern that is typical of liver tissue. This was, for example, reflected in a high activity of genes coding for liver-specific proteins, such as α 1-antitrypsin or fibrinogen (Figure 1). Only few genes were differentially expressed to such an extent that they were visible to the naked eye. These were upregulation of

Figure 1 Gene expression profile of laser-microdissected FAH (A) and unaltered liver parenchyma of the same animal (B), 14 days after islet transplantation. The overall staining intensity was slightly stronger in (B) than in (A). Genes, which code for liver-characteristic proteins, show a strong expression in both arrays, thus serving as an internal plausibility control. Few genes displayed a clearly differential expression in these arrays, which can be recognized with the naked eye, including apolipoprotein A-I (1) and A-IV (2). FAS was not visible in the unaltered liver tissue but displayed a distinct signal of moderate intensity, thus indicating an overexpression in the FAH (framed areas in the arrays and magnifications at the bottom).

FAS, apolipoprotein A-I, and apolipoprotein A-IV in FAH in both arrays, as well as IGFBP-1-precursor downregulation in FAH in one array.

FAS-RT-PCR

Quantitative RT-PCR displayed a 3.0- and 6.7-fold overexpression of FAS in the FAH of both animals, confirming the results of the arrays (Figure 2). FAS expression in the HCC was more variable, showing a

downregulation in four tumors (0.24–0.39-fold of the expression of the unaltered liver tissue), and an unaltered expression or slight overexpression in the other two (0.94- and 1.77-fold, respectively).

Histology and Immunohistochemistry

In the transplantation experiment, the usual distribution and arrangement of FAH were identified. Transplanted islets of Langerhans were situated in the terminal portal venules, and the FAH were recognized as glycogen- and fat-storing clear-cell foci, strictly confined to the borders of the draining liver acini. Regarding the morphology, no differences were observed between streptozotocin-diabetic Lewis and autoimmune-diabetic BB rats. A total of 80 and 45 FAH were noted in the streptozotocin-diabetic and BB rats, respectively, and then examined immunohistochemically. The preneoplastic foci in the diabetic and nondiabetic animals of the NNM-model were randomly distributed within the liver parenchyma. In this experiment, we identified 47 lesions for subsequent immunohistochemical analysis. Two spontaneous clear-cell foci were noted in the nondiabetic control rats. In addition, we found three HCA (two basophilic, one clear-cell) and one well-differentiated clear-cell HCC in the transplantation experiment, as well as two basophilic and one clear-cell well-differentiated HCC in the NNM-model.

FAS expression was always granular and cytoplasmic. In the non-diabetic control rats (Figure 3a), we encountered a weak to moderate and more evenly distributed staining pattern. In the diabetic animals, however, expression was often stronger and predominated in hepatocytes of the acinar zone 3 (Figure 3b).

The two spontaneous FAH in the control animals showed strong FAS overexpression when compared to the adjacent parenchyma (Figure 3c). The NNM-induced FAH had a morphology that was similar to these spontaneous foci, and 87% of them displayed FAS overexpression (Figure 3d). Two of these FAH showed intermingled basophilic cells, qualifying them as mixed-cell foci. These basophilic cells were immunohistochemically negative. FAS overexpression within the NNM-model could be demonstrated in both the diabetic and non-diabetic animals, although it was more pronounced in the hyperglycemic rats.

In the transplantation experiment, 98 and 96% of FAH in the streptozotocin- and autoimmune-diabetic rats showed strong overexpression of FAS, respectively. As we noted no differences in staining intensity between these two groups, a crucial role of streptozotocin was excluded. FAS overexpression was so strong that the FAH could be easily observed at very low magnification (Figure 4a). The transplanted islet within the FAH and the strict confinement of the FAH to the borders of liver acinus were

Figure 2 Quantitative RT-PCR (Light-Cycler) of FAS mRNA (left side) and the housekeeping gene β -actin (right side). For determining the amplification efficiency of the RT-PCR, serial dilutions of known concentrations of the plasmid probe of β -actin or serial dilutions of the cDNA of normal liver tissue (upper row) were simultaneously amplified with the mRNA of both, the laser-microdissected liver tissue of foci of altered hepatocytes (FAH) and unaltered liver tissue (control) of the same animal (bottom row). The example depicted in this diagram showed a 9.1-fold overexpression of FAS (for calculation method see Materials and methods).

well recognizable (Figure 4b). Staining was more intense in FAH with predominant glycogen than in those with fat accumulation, but this was merely a result of the smaller cytosolic compartment in fat-storing hepatocytes, caused by extending fat vacuoles.

FAS was overexpressed in the clear-cell but not in the basophilic tumors of both, the endocrine and the chemical model, irrespective of whether they were HCA or HCC and to which experimental group they belonged (Figure 4c,d). In most cases, clear-cell HCA and HCC also displayed a few intermingled basophilic cells that did not overexpress FAS.

Discussion

Array hybridization of early FAH revealed an overall similar gene expression pattern when compared to the unaltered normal tissue. In particular, there was no altered expression of certain oncogenes or tumor suppressor genes, although this was not expected at this early time-point 2 weeks after transplantation. Nevertheless, some genes did show an altered expression, which, in part, confirmed observations made in former studies. The upregulation of apolipoprotein A-IV (A-IV) has recently been shown in this model.³⁷ This was the first indication of alterations in the lipoprotein metabolism within

the FAH. Regarding carcinogenesis, however, down-regulation of IGFBP-1 is more important, as IGFBP-1 has been ascribed tumor suppressive potential via its supposed inhibitory effect on IGF-1. This confirms the results of a previous study that investigated the gene expression of the IGF axis components in detail and that, apart from an overexpression of IGF-1 and IGFBP-4, demonstrated downregulation of IGFBP-1 in this model.⁶

The most intriguing result of the array analysis was the discovery of FAS overexpression in the FAH, confirmed by RT-PCR. FAS gene expression in rat hepatocytes is regulated by both insulin and glucose. It has been suggested that insulin regulation of FAS is at least partly mediated by the basic/helix-loop-helix/leucine zipper transcription factor sterol regulatory element-binding protein-1c (SREBP-1c)³⁸ and that glucose induction of FAS is mediated through a SREBP-1c-distinct transcription factor, designated as either carbohydrate-responsive element-binding protein (ChREBP)³⁹ or carbohydrate-responsive factor.⁴⁰ Interestingly, glucose and insulin stimulate FAS expression synergistically, that is, high glucose levels increase the insulin stimulatory effect,³⁸ which explains the strong FAS overexpression in our hormonal model. It has also been proposed that the mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol (PI) 3-kinase pathway are involved in the

Figure 3 FAS immunohistochemistry. FAS protein is weakly expressed in liver tissue of nondiabetic control animals in a granular cytoplasmic pattern (a). In unaltered liver tissue of diabetic animals, the expression was often stronger and displayed a zonal distribution. Panel b shows the typical stronger expression in hepatocytes localized around the hepatic vein (left), that is, acinar zone 3, than in hepatocytes of the acinar zones 1 and 2 (portal tract at the right). Spontaneous as well as chemically induced FAH consist of enlarged hepatocytes with clear cytoplasm in hematoxylin and eosin staining, owing to an increase in glycogen storage. One of the two spontaneous glycogenotic FAH is depicted in (c) and shows strong FAS overexpression as the FAH in (d), which developed in an diabetic animal of experimental group IV in the NNM model. FAS immunohistochemistry. Original magnification: $\times 254$ (a), (c), (d), and $\times 127$ (b).

Figure 4 FAS immunohistochemistry. Already at very low magnification, FAH can be identified in the liver tissue of diabetic rats after islet transplantation, owing to their strong FAS overexpression (a). At higher magnification (b), the typical morphology of these FAH becomes clear. An islet graft (I) is situated in a terminal portal venule, branching off an portal tract (middle left). The hepatocytes of such an FAH are enlarged, owing to an increase in glycogen and fat storage (fat vacuoles are in particular displayed in the upper right part of the FAH). At this early stage, these FAH are confined to the anatomical borders of the liver acinus, draining the blood from the islet graft and are demarcated by hepatic veins (upper right). These early clear-cell FAH, few weeks after islet transplantation, show very strong FAS overexpression. In cases, when the lesion has lost its clear-cell (glycogenotic) phenotype, FAS expression diminished, as it is shown in the basophilic hepatocellular adenoma (upper three quarter) depicted in (c). However, if the glycogenotic phenotype prevailed, the preneoplastic lesion or neoplasm also maintained its FAS overexpression. Panel d shows such a well-differentiated, glycogenotic and fat-storing hepatocellular carcinoma with retained FAS overexpression. Original magnification: $\times 25$ (a), $\times 51$ (b), $\times 254$ (c) and (d).

SREBP-1c-mediated FAS gene expression^{41,42} and that hepatic glucokinase (glucose-specific hexokinase) is required for the synergistic action of ChREBP and SREBP-1c in lipogenic gene expression.³⁹ The high hexokinase activity in the transplantation model has already been demonstrated⁵ and we have recently also shown, that insulin signalling via the MAP kinase pathway is strongly increased in the FAH.⁷ Thus, our *in vivo* study confirms the observations made in cultured hepatocytes: high levels of insulin and glucose in conjunction with high glucokinase expression, possibly mediated by the MAP kinase pathway, lead to upregulation of the FAS gene.

Although we did not perform a quantitative analysis of the protein expression, the synergistic effect of glucose and/or insulin on FAS expression is, nevertheless, displayed by our immunohistochemical results. In the transplantation experiment, the staining signal was lowest in the liver tissue of the control animals (low insulin, low glucose), stronger in the nonaltered liver tissue of diabetic EG I and EG II animals (high glucose, low insulin), and highest in FAH (high glucose, high insulin). The stimulatory effect of hyperglycemia was also noted in the NNM-model, as FAS expression in the insulinomimetic FAH was higher in diabetic animals of EG IV than in normoglycemic rats of EG III.

FAS expression in carcinogenesis is of growing interest, as FAS has been reported to be overexpressed in several human malignancies.^{16–25,28} Interestingly, FAS is already overexpressed in some noninvasive precursors, such as intraepithelial neoplasia of the squamous^{24,25} or prostate²³ epithelium, intraductal mammary carcinoma,²⁰ or colorectal adenoma.^{16–18} Thus, it seems to be an early event in carcinogenesis of these entities and resembles the early overexpression seen in our model. As FAS is overexpressed in many different tumor entities with a different pathogenetic background, it may be regarded as an alteration of general importance in carcinogenesis. Lipids are necessary not only for energy metabolism; they also provide the pool of available substrates for the biogenesis of cellular membranes, which is particularly important for tumor cells as a prerequisite for their proliferative activity. Moreover, it has recently been shown that FAS expression mainly affects the phospholipid content of detergent-resistant membrane fractions, thus possibly altering microdomain-controlled cell functions, including signal transduction and intracellular trafficking.⁴³

To the best of our knowledge, FAS overexpression in HCC has not yet been reported. Thus, our observations are the first to give evidence of a FAS overexpression in hepatocellular preneoplastic foci and glycogenotic hepatocellular neoplasms. It still needs to be verified which mechanism triggers hepatocarcinogenesis in the NNM-model and in the spontaneous FAH in old control animals; however, in most aspects, these glycogenotic

FAH resemble the insulin-triggered transplantation model,³⁵ including the clear-cell morphology of the early preneoplastic lesions and most of the alterations in enzyme activities in carbohydrate metabolism.^{5,44} Thus, FAS overexpression further substantiates the insulinomimetic phenotype of clear-cell FAH in chemically induced hepatocarcinogenesis.

The variability of FAS overexpression in the hepatocellular tumors is in line with previous reports on other aspects of carcinogenesis in this model. In later stages of the carcinogenic process, which were not the primary subject of this study, the preneoplastic lesions and tumors become more diverse morphologically and biologically.⁴ The clear-cell character in some lesions gets lost with increasing basophilia of these cells, and other altered signalling pathways and protein expressions, such as an implication transforming growth factor α , have been noted; thus, these lesions do not reflect the insulinomimetic phenotype of the early FAH anymore. In this context, loss of FAS overexpression in the basophilic cell population is not surprising.

In humans, epidemiological studies have revealed an increased risk of primary liver cancer in patients with diabetes mellitus.^{45,46} The situation in type II (non-insulindependent) diabetes mellitus, that is, hyperinsulinemic and hyperglycemic portal vein blood, is comparable to the FAH in our model. Thus, for these patients, the results of our studies, using this animal model, might provide a mechanism, which links the metabolic disorder to the carcinogenic process. Unfortunately, these studies did not distinguish between type I and type II diabetes. According to our observations, we would expect that particularly type II diabetic patients carry an increased risk of hepatocellular neoplasms.

Conclusively, we have shown FAS overexpression in clear-cell preneoplastic foci of spontaneously developing, as well as hormonally and chemically induced rat hepatocarcinogenesis, indicating a possible implication of FAS in the development of hepatocellular tumors. Moreover, FAS overexpression in the transplantation experiment can be explained as the result of a combined influence of insulin and glucose on FAS gene expression, which confirms observations that have formerly only been made *in vitro*.

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