Histochem Cell Biol (2007) 128:431–443
DOI 10.1007/s00418-007-0338-y

ORIGINAL PAPER

Kinetics of albumin- and alpha-fetoprotein-production during rat liver development

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Accepted: 27 August 2007 / Published online: 19 September 2007

Abstract Synthesis of most of the plasma proteins is one of the main functions of the hepatocytes. Albumin synthesis is quantitatively the most abundant. In the present study we investigated albumin- and alpha-fetoprotein-gene-expression, and the function of the secretory apparatus during rat liver development. To this purpose we used the method of radioactive biosynthetic labeling of newly synthesized albumin and alpha-fetoprotein (AFP) to monitor the secretory capacity of endodermal cells derived from ventral foregut region (embryonic day 10, E10), and of embryonic and fetal hepatoblasts. Synthesis and secretion of albumin and AFP were already detected in the low numbered ventral foregut endodermal cells; fibrinogen synthesis was detectable in the E12 hepatoblasts, which were in higher number. The whole secretory machinery was functional from the earliest stages of liver development, and the speed of secretion was comparable with that of the adult hepatocytes. There was almost 4-fold increase of hepatoblasts cell volume in fetal stage compared with embryonic stage. The model used suggests that the hepatocyte secretory apparatus is already functional before the emergence of the liver bud. This is the first comparative report to analyze the hepatocyte secretory function, cell proliferation and cell volume during liver development.

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Keywords Secretory apparatus · Albumin · Differentiation · Cell volume · Proprotein convertases · Subtilisin-like proteinases

Introduction

Albumin-production is one of the main functions of the liver. Liver cirrhosis in the advanced state is characterized by protein wasting, as indicated by the loss of muscle mass, hypoalbuminemia, and an abnormal amino acid profile. The protein wasting condition cirrhosis is associated with a poor prognosis and reduced survival. Albumin synthesis appears to parallel liver function, i.e., the more compromised is the liver, the less is the albumin production rate (Tessari 2003).

At day 12 (E12) of the rat embryonic development the liver anlage appears, and albumin- AFP-mRNA, as well as their corresponding proteins, are expressed (Shiojiri et al. 1991). Explanted endoderm derived from mouse embryos at E8–E8.5 (corresponding to E9.5–E10 in the rat) revealed that AFP-mRNA is expressed in the ventral and dorsal endoderm. In contrast, albumin-mRNA-expression was detected only after a coculture with cardiac mesoderm (Gualdi et al. 1996), or after stimulation with different types, and concentrations of fibroblast growth factors (Jung et al. 1999). Recent experiments provided insight into albumin-synthesis. Albumin was detected by immunofluorescence in sectioned mouse embryos at 9–10 somites stages as well as in single cells on cytospin slides generated from ventral foregut region at 8, 10, and 14 somites (Serls et al. 2005). Muglia and Locker (1984) demonstrated that in the developing liver AFP-mRNA reaches a peak at E16 and then decreases, while albumin-mRNA peaks at E18 and persists in the following stage. Otherwise it has been

claimed that albumin-gene-expression increases gradually during liver development (Petkov et al. 2004), and the ratio of AFP synthesis to albumin synthesis, as well as the ratio of AFP-mRNA to albumin-mRNA, suggest a gradual decrease during liver development (Liao et al. 1980).

Since albumin production rate is a crucial parameter of the liver function (Tessari 2003), it was our interest to investigate the development of the albumin production capacity during liver development. The kinetics of synthesis and secretion of albumin and AFP was related to the growth of the liver considering both cell proliferation and increase of the cellular volume. Production of albumin and AFP were monitored in cultured cells derived from ventral endoderm and in cultured hepatoblasts isolated at different developmental stages using a continuous radioactive biosynthetic labeling combined with pulse chase experiment.

The data presented in this study indicate that E10 in the rat is the earliest time point for the emergence of functional hepatoblasts. In both embryonic and fetal stages 50% of hepatic cells were involved in albumin- and AFP-gene-expression.

Materials and methods

Animals and preparation of embryos

About 300–400 g Pregnant Wistar rats were purchased from Harlan Winkelmann (Borchen, Germany). The rats were prepared on the same day of delivery or kept at 19–23°C under standard conditions with 12-h light/dark cycles and access to food and water till desired development stage achieved. All animals received humane care in accordance with the institution's guidelines, the German Convention for Protection of Animals and the National Institutes' of Health guidelines. The whole experimental procedure was evaluated and continuously controlled by the local ethical committee. The Rats were anesthetized by intraperitoneal injection of pentobarbital (Narcoren, Merial, Hallbergmoos) (260 mg/kg body weight). After Narcoren anesthesia, the uterus was removed and placed in Petri dish with cold PBS (with Ca^{++} and Mg^{++}). The embryos from E10, E12, E14, E16, E18, and E20 were removed, collected in ice-cold MEM Hanks with stable glutamine.

Isolation of endodermal cells, primary hepatoblasts, and hepatocytes

The ventral foregut endoderm from embryos at E10 and the liver from embryos at E12 and E14 were dissected under binocular microscope and incubated in MEM Hanks medium with 25 mM Hepes, stable glutamine-supplemented with 0.05% DNase. The liver from E16, E18, E20, and newborn rats was dissected macroscopically, and collected in 1 ml MEM Hanks with 25 mM Hepes, stable glutamine-supplemented with 0.05% Dnase, and 0.05% collagenase H. A single cell suspension was performed after two-times incubation at 37°C for 15 min followed by several times pipetting with a melted and recooled 1 ml and 100 µl pipette tip. Cells were collected after centrifugation (243 g, 5 min, RT). Cell viability was assessed by trypan blue staining and the cell number was determined in a haemocytometer. Cells were suspended in Williaḿs E medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine, 0.1 µg/ml insulin, 0.1 µg/ml glucagon and 0.1 µg/ml dexamethasone. Subsequently, cells were seeded onto collagen type I-coated 24 well plate (BD, Bedford, MA, USA) and 8-chambered slides (Nunc, Roskilde, Denmark) and incubated at 37°C humidified 5% CO2 incubator. After 24 h, the cells were washed twice with PBS and kept in culture in serum-free hybridomed medium (DIF) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine, 0.1 µg/ml insulin, 0.1 µg/ml glucagon and 0.1 µg/ml dexamethasone.

The hepatocytes were isolated from adult rat by a perfusion technique as described elsewhere (Ramadori et al. 1990).

Whole- mount in situ hybridization

Whole-mount in situ hybridization was essentially done as described previously (Wilkinson and Nieto 1993) with modifications as reported by Belo et al. (2000). Rat embryos (E10) were fixed overnight immediately after dissection in 4% paraformaldehyde dissolved in 1 × PBS buffered to pH 7.4. Embryos were washed twice with PBST (PBST = PBS with 0.1% Tween-20) and dehydrated through an increasing methanol gradient, and stored in methanol at –20°C. Embryos were rehydrated through a reciprocal methanol gradient, and then washed three times for 5 min with PBST on ice. Afterwards, embryos were washed with 4.5 µg/ml proteinase K in PBST for 30 min. The digestion was stopped by washing in freshly prepared 2 mg/ml glycine in PBST, followed by two successive washes each 5 min in PBST at room temperature. The embryos were refixed in 0.2% glutaraldehyde/4% paraformaldehyde for 1 h at room temperature and rinsed three times in PBST. A wash for 5 min was performed with 50% PBST/50% hybridization buffer (50% formamide, 4 × SSC, 0.1% (w/v) dextran sulfate, 0.05% (w/v) tRNA yeast, 10 mM dithiothreitol, 10% (v/v) salmon sperm 10 mg/ml), followed by a wash

with 100% hybridization buffer. After 3 h of prehybridization at 65°C, hybridization was carried out overnight; with antisense and sense riboprobes at 200 ng/ml in hybridization buffer. After hybridization, the hybridization buffer was removed and the embryos were washed with 800 µl of fresh hybridization buffer for 5 min at 70°C. Afterwards 400 µl of 2 × SSC, pH 4.5 (without removing hybridization buffer) were added and the wash was repeated twice by adding 2 × SSC. The mix was removed and the embryos were further rinsed twice with 2 × SSC, pH 7/0.1% CHAPS at 70°C for 30 min each time, followed by two successive Washes in maleic acid buffer, two times each, first for 10 min at RT and second for 30 min at 70°C. The final wash was carried out in PBS for 10 min at RT and in PBST for 5 min at RT. Signal was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and NBT/BCIP substrate (Roche, Germany).

Single and double in situ hybridization

In situ hybridization experiments were performed according to a protocol described by (Wilkinson and Nieto 1993). Ten micrometer sections of frozen embryos from developmental stages of E12, E14, E16, E18 E22 and adult liver were fixed for 1 h with 4% paraformaldehyde in PBS followed by two successive washes each 15 min with PBS treated with 0.1% active DEPC at RT. A 100 µl hybridization buffer was given to the sections, covered and then kept in humid chamber for prehybridization for 2 h at 65°C. The hybridization was carried out overnight with labeled antisense and sense riboprobes of albumin and AFP at 500 ng/ml in hybridization buffer. After hybridzation two successive washes were performed for 50 min at 60°C, first with 2 × SSC and second with 0.1 × SSC. RNase treatment was achieved with 10 µg/ml RNase A (Roche, Germany) in 2 × SSC for 30 min at 37°C, followed by a wash with 0.1 × SSC for 50 min at 60°C. For a single in situ hybridization albumin and AFP mRNA was detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody (Sigma, Germany) and NBT/BCIP substrate (Roche, Germany). For double in situ hybridization, AFP mRNA positive slides were further examined with alkaline phosphatase-conjugated anti-Fluorescein in an attempt to detect albumin expression. For this purpose alkaline phosphatase enzyme was deactivated by incubating the slides in MABT (100 mM maleic acid pH7.5, 150 mM NaCl, 0.1% (v/v) Tween-20) at 65°C for 30 min. After two rinses with MABT at RT, slides were treated with glycine-buffer (0,1 M glycine-HCl pH 2,2) for 30 min followed by a wash in MABT at RT (Pringle et al. 2003). Slides were then ready for immunoreaction with alkaline phosphatase-conjugated anti-Fluorescein antibody (Sigma, Germany). Signal detection was performed as described by Neubauer et al. (1996). In vitro in situ hybridization was also performed in 8-chambered slides after 2 days culture of hepatocytes, hepatoblasts, and endodermal cells generated from ventral foregut. Fixation with 4% paraformaldehyde, prehybridization, hybridization, and signal detection were carried out as described above.

Proliferating cell nuclear antigen (PCNA)-immunohistochemical detection

Proliferating cell nuclear antigen (PCNA)-expression was assessed using peroxidase immunostaining (Micsenyi et al. 2004). Five micrometer cryosection of rat embryos from developmental stage E12, E14, E16, E18 as well as newborn and adult liver were fixed with 4% formaldehyde freshly prepared from paraformaldehyde for 30 min at RT followed by an incubation step in 70% ethanol at –20°C. Subsequently the slides were rinsed twice in PBS. Slides were microwaved for 40 min in citrate buffer at pH 6 followed by incubation for 10 min in Triton buffer. Endogenous peroxide was inhibited by incubating the slides with phosphate-buffered saline containing glucose-oxidase/H_2O_2 for 30 min at 37°C. After two successive washes in PBS, sections were blocked with FCS for 20 min, washed again and incubated overnight at 4°C in PBS diluted (1:100) mouse monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) antibody (Novocastra, UK). The next day, sections were washed and incubated with the secondary anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Dako, Glostrup Denmark) (1:100) for 1 h at RT. A signal was detected using diaminobenzidine/H_2O_2 (0.05% (w/v)/0.01% (v/v)). The sections were counter-stained with hemalaum (Merck, Darmstadt, Germany).

Quantitative morphological studies

Quantitation of albumin-, AFP-, and PCNA- positive cells detected by in situ hybridization and PCNA immunostaining was performed by counting of positive cells under microscope using a shaded ocular, and by application of Image J software (Wayne Rasband, NIH, USA) (Sheikh et al. 2006), and relating the positive cells to the whole cell counts of the analyzed image area. Measurement of the cellular volumes of albumin-expressing cells was carried out on embryonic liver sections and 8-chambered slides hybridized with the antisense albumin probe. The cell volumes were measured based on calibrated slides of Zeiss

(Göttingen, Germany) and by application of the Axiovision software (Zeiss). The morphological analyses were performed on an Axiovert 200M, with Apotome function (Zeiss), using differential-contrast images. At each analyzed time points in the in vivo and the in vitro samples 30 cells were measured, and the differences in the cell volumes between time points of embryonic and fetal development were analyzed statistically. Comparisons between two groups were performed by Student's t-test. For multiple groups, data were analyzed by ANOVA. Differences were considered significant at probability levels of $P < 0.05$ using the Fisher's protected least 10.

Immunocytochemical analysis

For immunofluorescence studies, endodermal cells from ventral foregut region and hepatoblasts from E12 were cultured for 2 days on 8-chambered slides and fixed in acetone for 10 min at room temperature, rinsed in phosphate-buffered saline. Blocking of non-specific binding with 1% (w/v) bovine serum albumin and 10% (v/v) goat serum (DAKO) in PBS was carried out for 1 h at RT. Endodermal cells and hepatoblasts were immunostained by rabbit polyclonal antibody against Hepatic Nuclear Factor 4-alpha (Santa Cruz, CA, USA) (1:50) alone, or in combination with a mouse monoclonal antibody against a 58 K Golgi protein (Bloom and Brashear 1989; Abcam, Cambridge, UK) overnight at 4°C. The rabbit polyclonal antibody was detected with Alexa 555-conjugated secondary anti-rabbit antibody, mouse monoclonal with Alexa 488- conjugated secondary anti-mouse antibody (Molecular Probes, Leiden, the Netherlands) diluted 1:400 and 1:200, respectively, in PBS. Eight-chambered slides were counter-stained with 4'-6-Diamidino-2-phenylindole (DAPI) (Molecular Probes) and analyzed with epifluorescence microscopes (Axioskop 50 or Axiovert 200 M, Zeiss).

Isolation of total RNA, cDNA, and riboprobes for albumin and AFP

Total RNA was isolated from cultured hepatoblasts, hepatocytes and whole liver at different developmental stages by means of guanidine isothiocyanate extraction, cesium chloride density-gradient ultracentrifugation and ethanol precipitation according to method of Chirgwin (Chirgwin et al. 1979) as described elsewhere (Ramadori et al. 1985). cDNAs were obtained by using M-MLV Reverse Transcriptase (RT) Kit (Invitrogen, Carlsbad, CA, USA). cDNA from explanted endodermal cells from ventral foregut and hepatoblasts at E12 were prepared by Fastlane Cell cDNA kit (Qiagen, Hilden, Germany). The riboprobes were synthesized as digoxygenin and fluorescein labeled RNA. The riboprobes were prepared by an in vitro transcription reaction using a cDNA, digoxigenin/fluorescein RNA-labeling kit and T7 RNA polymerase (Roche, Mannheim, Germany). The cDNA Probes for albumin and AFP were amplified by RT-PCR using the primers listed in Table 1 and purified by Gel Extraction Kit (Qiagen, Hilden, Germany). For single and in vitro in situ hybridization both riboprobes (albumin and AFP) were labeled with digoxygenin. In case of double in situ hybridization albumin was labeled with fluorescein and AFP with digoxygenin.

PCR analysis of total RNA

mRNA expression was measured quantitatively during liver development by real-time RT-PCR experiments using an ABI Prism 7,000 thermal cycler (Qiagen), SYBR® Green PCR Mastermix (Invitrogen), cDNA, and specific primers (Table 2). The gene expression levels of target genes (Q) was determined based on the threshold PCR cycle-values (Ct target) (following the instructions by Applied Biosystems) and it was normalized with the

Table 1 List of primers used for preparation of albumin and AFP sense and antisense (for = Forward, rev = reverse) probes. When T7 promoter (underlined sequence) is upstream of the reverse primer, we obtain a cDNA construct for the synthesis of an antisense probe. When a T7 promoter is upstream of the forward primer, we generate a cDNA construct for the synthesis of sense probe

Primer	Sequence
Antisense AFP for	GGCGATGTCCATAAACACGTTC
Antisense AFPT7 rev	TAATACGACTCACTATAGGGCCGGTTTGTCGCCATTTTC
Sense AFPT7 for	TAATACGACTCACTATAGGGGGCGATGTCCATAAACACGTTC
Sense AFP rev	CCGGTTTGTCGCCATTTTC
Antisense Alb for	GGATTCCAAAACGCCGTTCT
Antisense AlbT7 rev	TAATACGACTCACTATAGGGCCTCAGTGGCGAAGCAGTTATC
Sense AlbT7 for	TAATACGACTCACTATAGGGGGATTCCAAAACGCCGTTCT
Sense Alb rev	CCTCAGTGGCGAAGCAGTTATC
T7 Promoter	TAATACGACTCACTATAGGG

Table 2 Primers used for PCR analysis (for = Forward, rev = reverse)

Primer	Sequence
Albumin for	GGA TTC CAA AAC GCC GTT CT
Albumin rev	CCT CAG TGG CGA AGC AGT TAT C
AFP for	GGC GAT GTC CAT AAA CAC GTT C
AFP rev	CCG GTT TGT CGC CAT TTT C
HNF4alpha for	CTT CTT TGA CCC AGA TGC CAA G
HNF4alpha rev	GCC GGT CGT TGA TGT AAT CCT
Beta-catenin for	CGC ACC ATG CAG AAT ACA AAT G
Beta-catenin rev	GGA TGC CGC CAG ATT TAA AGA T
Prox1 for	GCT CCA ATA TGC TGA AGA CC
Prox1 rev	ATC GTT GAT GGC TTG ACG TG
BMP4 for	TTC CCT CAA GGG AGT GGA AAT TC
BMP4 rev	CCA TCG TGG CCA AAA GTG A
Foxa2 for	CAT GGT GAA ATC CAG GTC TCG
Foxa2 rev	TGG AAC TCT GGC ATT CTA GCC
Furin for	AAT CCC AAC CAC ATC CAC AT
Furin rev	GCT GAT GGA CAG CGT GTA GA
GATA-4 for	TTG ATC TCC GTT TTC GCG AC
GATA-4 rev	GCT CCC CTT TAT TTG CAA GTC A
PC 7/8 for	ACG GAT GCC TTT TTA TGC AG
PC 7/8 rev	GTT CCT GTG TGG CCT TCA GT
Ribosomal 18S for	CGG CTA CCA CAT CCA AGG AA
Ribosomal 18S rev	TTT TCG TCA CTA CCT CCC CG

threshold PCR cycle-values of the endogenous control of ribosomal RNA 18S (Ct 18S), using the following formula:

$Q = 2^{-\Delta Ct}$, where $\Delta Ct = Ct_{target} - Ct_{18S}$.

Biosynthetic labeling, immunoprecipitation and SDS-PAGE of newly synthesized proteins

Newly synthesized proteins were radioactively labeled with ^{35}S-methionine as described before (Ramadori et al. 1990). Briefly, endodermal cells derived from ventral foregut and hepatoblasts at different developmental stages were kept in culture for 2 days. Afterwards, cells were washed three times with methionin-free RPMI medium and labeled in RPMI medium supplemented with ^{35}S-methionin (100 µCi/well) for 2 h. Cells derived from ventral foregut were labeled overnight . For pulse chase experiment according to the protocol described by (Tworkowski et al. 2002) the hepatoblasts from E14 and hepatocyte were pulsed for 1 h in RPMI medium supplemented with 100 µCi/well ^{35}S-methionine, followed by three washes to remove the radioactive traces. Cells were further cultured in radioactive-free RPMI medium supplemented with 2 mM "cold" L-methionin and then incubated during a chase period of 15, 30, 45, 60, 90, 120, 240 min. After labeling and at each period of chase, supernatants were harvested and diluted to 50% with lysis buffer with sodium dodecylsulfate (0.5% (w/v) deoxycholic acid (DOC), 1% (v/v) Triton x-100, 10 mM ethylene-diaminetetraacetic acid (EDTA), 1% (w/v) sodium dodecylsulfate, dissolved in PBS. pH 7.4). The cells were lysed after freeze-thawing and scraping in lysis buffer without sodium dodecylsulfate supplemented with 1% (v/v) phenylmethylsulfonyl fluoride (2 mM PMSF in ethanol, pH 7.4). Cell-lysates were then harvested and diluted to 50% with lysis buffer with sodium dodecylsulfate. The count of the total labeled proteins was taken after a trichloroacetic acid precipitation. For immunoprecipitation of albumin, AFP, and fibrinogen (Dako, Glostrup, Denmark), supernatants and cell-lysates, the same counts of total labeled proteins were used. The samples were incubated first with 50 µl protein A for 30 min on ice. After centrifugation, the pellet was discarded and precleaned samples were incubated overnight at 4°C with rabbit polyclonal anti-albumin, or anti-AFP or anti-fibrinogen. Immunocomplexes were precipitated by adding 50 µl of protein A and incubation for 1 h on ice. Immunoprecipitates were centrifuged, washed first once with lysis buffer with sodium dodecylsulfate, supplemented with 0.5% BSA followed by four washes with lysis buffer with sodium dodecylsulfate and finally resuspended in 25 µl loading buffer supplemented with fresh β-mercaptoethanol. Immunoprecipitated albumin, AFP, and fibrinogen were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Samples were boiled for 5 min, centrifuged at 14,000 g for 15 min, where pellet was discarded and supernatants were loaded on the gel. The electrophoresis was performed at 200 V for 4 h. Afterwards gels were fixed overnight, incubated for 1 h in Amplify™ Fluorographic Reagent (GE Healthcare, Freiburg, Germany), covered with Cellophane and then dried. Dried gels were analyzed by autoradiography.

Results

Analysis of albumin- and AFP-gene-expression in ventral endoderm and during rat liver development

Whole mount in situ hybridization revealed that AFP-mRNA is strongly expressed in the ventral foregut region (Fig. 1A). A single cell suspension was prepared from ventral foregut endoderm and the mRNA-expression of albumin and AFP was analyzed. In the endodermal cells the expression of endoderm and hepatic marker genes as well as of transcription factors involved in liver development was

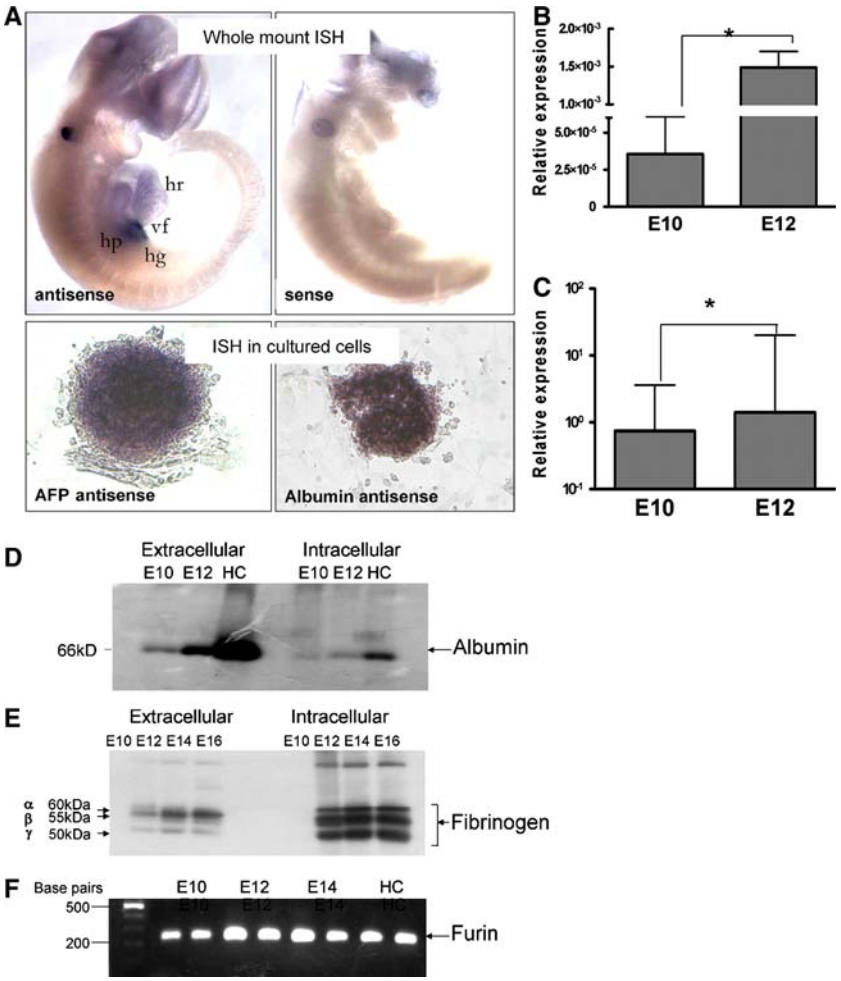

Fig. 1 Albumin and AFP- mRNA-expression, protein synthesis and secretion in cultured endodermal cells derived from ventral foregut region in rat embryos at 10 days of gestation. (**A**) In situ hybridization of albumin and AFP in endodermal cells of ventral foregut region. *Upper panel*: Whole mount in situ hybridization using DIG-labeled antisense RNA probes showed the expression of AFP in ventral foregut (hr: heart, vf: ventral foregut, hg: hindgut, hp: hepatic primordium), while the sense RNA probe was not reactive. *Lower panel*: In vitro in situ hybridization of albumin and AFP performed in cultured endodermal cells isolated from ventral foregut region, showing the reactivity of both antisense albumin and antisense AFP RNA probes. Real-time-RT-PCR analysis confirmed the expression of albumin- (**B**) and AFP (**C**) -mRNA in cultured endodermal cells (E10) and hepatoblasts (E12). The expression was normalized with the endogenous control of ribosomal 18S rRNA (relative expression). Error bars represent S.E.M, n = 3. Statistically significant difference (marked with asterisks, $P < 0.05$ Studenťs *t*-test) was found between the expression levels in cultured endodermal cells (E10) and hepatoblasts (E12). (**D**) Radioactive biosynthetic labeling showing the synthesis and secretion of albumin by endodermal cells, while fibrinogen secretion was not detected in them (**E**). As development progressed (E12; E14; E16) besides albumin (**D**), also fibrinogen synthesis and secretion was detected in hepatoblasts (E12-16) (**E**). (**F**) RT-PCR detection of furin, a subtilisin-like proprotein convertase, involved in albumin processing in cultured ventral foregut endodermal cells (E10), in hepatoblasts (E12–E14) and in adult rat hepatocytes (HC)

confirmed. HNF4α-positivity was detected in cultured clustered endodermal cells by immunofluorescence (Fig. 2 B–C). In HNF4α-positive cultured clustered endodermal cells of 58K Golgi marker protein was detected (Fig. 2C). HNF4α-, Prox1-, Beta-catenin-, FOXA2-, BMP-4, and GATA-4-mRNA were detected in ventral endoderm and hepatoblasts by RT-PCR. BMP-4 and FOXA2-displayed a low mRNA expression (Fig. 2A).

Albumin- and AFP-mRNA was detected in cultured endodermal cells by in vitro in situ hybridization (Fig. 1A (lower panel)). Albumin- and AFP-mRNA-expressing-cells were surrounded by negative cells. As negative control, sense albumin probe was used, with no reactivity (not shown). Albumin- and AFP-mRNA-expression in ventral endoderm was confirmed by real time RT-PCR experiment (Fig. 1B, C). In whole liver (in vivo) the levels of albumin-mRNA-expression showed an increase from the embryonic (E12) through fetal toward adult stages. The increase of albumin from embryonic to fetal stages was interrupted by a decrease after E18, followed by a second increase after

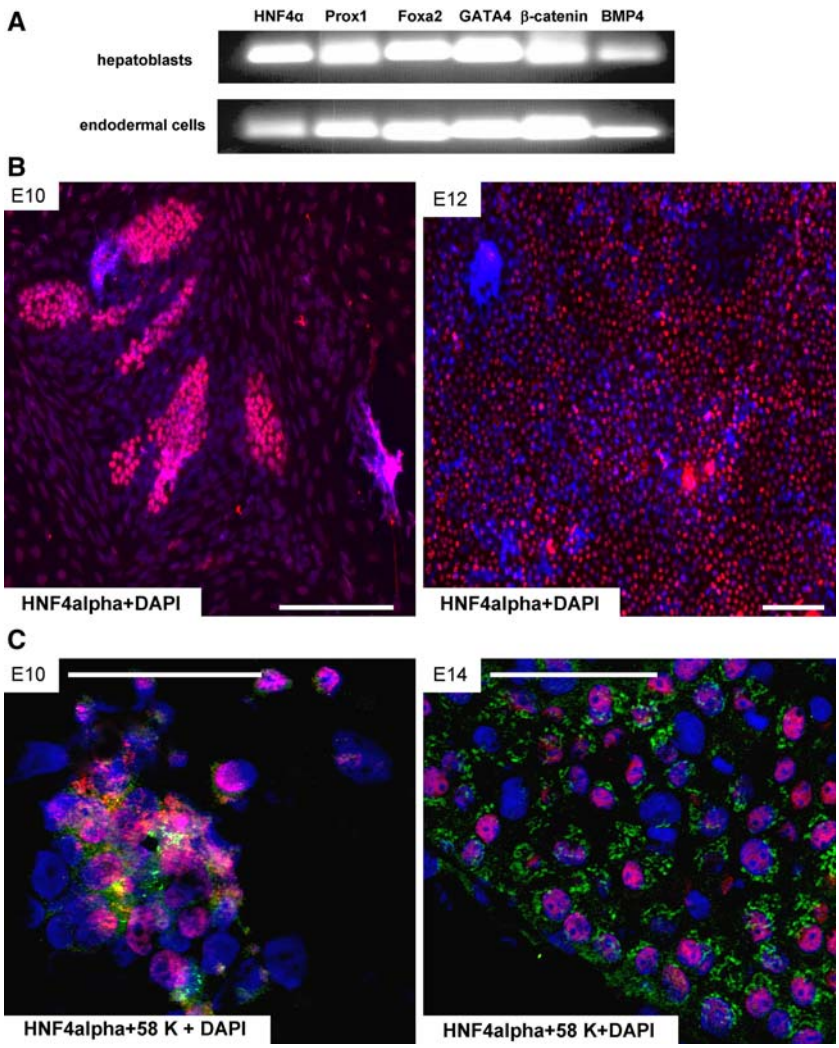

Fig. 2 (**A**) RT-PCR analysis of cultured endodermal cells and hepatoblasts for transcripts for HNF4-alpha, Prox1, Foxa2, GATA4, Beta-catenin and BMP-4. (**B**) Immunofluorescence staining displays HNF4-alpha-expression (red) in cultured clustered endodermal cells (E10) and in hepatoblasts (E12). Counterstaining of nuclei with DAPI (blue). Original magnification 100×. (**C**) Immunofluorescence staining of HNF4-alpha (red)- and a 58K Golgi-protein (green) in cultured-clustered endodermal cells (E10) and in hepatoblasts (E14). Counterstaining of nuclei with DAPI (blue). Original magnification 400×. Bars represent 50 μm

birth. In vitro, albumin expression showed a continuous increase interrupted by a steady-state from E18 to birth, and followed by further increase in adult hepatocytes (Fig. 3A).

In vivo AFP was the highest expressed at E12 followed by a decrease at embryonic stages, and a peak at E18. After E18, AFP expression continuously decreased toward the adult stage. In vitro AFP expression increased in cultured hepatoblasts during embryonic stages (E12-16), and the expression decreased in fetal hepatocytes. In adult hepatocytes no significant expression was found (Fig. 3A).

Analysis of albumin and AFP synthesis and secretion in ventral endoderm and during rat liver development

So far, immunological techniques failed to detect albumin at protein level in the ventral endoderm. Hence, a more sensitive method, the radioactive biosynthetic labeling was used to assess synthesis and secretion of albumin. Endodermal cells derived from ventral foregut were kept in culture for 2 days, and then overnight radioactively labeled with ^{35}S methionin contained in the culture medium. Albumin and fibrinogen were immunoprecipitated from supernatants (extracellular) and cell-lysates (intracellular). Endodermal cells were found to synthesize and secrete biosynthetically labeled albumin (Fig. 1D). Compared to hepatoblasts (E12) 10-times volume (500 μl) of supernatants and cell-lysates of labeled endodermal cells was required and low band intensity for albumin was observed (Fig. 1D). Fibrinogen synthesis and secretion was not detectable in endodermal cells, while it became detectable in E12 hepatoblasts (Fig. 1E), together with the increase of the number of cells plated. We observed an increase of the amount of synthesized and secreted fibrinogen during the liver development, together with the increase of the number of hepatoblasts.

The use of radioactive biosynthetic labeling followed by specific immunoprecipitation is the only method, which allows the monitoring of the synthesis and secretion of

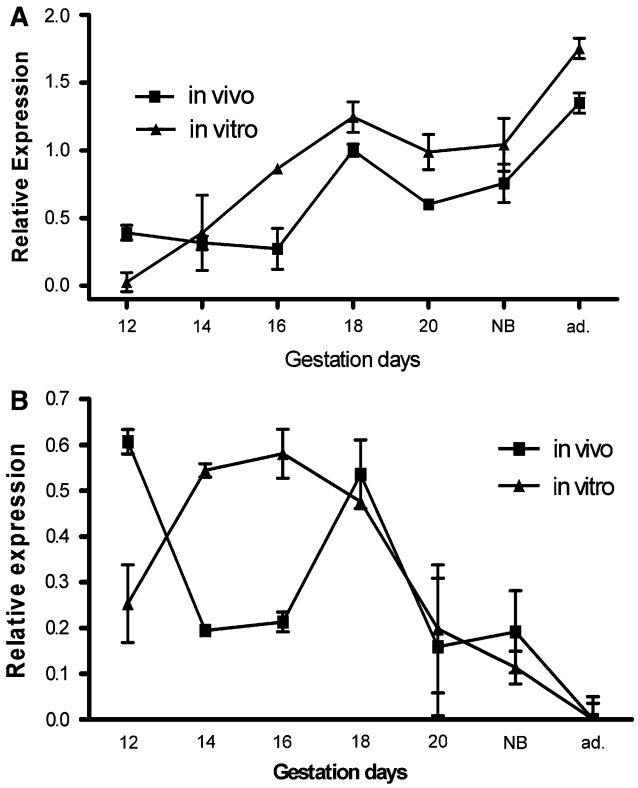

Fig. 3 Quantitative analysis of albumin (**A**) and AFP (**B**) mRNA-expression in whole liver (in vivo) and in cultured hepatoblasts (in vitro) during liver development using real time RT-PCR. The expression was normalized with the endogenous control of ribosomal 18S RNA (relative expression). Error bars represent S.E.M., $n = 3$. (NB = new born, ad = adult)

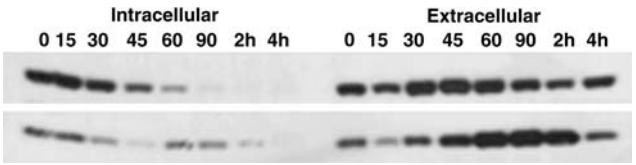

Fig. 4 Kinetics for albumin synthesis and secretion in hepatoblasts (E14) and adult hepatocytes estimated by pulse chase experiment. The secretion speed in E14 hepatoblasts (upper panel) was comparable with that of the adult hepatocytes (lower panel). At 60 min the apparent increase of labeled albumin protein in the intracellular pool of adult hepatocytes is due to the contamination of the extracellular pool, which dramatically increased at this time point

albumin and AFP in ventral endoderm. This is the first demonstration of such a synthetic and secretory function in the endodermal cells (E10) which express simultaneously endoderm and liver markers genes.

The albumin processing activity of ventral foregut endodermal cells was confirmed by detecting the expression of subtilisin-like proprotein convertases reported before, to be involved in proteolytic processing of albumin (Mori et al. 1999; Roebroek et al. 2004). PCR results showing the expression of furin in ventral foregut endodermal cells, in hepatoblasts and in adult hepatocytes are demonstrated on Fig. 1F.

Pulse chase experiments

To test the transport of a very abundant protein like albumin we used the pulse chase method. As shown in Fig. 4 the secretion kinetics of albumin in hepatoblasts was comparable with that of in adult hepatocytes. These data show that not only the capacity of the synthetic but also of the secretory apparatus is comparable in hepatoblasts and adult hepatocytes. The speed of secretion of albumin was the same in E14 hepatoblasts as in adult hepatocytes. In fact, most of the synthesized protein is secreted within 30 min.

Distribution of albumin- and AFP-producing cells during rat liver development

The embryonic liver is formed at E12. At this developmental stage, albumin- and AFP- mRNA were expressed and their proteins were synthesized and secreted. The ratio of albumin- and AFP-expressing cells to total liver cells and to proliferating liver cells was analyzed during the liver development. For this purpose, at each developmental stage, in situ hybridization was performed using albumin and AFP riboprobes combined with proliferating cell nuclear antigen (PCNA) immunostaining. Albumin- and AFP-positive cells were homogeneously distributed during liver development (Fig. 5A–B)). At embryonic and fetal stages about 50% of liver cells expressed albumin and AFP. No significant difference was found in this sense at embryonic and fetal stages by E12, 14, 16, 18, and 22 (By comparison with one-way ANOVA $P = 0.8018$ regarding the AFP^+ cells/total cells, and $P = 0.4367$ regarding the $Albumin^+$ cells/total cells; Fig. 5D). Cell proliferation in developing liver was assessed by immunohistochemical reactions with proliferating cell nuclear antigen (PCNA) in embryonic and fetal livers at E12, E14, E16, E18 and in livers after birth and adult stages. High number of PCNA-positive cells was observed at E12, E14, and E16. Fewer PCNA-positive cells were observed at E18 and at birth. Far fewer PCNA-positive cells were observed in adult liver (Fig. 5 C). In developing liver, the ratio of albumin- and AFP-expressing cells to proliferating cells showed an increase during embryonic and fetal stages. At E18 the ratio of AFP-expressing cells to proliferating cells reached the maximum, followed by a decrease at birth, in contrast, the ratio of albumin-expressing cells to proliferating cells increased exponentially (Fig. 5E). The co-localization of

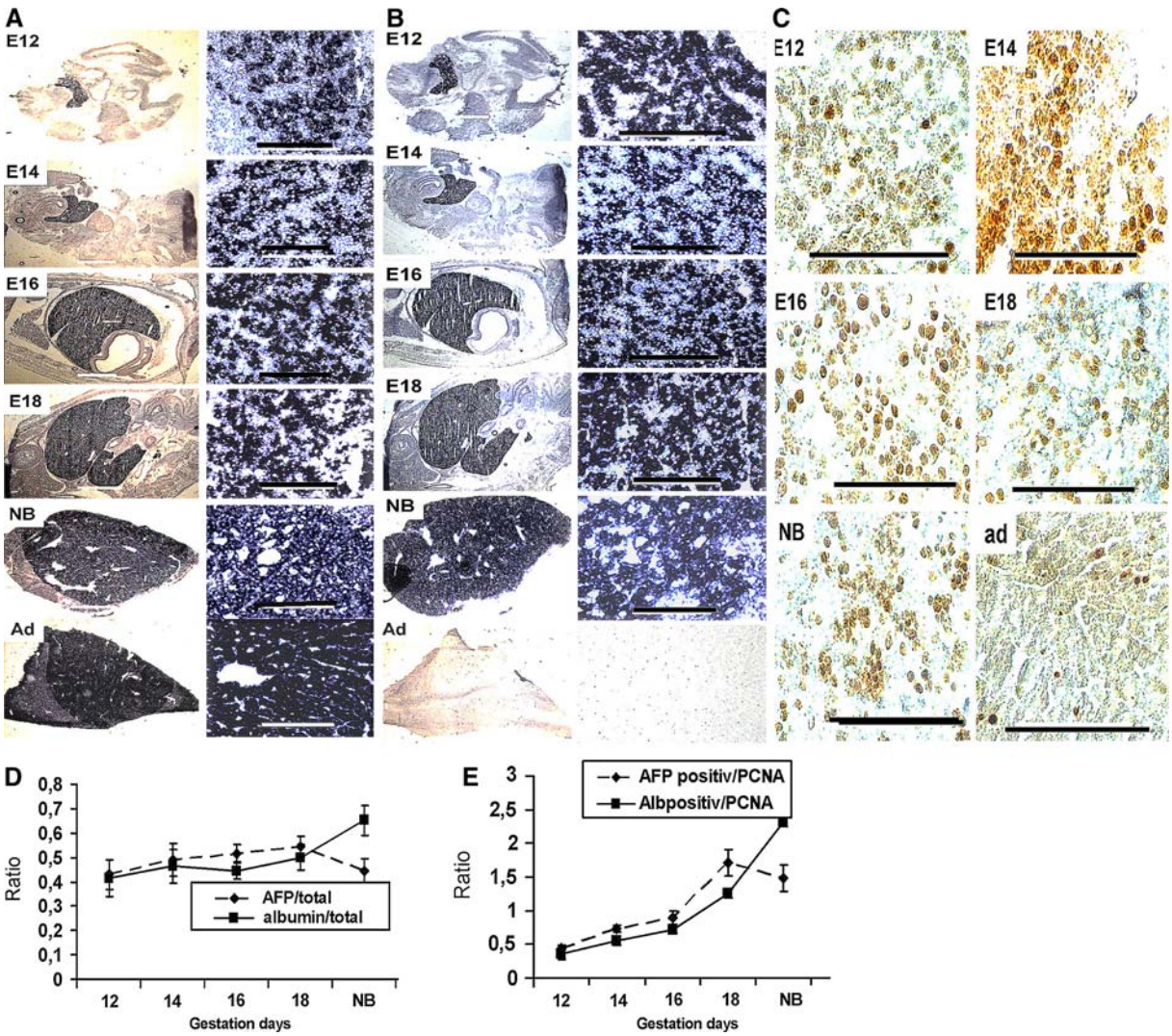

Fig. 5 Albumin- (**A**) and AFP- (**B**) mRNA-expression in developing liver assessed by in situ hybridization using DIG-labeled antisense RNA probes (Original magnification 50×; 200×, Bars = 100 μm). (**C**) Proliferation of hepatoblasts estimated by the number of cells positively stained for proliferating cell nuclear antigen (PCNA) in developing liver. Immunohistochemical reaction was detected by peroxidase-labeled secondary antibody (Original magnification 400×, bars = 100 μm). (**D**) The ratio of albumin- and AFP-expressing cells to total cells and (**E**) the ratio of albumin- and AFP-expressing cells to $PCNA^+$ cells during liver development. Albumin- and AFP-positive cells were identified by in situ hybridization, and PCNA-positive cells by immunohistochemical staining. The positive cells were counted under microscope using a shaded ocular, and by application of Image J software. Error bars represent S.E.M., $n = 3$. The significance ($P < 0.05$) was analyzed by ANOVA

the two plasma proteins albumin and AFP was observed at all developmental stages. At birth, albumin was produced by hepatocytes, which did not express AFP anymore (Fig. 6A). The co-expression of albumin- and AFP-genes in hepatoblasts was confirmed by in vitro in situ hybridization performed in cultured hepatoblasts at E12, E14 and E18 (Fig. 6B). Thus, the co-expression of albumin and AFP was not restricted to the fetal stage. Both plasma proteins were co-expressed by hepatoblasts from E12, the time when the liver anlage appeared, until birth.

Cellular volume increase of albumin-expressing cells during rat liver development

The cellular volume of albumin-expressing cells (identified by in situ hybridization) was measured at embryonic and fetal stages in vivo, and also in vitro 2 days after the isolation of foregut endodermal cells and hepatoblasts. In vivo at E12 the volume of the liver was 3×10^{-3} mm^3, the albumin-expressing hepatoblasts showed an average of 971.8 ± 287.4 μm^3 volume. The cell volume significantly increased at E16, and further increased at the fetal

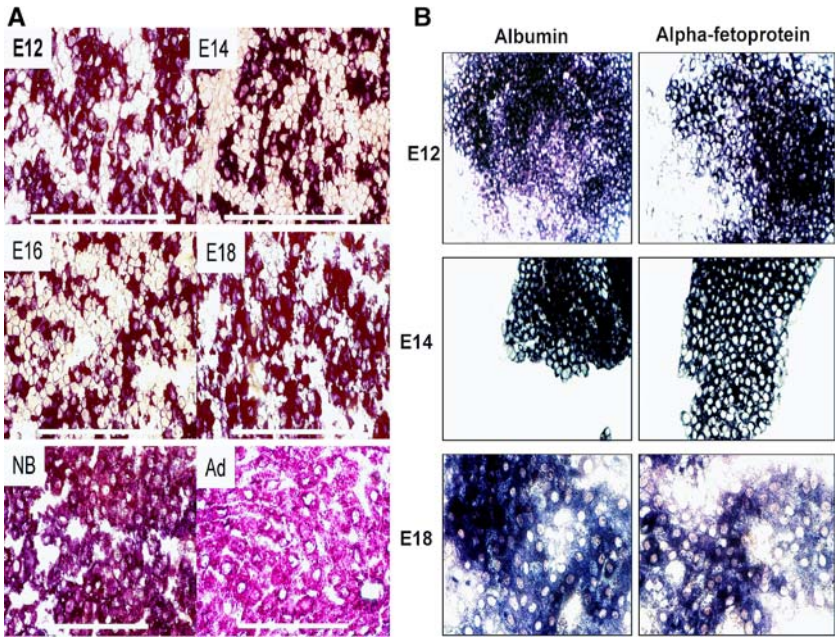

Fig. 6 Co-localization of albumin- and AFP-mRNA during liver development measured by double in situ hybridization (**A**) and in vitro in situ hybridization (**B**) using DIG-labeled antisense RNA probe of AFP (blue) and Fluorescein-labeled antisense RNA probe of albumin (red) for double in situ hybridization (Bar = 100 μm, Original magnification 400×). For in vitro in situ hybridizations albumin and AFP mRNA were detected by using DIG-labeled antisense RNA probe (Original magnification 200×)

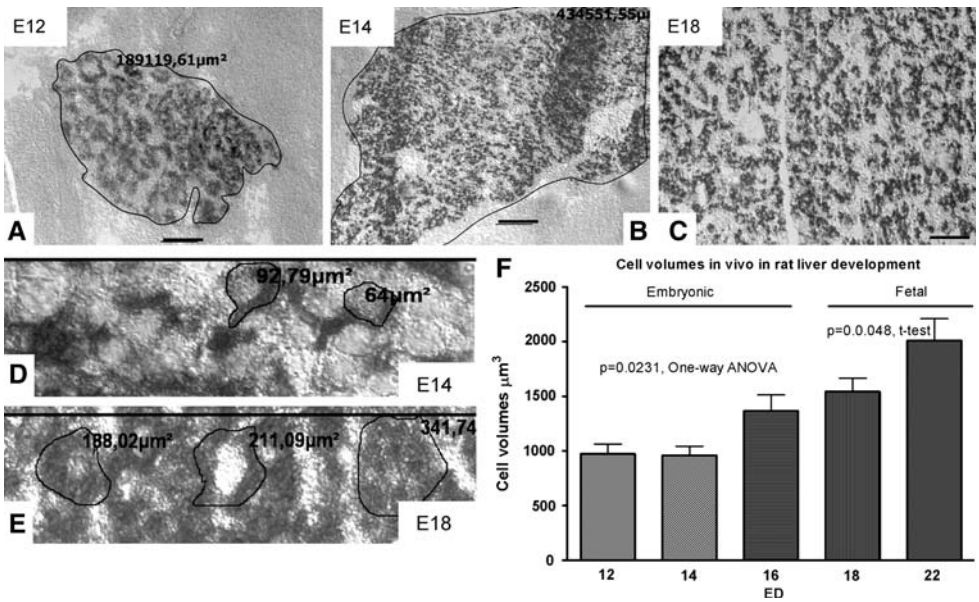

Fig. 7 Measurement of the cellular volumes at different stages of rat liver development in vivo. Differential-contrast (DIC) images of albumin in situ hybridization at E12 (**A**), E14 (**B**) and E18 (**C**), using 100× original magnification. For the cell volume measurement the thickness of the measured areas of the section was measured, which was found to be between 8–11 μm. The surface of the albumin-positive cells was estimated based on calibrated units. The cell volumes were calculated from the cell surface and the thickness of the measured area. (**D**, **E**) Examples for cell volume analysis at E14 (**D**) and at E18 (**E**) on 400× original magnification DIC images. Bars represent 100 μm. (**F**) Analysis of cell volumes of albumin-expressing cells at different stages of rat liver development

stage (Fig. 7). In vitro at E10 in the isolated cells of the foregut endoderm, albumin-expressing population was found with epithelial morphology. The average volume of these cells was 379.2 ± 109.0 μm^3. At E12 the isolated hepatoblasts showed a volume of 946.6 ± 391.0 μm^3, which is comparable with the in vivo volume of the hepatoblasts. Hepatoblasts showed a moderate increase of the cellular volume at embryonic stages (E10–16), and in the fetal stage the cellular volume increased up to 3.2 ± 0.8 mm^3 , which is an 8.6-fold increase compared with the volume of the foregut endodermal cells (E10) (Fig. 8).

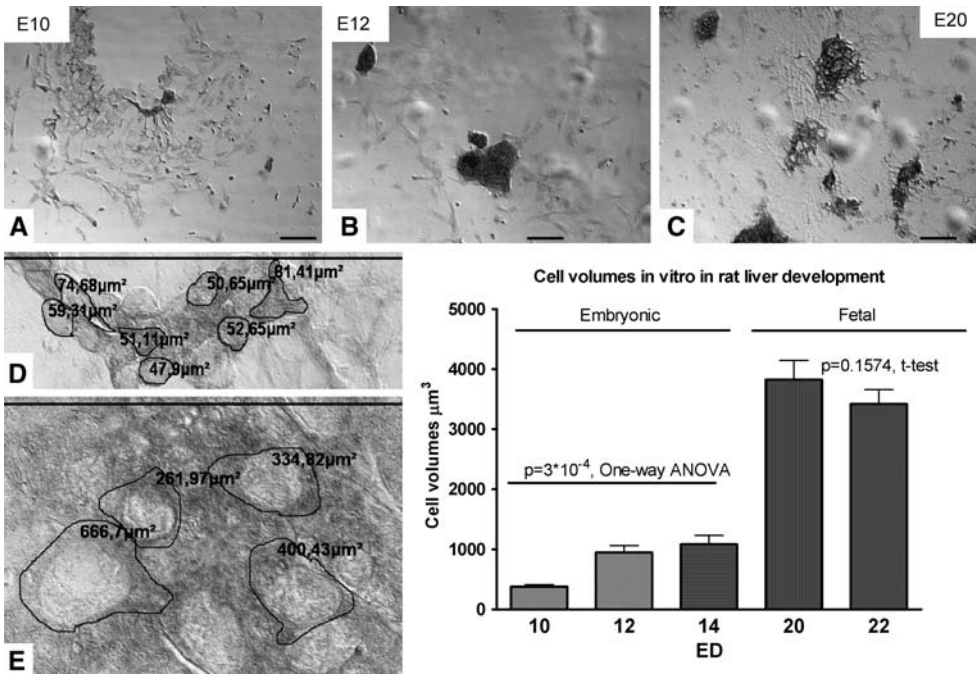

Fig. 8 Measurement of the cellular volumes at different stages of rat liver development in vitro. Differential-contrast (DIC) images of albumin in situ hybridization in isolated foregut endodermal cells E10 (**A**), and in isolated hepatoblasts E12 (**B**), E20 (**C**), at 100× original magnification. For the cell volume measurement the thickness of the measured area of the chambered-slide was measured, which was between 5–7 µm. The surface of the albumin-positive cells was estimated based on calibrated units. The cell volumes were calculated from the cell surface and the thickness of the measured area. (**D**, **E**) Examples for cell volume analysis at E10 (**D**) and at E20 (**E**) on 400× original magnification DIC images. Bars represent 100 µm. (**F**) Cell volumes of isolated and cultured albumin-expressing cells at different stages of rat liver development

Discussion

Identification of albumin- and AFP-expressing cells in the ventral endoderm

In the current study the earliest stage in the rat liver development when the main hepatic function was detected was the emergence of albumin- and AFP-expressing cells in the ventral foregut region of E10 rat embryos (0–4 somites). This finding represents a considerably earlier stage than the one known before. Furthermore, in vitro, endodermal cells generated from ventral foregut showed this function as well. This finding is consistent with the studies using four–six somites mouse embryos (E8–E9.5). It has already been shown that AFP-mRNA is detectable by in situ hybridization performed in explants derived from the ventral endoderm region (Gualdi et al. 1996). Previous studies using rat embryos claimed that AFP is expressed first, followed by the expression of albumin 1 day later (Shiojiri et al. 1991; Muglia and Locker 1984). These studies reported that albumin and AFP mRNA were not detectable in the E10 RNA isolation taken from the foregut region, even when very long exposure times were used. In contrast, in 12-day liver RNA, the expression of both genes was observed. So far, using in situ *hybridization*, neither albumin-mRNA nor AFP-mRNA could be detected at E9.5-E10 in the rat. AFP-mRNA was detected in the ventral endoderm at E10.5 but its protein was observed only 1 day later (E11.5). Albumin-mRNA was identified only at 11.5 days of gestation, but its protein was found 1 day later (E12.5) (Shiojiri et al. 1991). AFP-mRNA was detected in the ventral and dorsal endoderm. However, albumin-mRNA was detected only in explants generated from ventral endoderm containing presumptive cardiac mesoderm or after stimulation with different types and different concentration of FGFs (Jung et al. 1999; Gualdi et al. 1996). In this study, we demonstrate that not stimulated cultured cells derived from ventral foregut region already express albumin-mRNA, at the same time as AFP. The current study clearly demonstrates that the earlier detection (E10) of albumin-expression and production in the rat embryo is not due to the lack of expression, but more due to the less sensitivity of the previously used methods.

Previous histological studies failed to functionally investigate the synthesis and secretion of albumin in viable endodermal cells. Since albumin production represents a crucial parameter of liver function (Tessari 2003), the investigation of the development of this liver function is mandatory. In this study, we established a reliable and

sensitive method for the first time, to analyze the albumin synthesis and secretion capacity in endodermal cells generated from ventral foregut region. It seems that the machinery of albumin gene expression, synthesis and also the secretion acts at the earliest developmental stage, when a hepatic specification of endoderm appears.

The synthesis and secretion of fibrinogen was detected 2 days later. This difference may be due to the increased number of cells plated at E12, than at E10. The difference in the cell numbers is well visible on Fig. 8.

It was proved in the current study that the enzymes involved in albumin processing are also expressed in the ventral foregut endodermal cells (furin was demonstrated on Fig. 1F).

Serum albumin is synthesized as a larger precursor form: proalbumin, which undergoes proteolytic processing at a dibasic site by a hepatic proprotein convertase within the secretory pathway to generate the mature form. Although furin, a member of the subtilisin-like proprotein convertase (SPC) family, was thought to be the only candidate hepatic convertase for proalbumin, SPC family members other than furin were also suggested to be involved in proalbumin processing (Mori et al. 1999). In the ventral foregut endodermal cells besides furin the expression of PC 7/8 was also seen (not shown).

A further morphological proof for the existence of the secretory apparatus is the immunocytochemical detection of a Golgi-marker protein 58 K in HNF4alpha-expressing ventral foregut endodermal cells. 58 K was originally identified from the Golgi membranes isolated from rat liver. Upon subfractionation of the membranes, 58 K was identified as a peripheral membrane protein exposed to the cytoplasmic side of the Golgi (Bloom and Brashear 1989)

The endodermal cell populations, which develop into the hepatic bud, were identified after assessment of expression of endoderm and liver markers genes. Endodermal cells expressed HNF4alpha, Prox1, Beta-catenin, BMP4, Foxa2, and GATA4. These factors play a crucial role in endoderm development and during hepatogenesis (Molkentin et al. 1997; Narita et al. 1997; Bossard and Zaret 1998; Rossi et al. 2001; Burke and Oliver 2002; Duncan et al. 1994; Tian and Schibler 1991).

Albumin and AFP production in the developing rat liver

Previously, the production of albumin and AFP was analyzed at embryonic or fetal stages by direct measurement of serum concentration in yolk sac (Gitlin 1973). Serum concentration of AFP in fetal liver and yolk sac underwent a decrease followed by a rapid fall after birth to reach a low level in adult state. In contrast, albumin concentration increased from low levels early in fetal development to high levels after birth and through adult life (Abelev 1974; Ruoslahti and Terry 1976). The radioactive biosynthetic labeling method offered the only available option to simultaneously assess the synthesis and release of secretory proteins in viable hepatoblasts. During embryonic stage, albumin and AFP proteins were detected in intracellular and extracellular sites of hepatoblasts, the synthesis and secretion machinery was fully functional, and was comparable with that of adult hepatocytes.

Relationship between albumin- and AFP- producing cells with proliferating, and total liver cells in developing rat liver

At 12 days of gestation, the embryonic liver is formed; albumin- and AFP-genes are expressed by hepatoblasts, and they synthesize and secrete secretory proteins including albumin, AFP, and fibrinogen. In this study we demonstrated that during embryonic stage about 50% of liver cells are engaged in both albumin- and AFP-gene-expression. The ratio of albumin- and AFP-expressing cells to total liver cells did not change significantly during embryonic and fetal stages ($P = 0.4$ and 0.8 respectively, with one-way ANOVA). In addition, the ratio of albumin- and AFP-expressing cells to proliferating cells increased during embryonic stage. As it is seen on Fig. 5C the number of $PCNA^+$ cells decreased at fetal stages. At E18 the ratio of AFP-expressing cells to proliferating cells reached its maximum, while the ratio of albumin-expressing cells further increased. After E18 the number of PCNA-positive cells decreased, and more cells expressed albumin than AFP. In addition, at fetal stage another process was found to dominate the liver development, the increase of the cellular volumes.

The volume of the liver expands 84-fold during liver development, and this correspond to eight doubling of hepatoblasts (Greengard et al. 1972; Vassy et al. 1988). Already 2.22-fold increase of the total volume of albumin-expressing cells in vivo was observed between E12 and E14 in the current study. It is noteworthy that while an increase of liver size and an increase of the ratio of albumin- and AFP-expressing cells to proliferating cells occur, constantly about 50% of liver cells are engaged in production of albumin and AFP during liver development. In vitro the cell volumes at fetal stage were nearly 4-fold increased compared to embryonic stage (in vivo this difference was by 2-fold).

This study demonstrated that 10 days of gestation in the rat (0–4 somites) is the earliest gestational stage for emergence of functional hepatoblasts. The secretory function providing transport of abundant synthetic products like albumin for the organism is already detectable in the

ventral endoderm cells, and works at a comparable speed in embryonic hepatoblasts as in adult hepatocytes. Moreover, the albumin-processing enzymes are also present in the ventral foregut endodermal cells. During embryonic and fetal stages 50% of the liver cells were engaged with secretory functions, in the embryonic stage the main way of the increase of the liver volume was cell division, while in fetal stage the increase of the cellular volume played more important role than cell division. In fetal stage the cells with increased volume were still mononuclear. This is the first report showing that the main hepatocyte function precedes the emergence of the liver bud.

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