Characterization of foetal hepatic cells during rat liver development

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Abbreviations

AFP	Alpha-fetoprotein
ANOVA	Analysis of variance
APS	Ammonium persulfate
BEC	Biliary epithelial cells
BLDs	Bioartificial liver devices
BMPs	Bone morphogenetic proteins ,
CoH	Canals of hering
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CK7 and CK19	Cytokeratins 7 and 19
DAB	3,3`-Diaminobenzodine
DAPI	4'-6-Diamidino-2-phenylindole
DIV	Days in vitro
DEPC	Diethylpyrocarbonat
DOC	Deoxycholic acid
DMF	N-N-Dimethylformamid
EDTA	Ethylenediaminetetraacetic
Epo	Erythropoietin
E	Days of gestation
ER	Endocytoplasmic reticulum
FCS	Fetal calf serum
Foxa2	Forkhead box proetins A2
FGFs	Fibroblast growth factors
GER	Glatt endoplasmic reticulum
GATA4	GATA binding protein 4
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-CSF	Granulocyte colony-stimulating factor
IL6	Interleukin-6
IL6 rec	Interleukin-6 receptor
LM+SDS	Lysis Mix with Sodium n-Dodecyl Sulfate
LM-SDS	Lysis Mix without Sodium n-Dodecyl Sulfate
LDL	Low density lipoprotein
MEM	Minimal essential medium
Na-As-Bi-P	Naohtol-AS-BI-Phosphat
OSM	Oncostatin M
PCNA	Proliferating cell nuclear antigen
PFA	Paraformaldehyde

Prox1	Prospero-Related Homeobox- transcription Factor-1
RT-PCR	Reverse trancriptase-polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
RER	Rough endoplasmic reticulum
SCF	Stem cell factor
TNF-R1	Tumor Necrosis Factors- receptor 1
TEMED	N,N,N´,N´-Tetramethylethylenediamine
TCA	Trichloracetic acid

ABSTRACT

During embryonic development the embryonic/foetal liver is the site where hepatogenesis and hematopoiesis take place. Hepatoblasts have been partly characterized in several previous studies. However, their characterization in the developing liver has not been previously studied. Therefore, the purpose of the current work was to characterize, in a rat model, hepatoblasts in *vitro* and in *vivo* during liver development. We consider the progress from early developmental stage, 10 days of gestation (E10) when the liver first starts to develop, to adulthood.

Albumin and alpha-fetoprotein (AFP) are the main hepatic markers and are the earliest synthetic products of hepatoblasts during liver development. In this study, we established a reliable method for the first time using a sensitive radioactive biosynthetic labelling, to analyse the albumin and AFP synthesis and secretion capacity of endodermal cells derived from ventral foregut region (E10). It seems that the whole program controlling the regulation of gene expression, synthesis and secretion of albumin and AFP already acts at the earliest developmental stage, when specification of hepatic endoderm appears. The present study shows that explanted endodermal cells from ventral endoderm can express HNF4-alpha, Prox1, beta-catenin, BMP-4, Foxa2, and GATA-4.

In the second part of this work we were interested in the development of the liver after it was clearly identifiable as a separate organ (from E12 to adulthood). We demonstrated that during the embryonic and foetal stages about 50% of liver cells are engaged in both albumin and AFP gene expression. In addition the ratio of albumin and AFP producing cells to proliferating cells increases during embryonic stage. At 18 days of gestation the ratio of albumin and AFP producing cells to proliferating cells reaches its maximum.

Quantitatively we found that at 18 days of gestation, albumin and alpha-fetoprotein mRNA reaches a maximum and a high rate of synthesis and secretion of albumin and AFP was observed. Additionally, it was observed that at the embryonic stage (from E12 up to E16) albumin and alpha-fetoprotein were synthesized and secreted at different rates. From 18 days of gestation to birth the kinetics of synthesis and secretion of albumin is similar to the kinetics in mature hepatocytes.

In the rat embryonic (E12 and E14) and foetal (E18) liver three cell populations were identified. Two of these had a unipotent character, developing into either hepatic lineage (Prox1 positive cells/CK-19 negative cells) or into intrahepatic bile duct lineage (Prox1 negative/CK19 positive cells). The third population retained its bipotent character (CK19 and prox1 positive cells), being able to differentiate into hepatic or bile duct epithelial cells. At this stage CK- 7 was also first detected.

Lastly hematopoiesis in the embryonic liver was investigated. A high expression of genes coding for factors which regulate hematopoiesis, such as GM-SCF, G-CSF, SCF and Epo, was observed at 12 and 14 days of gestation.

1. INTRODUCTION

Liver transplants are currently the only available and effective treatment for patients with end-stage liver failure. There is, however, an increasing shortage of donor livers for clinical therapies. Improved cell therapies may serve as an alternative approach for the treatment of these patients, and could offer a ray of hope for many suffering from liver diseases. Recent studies have proposed different types of stem/progenitor cells for transplantation. Suggestions include: Embryonic stem cells (Kania et al., 2003); hematopoietic stem cells (HSCs) (Avital et al., 2002; Alison et al., 2004; Kang et al., 2005); mesenchymal stem cells (Avital et al., 2002; Alison et al., 2004; Kang et al., 2005; Hong et al., 2005; Teramoto et al., 2005); ovale cells, also referred to as intrahepatic adult stem cells (Lazaro et al., 1998; He et al., 2004; Matsusaka et al., 2000; Yoon et al., 2004); and hepatoblasts (Mahieu-Caputo et al., 2004; Rogler, 1997; Stamp et al., 2005). Furthermore bioartificial liver devices (BLDs), gene therapy and administration of exogenous factors to stimulate normal physiological responses have been discussed.

Hepatoblasts might also be a candidate for therapeutical use in liver failures. They have been partially characterized in several previous studies (Sigal et al., 1994; Tanimizu et al., 2004; Rogler, 1997; Stamp et al., 2005), but their characterization in the developing liver remains unclear. In the current study we focussed our attention on the characterization of hepatoblasts in *vivo* and in *vitro* in the rat, from early developmental stage (10 days of gestation, the time when the liver starts to develop) to adulthood.

1.1. The liver

1.1.1. Structure and function

The liver is a large parenchymal organ consisting of several separate lobes. Its weight (1.5-1.8 kg) represents about 2% of the total body weight in the human. The liver is the central organ of nutrient digestion and processing, where most of the individual metabolism occurs. Furthermore it is responsible for the synthesis of the serum proteins which regulate the oncotic pressure and the retention of water within the vessels. The liver is the only organ with two separate afferent blood supplies (Fig. 1). The hepatic artery provides oxygenated blood and the portal vein brings in venous blood rich in nutrients and hormones from intestine and pancreas (Desmet V.J. et al., 1994).The main cell types resident in the liver are hepatocytes , bile duct epithelium, stellate cells (Ito cells), kupffer cells, vascular endothelium, fibroblasts, and leukocytes (Ramadori and Saile, 2002).

1.1.2. Hepatocytes

The hepatocytes are one of the largest cells populations of the body. Individually they have a size of 20-30 µm with a volume of 11,000 μm^3 (estimations vary between 10,000 and 60,000 μm^3). Their size however can vary considerably depending on age, location, the blood flow and metabolic load. A hepatocyte is polyhedric and possesses 5-12 facettes. Of these, one to three is in contact with sinusoidal blood, whereas four to nine are in contact with the biliary poles of the neighboring cells.

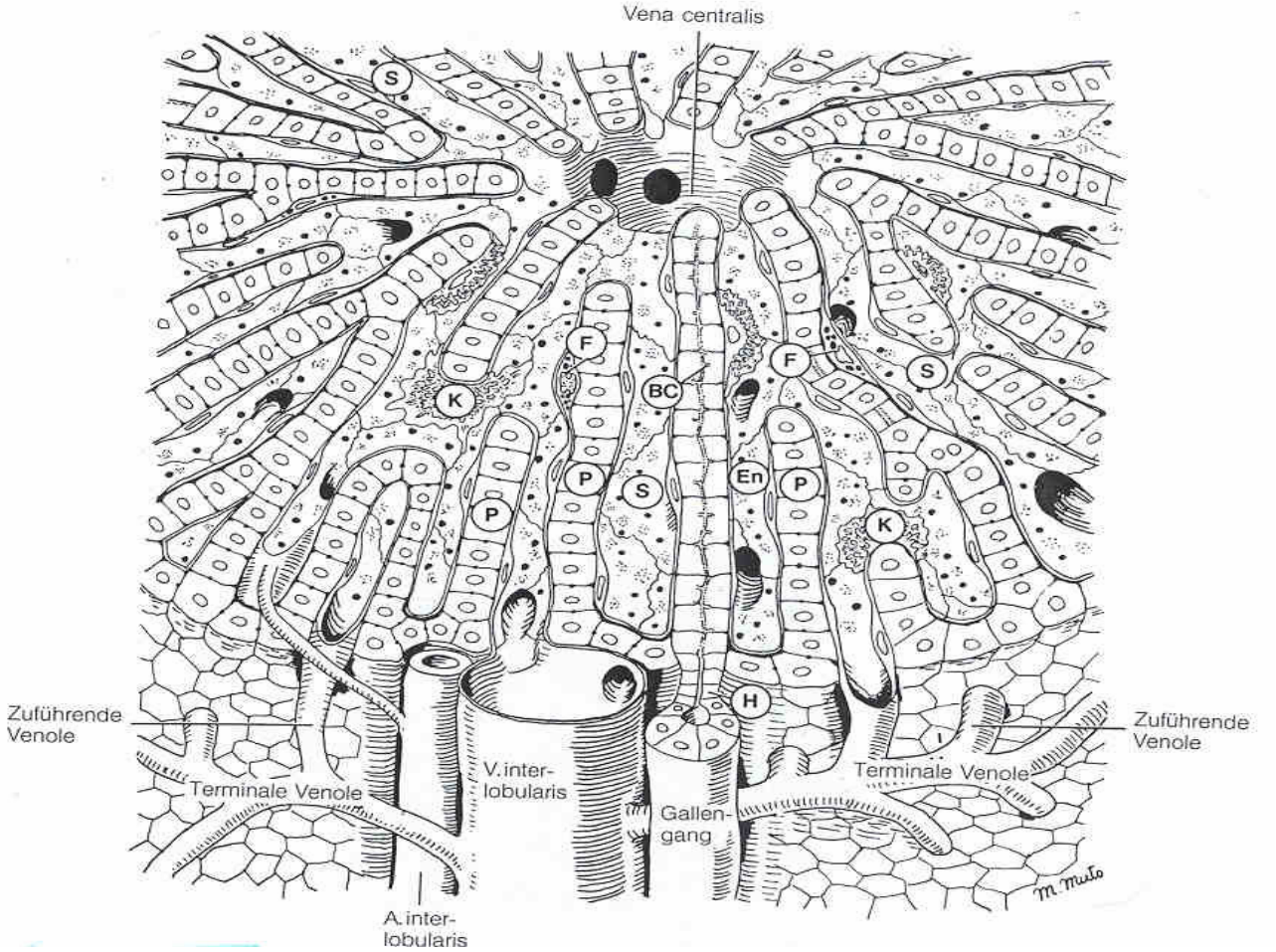


Figure 1: Three dimensional presentation display vascular supply and sinusoidal structure of the liver. Blood from Portal vein (V.interlobularis) and hepatic artery (A. interlobularis) enters the hepatic sinusoids one or two cells from the edge of the lobule, mixes in the sinussoids(S), delivers oxygen and nutrients to the liver cells, picks up carbon dioxide and metabolic products from the liver cells, and drains into the central vein (Vena centralis). Liver plate (P) is a single layer of hepatocyte. Endothelial (En) cells form walls of the sinusoids and make openings between sinusoids and hepatocytes. Kupffer cells (K) are located in the sinusoids and Ito cells (F) are located in spaces between endothelial cells and hepatocytes. Bile canaliculi (BC) drain bile into interlobular bile ducts in a direction opposite from the blood flow. H: canal of Hering (Grompe M. and Finegold M. J., 2001).

The hepatocyte represents the main cell type in the liver (about 90% of the weight of the liver). It is one of the most metabolically active cell types of the body. Their ability to manage several complex functions at the same time is due to the fact that they contain a large number of organelles. The most abundant are the endoplasmic reticulum (ER), mitochondria, lysosomes and peroxysomes (Fig. 2).

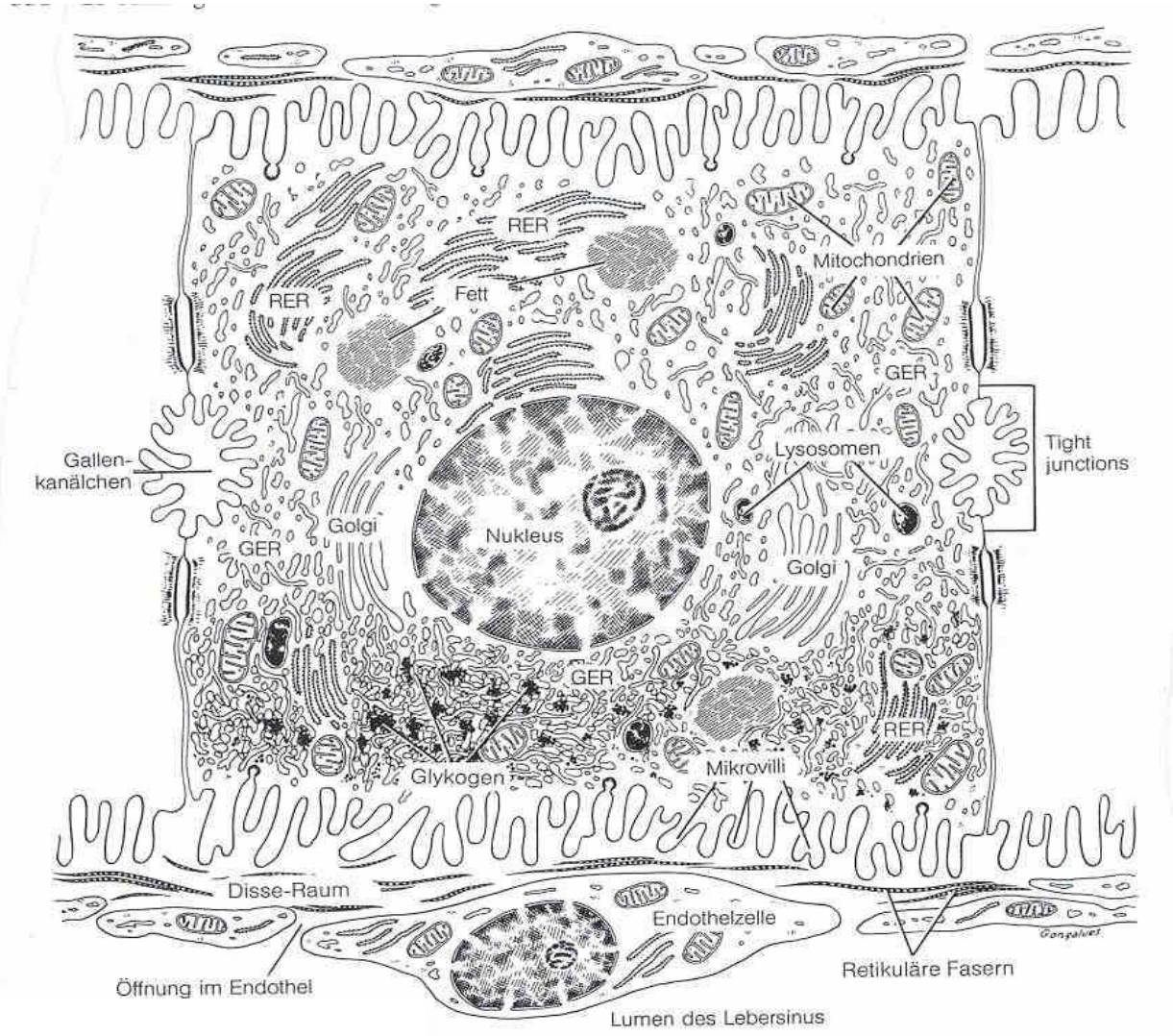


Figure 2: Structure of hepatocyte. *RER*: rough endoplasmic reticulum. *GER*: glatt endoplasmic reticulum. (Junqueira L. C. and Carneiro J., Histologie, 1996).

1.1.3. Physiology of the liver

The liver has many functions that involving storage, metabolism, production and secretion. Furthermore, the hepatocytes are involved in the synthesis of bile acids, bile formation, and the processing of absorbed nutrients and xenobiotics. They are also responsible for maintenance of glucose, amino acid, ammonia and bicarbonate homeostasis in the body. They participate in the synthesis of most plasma proteins, and the storage and processing of signal molecules.

1.1.3.1. Glucose metabolism

The liver plays a pivotal role in glucose metabolism of the organism. It regulates the blood glucose level by glycogenolysis or gluconeogenesis in case of need and glycogen synthesis or glycolysis and lipogenesis when it is present in excess. Several factors are responsible for controlling the reversible switch between glycogenolysis/gluconeogenesis and glycogen synthesis/ glycolysis, such as substrate concentrations, hormone levels, hepatic nerves, the hepatocellular hydration and zonal hepatocyte heterogeneity (Jungermann and Thurman, 1992; Kaiser, 1998). Glycogen synthesis and glycolysis are predominantly regulated by the portal glucose concentration, with insulin and parasympathic nerves being auxiliary factors. Glycogenolysis and gluconeogenesis on the other hand, are initiated by glucagon and sympathic nerves but inhibited by high portal glucose concentration.

1.1.3.2. Lipid/Lipoprotein and cholesterol

The liver plays a central role in synthesis and metabolism of fatty acids, lipids and phospholipids, which are delivered into the blood as plasmalipoprotein. The liver has also the ability to synthesize cholesterol.

The hepatocyte possess the low density lipoprotein (LDL) receptor, which is capable of cholesterol uptake. Excess of cholesterol is the basis for bile acid synthesis. It is directly shifted from the cell and secreted into the bile.

1.1.3.3. Amino acid, protein synthesis and ammonia metabolism

The liver participates in the amino acid homeostasis in the body. Excess amount of amino acids leads to their breakdown and utilization for protein synthesis as well as glycogen synthesis and simultaneously, inhibits amino acid generation by proteolysis. The liver continuously produces and secretes proteins including most of the plasma proteins. The major secreted protein is albumin (50% of the secreted proteins)(Quinlan et al., 2005), which is secreted rapidly and is difficult to find it intracellularly. The portal blood contains high concentration of ammonia, which is derived from the intestinal mucosa, glutamine and intestinal microorganisms. However, ammonia is also produced by the hepatocytes during the processing of amino acids. The detoxification of ammonia occurs by both liver-specific urea synthesis and glutamine synthesis.

1.2. Embryology of the rat and liver development

The embryonic development of the rat is similar to the vertebrates where the sperm cell penetrates into the ova (fertilization). After that, the chromosomes of the two germ cells merge forming the metaphase plate of the first cleavage spindle. After several cleavages the blastocyte stage is reached between 80 and 110 hours after mating. On the fifth day of gestation begins the implantation stage, in which the blastocyte loses the zona pellucida. During the sixth and seventh gestation days, the blastocyte approaches the uterine surface, increase in size and then adheres to the uterine epithelium in several places (Hebel R. and Melvin W., 1986).

The gastrulation begins on the eigth day of gestation (E8). It starts with the formation of the yolk sac and the three germ layers ecto, meso and endoderm. The formation of somites begins at the end of E9 and during E10. In 2 somites embryos, the foregut is a cone shaped elevation of the endoderm and the primordial germ cells are recognizable within the hindgut epithelium. At 10 day of gestation (4 somites stage), the foregut and hindgut become visible and the process of liver development starts. It begins with a connection of the foregut endoderm with the developing heart. Bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) signals arise from the septum transversum and the developing heart respectively, and transcription factors such as GATA, Foxa1 and Foxa2 (Zaret, 1996); Zhao et al., 2005; Serls et al., 2005; Deutsch et al., 2001) activate liver specific genes which regulate the hepatogenesis. On day 12 of gestation the liver is developed (Fig. 3).

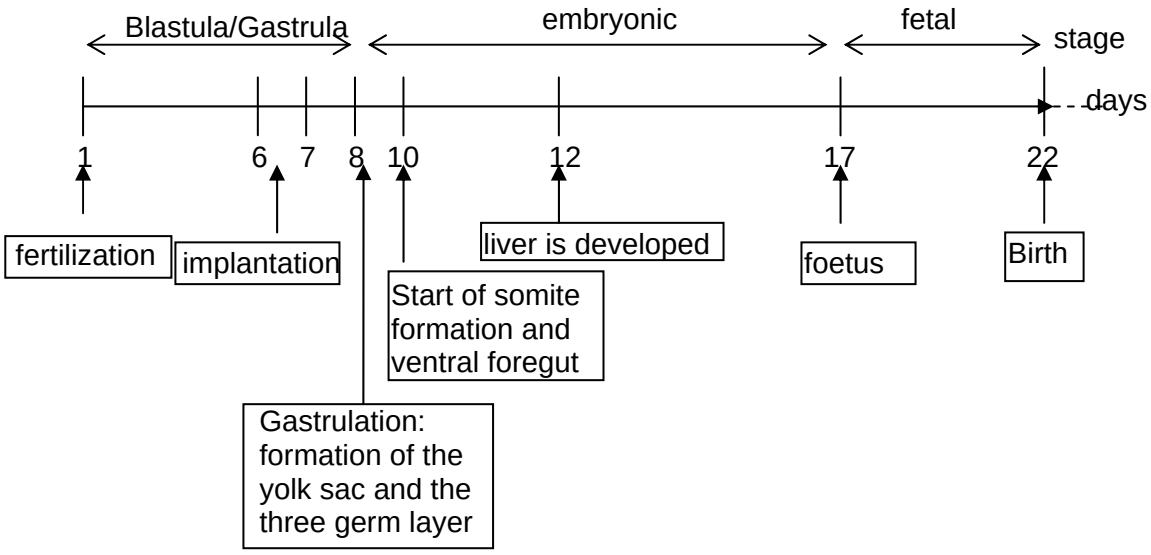


Figure 3: Embryonic development of the rat (Hebel R. and Melvin W., 1986).

1.3. Embryonic/foetal liver and hematopoiesis

The embryonic liver represents the major site of hematopoiesis during embryonic development (Chang et al., 2005; Fukuda, 1974; Chang et al., 2005)). Along with maturation of the bone marrow and spleen, hematopoietic cells relocate from the liver to their final destinations, while the liver starts organizing its own structure and develops numerous metabolic functions toward adult.

During primitive (or embryonic) erythropoiesis (from E9 to E13 in the rat), which takes place first in yolk sac and then continue in embryonic liver, erythrocytes are distinguishable from those in definitive (or adult) erythropoiesis (from 13 to 20 in the rat) by their morphology. Primitive erythrocytes are nucleated cells containing embryonic as well as adult hemoglobin, whereas definitive erythrocytes are small nonnucleated red cells committed only to adult hemoglobin synthesis (Lin et al., 1996)**.**

In yolk sac and early hepatic hematopoiesis, primitive macrophages develop from hematopoietic stem cells. They enter the blood stream and migrate into the embryonic liver before the initiation of hepatic hematopoiesis. They become mature and then transform into Kupffer cells. Myelopoiesis and monocytopoiesis are not active in yolk sac. Monocytic cells develop during hepatic hematopoiesis after the development of primitive macrophages, then move into the bone marrow in late ontogeny, forming a monocyte-derived macrophage population in tissues (Naito et al., 1990; Takahashi et al., 1996).

1.4. Specific markers for hepatic and intrahepatic bile duct phenotype

1.4.1. Albumin and alpha-fetoprotein (AFP)

Albumin and alpha-fetoprotein (AFP) are two secretory plasma proteins that represents the members of albuminoid gene family consisting of four members to date: albumin, vitamin D-binding (Gc) protein, alpha-fetoprotein and alpha-albumin (McLeod and Cooke, 1989; Lichenstein et al., 1994). The genes encoding albumin and AFP are located in the same chromosome, positioned close to each other and have a common direction of transcription, despite their different regulation process at the transcription level (Belanger et al., 1994; Sargent et al., 1981; Jagodzinski et al., 1981). Albumin and alpha-fetoprotein are highly homologous in primary structure. They consist of three homologous domains and perform similar functions like maintenance of oncotic pressure, binding and delivering of endogenous and exogenous compounds including fatty acids, metal ions, hormones and drugs to different tissues.

Albumin is a 66 kD, single chain polypeptide that is produced in liver and forms a large proportion of all plasma proteins. It is also produced by others organs such as mammary gland, tongue, intestine, lymph gland, testicle and uterus, but in less amount than the liver (Shamay et al., 2005).

Compared to albumin, alpha-fetoprotein is a single chain glycoprotein that is normally produced in the foetus during its development but its gene expression is repressed reversibly in adult liver. It can be expressed by oval cells during the course of liver regeneration induced by partial hepatectomy, when up to 2/3 of the organ is removed surgically or by acute CCl_4 intoxication that causes necrosis of the hepatocytes bordering central veins (Tournier et al., 1988), (Bisgaard et al., 1994). It can be used in adults as a tumour marker in the case of primary hepatic tumors. The

main product of AFP gene transcription in foetal liver is a 2.1 kb mRNA (corresponding to polypeptide chain of 68kD and 70kD) (Lemire and Fausto, 1991; Lazarevich, 2000). Other sequence of AFP mRNA (1.7kb, 1.4kb and 1 kb) were detected in foetal, regenerating liver and in carcinogenesis (Petropoulos et al., 1985), (Wan and Chou, 1989).

1.4.2. Prospero-related homeobox transcription factor-1 (Prox1)

Porspero-related homeobox 1 (Prox1) is a divergent transcription factor with two highly conserved domains, a homeobox and a prospero domain. It was first detected in the hepatic endoderm of the mouse at the 7 to 8 somites stage (E8.5) (Sosa-Pineda et al., 2000). In the endoderm, Prox1 expression is confined to a short segment that gives rise to liver and pancreas, where it remains expressed into adulthood (Burke and Oliver 2002; Dudas et al. 2004). Prox1 plays no role in hepatic specification. It is required for the migration of hepatocytes into the septum transversum. In *Prox1* deficient mice, the hepatoblasts fail to migrate into the neighbouring mesenchyme. They are abnormally clustered near the hepatic diverticulum, and remain invested by a continuous basal lamina. *Prox1* null mice die around E14.5, and show a 70% reduction of the liver size (Sosa-Pineda et al. 2000).

Prox1 is an early marker of hepatoblasts in mouse and rat embryos (Dudas et al. 2004). In both mice and rats Prox1 expression in hepatocytes persists into adulthood. It is not expressed in bile duct epithelial cells, which are positive for cytokeratin-7 (CK-7) (Dudas et al. 2004), nor is it expressed in liver endothelial cells, which are positive for CD31. The expression pattern of Prox1 is highly conserved in vertebrates and the human (Dudas et al. 2004).

1.4.3. Cytokeratins-7 and -19 (CK-7 and CK-19)

Cytokeratins are the largest subfamily of intermediate filament proteins and include more than 20 different gene products. Different epithelial types are characterized by the expression of specific cytokeratin (CK) subtypes. Cytokeratins can therefore serve as a 'lineage marker' of epithelial cells. In the liver CK-7 and CK-19 are the two cytokeratins expressed exclusively in the intrahepatic and extrahepatic bile duct epithelial cells. They are known to undergo extensive changes in expression with alteration of the hepatocyte phenotype *in vitro* (Sasaki et al., 2001; Van Eyken et al., 1987; Saunders et al., 2000).

The canals of Hering (CoH) begin in the lobules, are lined partially by cholangiocytes and partly by hepatocytes, and conduct bile from bile canaliculi to terminal bile ducts in portal tracts. They are not readily apparent on routine histological staining but are highlighted by the biliary cytokeratins CK-19 and CK-7. The canals represent the true hepatocytic-biliary interface that lies within the lobule and not at the limiting plate. The CoH are destroyed early in primary biliary cirrhosis, perhaps explaining lobular "hepatitis" in this disease. They may also be the primary sites of scarring in methotrexate toxicity. Most intriguingly, the CoH have been speculated to harbor intraorgan stem cells of the liver, perhaps forming the hepatic stem cell "niche" and have been demonstrated to proliferate in disease states (Saxena and Theise, 2004)**.**

1.5. Background

During rat embryogenesis the liver bud begins to develop at 10 days of gestation (E10), when the foregut and hindgut become visible followed by a connection of the foregut endoderm with the developing heart that leads to initiation of hepatic specification of the ventral foregut endoderm. Much

is known about how signaling molecules such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transcription factors such as GATA-4, Foxa-1 and Foxa-2 induce the liver development (Zaret, 1996); Zhao et al., 2005; Serls et al., 2005; Deutsch et al., 2001). The characterization of endodermal cells generated from ventral foregut which are involved in this process, has been neglected. In addition, the earliest embryonic developmental stage for the emergence of hepatoblasts that can express albumin and AFP remains controversial.

1.5.1. Kinetics for albumin and AFP mRNA expression during liver development.

Albumin and alpha-fetoprotein represent the main hepatic markers. Densitometric analysis after hybridization with albumin and AFP cDNA probes revealed that albumin and AFP mRNA could not be detected in the foregut region in rat embryos (Muglia and Locker, 1984). In contrast, AFP mRNA was clearly detected by *in situ* histological analysis in the ventral endoderm at E10.5 (10.5 days of gestation). At E11.5 both AFP mRNA and its coding protein were present in hepatoblasts. At this stage albumin mRNA was also identified but its protein product appeared one day later (at E12.5) (Shiojiri et al., 1991).

Recent experiments in mouse embryos from E8-E9.5 (corresponding to E9.5-E11 in the rat) provided much insight into albumin and AFP gene expression. Albumin becomes detectable by immunofluorescence in sectioned embryos from 9-10 somites stages as well as in single cell cytospin slides generated from dissected ventral foregut endoderm at 8, 10 and 14 somites (Serls et al., 2005). Tissue explants generated from ventral endoderm region at 4 to 6 somites stages have revealed that AFP mRNA is expressed in the ventral and dorsal endoderm. In contrast, albumin mRNA expression is induced through a co-culture with cardiac mesoderm (Gualdi et al., 1996) or after stimulation with different types,

and concentration of fibroblast growth factors (FGFs) (Jung et al., 1999; Deutsch et al., 2001); (Sekhon et al., 2004).

The embryonic liver appears on day 12 of gestation, the day when albumin and AFP mRNA as well as their corresponding proteins are expressed. It has been demonstrated that the expression of albumin and AFP transcripts change during liver development. AFP mRNA reaches a peak at 16 days of gestation and then decreases, while albumin mRNA reaches a peak at 18 days of gestation and persists at the following stages (Muglia and Locker, 1984). In other terms, it has been claimed that albumin gene expression increases gradually during liver development, and the ratio of AFP synthesis to albumin synthesis as well as the ratio of AFP mRNA to albumin mRNA suggests a gradual decrease during liver development (Petkov et al., 2004); Liao et al., 1980).

1.5.2. Distribution of albumin and AFP producing cells during liver development

During liver development albumin and AFP producing cells (hepatoblasts) undergo a proliferation process that leads to an increase in liver size (Micsenyi et al., 2004). It has been demonstrated using foetuses from 17 to 19 days of gestation and newborn animals that albumin and AFP genes are expressed simultaneously by all hepatocytes. However, in the first weeks of postnatal life albumin and alpha-fetoprotein genes are highly expressed by periportal and perivenous hepatocytes respectively (Poliard et al., 1986).

1.5.3. Synthesis and secretion of albumin and AFP

Albumin and AFP as secretory proteins are in general synthesized on polysomes bound to the rough endoplasmic reticulum (RER) and cotranslationally transferred to the lumen of this compartment. Proteins

are then transported from ER to the Golgi apparatus and secreted. (Lodish et al., 1983) and (Fries et al., 1984) have demonstrated it as a rate limiting step. The kinetics for the intracellular transfer of apoB-100 in comparison to albumin and transferrin was investigated in hepatoma cell-line HepG2 by using a pulse chase methods combined with subcellular fractionation (Bostrom et al., 1986).

1.5.4 Cytokeratin-7 and -19 expression during intrahepatic bile duct development

In several studies, the bile duct development is based on the expression of the main bile duct marker (cytokeratin-7 and -19). The early hepatoblasts are supposed to be bipotent, giving rise to hepatocytes and intrahepatic cholangiocytes. Intrahepatic bile ducts start to differentiate from periportal hepatoblasts, which express AFP and albumin at embryonic day E15.5 in the rat, some of those cells were CK-19 positive and AFP negative (Shiojiri et al. 1991). Cytokeratin-7 (CK-7) is expressed in biliary epithelial cells in the late gestational stage (Shiojiri et al. 1991; Pack et al., 1993). It has been claimed that in foetal rat liver, cells expressing cytokeratin-19 appeared at 17 and 18 days of gestation. However, the expression of cytokeratin-7 begins later than that of cytokeratin-19 and it is present only in cholangiocytes throughout pre- and postnatal ontogenesis (Kiiasov et al., 1997).

(Gall and Bhathal, 1989) have demonstrated that intrahepatic bile ducts develop by a reorganization and modulation of the periportal hepatoblasts to biliary epithelial cells (BEC). They thought that bile duct development takes place in the 19 day foetus around the larger branches of the portal vein, with the formation of lumina surrounded by cuboidal or elongated hepatoblast-like cells on the portal aspect and readily distinguished hepatoblasts on the lobular aspect. On 21 day of gestation these

structures had developed into canals of Hering lined jointly by recognizable liver cells and BEC. The number of canals of Hering per portal tract peaked on 22 day of gestation and diminished in number at birth. Bile ducts lined completely by BEC were first found on 20 day of gestation. At this developmental stage prekeratin antigens were first detected in duct-like structures not only in phenotypic BEC but also in adjacent cells with a hepatoblast phenotype. Such intermediate cells were present until birth.

1.5.6 Cytokines regulating hematopoiesis in embryonic and adult state

Hematopoiesis is controlled by different regulators, namely granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and Erythropoietin (Epo). GM-CSF and SCF play regulatory functions in the early steps of erythropoiesis (Chui and Loyer, 1975; Pillarisetty et al., 2003; Sansone et al., 1979; Praloran, 1991). GM-CSF enhances the differentiation of granulocyte. This GM-CSF mediated differentiation is associated with the up-regulation of G-CSF receptor mRNA (Collins et al., 2001).

Erythropoietin (Epo) is the main factor which acts positively on the last steps of the production of erythrocytes. It is specific for the erythroid progenitor cells and has only little effect on other cells. Erythropoietin acts on these progenitors through surface receptors specific for Erythropoietin and induces the proliferation and differentiation of erythroid progenitors leading finally to reticulocytes (Johnson and Barker, 1985; Wong et al., 1983). Macrophages play a supportive role in erythropoiesis. Erythroid colony-forming units adhere to a macrophage and differentiate to erythroblasts in the presence of erythropoietin (Epo), resulting in the formation of an erythroblastic island (Sasaki et al., 1993).

1.6. Objective of the work

The aim of this study is the characterization of embryonic/foetal liver cells during liver development, from the earliest stage of liver development (the time of hepatic specification) up to adulthood. Compared to mature liver, the embryonic/foetal liver were characterized as follows:

1) Cultured endodermal cells derived from ventral foregut region were used to investigate albumin and AFP synthesis and secretion. Radioactive biosynthetic labelling proved to be a very sensitive method for this purpose. The endodermal cells derived from ventral foregut region were further characterized by measuring the expression of hepatic and endodermal markers.

2) Cellular analysis was performed on the developing liver. The ratios of albumin and AFP expressing cells to both total liver cells and to proliferating cells were measured at all developmental stages.

3) The kinetics of synthesis and secretion of albumin and AFP in hepatoblasts at all developmental stages was assessed, with a view to estimating the secretion-velocity and to see whether the secretion apparatus works as in adult liver.

4) Hepatoblasts from 12 and 14 days of gestation were passaged three times in order to determine if their hepatic character was lost during the passage. For this purpose the expression of albumin and AFP was measured after each passage.

5) Hepatoblasts derived from embryonic and fetal stages were stimulated with interleukin-6 (IL-6), after which the synthesis and secretion of albumin

and AFP were examined. The expression level of interleukin-6 receptor mRNA (IL-6rec) was also measured in unstimulated hepatoblasts.

6) The bile duct development was investigated by assessing the *in vivo* and *in vitro* gene expression of prox1, cytokeratin-7 and -19 during liver development.

7) The expression of cytokines (granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and Erythropoietin (Epo) involved in the regulation of hematopoiesis was also examined during liver development.

2. MATERIALS AND METHODS

2.1 Animals

Pregnant Wistar rats (Rattus norvegicus, Sprague Dawley) 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-hour light/dark cycles and access to fresh water and till desired development stage achieved. The Rats were anesthetized by intraperitoneal injection of pentobarbital (400 mg/kg body weight). 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. In this study about 400 pregnant rats were used, corresponding to 5500 - 6000 embryos.

2.2. Chemicals, Enzymes and Kits

All chemicals, Enzymes and Kits used in this study are listed below.

Agarose	Invitrogen, Karlsruhe
Amplify^{TM} Fluorographic Reagent	GE Healthcare, UK
Acetone	Merck, Darmstadt
Ammonium persulfate (APS)	Merck, Darmstadt
Acetic acid	Merck, Darmstadt
Ampuwa water	Fresenius Kabi, Homburg
Acrylamid/Bisacrylamid	Sigma, München.
Bromophenol blue	Sigma, München
5-bromo-4-chloro-3-indolyl-phosphate (BCIP)	Roche, Mannheim

Blocking reagent (DIG Nucleic Acid Detection Kit)	Roche, Mannheim
BSA (bovine serum albumin)	Serva, Heidelberg
3-[(3-Cholamidopropyl)dimethylammonio] -1-propanesulfonate (CHAPS)	Biorad, München
Cesium chloride ($CsCl_2$)	Invitogen, Karlsruhe
Calcium chlorid ($CaCl_{2)}$.	Merck, Darmstadt
Collagen type I	Becton Dickinson (Bedfort, USA)
Citric acid –monohydrate	Sigma, München
Collagenase H	Boehringer Mannheim, 1074 032
DAB (3,3`-Diaminobenzodine)	Sigma, München
Dexametasone	Sigma, München
N-N-Dimethylformamide (DMF).	Carl Roth, Karlsruhe
Dextran sulfate sodium salt	GE Healthcare, UK
Digoxygenine-U-dNTP	Sigma, München
Dihydroxyaceton	Sigma, München
DMEM medium	PAA, Pashing
(Diethylpyrocarbonat) DEPC	Sigma, München
Dithiothreitol (DTT)	Sigma, München
DNase I (10000 Units)	Boehringer Mannheim
DNA Molecular Weight Marker XVII, 500bp Ladder	Boehringer, Mannheim
Deoxycholic acid (DOC)	Sigma, München
Ethidiumbromide	Sigma, München
(Ethylenediaminetetraacetic acid) EDTA	Merck, Darmstadt
Ethanol absolut p.A.	Merck, Darmstadt
80% Ethanol techn.(Alkopharm)	BrüggemannAlcohol, Heilbronn
Fetal calf serum (FCS)	Sigma, München

Formamide	Merck, Darmstadt
Fastlane Cell cDNA kit	Qiagen, Hilden
Formaldehyd 37 %	Merck, Darmstadt
Fluoromount-G	Southern Biotechnology
Full Range Rainbow protein molecular weight marker RPN 800	GE Healthcare, UK
Fluorescein-U-dNTP	Roche, Mannheim
D-Glucose	Merck, Darmstadt
Glucagon	Sigma, München
L-Glutamine	Fulka, Buchs Schweiz
Guanidinthiocyanate (GITC)	Invitrogen, Karlsruhe
Glycine	Sigma, München
Glutaraldehyde	Carl Roth, Karlsruhe
Glucoseoxidase	Sigma, München
10% goat serum	DAKO, Glostrup, Denmark
HCl (5N)	Merck, Darmstadt
HEPES	Sigma, München
Hydrogen peroxide 30% (H_2O_2)	Merck, Darmstadt
HybridoMed DIF 1000 medium	Biochrom KG, Berlin
Herings-Sperm DNA	Roche, Mannheim
Rat Interleukin-6(IL-6)	Pepro Tech Inc, NJ USA
Insulin	Sigma, München
Isopropanol	Merck, Darmstad
Kaisers-Glyceringelatine	Merck, Darmstadt
Levamisol	Sigma, München
6X Loading Dye Solution	MBI Fermentas
Mixed Bed Resin, AG 501-X8 (D)	Bio-Rad, München
Minimal essential medium (MEM) Hanks standard with stable Glutamine	Promocell, Heidelberg

Magnesium chloride ($MgCl_2$)	Merck, Darmstadt
L-methionine	Sigma, München
(^{35}S)Radioaktives methionine	GE Healthcare, UK
Methanol	Merck, Darmstadt
β-Mercaptoethanol	Merck, Darmstadt
Mayers Hemalaun solution	Merck, Darmstadt
M-MLV Reverse Transcriptase (RT) Kit	In vitrogen, Karlsruhe
5x first strand buffer	In vitrogen, Karlsruhe
DTT Dithiothreitol	In vitrogen, Karlsruhe
Oligo (dT)12-18 (500µg/ml)	In vitrogen, Karlsruhe
dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP)	In vitrogen, Karlsruhe
MMLV RT	In vitrogen, Karlsruhe
Naphthol AS-BI-phosphate disodium salt (Na-As-Bi-P)	Sigma, München
Narcoren	Merial, Hallbergmoos
Nitroblue tetrazolium salt (NBT)	Roche, Mannheim
N,N,N´,N´-Tetramethylethylenediamine (TEMED)	Sigma, München
New fuchsin	Sigma, München
Qiagen-II-Gel-Extraction-Kit	Qiagen, Hilden
PBS without Ca++, Mg++	Biochrom KG, Berlin, Germany
Taq PCR Master Mix	Qiagen, Hilden
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma, München
Penicillin/Streptomycin	Sigma, München
Paraformaldehyde (PFA)	Merck, Darmstadt
Propandiol	Merck, Darmstadt
Proteinase K	Roche, Mannheim

Protein A	Roche, Mannheim
Potassium Chloride (KCl)	Merck, Darmstadt
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt
Rnase inhibitor	Roche, Mannheim
RNase A	Roche, mannheim
RPMI medium (Methionine-free)	Biochrom KG, Berlin
Scintillation liquid	Zinsser Analytic, Francfurt
Sodium Dodecyl Sulfate (SDS)	Karl Roth, Karlsruhe
Sarcosyl (N-Laurylsarcosine	Sigma, München
Sodium-Acetate	Sigma, München
Sodium citrate	Sigma, München
Sodium Chloride (NaCl)	Merck, Darmstadt
Sodiumhydroxid (NaOH) pellets.	Merck, Darmstadt
Sodium azide	Merck, Darmstadt
di-sodium hydrogen phosphate (Na_2HPO_4)	Merck, Darmstadt
Sodiumhydroxide (5N)	Merck, Darmstadt
Sodiumnitrite (NaNO2)	Merck, Darmstadt
SYBR® Green PCR Mastermix	Invitrogen, Karlsruhe
tRNA yeast	Invitrogen, Karlsruhe
TRIS-HCl	Serva, Heidelberg
TRIS-Base	Carl Roth, Karlsruhe
T7 RNA Polymerase (5000 Units)	Roche, Mannheim
Triton X-100	Sigma, München
Tri-sodium citrate	Merck, Darmstadt
Trichloracetic acid (TCA)	Merck, Darmstadt
Trypan blue	Sigma, München
Tissue.Tek O.C.T.^{TM} Compound	SakuraFinetek, Zoeterwounde NL
10 x Trypsin – EDTA	Gibco (Karlsruhe, Deutschland)

Polyoxyethylenesorbitan monolaurate (Tween®20)	Sigma, München
William's E Medium	Biochrom KG, Berlin

2.3. Antibodies

Anti-alpha-Fetoprotein rabbit polyclonal	Dako, Glostrup, Denmark
Anti-albumin antibody rabbit polyclonal	Dako, Glostrup, Denmark
Anti-cytokeratin (CK-7) mouse monoclonal	Dako, Glostrup, Denmark
Anti-connexin 32 mouse monoclonal	Alpha Diagnostic Int, USA
Anti- cytokeratin (CK-19) mouse monoclonal	Novocastra, UK
Anti-HepPar1 mouse monoclonal	Dako, Glostrup, Denmark
Anti-Dioxygenine-alkaline phosphatase conjugated (Anti-DIG-AP)	Roche, Mannheim
Anti-Fluorescein-alcaline phosphatase conjugated (Anti-FL-AP)	Roche, Mannheim
Anti-NF4alpha antibody rabbit polyclonal	Santa Cruz, California
Anti-proliferating cell nuclear antigen antibody (anti-PCNA) mouse monoclonal	Novocastra, UK
Anti-Prox1 antibody rabbit polyclonal	Strathmann, Hamburg
Anti-mouse horseradish peroxidase (HRP)-conjugated antibody	Dako, Glostrup, Denmark
Alexa 555-conjugated secondary anti-rabbit antibodies	Molecular Probes, Leiden, Netherlands
Alexa 488-conjugated secondary Anti-mouse antibodies	Molecular Probes, Leiden, Netherlands
4'-6-Diamidino-2-phenylindole (DAPI)	Molecular Probes,Leiden, Netherlands

2.4. Oligonucleotides

General PCR reactions for preparation of riboprobes used for *in situ* hybridization and for quantitative analysis of genes expression were carried out with sequence-specific primers from Invitrogen (Karlsruhe, Germany). cDNA was constructed using Oligo(dT)$_{12\text{-}18}$ primer (Invitrogen, Karlsruhe, Germany). All primers used are listed below.

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

Table1: List of primers used for preparation of albumin and AFP sense and antisense (For = Forward, rev = reverse). 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

Table 2: Primers used for quantitative analysis by real-time PCR analysis (For = Fotward, Rev = reverse)

Primer	Sequence
Albumin For	GGATTCCAAAACGCCGTTCT
Albumin Rev	CCTCAGTGGCGAAGCAGTTATC
AFP For	GGCGATGTCCATAAACACGTTC
AFP Rev	CCGGTTTGTCGCCATTTTC
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-cateninRev	GGA TGC CGC CAG ATT TAA AGA T
Prox1 For	GCTCCAATATGCTGAAGACC
Prox1 Rev	ATCGTTGATGGCTTGACGTG
BMP4 For	TTCCCTCAAGGGAGTGGAAATTC
BMP4 Rev	CCATCGTGGCCAAAAGTGA
GATA-4 For	TTG ATC TCC GTT TTC GCG AC
GATA-4 Rev	GCT CCC CTT TAT TTG CAA GTC A
Foxa2 For	CAT GGT GAA ATC CAG GTC TCG
Foxa2 Rev	TGG AAC TCT GGC ATT CTA GCC
Interleukin receptor For	TTGCAATTCGAGCTTCGATACC
Interleukin receptor Rev	TCGCAAGGCATCATGGATG
Ribosomal 18S For	CGGCTACCACATCCAAGGAA
Ribosomal 18S Rev	TTTTCGTCACTACCTCCCCG
GAPDH For	TCC TGC ACC ACC AAC TGC TTA G
GAPDH Rev	TTC TGA GTG GCA GTG ATG GCA
GM-CSF For	GCTCTGGAGAACGAAAAGAACG
GM-CSF Rev	TGCTTGTATAGCTTCAGGCGG
G-CSF Rev	AGGCACTTTGTCTGCTGCAAG

SCF For	AACCTGCAGCCCGTAGTTTA
SCF Rev	AGTGGCTGATGCTACGGAGT
Erythropoietin For	TCCCACCCTGCTGCTTTTACT
Erythropoietin Rev	CCCATTGTGACATTTTCTGCC

2.5. Other Materials

Dako pen, Dako, Glostrup, Denmark
24-well plates, Petri dishes (100 mm), Greiner, Frickhausen, Germany
6-well plates, 96-well microtiter plates, Lab-Tek chamber slides, Nunc, Naperville, IL,USA
Cover-slips, 24x55 mm, Menzel-Gläser, Braunschweig, Germany
Culture dishes (35, 100 and 150 mm), Becton Dickinson Labware, Lincoln Park, NJ, USA
Intravenous cannula with injection port, Braun, Melsungen, Germany
Intravenous cannula with injection port, Klinika Medical, Usingen, Germany
Latex powder-free gloves, Kimberly-Clark, Zaventem, Belgium
Microscope glass slides, 76x26 mm, Menzel-Gläser, Braunschweig, Germany
Nitrile gloves Nitra Tex, Ansell, Kulim, Malaysia
Nitrocellulose Transfer Membrane, Sartorius, Göttingen, Germany
Polyallomer thin-walled centrifuge tubes (5 ml), Beckman, Munich, Germany
Safe-Lock tubes (0.2, 0.5, 1.5 and 2 ml), Eppendorf, Hamburg, Germany
Scintillation vials (5 ml), Zinsser Analytic, Frankfurt, Germany
Serological pipettes (2, 5, 10, 25 ml), transfer pipettes, plastic tubes (15 and 50 ml),
Sarstedt, Nümbrecht, Germany

Sterile filter Nalgene, 0.2 µm, Sartorius, Göttingen, Germany
Sterile filter pipette tips, Biozym, Oldendorf, Germany
Whatman 3MM paper, Schleicher and Schuell, Dassel, Germany
X-ray films HyperfilmTM, Amersham Biosciences, Freiburg, Germany
X-ray films X-Omat AR, Kodak, Rochester, NY, USA
X-ray films, Fuji, Düsseldorf, Germany
X-ray films, Konica, Hohenbrunn, Germany

2.6. Instruments

Automatic pipettes, type Reference®, Eppendorf, Hamburg, Germany
Automatic pipettes, type Pipetman, Gilson, Bad Camberg, Germany
Bench-top centrifuges, high speed centrifuges, ultracentrifuges and rotors:

Beckman model J2-21 centrifuge Beckman rotor JE-6B	Beckman, Munich, Germany
Centricon T-2070 ultracentrifuge Centricon rotor TST55.5 – 55000 rpm	Kontron Instruments, Neufahrn, Germany
Eppendorf bench-top centrifuge, type MiniSpin 5415C	Eppendorf, Hamburg Germany
Hettich Mikro Rapid/K centrifuge Hettich Rotina 3850 centrifuge Hettich Rotina 48RS centrifuge Hettich Rotixa/RP centrifuge	Hettich, Tuttlingen, Germany
Dissection tools: Micro-Dissecting Scissors, Micro-Forceps	Martin Surgery, Tuttlingen
Minifuge GL centrifuge	Heraeus-Christ, Osterode, Germany

Sigma 3K30 centrifuge
Rotor Nr 12156 – 16500 rpm — Sigma Laboratory Centrifuges
Rotor Nr 12153 – 22000 rpm — Osterode, Germany

Digital photocamera Canon EOS D60 and software Remote Capture 2.5, Canon, Tokyo, Japan
Eagle Eye™ system with built-in ultraviolet emitter, video camera and frame integrator, Stratagene, Amsterdam, Netherlands
Electrophoresis power supply Power Pac 300, Bio-Rad, Munich, Germany
Electrophoresis power supply ST305, Invitrogen, Karsruhe, Germany
End-over-end rotator, W.Krannich, Göttingen, Germany
Gamma-counter, Mini-instruments, Burnham-on-Crouch, UK
Gas controlled incubators, Heraeus-Electronic, Hannover, Germany
Geiger hand gamma-counter, Berthold, Oak Ridge, TN, USA
Gel dryer, LKB Bromma, Bromma, Sweden
Hybridization oven, Biometra, Göttingen, Germany
Ice machine, Ziegra, Isernhagen, Germany
Liquid scintillation counter Wallac 1409, EG&G, Turku, Finland
Magnetic mixer with warming, type M21/1 Framo-Gerätetechnik, Eisenach, Germany
Microplate reader MRX, Dynatech Laboratories, Chantilly, VA, USA
Microscope Axioscop with fotocamera MC 100 Spot, Zeiss, Oberkochen, Germany
Microscope Axiovert 25, Zeiss, Oberkochen, Germany
Microwave, Whirlpool, Comerio, Italy
Mini-vertical gel electrophoresis unit Hoefer Mighty Small II, Amersham Biosciences, Freiburg, Germany
Peristaltic pump P-1, Amersham Biosciences, Freiburg, Germany
pH-Meter, Glas-Gerätebau, Bovenden, Germany
Pipette holder with safety valve, filter and wall holder; Hirschmann Laborgeräte, Eberstadt, Germany
Rocking platform, Biometra, Göttingen, Germany

Savant Speed Vac® concentrator, ThermoLife Sciences, Egelsbach, Germany
Scanning densitometer, Molecular Analyst, Bio-Rad Hercules, CA, USA
Sonicator Sonoplus HD 70, Bandelin, Berlin, Germany
Standard dual cooled gel electrophoresis unit Hoefer SE 600, Amersham Biosciences, Freiburg, Germany
Sterile bench, The Baker Company, Sanford, ME, USA
Thermomixer 5436, Eppendorf, Hamburg, Germany
Thermostat, Heraeus, Hanau, Germany
Thermostatic calculator, model 2219 Multitemp II, LKB Bromma, Bromma, Sweden
Transfer electrophoresis unit Hoefer TE 50X, Amersham Biosciences, Freiburg, Germany
Transfer electrophoresis unit Mini Trans-Blot®, Bio-Rad, Munich, Germany
Ultra-pure water system Milli-Q, Millipore, Molsheim, France
UV spectrophotometer, RNA/DNA Calculator GeneQuant II, Pharmacia Biotech, Freiburg, Germany
Vortex, Genie 2™, Bender and Hobein, Zurich, Switzerland
Vortex, with platform, Schütt Labortechnic, Göttingen, Germany
Water bath, W.Krannich, Göttingen, Germany
X-ray film cassettes 10x18, Siemens, Munich, Germany
X-ray film-developing machine SRX-101A, Konica Europe, Hohenbrunn, Germany

2.7. Embryos, hepatoblasts isolation and culture

2.7.1. Dissection of the embryos

Pregnant rats from different gestation stages were prepared under Narcoren anesthesia (pentobarbital (400 mg/kg body weight). 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 minimal essential medium (MEM) Hank´s standard with glutamine (preparation-medium I). 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 (preparation-medium II). The liver from E16, E18, E20 and new born were dissected macroscopically and collected in 1ml MEM Hanks with 25 mM Hepes, stable glutamine supplemented with 0.05% DNase and 0.05% collagenase H (Preparation-Medium III). A single cell suspension was obtained after two times incubation at 37°C for 15 min followed by several times pipeting with a melted 1 ml and 100 µl pipet tip. Cells were collected after centrifugation (243 g, 5 min, RT). Cell viability was assessed by trypan blue staining and cell number was determined in a Neubauer chamber. Cells were diluted in William´s E medium supplemented with 10% foetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% L-glutamine, 0.1 µg/ml insulin, 0.1 µg/ml glucagon and 0.1µg/ml dexamethasone. Subsequently cells were seeded onto collagen type I coated 24 well plate or lab-tecks 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, 0.1µg/ml insulin, 0.1 µg/ml glucagon and 0.1 ug/ml dexamethasone.

2.7.2. Medias

Preparation-Medium I:

Minimal Essential Medium (MEM) Hanks standard with stable glutamine

Preparation-Medium II:

Minimal Essential Medium (MEM) Hanks with 25 mM HEPES and stable glutamine, supplemented with 0.05% DNase

Preparation-Medium III:

Minimal Essential Medium (MEM) Hanks with 25 mM HEPES and stable glutamine, supplemented with 0.05% DNase and 0.05% Collagenase H

WE with 10% FCS culture medium:

William's E Medium (1x) with
10% fetal calf serum (FCS)
Dexamethasone (0.1 µg/ml)
Glucagon (0.1 µg/ml)
Insulin (0.1 µg/ml)
100 U/ml penicillin
100 µg/ml streptomycin
1% L-glutamine

DIF culture medium:

HybridoMed DIF 1000 (1x) with
Dexamethasone (0.1 µg/ml)
Glucagon (0.1 µg/ml)
Insulin (0.1 µg/ml)
100 U/ml penicillin
100 µg/ml streptomycin
1% L-glutamine

PBS-Buffer (with Mg^{2+}/Ca^{2+}):

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	7.5 mM
KH_2PO_4	1.47 mM
$CaCl_2$	1 mM
MgCl	0.5 mM

The pH of PBS-Buffer was set up to 7.4 with HCl. Finally, the buffer was filtered through 0,45 µm Stericup (Millipore) under sterile conditions and stored at 4 °C.

2.8. Primary hepatocyte isolation and culture

2.8.1. Isolation and culture

Hepatocytes were isolated from Wistar rats by a perfusion technique as described elsewhere (Ramadori et al., 1990). Briefly, the liver was shortly perfused with a calcium-free saline (Buffer I) and then with a solution

containing 0.05% collagenase H (Buffer II). The liver was removed and cell suspension was collected in Buffer III. Hepatocytes were seperated from non-parenchymal cells by centrifugation at 243 g for 5 min at 4°C. Cell viability was assessed by trypan blue staining and the cell number was determined in a Neubauer chamber. The hepatocytes (5 x 10^4 cells/cm^2) were plated onto collagen type I-coated 24 well plates or lab-tecks in DMEM medium suplemented with 4.5 g/l glucose 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. After 2 h, the medium was changed and the hepatocytes were kept in culture at 37°C in a humidified 5% CO2 incubator.

2.8.2. Buffers

All solutions for hepatocyte isolation were prepared in double distilled water and sterile filtered through 0.45 µm Stericup (Millipore) and stored at 4 °C.

Buffer I

NaCl	8.3 g/l
KCl	0.5g/l
HEPES	2.4g /l

pH was adjusted to 7.4 with HCl

Buffer II

NaCl	3.9 g/l
KCl	0.5 g/l
$CaCl_2\ 2H_2O$	0.7 g/l
HEPES	24 g/l

pH was adjusted to 7.6, before use 0.05% Collagenage H was added

Buffer III

NaCl	8.3 g/l
KCl	0.5 g/l
$CaCl_2\ 2H_2O$	0.18 g/l
HEPES	2.4 g/l

pH was adjusted to 7.4

2.9. Coating of plate with Collagen type I

Collagen type I (Becton Dickinson) was diluted in 0.02 N acetic acid. The plates were coated with the concentration of 10 µg/cm^2 and incubated for 1 h at 37°C. Afterwards the rest of acetic acid was removed and the plates were washed twice with sterile H_2O. The plates were air-dried under clean bench and stored at 4°C until use.

2.10. In situ hybridization

2.10.1. Preparation of the riboprobes

The riboprobes were prepared as digoxygenine and fluorescein labeled RNA. For single *in situ* hybridization both riboprobes (albumin and AFP) were labelled with digoxygenine. For double *in situ* hybridization albumin was labelled with fluorescein and AFP with digoxygenine.

2.10.2. First-strand cDNA synthesis using M-MLV reverse transcriptase (RT)

cDNA synthesis using a total RNA from E14 liver embryos was carried out as follows:

1 µg total RNA from E14 liver embryos dissolved in final volume of 10µl sterile, distilled H2O.

Heat mixture to 65°C for 10 min and quick chill on ice

Collect the contents of the tube by brief centrifugation

Add 8 µl 5 x first strand buffer

4 µl 0.1 M DTT

8 µl Oligo $(dT)_{12\text{-}18}$ (500 µg/ml)

8 µl 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP)

2 µl MMLV RT

Mix contents of the tube by pipetting gently up and down and incubate at 37°C for 1h

Inactivate the reaction by heating at 90°C for 5 min.

The cDNA can now serve as template for amplification in PCR.

2.10.3. Amplification of DNA by polymerase chain reaction (PCR)

AFP and albumin sense and antisense probes were prepared by polymerase chain reaction (PCR) using Taq PCR Master Mix (Qiagen). The used primers are listed in table 1. When T7 promoter 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

PCR reaction:

25 µl Taq PCR Master Mix
5 µl (5 µM) forward primer
5 µl (5 µM) reverse primer
5 µl cDNA
Volume was adjusted to 50 µl with sterile H_2O
The PCR was carried out for 40 cycles under the following conditions:

95°C	10 min	Denaturation step before the first cycle
95°C	30 second	Denaturation
65°C	1 min	Annealing
72°C	1 min	Extension
72°C	10 min	Final Extension

2.10.4. Agarose gel electrophoresis

For preparation of a 1.5% agarose gel, 1.25 g of agarose was dissolved by heating in 100 ml of 1X TAE buffer. For visualization of the bands, 8 µl of ethidium bromide (10 mg/ml) was added to the mixture. After mixing,

the gel was poured into the prepared gel plate. The samples were prepared for loading by mixing 25 µl of PCR product with 3 µl of 6 X loading dye solution (MBI Fermentas). After polymerization, the samples were loaded and the gel run was performed in 1 x TAE buffer at 100 V. The positive bands were excised and the amplified cDNA was purified by Gel Extraction Kit (Qiagen).

1 X Tris/acetate/EDTA (TAE) buffer:

Tris base 4.84 g
EDTA 0.36 g
Acetic acid 1.14 ml
Dissolved in 1l distilled H_2O
pH was adjusted to 8.3 with acetic acid

2.10.5. In vitro transcription

The riboprobes albumin and AFP (sense and antisense) were labeled by using *in vitro* transcription reaction. This was set up at room temperature in the following order.

1 µg PCR product (DNA)
2 µl Digoxygenine-U-dNTP or fluorescein-U-dNTP
2 µl 10 x transcription buffer
1 µl RNase inhibitor
2 µl T7-RNA polymerase (5000 Units)
add H_2O to make a final volume of 20µl and incubate the reaction at 37^oC for 2h. Incubation with 2 µl DNase I (10000 Units) at 37°C for 15 min. The reaction was stopped with 2 µl 0.2 M EDTA.

2.10.6. Whole-mount *in situ* hybridization with digoxygenine-labelled AFP riboprobe

Whole-mount *in situ* hybridization was essentially done as described previously (Harland, 1991) with modifications as reported by (Belo et al., 2000). 1 to 4 somites stages rat embryos (E10) were fixed immediately after dissection for overnight in 1 x PBS to pH 7.4 buffered 4% paraformaldehyde. Embryos were washed twice with PBSw and dehydrated through an increasing methanol gradient, and stored in 100% methanol at -20°C. Embryos were rehydrated through a reciprocal methanol gradient, and then washed three times for 5 min with PBSw on ice. Afterwards embryos were washed with 4.5 µg/ml proteinase K in PBSw for 30 min. The digestion was stopped by washing the embryos in freshly prepared glycine solution, followed by two successive washes each 5 min in PBSw at room temperature. The embryos were refixed in 0.2% glutaraldehyde / 4% paraformaldehyde for 1 h at room temperature and rinsed three times in PBSw. A wash for about 5 min was performed with 50% PBSw / 50% hybridization buffer, followed by 100% hybridization buffer. After 3 h prehybridization at 65°C, the hybridization was carried out with antisense and sense riboprobes at 200 ng/ml in hybridization buffer overnight.

2.10.6.1. Post-hybridization washes

After overnight 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 x SSC buffer, pH 4.5 (without removing hybridization buffer) were added and the wash was repeated twice by adding 2 x SSC buffer. The mix was removed and the embryos were further rinsed twice with 2 x SSC buffer, pH 7 / 0.1% CHAPS at 70°C for

30 min each time, followed by two successive Washes in maleic acid buffer, first for 10 min at RT and second for 30 min at 70°C. A final wash was carried out in PBS for 10 min at RT and in PBSw for 5 min at RT.

2.10.6.2. Blocking of embryos and antibody binding

The embryos were incubated in 1 ml 1x blocking solution for 2 h at 4°C with rocking. Afterwards, the embryos were incubated with antibody solution anti-digoxygenine overnight at 4°C with rocking.

2.10.6.3. Post-antibody washes and signal detection

After overnight incubation, the embryos were washed as follows:

1- Fast wash embryos with 0.1% BSA in PBSw

2- 5 washes, 45 min each, with 5 ml 0.1% BSA in PBSw

3- Wash twice, 30 min each in PBSw

4- Wash the embryos in detection buffer two times, 10 min each at RT with rocking.

5- Incubate the embryos in staining solution (NBT/BCIP) in the dark overnight at 4°C with slight rocking

6- Stop staining reaction by washing at least with three changes of PBS

7- After staining, dehydrate through methanol series and store in methanol at -20°C.

2.10.7. Single and double in situ hybridization

In situ hybridization experiments were performed according to a protocol described by (Harland, 1991) and (Pringle et al., 2003). The riboprobes were prepared as described previously. For single *in situ* hybridization both riboprobes (albumin and AFP) were labelled with digoxygenine. In

case of double in situ hybridization, AFP was labelled with digoxigenine and albumin with fluorecein. 10 µm sections of frozen embryos from developmental stages E12, E14, E16, E18 as well as new born 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. 100 µl hybridization buffer were 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 labelled antisense and sense riboprobes of albumin and AFP at 500 ng/ml in hybridization buffer.

2.10.7.1. Post-hybridization washes and signal detection

After overnight hybridization the sections were washed in the following order:

1- Two successive washes at 60°C, each 50 min, first wash with 2 x SSC and the second with 0.1 x SSC.
2- RNase treatment was achieved with 10 µg/ml RNase A (Roche) in 2 x SSC buffer for 30 min at 37°C,
3- A wash with 0.1 x SSC for 50 min at 60°C.
4- Incubation of slides with blocking solution for 30 min at RT
5- For a single *in situ* hybridization, sections were incubated with alkaline phosphatase-conjugated anti-digoxygenine antibody for 2 h at RT
6- Wash slides twice, 15 min each, in washing buffer at RT
7- Incubate slides with detection buffer for 5 min at RT
8- Incubate the sections in staining solution (NBT/BCIP) in the dark overnight at RT
9- After staining, wash slides in distilled water for 10 min at RT

Slides were covered using a pre-warmed at 60°C liquid Kaiser gelatin.

2.10.7.2. Deactivation of the first alkaline phosphatase enzyme

For double *in situ* hybridization, AFP mRNA positive slides (blue color) were further examined with alkaline-phosphate- conjugated anti-Fluorescein in an attempt to detect albumin expression. For this purpose alkaline phosphatase enzyme was deactivated by incubating the slides in washing buffer at 65°C for 30 min. After two rinses with washing buffer at RT, slides were treated with glycine-buffer (0,1 M glycine-HCl pH 2,2) for 30 min followed by a wash in washing buffer at RT. Slides were now ready for alkaline phosphatase-conjugated anti-Fluorecein antibody. The incubation with antibody and the wash- steps were carried out as described previously. Signal detection was assessed using alkaline phosphatase-development solution (red colour) (Neubauer et al., 1996). The slides were incubated overnight in alkaline phosphatase-development solution in the dark at room temperature followed by washing in distilled water for 10 min at RT.

2.10.8. In situ hybridization in explanted cells

In situ hybridization was also performed in 8-chambered lab-tecks after two days in cultue of hepatocyte, hepatoblasts and endodermal cells generated from ventral foregut endoderm. Fixation with 4% paraformaldehyde, prehybridization, hybridization and signal detection were performed as described previously.

2.10.9. Solutions and buffers

PBS:

PBS was mixed with 0.1% (v/v) DEPC, incubated overnight and then sterile autoclaved. Before use, PBS was retreated with fresh 0.1% DEPC.

PBSw:

PBS with 0.1% (v/v) Tween-20

Glycine solution:

0.2% (w/v) glycine was dissolved in PBSw

4% Paraformaldehyde:

4 g Paraformaldehyde were dissolved in 100 ml RNase free PBS under basic condition (500 µl NaOH) at 60 °C. pH was adjusted to 7.4

20x SSC Buffer:

3 M NaCl

0.3 M Tri-sodium citrate

dissolved in H_2O (treated with 0.1% DEPC), pH 7

Deionized formamide:

500 ml Formamide was mixed with 50 g mixed Bed Resin. The mixture was stirred for 30 min in the dark at room temperature. Afterwards, it was sterile filtered, dispensed into 50 ml aliquots and stored in the dark at -20°C.

Hybridization buffer:

50% (v/v) deionized Formamide

4 x SSC Buffer

10% (v/v) Herings-Sperm 10 mg/ml

10 mM dithiothreitol

0.05% (w/v) tRNA yeast

0.1% (w/v) dextran sulfate

dissolved in H_2O treated with 0.1% (v/v) DEPC

Maleic acid:

0.1 M Maleic acid

0.15M NaCl

dissolved in 1l H_2O

pH was adjusted to 7.5 by NaOH pellets.

Washing buffer:

0.3% (v/v) Tween dissolved in maleic acid.

10 x Blocking solution:

5 g Blocking reagent were dissolved in 50 ml maleic acid by stirring and heating at 65°C. 10 x blocking solution stock was autoclaved and stored at -20°C.

Antibodies solution:

The antibodies anti-digoxygenin-alkaline phosphatase conjugated (Anti-DIG-AP) and anti-fluorescein-alkaline phosphatase conjugated (Anti-FL-AP) were centrifuged for 5 min, at 10000 rpm. The supernatants were diluted to a concentration of 1:500 in 1x blocking solution.

Detection buffer:

100 mM tris-HCl

100 mM NaCl

dissolved in H_2O, pH 9.5

0.024% (w/v) levamisol was added fresh before use.

Staining solution:

250 µl nitroblue tetrazolium salt (NBT) and 188 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were dissolved in 60 ml detection buffer with Levamisol. For double *in situ* hybridization, alkaline-phosphatase-development solution was also used (Neubauer et al., 1996).

Alkaline phosphatase-development solution:

Solution A:

- 70 ml development buffer containing:

4.9 g Tris-Base

8.7 g NaCl

1.5 g Tris-HCl

pH was adjusted to 9.7 with 1 N HCl

Fill to 1 l distilled H_2O

- 25 ml propandiol

- 40 mg levamisol

Solution B:

50 mg Naphthol-AS-BI-phosphate disodium salt (Na-As-Bi-P) dissolved in 0.6 ml N-N-Dimethylformamide (DMF).

Solution C:

20 mg sodiumnitrite ($NaNO_2$) dissolved in 0.5 ml H_2O. Afterwards 0.2 ml Neufuchsin was added.

Alkaline phosphatase-development solution was freshly prepared as follows:

Solution C was mixed with solution A before adding solution B. pH was adjusted to 8.8 with 2N HCl. The mixture was filtered bfore use.

2.11. Proliferation assay using PCNA immunostaining

2.11.1. PCNA-staining by immunoperoxidase technique

PCNA immunohistochemistry experiment was performed by immunoperoxidase technique to detect proliferating cell nuclear antigen (PCNA) (Micsenyi et al., 2004). 5 µm cryosection of rat embryos from developmental stage E12, E14, E16, E18 as well as new born and adult liver were fixed with 4% paraformaldehyde for 30 min at RT followed by an incubation step in 70% ehtanol at -20°C. Subsequently the slides were rinsed twice in phosphate-buffered saline (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 inactivated after incubation in glucose solution for 30 min at 37°C. After two successive washes with PBS, sections were blocked with FCS for 20 min, washed again and incubated overnight at 4°C in PBS diluted (1:100) mouse anti-proliferating cell nuclear antigen (anti-PCNA) antibody. The next day, sections were washed and incubated with the secondary anti-mouse horseradish peroxidase (HRP)-conjugated antibody (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 hemalaun. After several washes with H_2O, the slides were covered using a pre-warmed at 60°C liquid Kaiser gelatin

2.11.2. Buffers

Citrate buffer:
1.92 g citric acid -monohydrate dissolved in 1l H_2O
pH was adjusted to 6 with 2 N NaOH

Triton buffer:
0.3% (v/v) Triton
0.03% (v/v) H_2O_2
Dissolved in PBS

Glucose solution:
0.1 mM Glucose
0.19 units Glucoseoxidase
1 M Sodium azide
Dissolved in PBS

2.12. Quantitative morphological studies

Quantitation of *in situ* hybridization of albumin and AFP and of PCNA immunohistochemical reactions were performed by counting of positive cells under microscope using a shaded ocular, or by application of Image J software (Wayne Rasband, NIH, USA), and relating the positive cells to the whole cell counts of the analyzed image area.

2.13. RNA extraction

2.13.1. Isolation of RNA by density-gradient ultracentrifugation

Total RNA was isolated from whole liver (*in vivo*) and cultured hepatoblats and hepatocyte (*in vitro*) at different development satge as well as from

new born and auldt liver 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). Frozen tissues as well as cells were homogenized in ice-cold GITC buffer. 2 ml of $CsCl_2$ buffer was poured into 5 ml polyallomer ultracentrifuge tubes (6 per preparation). The cleared guanidine lysed samples were carefully layered on top of the $CsCl_2$ buffer. The samples were centrifuged overnight (16 h) at 35,000 rpm in a Kontron TST55 rotor at 20°C. The supernatants were carefully removed by aspiration and the transparent RNA pellets were gently washed twice with 200 µl of 70% ethanol at room temperature. The pellets were reconstituted in 200 µl of RNase-free water by pipetting and transferred into sterile 1.5 ml reaction tubes. The procedure was immediately continued by RNA precipitation. The RNA was precipitated with 400 µl of 100 % ethanol in the presence of 20 µl sodium-acetate solution overnight at –20°C. The RNA precipitates were pelleted by centrifugation for 30 min at 12,000 rpm and 4°C. Supernatants were discarded and pellets were washed with 200 µl of ice-cold 70% ethanol to remove all traces of sodium-acetate. After subsequent recentrifugation as described, the supernatants were discarded and the pellets were air dried. Afterwards, the pellets were dissolved in 100 µl of RNase-free water. To determine the concentration and purity of the obtained RNA, the aliquot of RNA sample was diluted 1:100 in RNase-free H_2O and the extinction at 260 nm and 280 nm was measured spectrophotometrically (GeneQuant II, Pharmacia Biotech).

2.13.2. Buffers

0.25 M sodium citrate:

7.36% (w/v) sodium citrate solution was prepared in RNase Free H_2O

pH was adjusted to 7 with 0.25 M citric acid

(Guanidine isothiocyanate) GITC buffer:

Guanidine isothiocyanate	4 M
0.25 M sodium citrate	0.5% (v/v)
N-laurylsarcosyl	0.5% (w/v)

Dissolved in RNase-free H_2O

The solution was sterile filtered and stored in the dark at 4°C. 0.1 M β-Mercaptoethanol was added just before use.

CsCl buffer:

Cesium chloride	5.7 M
Sodium citrate	0.25 M
EDTA	0.5 M

Dissolved in RNase-free H_2O.

pH was adjusted with 0.25 M citric acid to 7.5; the solution was sterile filtered and stored at room temperature.

2 M Sodium-acetate solution:

16.4% (w/v) Sodium-acetat dissolved in Rnase-free H_2O

pH was adjusted to 5.4 with acetic acid

2.14. Real-time PCR analysis

For the quantitative analysis of albumin, AFP, HNF4alpha, beta-catenin, Prox1, Foxa-2, GATA-4, BMP-4, Interleukin-6 receptor (IL-6rec), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and Erythropoietin (Epo) specific transcripts, real-time RT-PCR was performed. Total RNA was extracted from cultured hepatoblasts (*in vitro*) and whole liver (*in vivo*) at different development stages. Their cDNA was obtained by using M-MLV Reverse Transcriptase (RT) Kit as described

previously. cDNA from explanted endodermal cells derived from ventral foregut and hepatoblasts at E12 was prepared by Fastlane Cell cDNA kit (Qiagen). Realtime PCR experiments were performed using an ABI Prism 7000 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 AppliedBiosystems) and it was normalized with the threshold PCR cycle-values of the endogenous control of ribosomal RNA 18S (Ct_{18S}), using the following formula:
$Q=2^{-\Delta Ct}$, where $\Delta Ct= Ct_{target}-Ct_{18S.}$

2.15. Fluorescent immunostaining

Immunofluorescence study was performed in 5 µm cryos-ections of rat embryos and livers removed from newborn and adult rats as well as endodermal cells generated from ventral foregut (E10) and hepatoblasts from developmental stage E12, E14, E18, cultured for two days on 8-chambered lab-Tecks were fixed in acetone for 10 min at room temperature and rinsed in phosphate-buffered saline. Blocking of non-specific binding with 1% (w/v) bovine serum albumin and 10% (v/v) goat serum (DAKO, Glostrup, Denmark) in PBS was carried out for 1 h at RT. Endodermal cells generated from ventral foregut and hepatoblasts from E12 were immunostained by rabbit polyclonal antibody against HNF4alpha (1:50) overnight at 4°C. In cryosection of embryos and hepatoblasts from different developmental stages a double immunostainings was performed. The first reaction was done overnight at 4°C with rabbit polyclonal anti-Prox1 antibody diluted to 1:500, rabbit polyclonal anti-AFP antibody diluted to 1:100, mouse monoclonal antibodies anti-cytokeratin (CK) 7 (1:50), anti-connexin 32 (1:100), anti-CK19 (1:100) and HepPar1 (1:50). Rabbit polyclonal antibodies were

detected with Alexa 555-conjugated secondary anti-rabbit antibodies 1:400 diluted in PBS. Mouse monoclonal antibodies were visualized with Alexa 488-conjugated secondary anti-mouse antibodies 1:200 diluted in PBS. Sections and lab-Tecks were counter-stained with DAPI, covered using Fluoromount and analysed with epifluorescence microscopes (Axioskop 50 or Axiovert 200M, with Apotome function) (Zeiss, Jena, Germany).

2.16. Quantitative analysis of Prox1-, CK19- and AFP-specific immunohistochemical reactions in embryonal and foetal rat livers

The sections of embryonal (E14) and foetal livers (E18) were stained simultaneously with monoclonal anti-CK-19 antibody and with polyclonal anti-Prox1 or anti-AFP antibodies. The antibodies were visualised as described above, CK-19 was detected in green, while Prox1 or AFP in red, and all cell nuclei were counter-stained with DAPI in blue. Images of red, green and blue channels were taken by an Axiovert 200M (Zeiss, Jena, Germany) microscope with the Apotome function, equipped by a high resolution Axiocam camera. Double positive cells were determined by images of combined red green channels. All single channels and red green combined channels were analysed, and positive cells were counted by the ImageJ software (Wayne Ras-band, NIH, USA). Single and double positive cell counts were related to all cell nuclei in the image (determined by DAPI staining), and expressed in average % + standard error of measurement (SEM) %. In all quantitative immunohistochemical analysis 15 random taken samples from the investigated sections were analysed.

2.17. Radioactive biosynthetic labelling, immunoprecipitation and SDS-PAGE analysis

2.17.1. Radioactive biosynthetic labeling with ^{35}-S Methionine

Newly synthesized proteins were radioactively labelled with ^{35}S-methionine as described before (Ramadori et al., 1990). Briefly, endodermal cells derived from venral foregut, hepatoblats at different development stages and hepatocytes were cultured in DIF medium for 2 days in 24 well-plate. After this, cells were washed 3 times with methionine-free RPMI medium and incubated in RPMI medium supplemented with ^{35}S-methionine (100 µCi/well) for 2 h. Cells derived from venral foregut were incubated overnight.

2.17.2. Preparation of cell-lysates and measurement of total labeled proteins after trichloracetic acid precipitation.

After labelling, supernatants were harvested and diluted to 50% with lysis mix with SDS (LM+SDS). The cells were lysed after freeze-thawing and scraping in lysis mix without sodium dodecylsulfate (SDS) (LM-SDS) suplemented 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 mix with SDS (LM+SDS). Supernatants and cell-lysates were stored till use at -80°C. The count of the total labelled proteins was measured after a trichloroacetic acid precipitation as follows:

1- 5 µl of cell-lysates and supernatants from each sample were transferred to a small piece of whatman paper
2- The whatman papers were air-dried

3- Incubated in 10% (w/v) trichloracetic acid (10% TCA supplemented with 0.75% (w/v) L-methionine) for 10 min at RT
4- Incubated in pre-warmed 5% (w/v) TCA supplemented with 1.5% (w/v) L-methionine for 15 min at 80°C
5- Two times wash with 5% (w/v) TCA supplemented with 1.5% (w/v) L-methionine at RT
6- Wash with 96% ethanol at RT
7- Wash with ethanol/acetone mix 1:1 at RT
8- Wash with 100% acetone at RT
9- The wathman papers were air-dried.
10- The wathman papers were transferred to scintillation tubes and then filled with scintillation liquid (5 ml per tube)
11- Measurement of Beta radioactivity

The measured radioactivity correspond to total labelled proteins, which incorporate ^{35}S- methionine.

2.17.3. Immunoprecipitation of albumin and AFP by polyclonal anti-albumin and anti-AFP antibodies.

2.17.3.1. Preparation of protein A

Protein A was prepared in lysis mix with SDS supplemented with 0.5% BSA. 1 x volume protein A was washed twice with lysis mix with SDS (LM+SDS) supplemented with 0.5% BSA and then resuspended in the original volume. Protein A is now ready for use.

2.17.3.2. Immunoprecipitation

For immunoprecipitation of albumin and AFP, 50 µl of supernatants and cell-lysates (with the same count of total labeled proteins precipitated by trichloroacetic acid) from labeled hepatocyte and hepatoblasts at different developmental stages were used. From labelled cells generated from ventral foregut endoderm, 500 µl of supernatants and cell-lysates were taken for analysis. The samples were incubated first with 50µl protein A for 30 min on ice. After centrifugation, the pellet was discarded and precleared samples were incubated with a monospecific rabbit polyclonal anti-albumin and anti-AFP overnignt at 4°C. Immunocomplexes were precipitated by adding 50 µl of protein A and incubation for 1 h on ice. Immunoprecipitates were sedimented, washed first once with lysis mix with SDS, suplemented with 0.5% BSA followed by 4 washes with lysis mix with SDS and finally resuspended in 25 µl loading buffer suplemented with fresh β-mercaptoethanol. The samples were stored for 5 min or overnight at -80°C.

2.17.3.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Immunoprecipitated albumin and AFP were analysed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to (Laemmli, et al., 1970). The samples were thawed, resuspended after long time vortexing and boiled for 5 min at 90°C. After high speed centrifugation for 15 min, pellet was discarded and supernatants were loaded on gel. The electrophoresis was performed at 200 V for 4 h. Afterwards the gels were fixed overnight with fixation solution, incubated for 1 h in Amplify™ Fluorographic Reagent, covered with Cell-glas (Cellophane) and then

dried using Gel Dryer System (Biorad). Dried gels were placed in X-ray film cassette and exposed to X-ray film.

Solution A:

18.2% (w/v) Tris-base dissolved in distilled H_2O, pH 8.8

Solution B:

30% (w/v) acrylamide and 0.8% (w/v) Bis-acrylamide dissolved in distilled H_2O, stored in the dark at 4°C

Solution C:

7.8% (w/v) Tris-HCl, pH 6.8

20% (w/v) SDS

40% (w/v) ammonium persulfate (APS)

Running buffer:

3.028 g Tris-Base

14.4 g Glycine

5 ml of 20% SDS

fill to 1 l distilled H_2O

Fixation solution:

460 ml methanol

80 ml acetic acid

460 ml distilled H_2O

SDS-PAGE	Running gel (12%)	Stacking gel (3%)
solution A	18 ml	X
solution B	28.4 ml	2.7 ml
solution C	X	2 ml
distilled H_2O	24.6 ml	15.2 ml
20%SDS	360 µl	100 µl
TEMED	20 µl	20 µl
40%APS	60 µl	20 µl

2.17.4. Reagents and Buffers:

Radioactive methionine: ^{35}S-methionine

RPMI medium without methionine:

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.

Lysis mix without sodium-n-dodecyl sulfate (SDS) (LM-SDS):

0.5% (w/v) deoxycholic acid (DOC)

1% Triton x-100

10 mM ethylene-diaminetetraacetic acid (EDTA)

Dissolved in 500 ml PBS. pH was adjusted to 7.4

Lysis mix with SDS (LM+SDS) :

5 g SDS was added to LM-SDS

PMSF(phenylmethysulfonylfluoride):

35.5 mg PMSF dissolved in 1ml ethanol; before use 10 µl was added to 1 ml lysis mix without SDS.

Protein A (sigma):

LM+BSA:

0.5% (w/v) BSA were dissolved in lysis mix with SDS.

Loading Buffer:

12.5 ml of 20 % (w/v) SDS
157 mg TRIS-Base,
21 ml glycerine,
Bromophenolblue,
add 100 ml H_2O, pH was adjusted to 6.8.
Before use, 950 µl loading Buffer was supplemented with 50 µl β-mercaptoethanol.

trichloroacetic acid:

10% TCA: 10% (w/v) trichloroacetic acid and 0.75%(w/v) Methionine dissolved in H_2O

5% TCA: 5% (w/v) trichloracetic acid and 0.15% (w/v) Methionine dissolved in H_2O

Ethanol and acetone:

96% ethanol

100% Acetone

Ethanol/acetone (1:1)

2.18. ^{35}Smethionine pulse-chase labelling

A pulse chase experiment was carried out according to the protocol described by (Tworkowski et al., 2002)**.** Hepatoblasts and hepatocyte from different developmental stages E12, E14, E16, E18, and adult were cultured in DIF medium for two days in 24 well-plate. The cells were washed three times with methionine free RPMI medium and then pulsed for 1 h in RPMI medium suplemented with 100 µCi/well ^{35}S-methionine. Subsequently three washes with PBS were performed to remove traces of radioactive methionine. The cells were further cultured in radioactive free RPMI medium suplemented with 2 mM L-methionine and incubated during a chase period of 15, 30, 45, 60, 90, 120, 240 min. At each period of chase, cell-lysates and supernatants were collected, diluted to 50% with lysis mix with SDS (LM+SDS) and stored till use at -80°C. Albumin and AFP were immunoprecipitated and analysed as described previously.
Pulse chase analysis was further performed in hepatoblasts at 12, 14, 16 and 18 days of gestation after 2 and 9 days in culture. The hepatoblasts were pulsed for 1 h with ^{35}S methionine and incubated in radioactive free RPMI medium supplemented with 2 mM methionine during a chase period of 2, 4 and 8 h. Albumin immunoprecipitation was performed as described previously. In this case immunocomplexes were not analysed by SDS gel electrophoresis (SDS-PAGE), synthesis and secretion of albumin was

analysed by measuring the radioactivity of albumin immunocomplexes using a beta counter and the ratio of count of albumin immunocomplexes to the count of total labelled protein was diagramed at each chase period.

2.19. Interleukin-6 (IL6) treatment

For Interleukin-6 (IL6) treatment, hepatoblasts from developmental stages E12, E14 and E18 were used. After two days in culture in 24 well-plate, the cells were treated simultaneously with 100 ng/ml interleukin-6 (IL6) and radioactive labelled with 100 µci ^{35}S-methionine per well overnight. Cell-lysates and supernatants were collected. Synthesis and secretion of albumin and AFP was examined by immunoprecipitation of albumin and AFP combined with SDS-PAGE as described previously. Interleukin-6 receptor (IL6rec) mRNA expression in untreated hepatoblasts from E12, E14 and E18 was measured by real-time PCR. RNA was extracted from cultured hepatoblasts, cDNA was obtained by using M-MLV Reverse Transcriptase (RT) Kit and realtime PCR experiment was performed as described previously. Primers for Interleukin-6 receptor (IL6rec) are listed in table 2. GAPDH was used as housekeeping gene.

2.20. Analysis of albumin and AFP gene expression in hepatoblasts after passage.

Hepatoblasts isolated at developmental stages E12 and E14 were kept in culture and passaged for three times. At each passage, the cells were cultured in 24 well-plate and labeled with 100 µci^{35}S-methionine per well. Cell-lysates and supernatants were analysed by measurement of synthesis and secretion of albumin and AFP. Immunoprecipitation of albumin and AFP combined with SDS-PAGE were performed as described previously. At each passage 100 µl supernatant and cell-lysates were used for immunoprecipitation of albumin and AFP. As positive control 25 µl supernatants and cell-lysates of labelled hepatocyte were

used for albumin precipitation. Furthermore, expression of albumin and AFP mRNA at each passage was analysed by real-time PCR. At each passage RNA was extracted and cDNA was prepared by using M-MLV reverse transcriptase (RT) Kit. Real-time PCR experiment was performed as described previously. Primers for albumin, AFP are listed in table 2. GAPDH was used as housekeeping gene.

2.21. Statistical analysis

The data were analysed using Prism Graph pad 4 software (San Diego, USA) and Microsoft Office Exel 2003. All experimental errors are shown as S.E.M. Statistical significance was calculated by ANOVA and student´s *t*-test. Significance was accepted at $P< 0.05$.

3. RESULTS

3.1. Morphological observations

Endodermal cells were isolated from ventral foregut region at 10 days of gestation. Detailed morphological and functional analysis of endodermal cells has not yet been performed so far. Therefore, in the current work studying morphological features of endodermal cells preceded subsequent experiments.

It was observed that endodermal cells were composed of cells which displayed different morphological features. After six months in culture, three cell-populations could be identified. First cell-population (cell-population I) consist of cells with polyhedric morphology. Some cells of them are diploid. Second cell-population (cell-population II) consists of cells with round structure and some cells displayed double-nuclei. Third cell-population (Cell-population III) consists of cells, which show fibroblast-like morphology with spindle-shaped appearance (Fig. 4).

The hepatoblasts were isolated from embryonic/foetal liver at developmental stages E12, E14, E16, E18, E20 and new born liver and then cultured on coated plate for two days. The hepatoblasts grew as colony surrounded by fibroblasts-like cells. We noticed that the volume of hepatoblasts increases and the number of chromosomes in hepatoblasts becomes doubled (polyploidy) during liver development (Fig. 5).

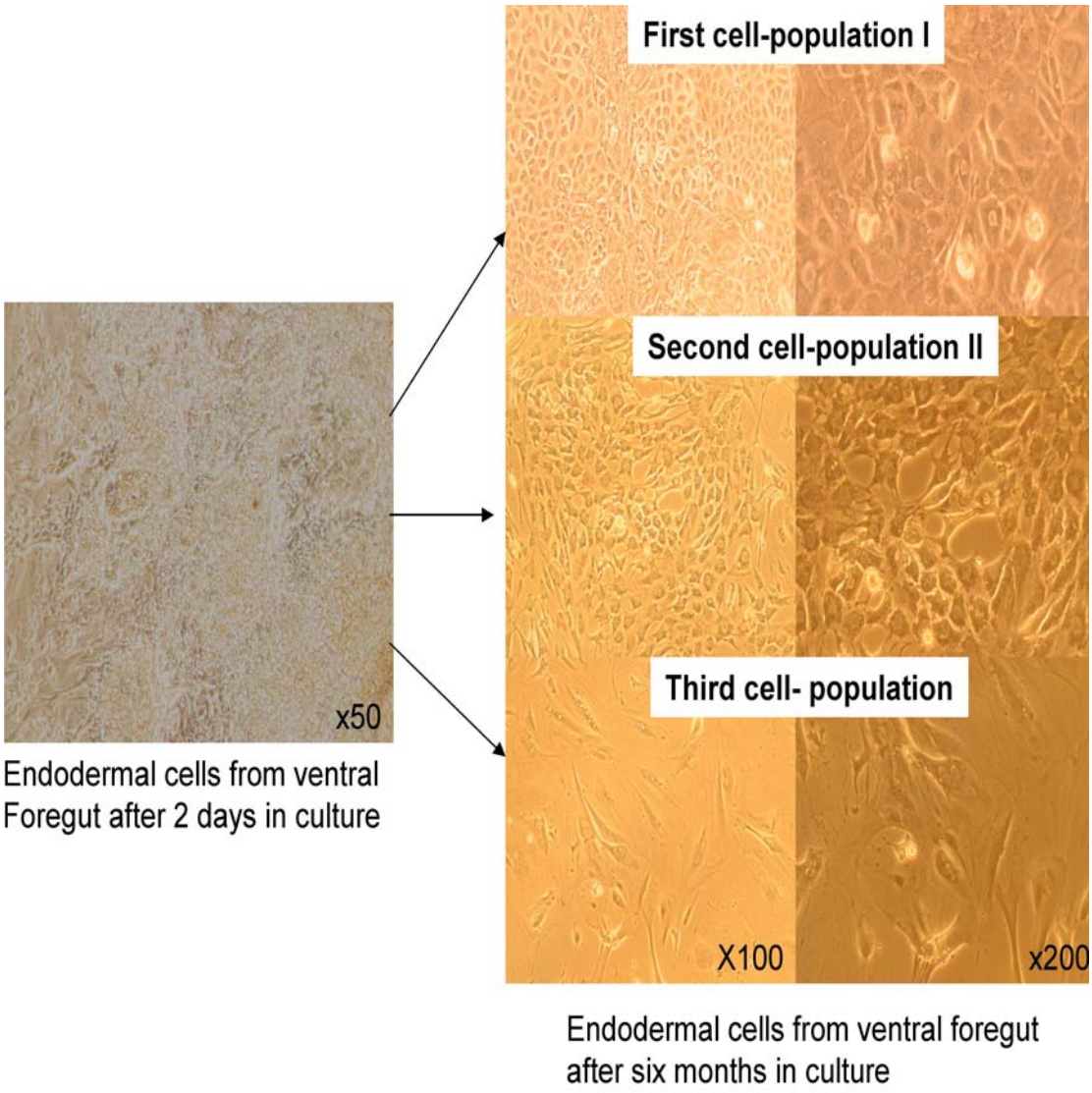


Figure 4: Morphological and cultural features of endodermal cells derived from ventral foregut region. Three cell-population were identified after six months in culture as assessed by phase-contrast microscopy. Original magnification x 50; x 100; x 200.

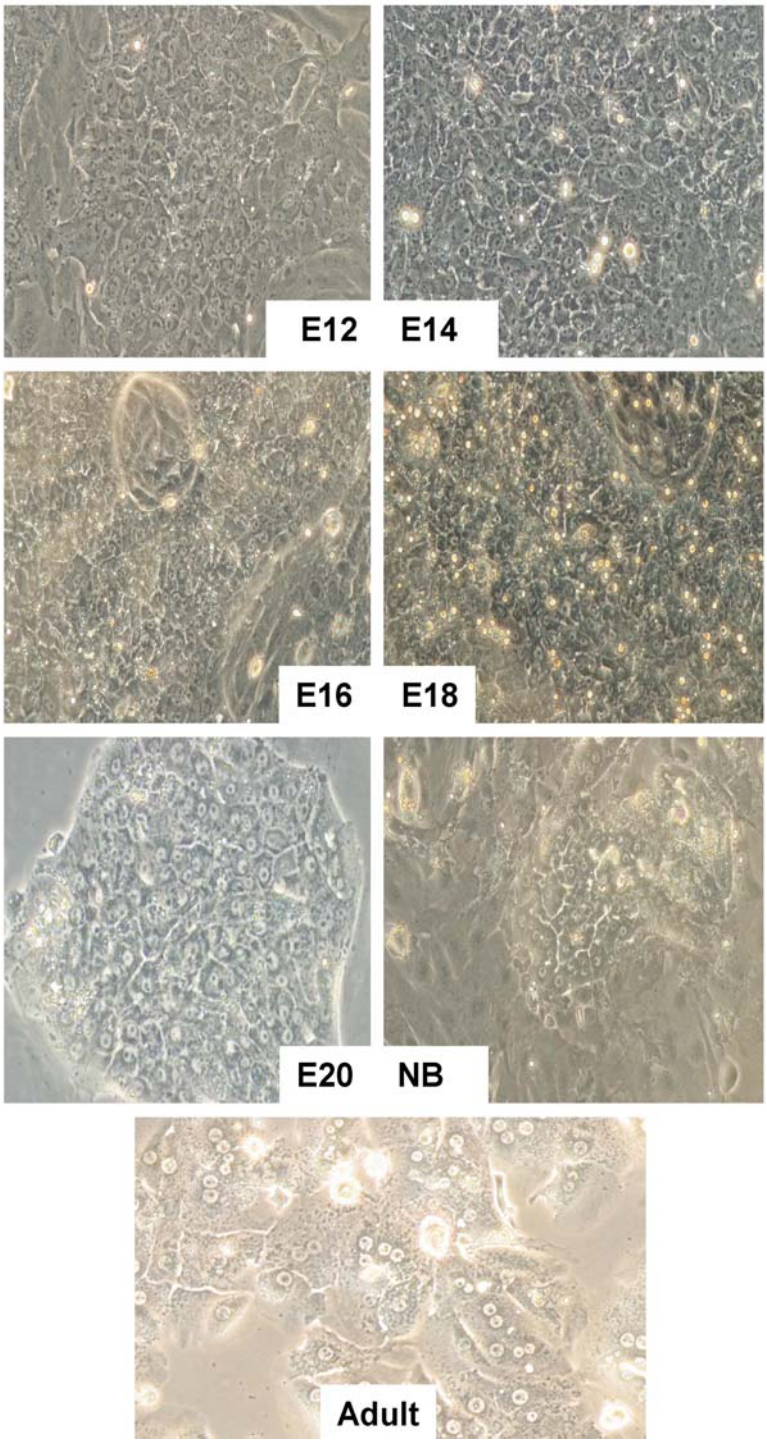


Figure 5: Phase-contrast microscopic pictures of primary rat hepatoblasts isolated at developmental stages E12, E14, E16, E18, E20 and primary hepatocytes derived from new born (NB) and adult liver. Original magnification x 200.

3.2. Albumin and AFP gene expression at the time of hepatic specification

During rat embryogenesis the liver bud begins to develop at 10 days of gestation (E10). This is the time when different signals like bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs) and transcription factors such GATA-4 and Foxa induce the differentiation of endodermal cells into embryonic hepatic cells (Serls et al., 2005; Deutsch et al., 2001; Zaret, 1996; Zhao et al., 2005). The first molecular evidence for liver development is the expression of albumin and AFP.

3.2.1. Identification of albumin and AFP mRNA expression in ventral foregut

At 10 days of gestation, the time of initiation of hepatic specification, AFP was strongly expressed in the ventral foregut region (Fig. 6A). A single cell suspension of ventral foregut endoderm was performed and the cells were cultured for two days. In vitro *in situ* hybridization revealed that the ventral foregut region contains albumin and AFP mRNA expressing cells (Fig. 6B), which are surrounded by cells which shown no albumin- and AFP-mRNA-expression. Albumin mRNA was expressed in cultured Hepatocyte, however, no reaction with the sense albumin probe was observed (Fig. 6C). Albumin and AFP mRNA expression in the ventral foregut region was confirmed with real time PCR experiment. As shown in figure 8 (A, B), the cells derived from ventral foregut region could express albumin and AFP mRNA.

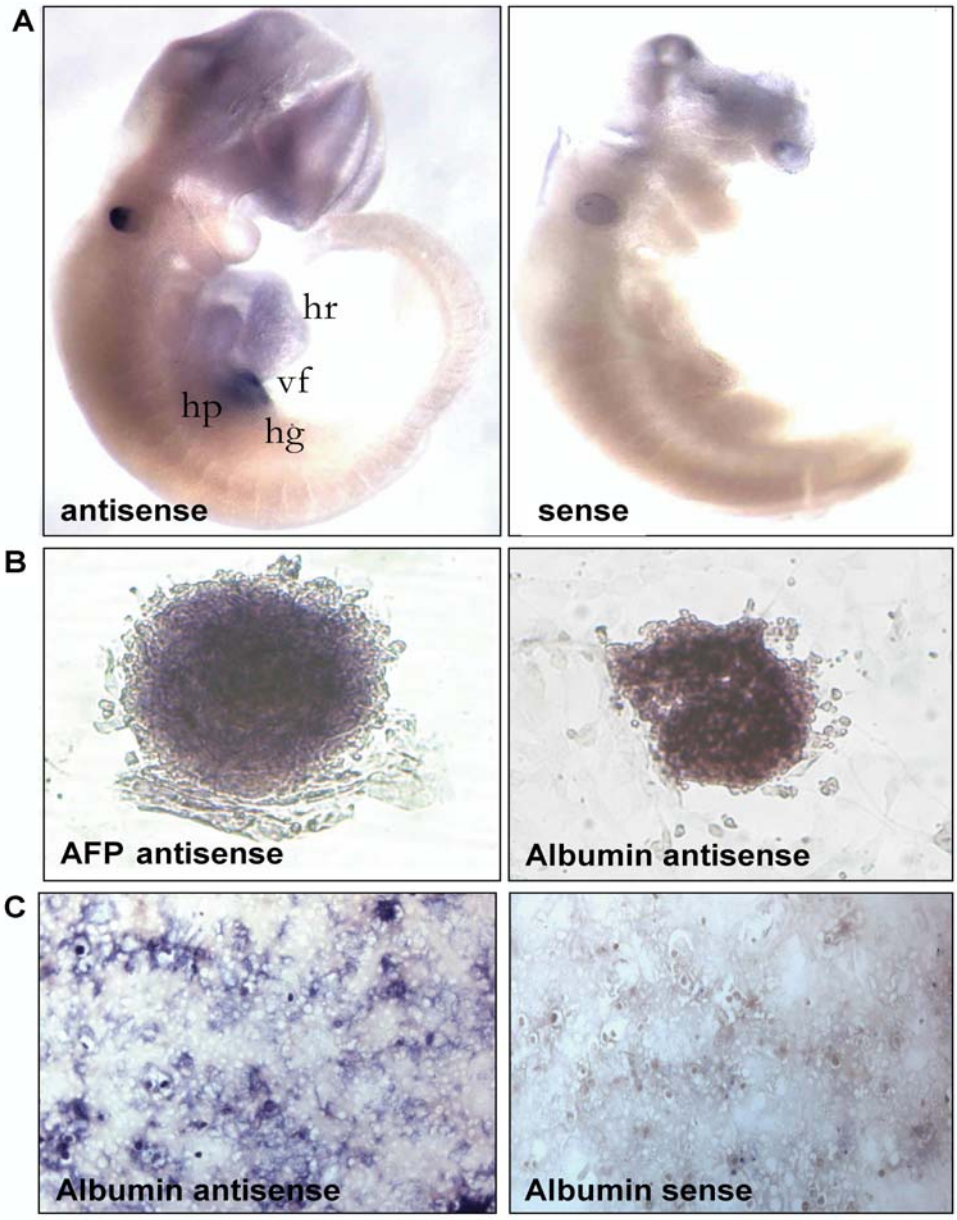


Figure 6: (**A**) AFP mRNA-Expression in ventral foregut region assessed by whole mount *in situ* hybridization. Albumin and AFP mRNA-expression in explanted endodermal cells (**B**) detected by in vitro *in situ* hybridization. Rat embryos at 10 days of gestation and cultured endodermal cells derived from ventral foregut were incubated with DIG-labelled sense and antisense probes of albumin and AFP as described in materials and methods. After incubation with alkaline phosphatase (AP)-conjugated anti DIG antibody, signals were visualised using BCIP/NBT. As positive control, the hepatocytes (**C**) were incubated with albumin sense and antisense probes. hr: heart, vf: ventrale foregut, Hg: hindgut, hp: hepatic primordium.

3.2.2. Synthesis and secretion of albumin and AFP by endodermal cells derived from ventral foregut.

So far, immunological techniques failed to detect albumin and AFP expression on protein level in viable endodermal cells generated from ventral foregut. Hence, a sensitive method, radioactive biosynthetic labelling, was used to assess synthesis and secretion of albumin and AFP proteins. Endodermal cells derived from ventral foregut were kept in culture for two days and then overnight radioactively labelled with ^{35}S methionine contained in the culture medium. Albumin and AFP were immunoprecipitated from 500 µl supernatants (extracellular) and cell-lysates (intracellular) of the cell culture using anti-albumin or anti-AFP polyclonal antibodies. As positive control we used 50 µl supernatants and cell-lysates from labelled hepatocyte and hepatoblats at 12 days of gestation. Cultured endodermal cells were found to synthesise and secrete biosynthetically labelled albumin and AFP (Fig. 7(A; B)). Interestingly, in 500 µl of supernatants and cell-lysates of labelled endodermal cells, a low synthesis and secretion of albumin and AFP proteins were observed. In contrast, in 50 µl of supernatants and cell-lysates of labelled hepatocyte and hepatoblats a high synthesis and secretion were observed. Endodermal cells derived from venral foregut could synthesise and secrete more AFP than albumin. This finding was confirmed at the RNA-level by real time PCR analysis (Fig. 8 (A; B))

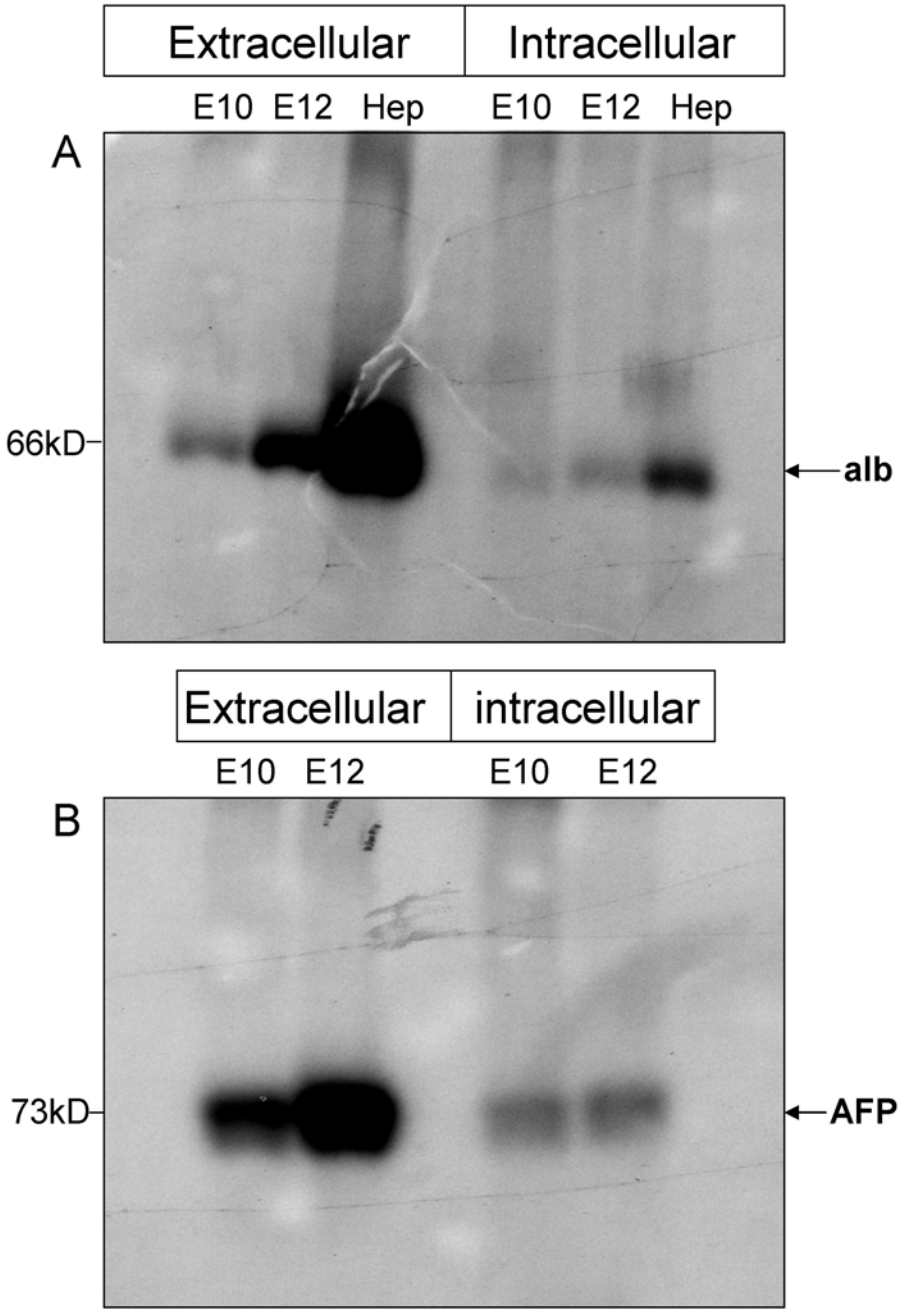


Figure 7: Synthesis and secretion of Albumin (**A**) and alpha-fetoprotein (**B**) by endodermal cells derived from ventral foregut region. Endodermal cells, hepatoblasts and hepatocytes were radioactive labelled with ^{35}S methionine. 500 µl of cell-lysates (intracellular) and supernatants (extracellular) were used for Immunoprecipitation of albumin and AFP from labelled endodermal cells. As positive control, we used 50 µl of cell-lysates and supernatants from labelled hepatoblasts and hepatocytes for immunoprecipitation of albumin and AFP. The immuncomplexes were analysed by SDS-PAGE.

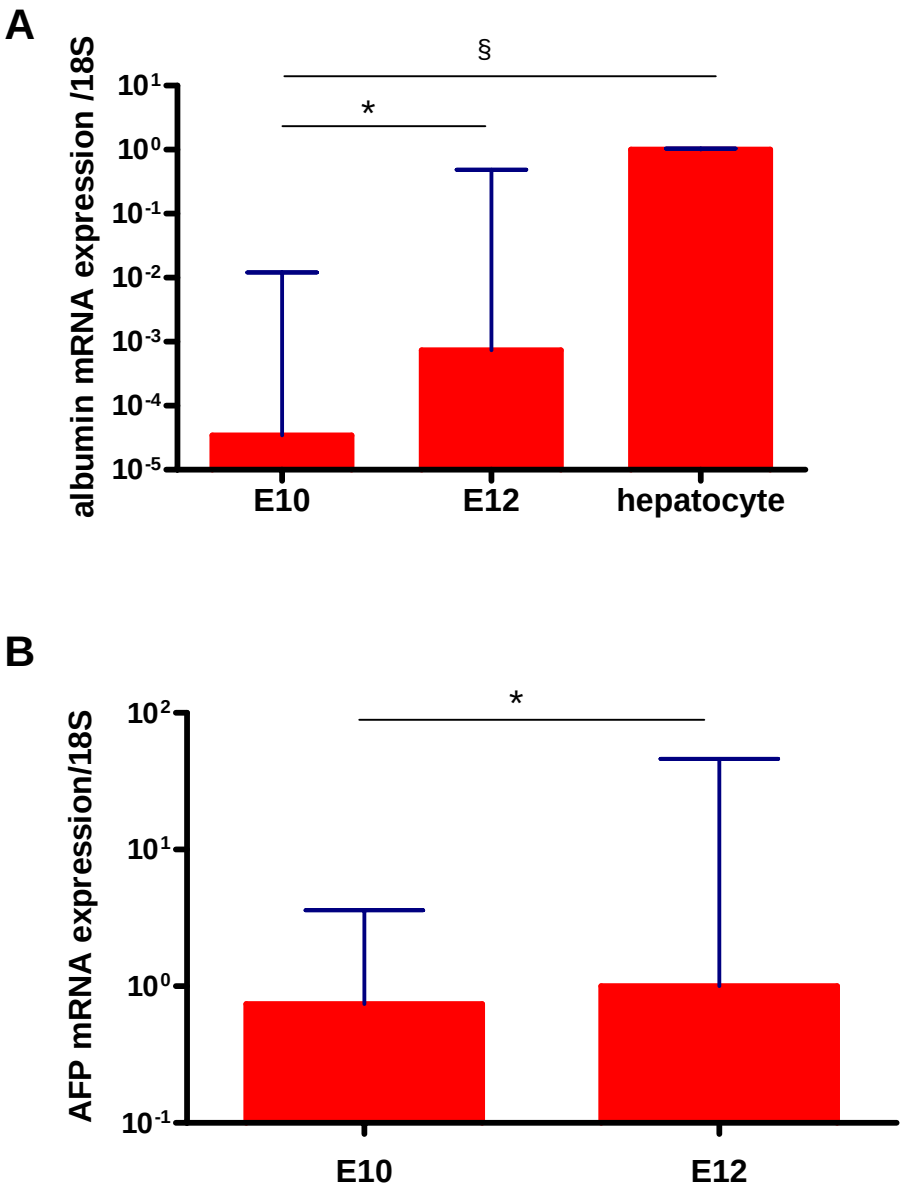


Figure 8: Real-time-PCR analysis of mRNA extracted from cultured endodermal cells, hepatoblasts and hepatocyte. Albumin (**A**) and AFP (**B**) mRNA were expressed in cultured endodermal cells derived from ventral foregut. As positive control, mRNA from hepatoblasts at 12 days of gestation and hepatocytes were used. cDNA from endodermal cells and hepatoblasts were prepared using Fastlane Cell cDNA kit (Qiagen). The expression was normalized with the endogenous control of ribosomal RNA 18S. Error bars represent S.E.M, n=3. Statistically significant difference (*P<0.05; §<0.05 student´s *t*-test) compared to positive control (hepatoblasts and hepatocytes).

3.3. Characterization of endodermal cells generated from ventral foregut

The endodermal cells derived from ventral foregut were characterized by assessing the expression of endodermal and hepatic markers as well as transcription factors involved in liver development. Positive cells for HNF4-alpha were detected in cultured clustered endodermal cells by immunofluorescence experiment (Fig. 9). In addition real-time-PCR analysis revealed that explanted endodermal cells could express HNF4-alpha, Prox1, beta-catenin, BMP-4, Foxa-2 and GATA-4 (Fig. 10).

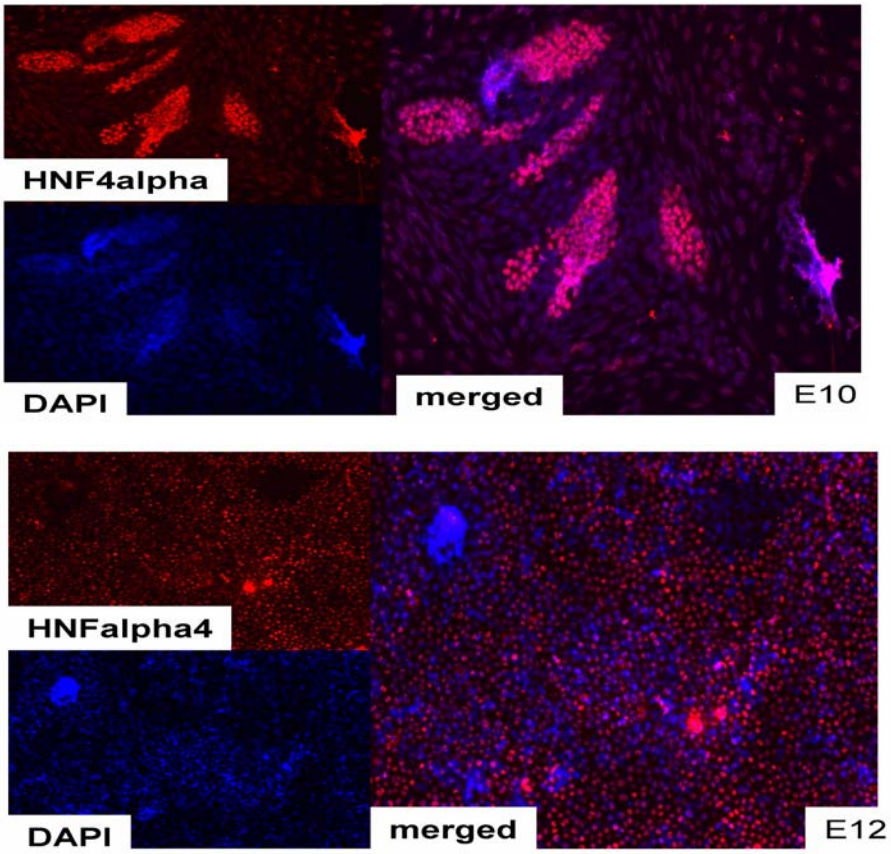


Figure 9: Immunofluorescence staining display HNF4-aplha-expression in cultured clustered endodermal cells and in hepatoblasts derived from 12 days of gestation. Counterstaining of nuclei with DAPI (blue). Original magnification x 100

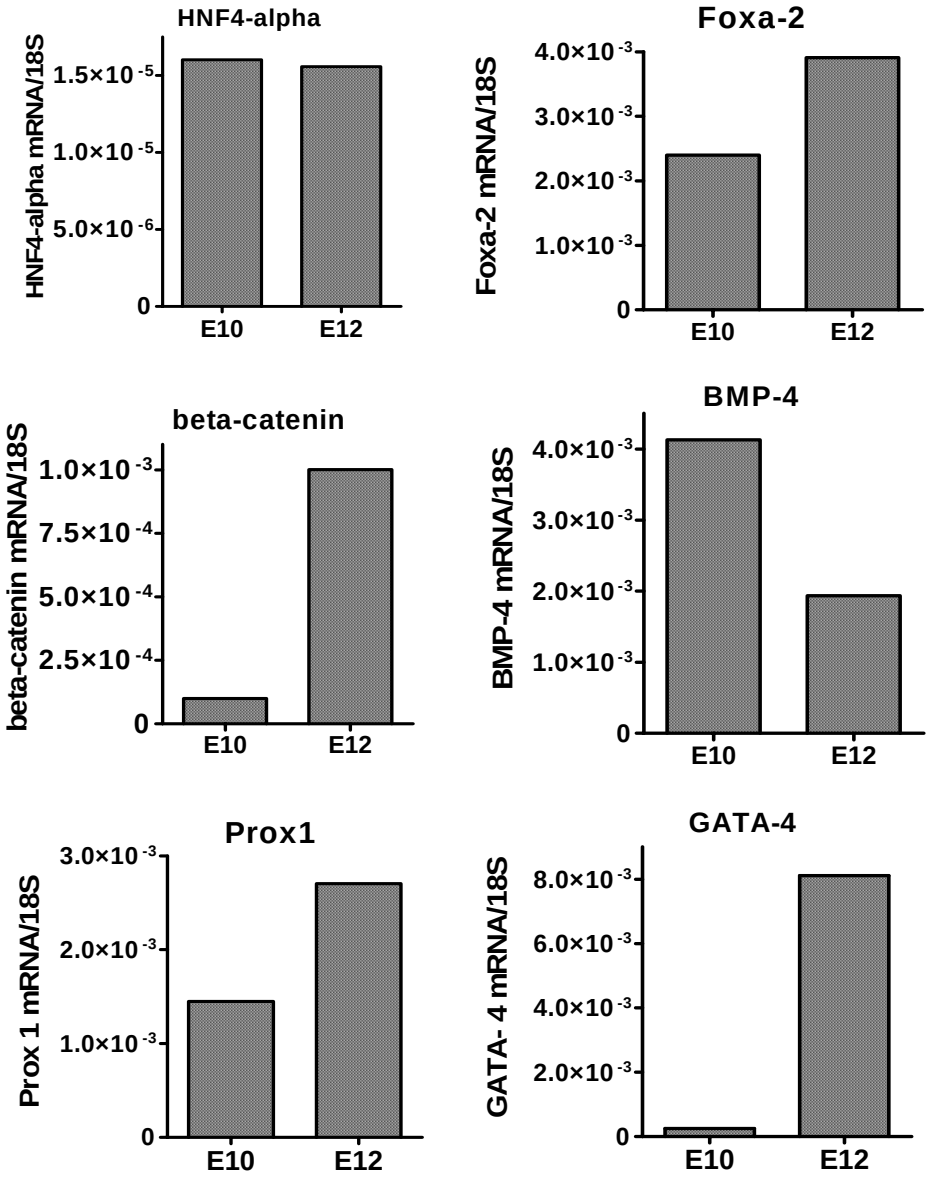


Figure 10: mRNA-expression levels of HNF4-alpha, beta-catenin, Prox1, Foxa-2, BMP-4 and GATA-4 in cultured endodermal cells and hepatoblasts from 12 days of gestation measured by real-time-PCR experiment. cDNA from cultured endodermal cells and hepatoblasts from 12 days of gestation were prepared using Fastlane Cell cDNA kit. The expression was normalized with the endogenous control of ribosomal RNA 18S.

3.4. Cellular analysis of albumin and AFP expressing cells during liver development

The embryonic liver appears at 12 days of gestation, when albumin and AFP mRNA as well as theirs coding proteins are expressed. The ratio of albumin and AFP producing cells either to total liver cells or to proliferating cells was analysed during liver development. For this purpose, at each developmental stage *in situ* hybridization was performed using albumin and AFP probes combined with proliferating cell nuclear antigen (PCNA) peroxidase immunostaining.

Albumin and AFP positive cells were distributed homogeneously during liver development (Fig. 11 and Fig. 12). A few negative cells which represents hematopoitic or endothelial cells were observed. Adult liver could not express AFP but albumin is high expressed. The same expression pattern has been previously observed in rat liver during prenatal (17 to 21 days of gestation) and postnatal life (1 to 5 weeks old neonates) (Poliard et al., 1986).

During embryonic and foetal stage (from E12 up to E18), about 50% of liver cells expressed albumin and AFP. From E18 up to birth, albumin and AFP positive cells developed inversely, we observed an increase of albumin positive cells and a decrease of AFP positive cells, which disappears in adult liver (Fig. 13).

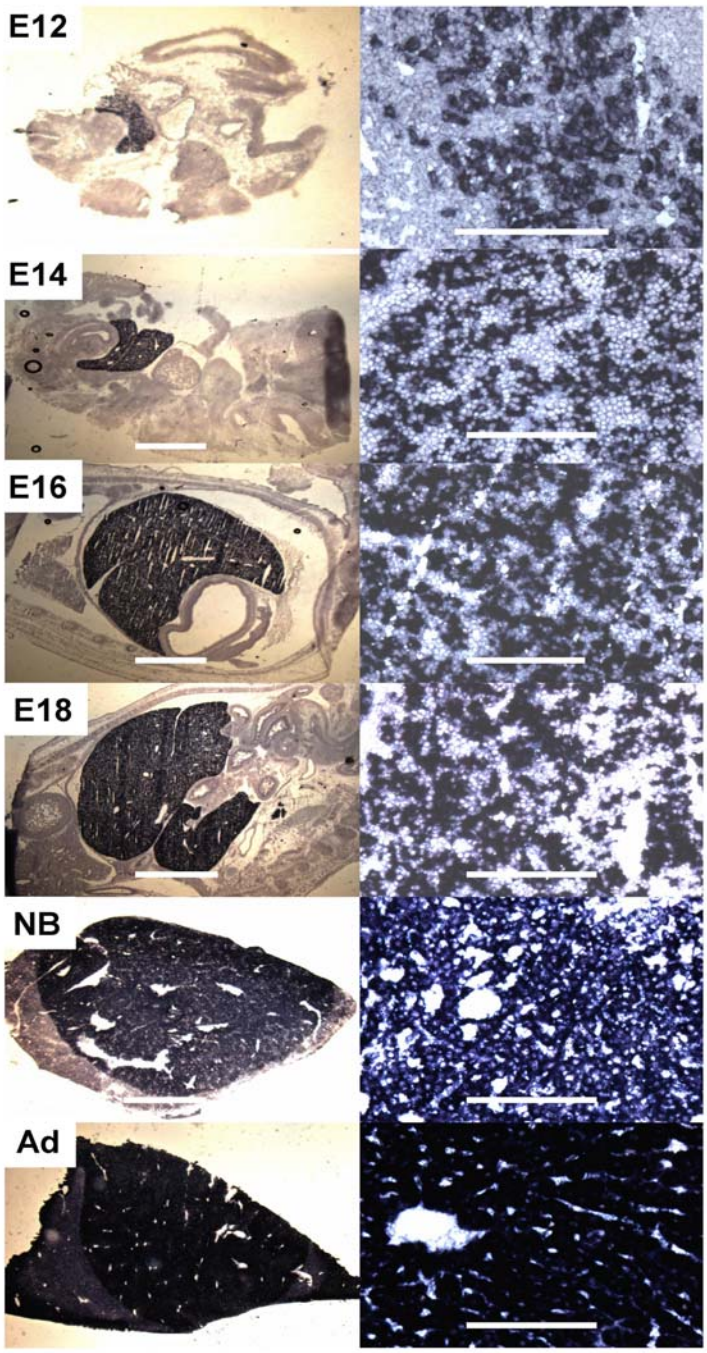


Figure 11: Albumin mRNA-expression during liver development assessed by *in situ* hybridization. Cryo-sections (10 µm) of rat embryos prepared at developmental stages E12, E14, E16, E18, new born and adult liver were incubated with DIG-labelled antisense riboprobe of albumin. After incubation with alkaline phosphatase (AP)-conjugated anti DIG antibody, the signals were visualised using BCIP/NBT.
(Bar = Magnification x 50; x 200).

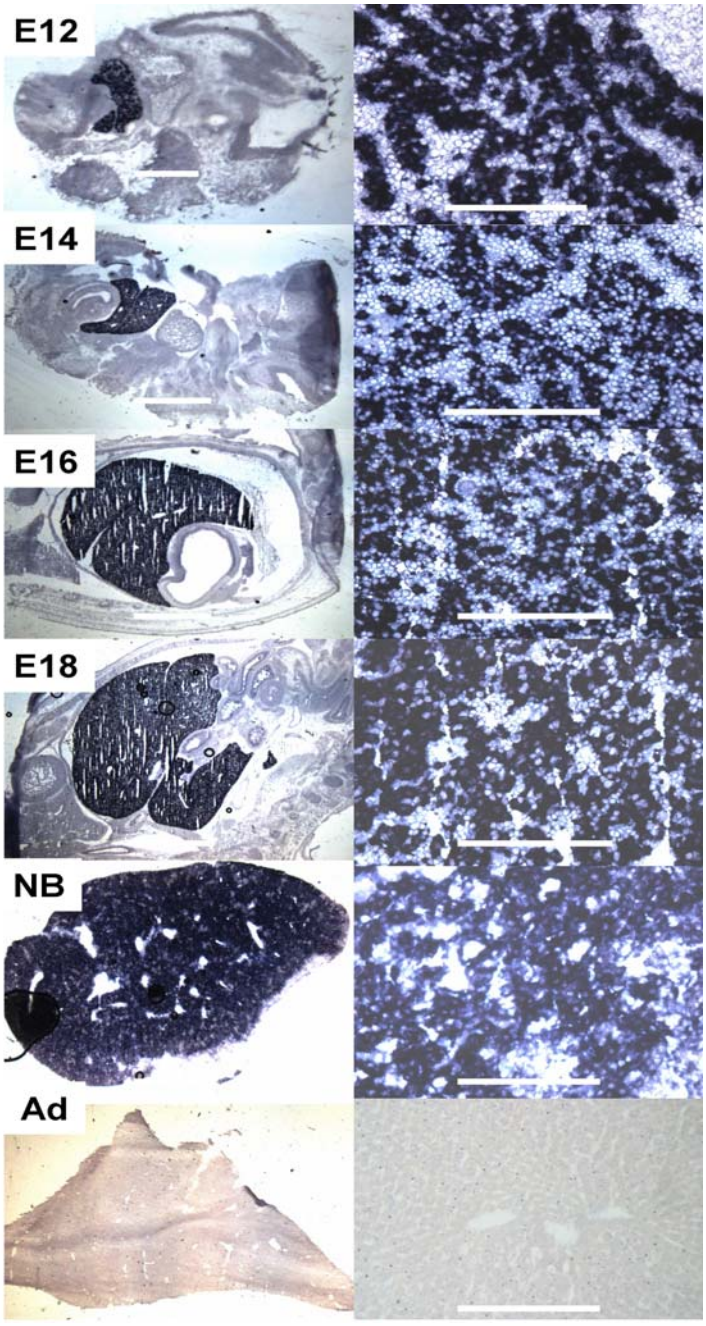


Figure 12: AFP mRNA-expression during liver development assessed by *in situ* hybridization. Cryo-sections (10 µm) of rat embryos prepared at developmental stages E12, E14, E16, E18, new born and adult liver were incubated with DIG-labelled antisense riboprobe of AFP. After incubation with alkaline phosphatase (AP)-conjugated anti DIG antibody, the signals were visualised using BCIP/NBT.
(Bar = Magnification x 50; x 200).

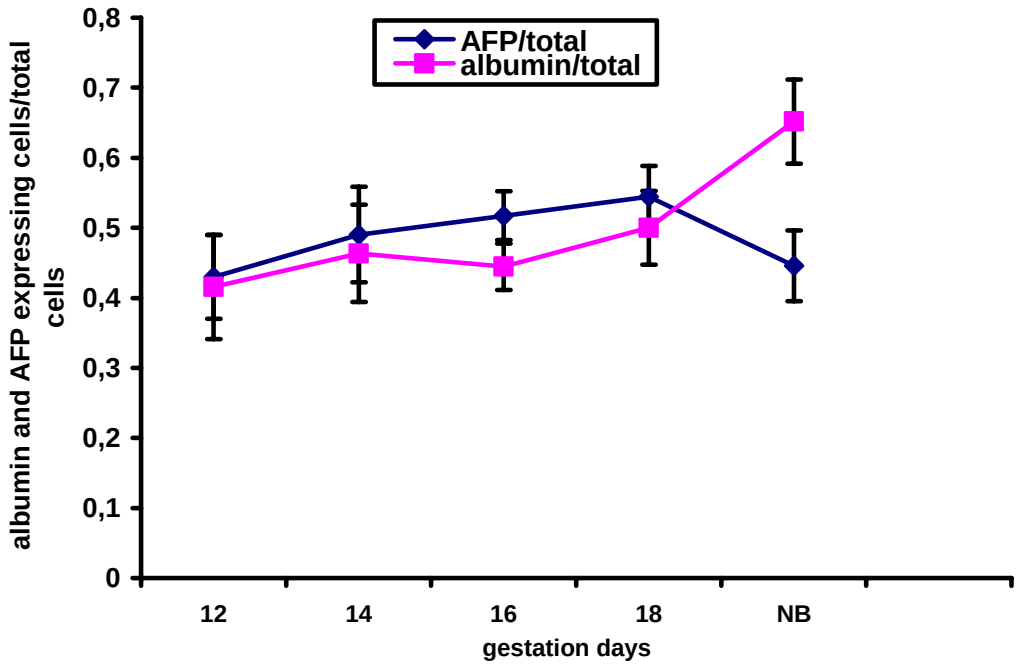


Figure 13: The ratio of albumin and AFP producing cells to total cells during liver development. Albumin and AFP positive cells were identified by *in situ* hybridization (as shown in figure 11 and 12) and counted under microscope using a shaded ocular, or by application of Image J software (Wayne Rasband, NIH, USA). Error bars represent S.E.M., n=3. The significance ($P<0.05$) was analysed by ANOVA

Cell proliferation in developing liver was measured by immunoreactions with proliferating cell nuclear antigen (PCNA) in embryonic and foetal livers at E12, E14, E16, E18 and in livers from new born and adult state. A high number of PCNA-positive cells were observed at E12, E14 and E16. Fewer PCNA-positive cells were observed at E18 and new born rats. Far fewer PCNA-positive cells were observed in adult liver (Fig. 14). In developing liver, the ratio of albumin and AFP producing cells to proliferating cells shows an increase during embryonic and foetal stages. At E18 the ratio of AFP producing cells to proliferating cells reaches the

maximum, followed by a decrease at birth. In contrast the ratio of albumin producing cells to proliferating cells increase continuously (Fig. 15)

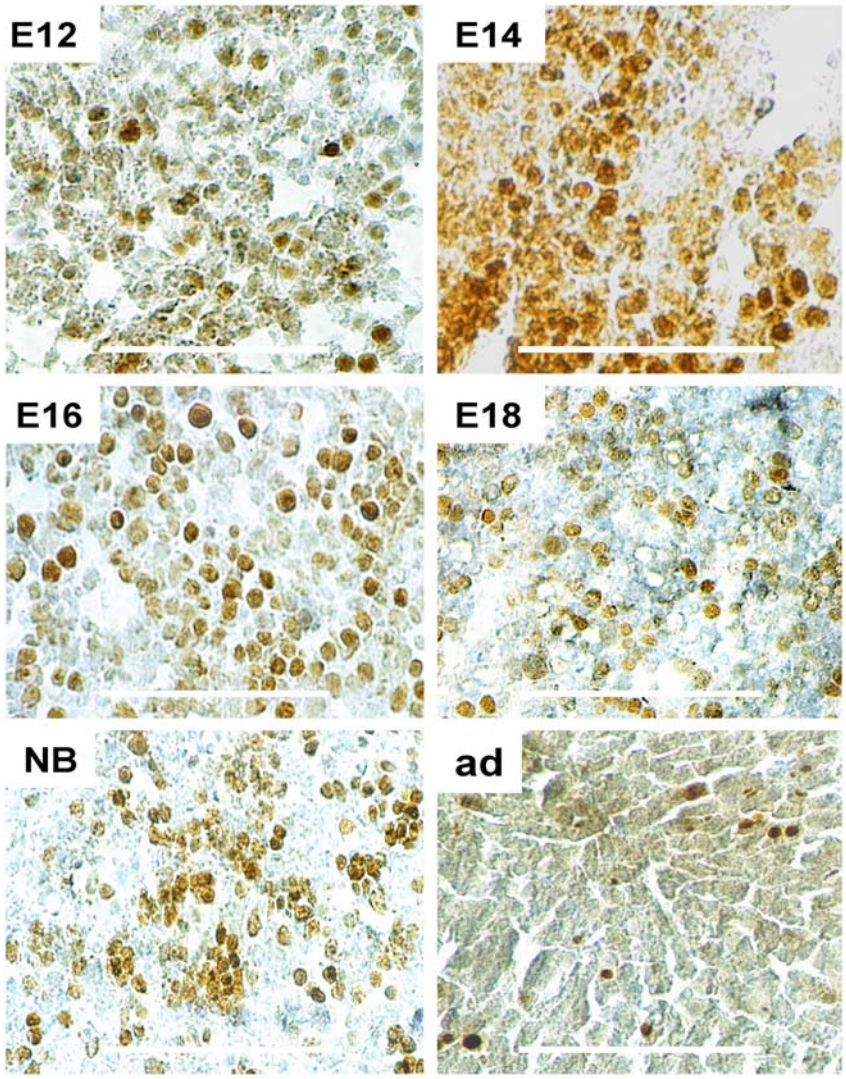


Figure 14: Proliferation estimated by the number of positive cells expressing proliferating cell nuclear antigen (PCNA) during liver development. Cryo-sections (5 µm) of rat embryos at developmental stages E12, E14, E16, E18, new born and adult liver were used. Immunreaction was detected by peroxidase-labelled second antibody. (Bar = Magnification x 200).

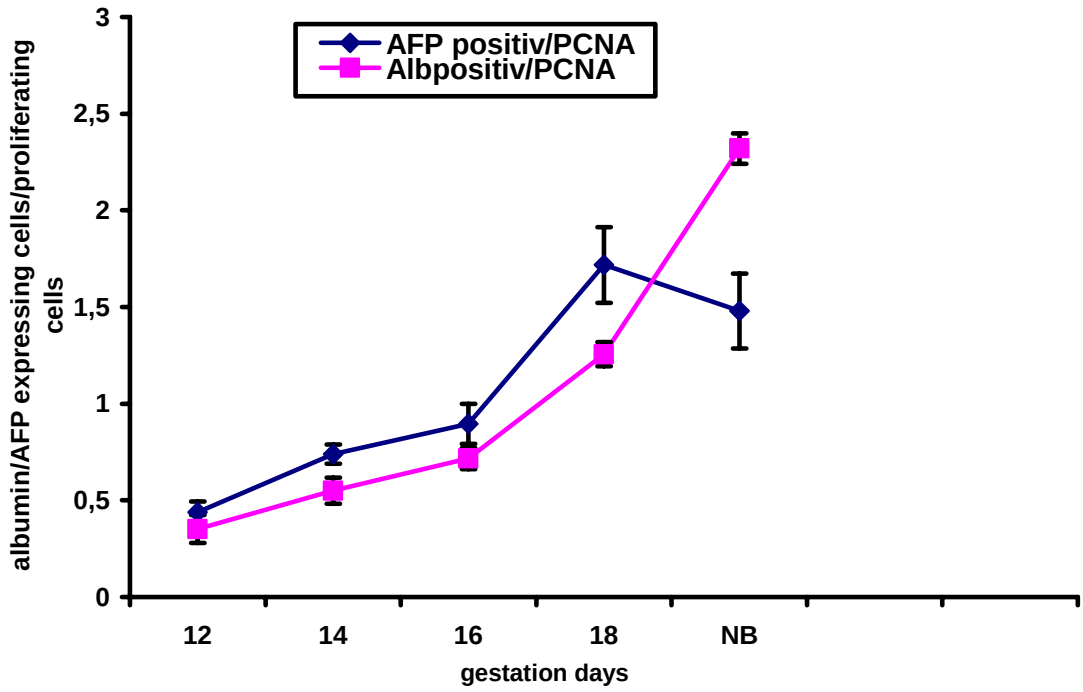


Figure 15: The ratio of albumin and AFP expressing cells to proliferating cells during liver development. Albumin/AFP and PCNA positive cells were identified by *in situ* hybridization (Fig. 11 and 12) and PCNA staining (Fig. 14) respectively. The positive cells were counted under microscope using a shaded ocular, or by application of Image J software (Wayne Rasband, NIH, USA). Error bars represent S.E.M., n=3. The significance ($P<0.05$) was analysed by ANOVA

The co-localisation (co-expression) of albumin and AFP was observed at all developmental stages (from E12 up to birth). At birth, albumin could be produced by hepatocytes which express AFP anymore (Fig. 16). The co-expression of albumin and AFP genes in hepatoblasts was confirmed by *in vitro in situ* hybridization, performed in cultured hepatoblasts at 12, 14 and 18 days of gestation. As shown in figure 17, the hepatoblasts could express albumin and AFP mRNA.

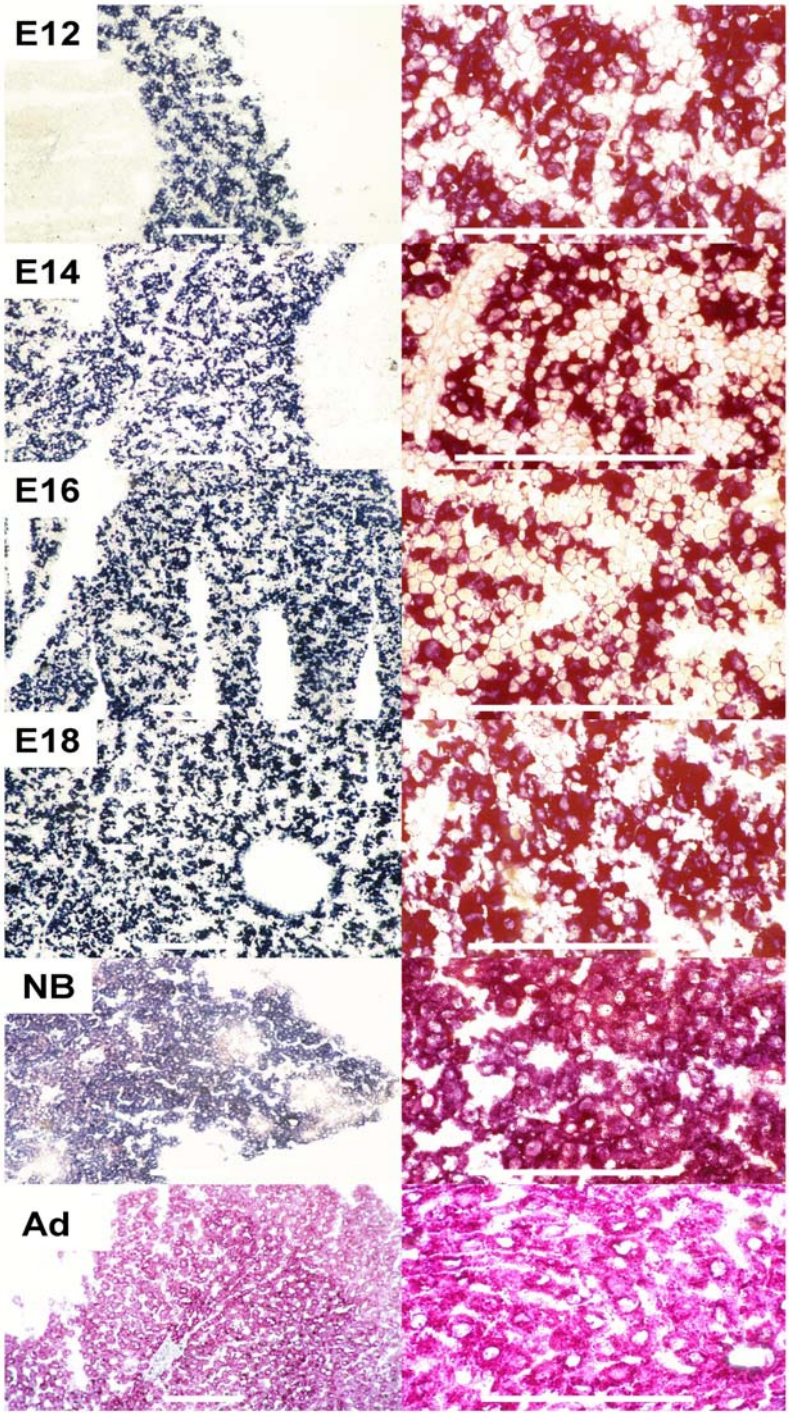


Figure 16: Co-localisation of albumin and AFP mRNA during liver development. Cryo-sections (10 µm) of rat embryos at developmental stages E12, E14, E16, E18 as well as new born and adult livers were incubated with DIG-labelled antisense riboprobe of AFP (blue) and Fluorescein-labelled antisense riboprobe of albumin (red).
(Bar = Magnification x 100; x 200).

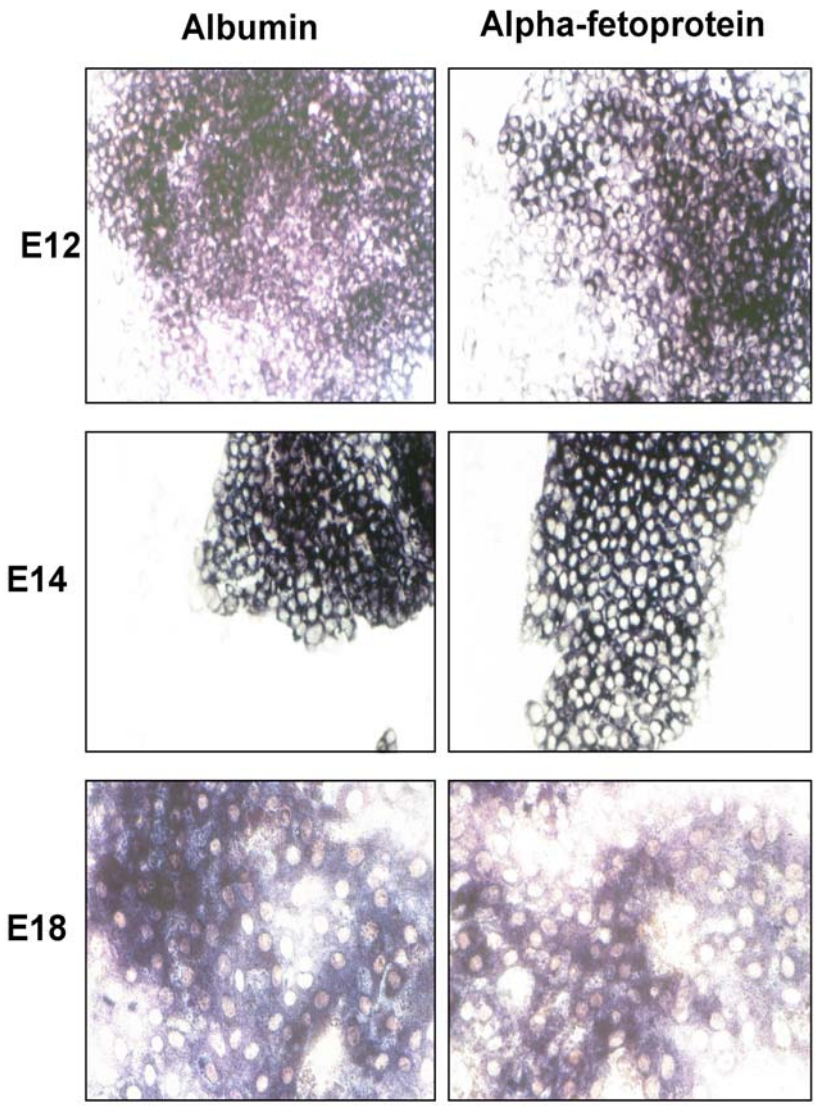


Figure 17: Albumin and AFP mRNA expression in cultured hepatoblasts during liver development. Hepatoblasts derived from developmental stages E12, E14 and E18 were cultured on lab-Tecks for two days and incubated with DIG-labelled antisense riboprobe of albumin and AFP. (Magnification x 200).

3.5. Quantitative analysis of albumin and AFP mRNA expression during liver development

In situ hybridization experiments have provided more insight about cellular distribution of albumin and AFP expressing cells during liver development. Quantitation of albumin and AFP mRNA was obtained by real-time PCR experiment using a reverse transcripted RNA extract from whole liver (*in vivo*) and from hepatoblasts (*in vitro*) derived from developmental stages E12, E14, E16, E18, E20, new born and adult.

In whole liver (*in vivo*), albumin mRNA was maintained from 12 up to 16 days of gestation. However, AFP mRNA displayed a high expression at E12 followed by a decrease at E14 and remained unchanged until 16 days of gestation. Both genes reach a peak at 18 days of gestation. A slight decrease at E20 followed by an increase of albumin mRNA at birth and during postnatal stage. AFP mRNA decrease continuously during fetal stage, at birth and during postnatal life (Fig. 18).

In hepatoblasts (*in vitro*), it was observed an increase of albumin and AFP mRNA during embryonic stage. AFP mRNA reached a peak at 16 days of gestation and then decreased, while albumin mRNA reached a peak at 18 days of gestation followed by a slight decrease at E20 and then increased at birth and during postnatal life (Fig. 19).

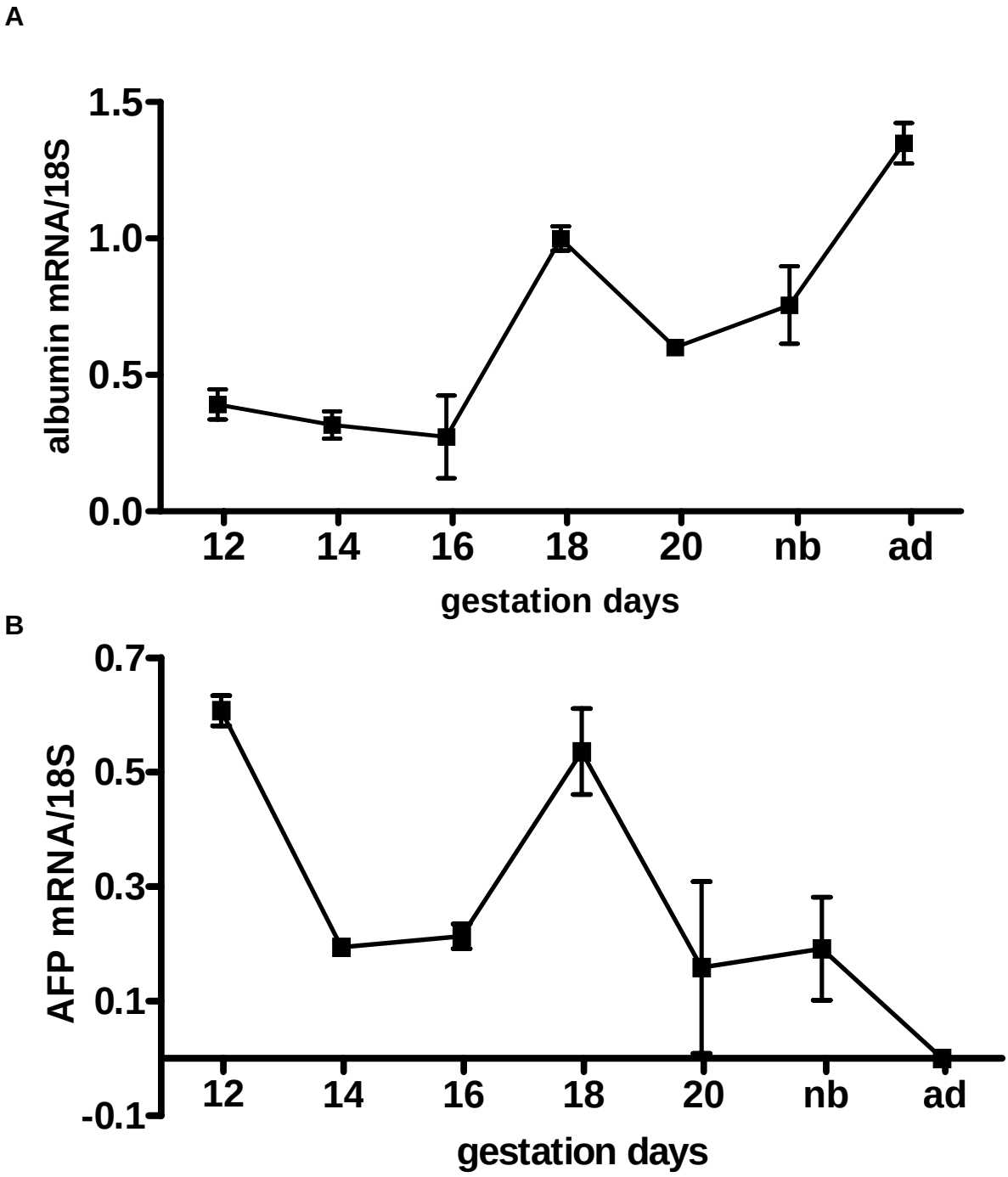


Figure 18 Quantitative analysis of albumin (**A**) and AFP (**B**) mRNA expression in whole liver (*in vivo*) during liver development using real-time-PCR experiment. The expression was normalized with the endogenous control of ribosomal RNA 18S. Error bars represent S.E.M., n=3. The significance ($P<0.05$) was analysed by ANOVA.

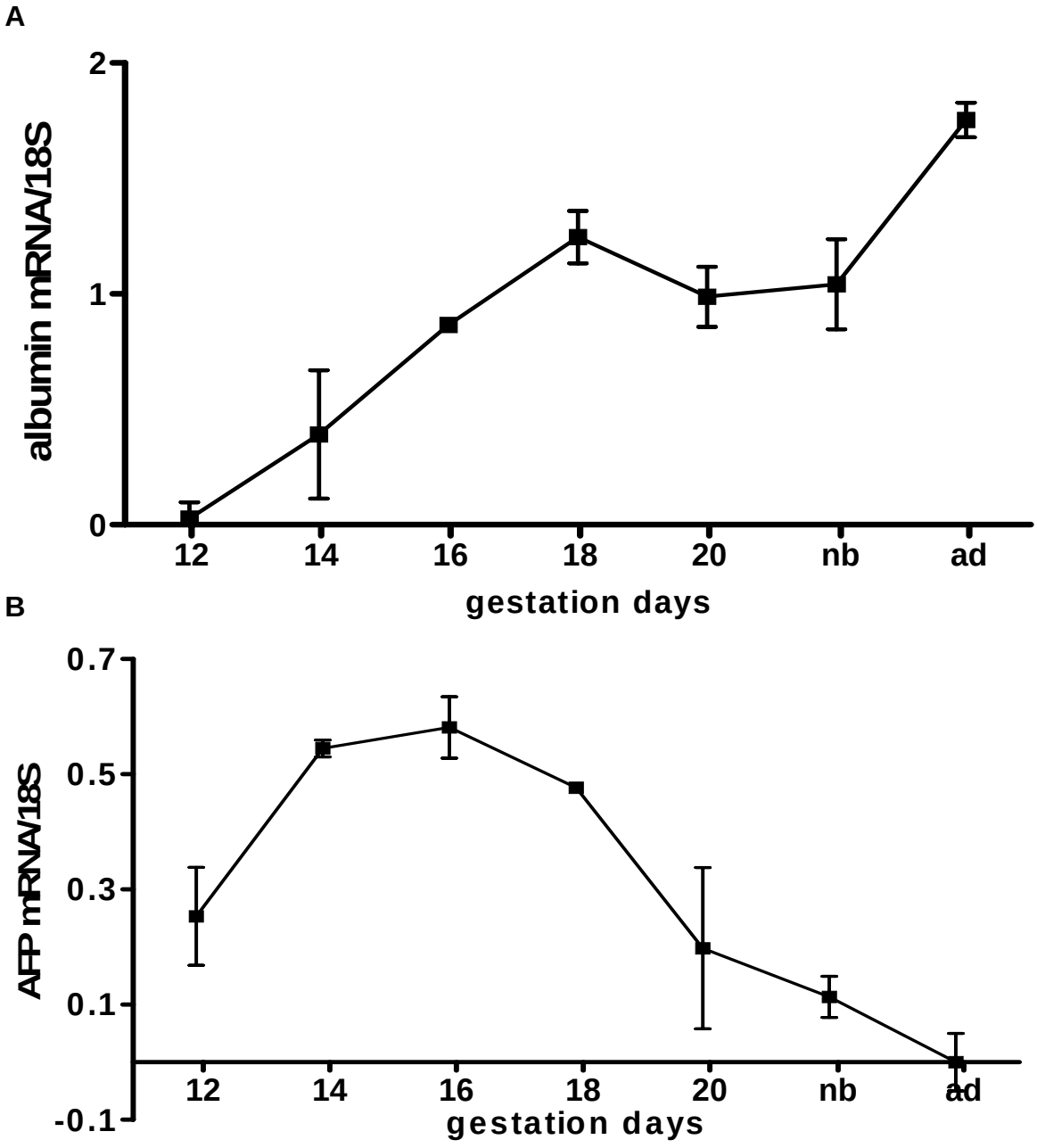


Figure 19: Quantitative analysis of albumin (**A**) and AFP (**B**) mRNA expression in cultured hepatoblasts (*in vitro*) during liver development using real-time-PCR experiment. The expression was normalized with the endogenous control of ribosomal RNA 18S. Error bars represent S.E.M., n=3. The significance was analysed by ANOVA ($P<0.05$)

3.6. Kinetics for synthesis and secretion of albumin and AFP during liver development

The kinetics for synthesis, as well as secretion of albumin and AFP was investigated by radioactive biosynthetic labelling method in viable hepatoblasts and hepatocytes isolated from livers at E12, E14, E16, E18, E20, new born and adult rats. Newly synthesized proteins were endogenously labelled for 2 h with ^{35}S methionine. Albumin and AFP were immunoprecipitated from supernatants (extracellular) and cell-lysates (intracellular). As shown in figure 20, during embryonic stage (E12 up to E16) synthesis and secretion of albumin and AFP remained relatively unchanged, approximately the same amount of albumin and AFP was released. At day 18 of gestation, an increase of synthesis and secretion of albumin and AFP was observed, followed by a decrease during prenatal stage (E20). At birth low levels of AFP were secreted, in contrast, albumin was secreted continuously. Hepatocyte generated from adult liver displayed a high synthesis and secretion of albumin, and a low secretion of AFP. The two polypeptides 68 kD and 70 kD, which correspond to 2.1 kb AFP mRNA, were synthesised and secreted by hepatoblasts at all developmental stage.

During liver development, the kinetics of secretion of albumin and AFP in hepatoblasts was measured using pulse chase experiment. Hepatoblasts were kept in culture for two days, pulsed for 1 h with 100 µCi/well ^{35}S-methionine and then chased for 15, 30, 45, 60, 90, 120, 240 min. Mature hepatocyte needed short time to synthesize and secrete albumin. However, hepatoblasts isolated at early development stage needed more time to synthesize and secrete albumin. At 14 and 16 days of gestation albumin needed 30 min to be released but hepatoblasts from 18 days of gestation are comparable to mature hepatocyte. They needed only 15 min to secrete new synthesized albumin (Fig. 21A). AFP was secreted in

hepatoblasts derived from developmental stage E12 after chase period of 45 min. At developmental stages E14, E16 and E18, AFP needed only 15 min to be secreted (Fig. 21B).

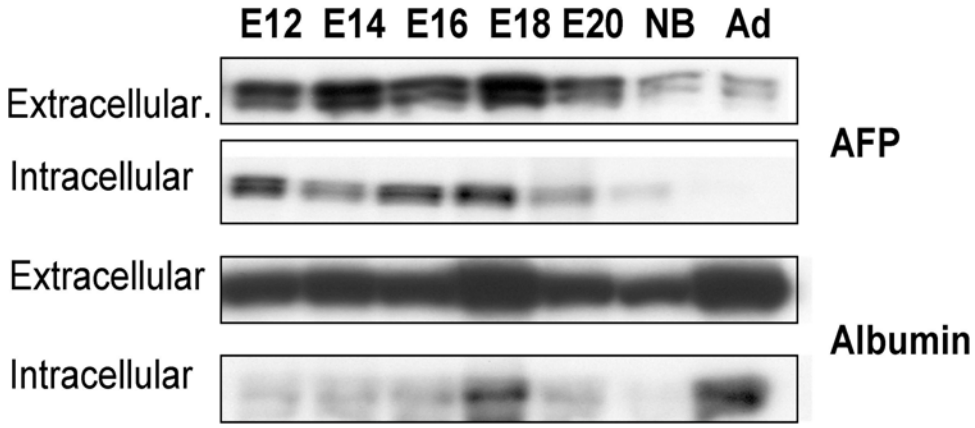


Figure 20: Synthesis and secretion of albumin and alpha-fetoprotein (AFP) in viable hepatoblasts during liver development. Hepatoblasts and hepatocytes were cultured for two days and then radioactive labelled with ^{35}S methionine. Albumin and AFP were immunopecipitated using polyclonal anti-albumin and anti-AFP antibodies. Immunocomplexes were analysed by SDS-PAGE. Cell-lysates (Intracellular) and supernatants (extracellular) were used for immunoprecipitaion by taking into consideration samples with similar count.

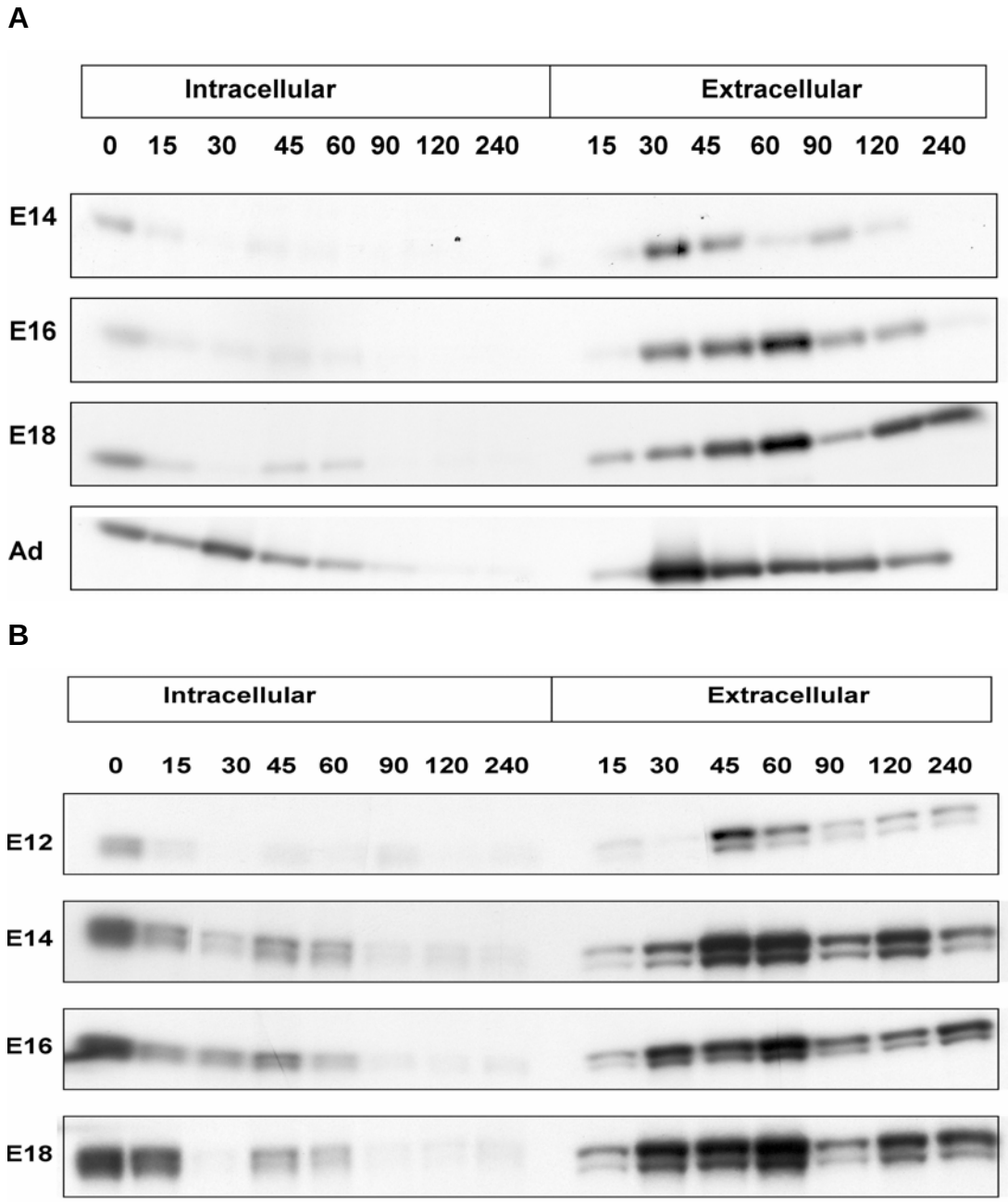
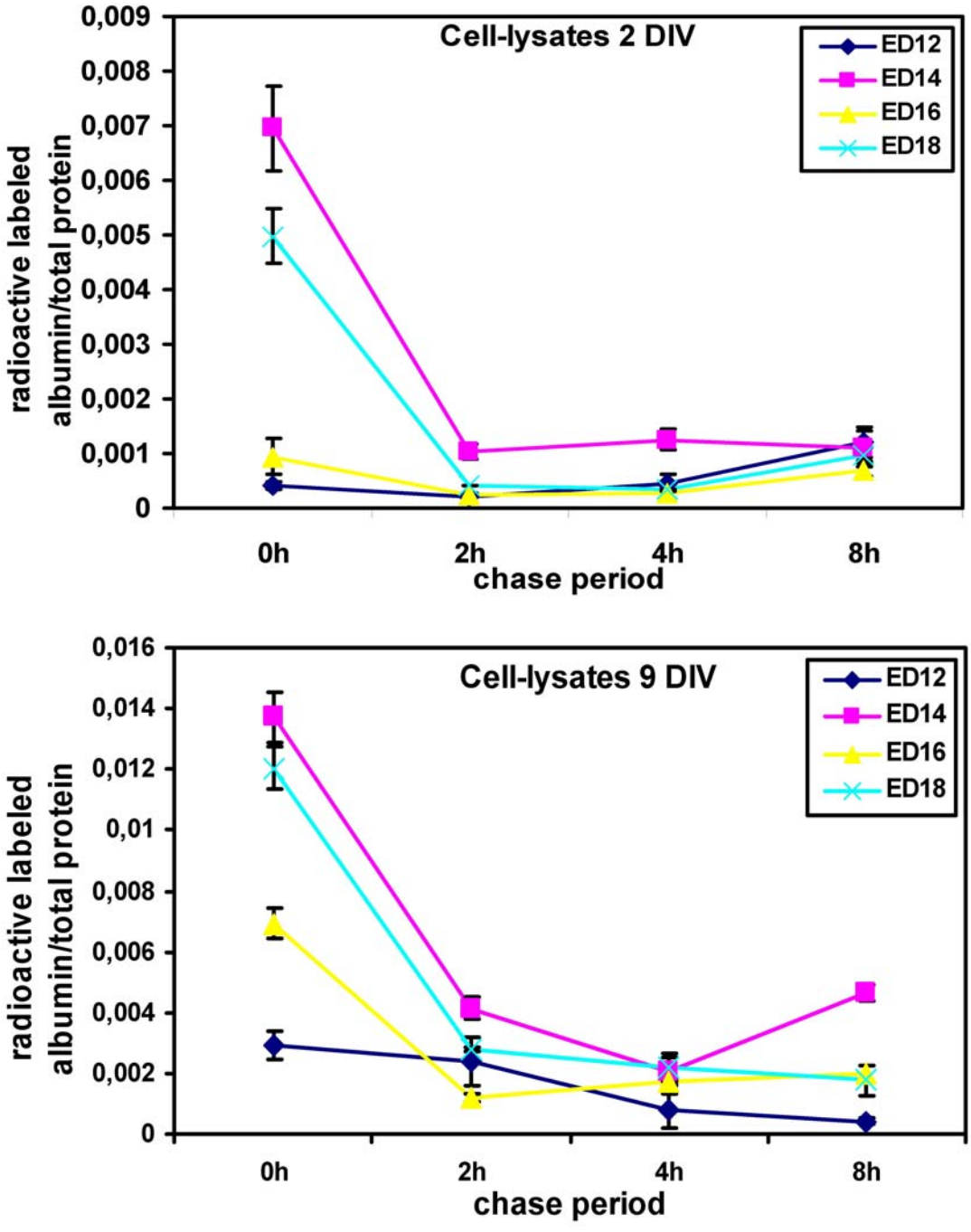


Figure 21: Kinetics for albumin (**A**) and AFP (**B**) in hepatoblasts during liver development estimated by pulse chase experiment. Hepatoblasts from developmental stages E12, E14, E16 and E18 as well as hepatocytes were kept in culture for two days, pulsed for 1 h with 100 µCi/well ^{35}S-methionine and then chased for 15, 30, 45, 60, 90, 120, 240 min. Albumin and AFP were immunopecipitated with polyclonal anti-albumin and anti-AFP antibodies. The immunocomplexes were analysed by SDS-PAGE. Cell-lysates (Intracellular) and supernatants (extracellular) were used for immunoprecipitaion by taking into consideration samples with similar count.

The kinetics for synthesis and secretion of albumin was measured in hepatoblasts isolated at 12, 14, 16 and 18 days of gestation after their cultivation for 2 and 9 days. The hepatoblasts were pulsed for 1 h with ^{35}S metionine followed by incubation for a chase period of 2, 4 and 8 hours. The extent of albumin synthesis and secretion was determined from its immunoprecipitable radioactivity and expressed as ratio to count of total labelled proteins precipitated by trichloroacetic acid. Hepatoblasts from embryonic liver at E12 displayed an increase of albumin synthesis and secretion during the culture (Fig. 22). We observed a decrease of intracellular albumin after a chase period of 2 h followed by continuous secretion during a chase period of 4 h and a decrease of secreted albumin after a chase period of 8 h. Interestinly, hepatoblasts isolated from developmental stages E14, E16 and E18 and cultured for 9 days could secrete completely albumin after a chase period of 4 h. In contrast, hepatoblasts isolated from developmental stage E12 could secrete continuously albumin even after a chase period of 8 h (Fig. 22).

A


Cell-lysates 2 DIV
ED12
ED14
ED16
ED18
radioactive labeled albumin/total protein
0,009
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0,007
0,006
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0,004
0,003
0,002
0,001
0
0h
2h
4h
8h
chase period
Cell-lysates 9 DIV
ED12
ED14
ED16
ED18
radioactive labeled albumin/total protein
0,016
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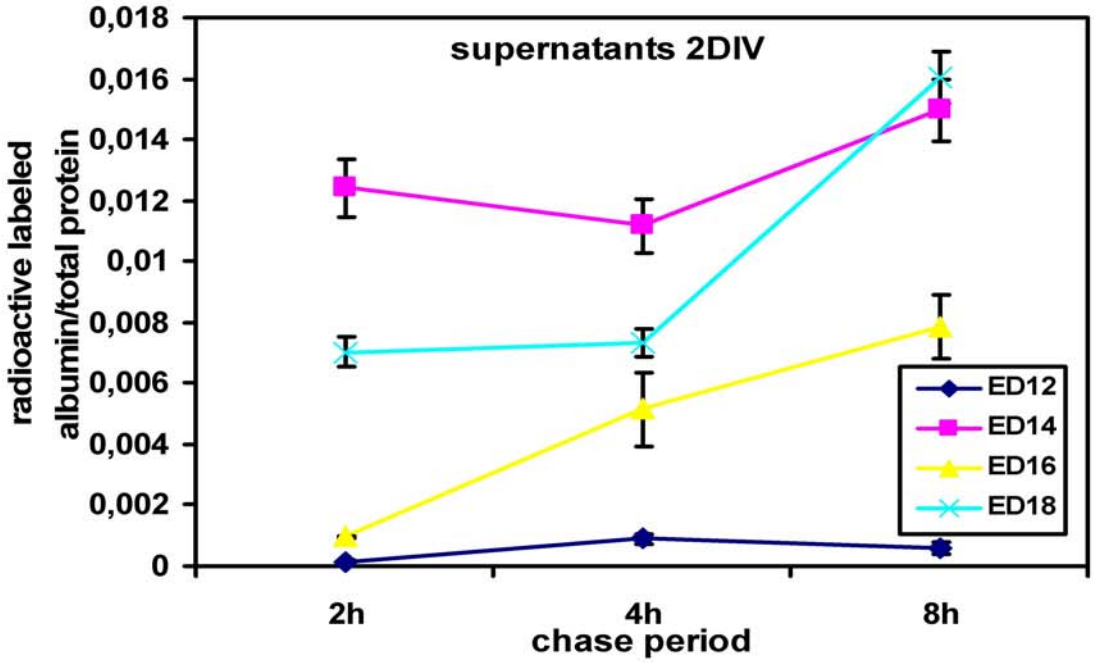
B


supernatants 2DIV
radioactive labeled albumin/total protein
0,018
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ED12
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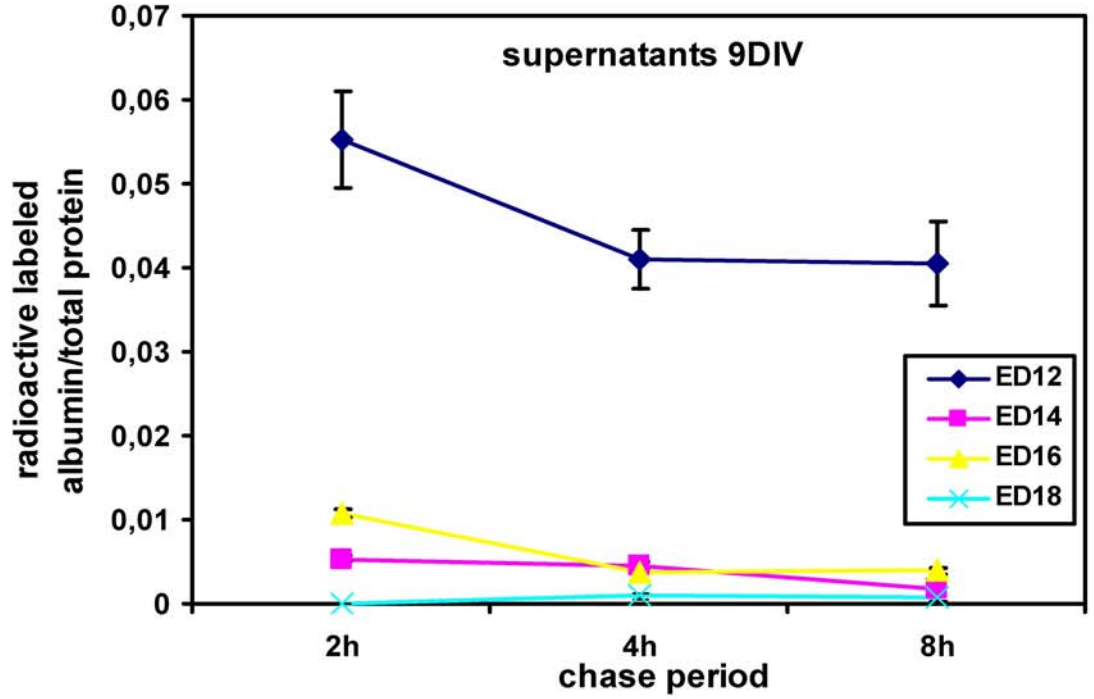
ED16
ED18


supernatants 9DIV
radioactive labeled albumin/total protein
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8h
chase period
ED12
ED14
ED16
ED18

Figure 22 : Kinetics of albumin in hepatoblasts isolated at developmental stages E12, E14, E16 and E18 after 2 and 9 days in culture (DIV : days in vitro), measured by pulse chase experiment. Hepatoblasts were pulsed for 1 h with 100µCi/well ^{35}S-methionine and then chased for 2h, 4h and 8h. Albumin was immunopecipitated from cell-lysates (**A**) and supernatants (**B**) which have a similar radioactive count measured after precipitation of total labelled proteins by trichloroacetic acid. The extent of albumin synthesis and secretion was determined from its immunoprecipitable radioactivity and expressed as ratio to count of total labeled proteins. Error bars represent S.E.M., n=3. The significance ($P<0.05$) was analysed by ANOVA

3.7. Expression of prox1, cytokeratin-7 and -19 during liver development.

Prox1 is not expressed in CK-7 positive bile duct epithelial cells of the adult liver (Dudas et al. 2004). However, CK-7 is a late marker of the bile duct development, and it becomes detectable only in the foetal stage (Shiojiri et al. 1991). Prox1 is an early marker of hepatoblasts, which co-express it with albumin and AFP (Dudas et al. 2004). Nevertheless, until now, it was not described how long Prox1 remains detectable in the nuclei of those hepatoblasts that differentiate toward bile duct cells. CK-19 was used to analyse embryonic and foetal hepatic cells, which may differentiate towards the biliary lineage. It was also not known whether CK-19 positive embryonic liver cells are always Prox1 positive and/or AFP positive.

At 14 and 16 days of gestation the majority of Prox1-positive cells in the developing rat liver showed cytokeratin-19 in their cytoplasm (Fig. 23.(A,B)), but cytokeratin-7 was not detected (Fig. 23 C). The co-expression of AFP and Prox1 in hepatoblasts isolated at E14 has been reported before (Dudas et al. 2004). At E16 some small CK-.19 positive, Prox1 negative and AFP negative cells were detected (Fig. 24 (A,C)). Prox1 was found mainly in AFP positive and CK-19 negative cells (Fig. 24

B). At E18 the antigenic properties of Prox1 and CK-19 become clearly separated (Fig 26). We observed a colocalisation of Prox1 and CK-19 in some cells, they were only represented at 3.66% (by relating the Prox1 positive/CK-19 positive cells to the whole population (Table 3), and the majority of the cells were either Prox1 positive or CK-19 positive (Fig. 25A). Co-localisation of CK-19 with AFP at E 18 was found only in 3.8% of the whole cell population (Fig. 26) (Table 3). CK-7 was first detected at E18 in Prox1 negative cells (Fig. 25B). The co-localisation of prox1 and CK-19 at embryonic stages (E14 and E16) and their expression separately in different cell-types at foetal stage (E18) was confirmed in cultured hepatoblasts. At the foetal stage (E18), we identified three cell populations, Prox1 positive/CK-19 positive cells, Prox1 negative/CK-19 positive cells and Prox1 positive/ CK19 negative cells (Fig. 27).

Cell nuclear Prox1 immunostaining co-localisation with the cytoplasmatic reaction of HepPar1 and the connexin 32-positive gap junctions indicate that hepatoblasts from E18 display a mature hepatocyte phenotype (Fig. 25 (C,D)). Prox1 remained a stable cell nuclear marker in adult hepatocytes, and was absent in bile ducts, while the latter were CK-19 or CK-7 positive (Fig. 28 (A,B)). The hepatocyte immunophenotype was confirmed by positive reactions with anti-HepPar1 and anti-connexin 32. Prox1 was detected in parenchymal cells connected by connexin 32 containing gap junctions, while sinusoidal cell nuclei were negative (Fig.28 (C,D,E))

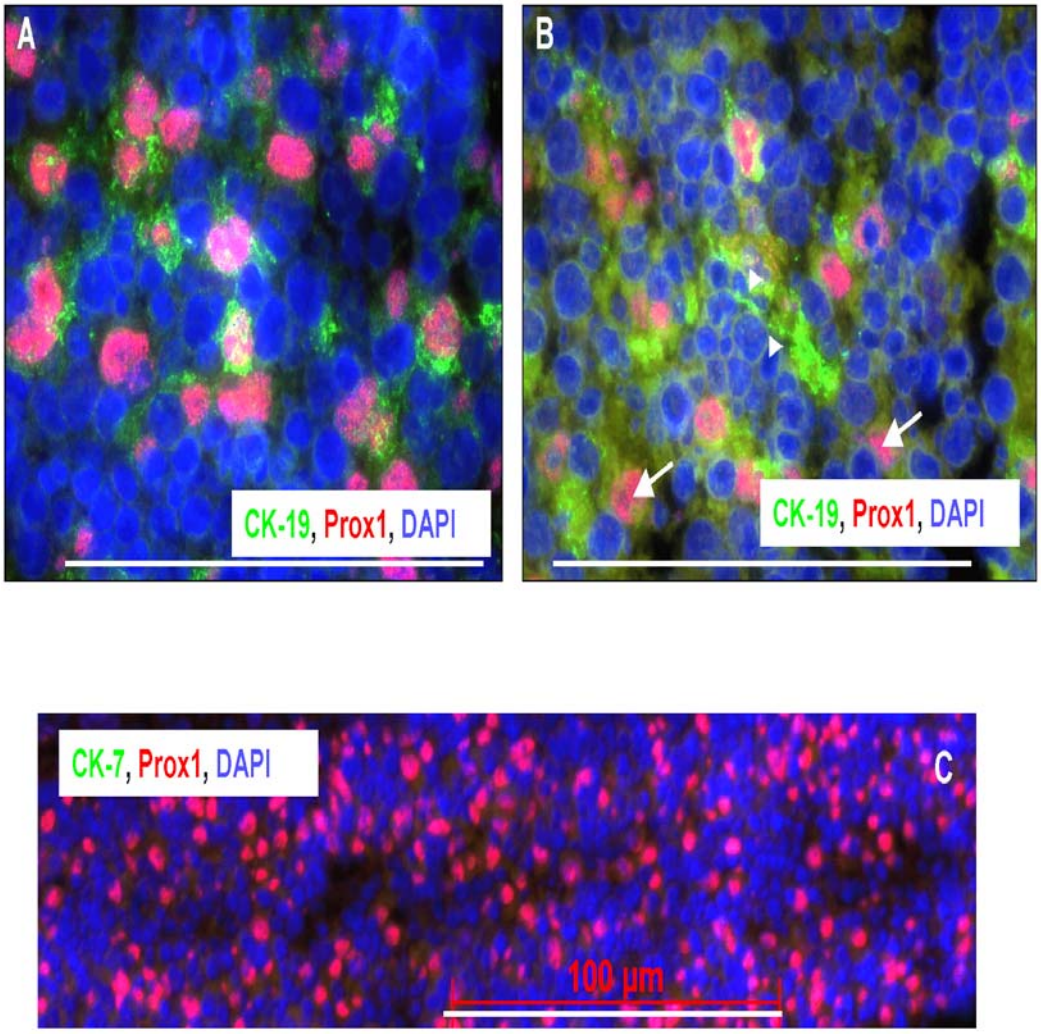


Figure 23: Prox1-, CK-19-, and CK-7-specifc immunohistochemical reactions in embryonic rat liver at E14 and E16. (**A**) At E14, cells with Prox1(*red*) positive nuclei show CK-19 (*green*) in the cytoplasm (Bar = 50 µm). (**B**) At E16, the Prox1-positive hepatoblasts partly show CK-19 staining, although in several cells the stainings are separated (showing CK-19 without Prox1 (arrowheads) or Prox1 without CK-19 (arrows) (Bar = 50 µm). (**C**) Liver section at E16 with immunofluorescent staining for CK-7 and Prox1. CK-7 (*green*) positive reaction is still not detected. Counterstaining of nuclei was performed with DAPI (blue) (Bar = 100 µm).

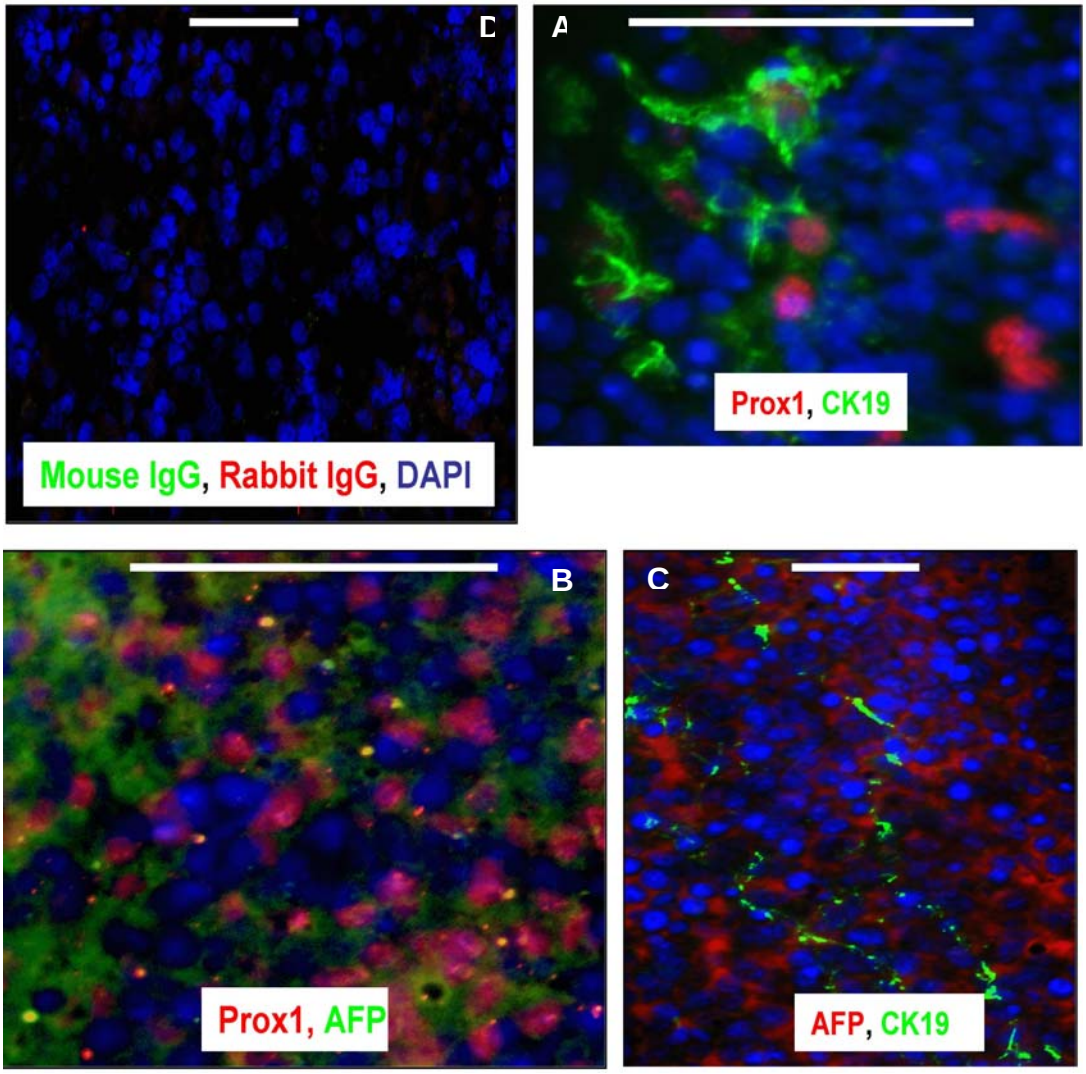


Figure 24: Immunohistochemical reactions with anti-Prox1 (*red*), anti-AFP (*red and green*) and anti-CK-19 (*green*) antibodies. counterstaining of nuclei with DAPI (*blue*). Prox1 negative/CK-19 positive(**A**) and Prox1 positive/AFP positive(**B**) cells are detected (Bar = 50 µm), the small CK-19 positive cells are AFP negative(**C**) (Bar = 100 µm). In negative control (performed with rabbit and mouse IgG, and secondary antibodies) of liver section at ED 16 no red or green reactions were observed (Bar = 100 µm).

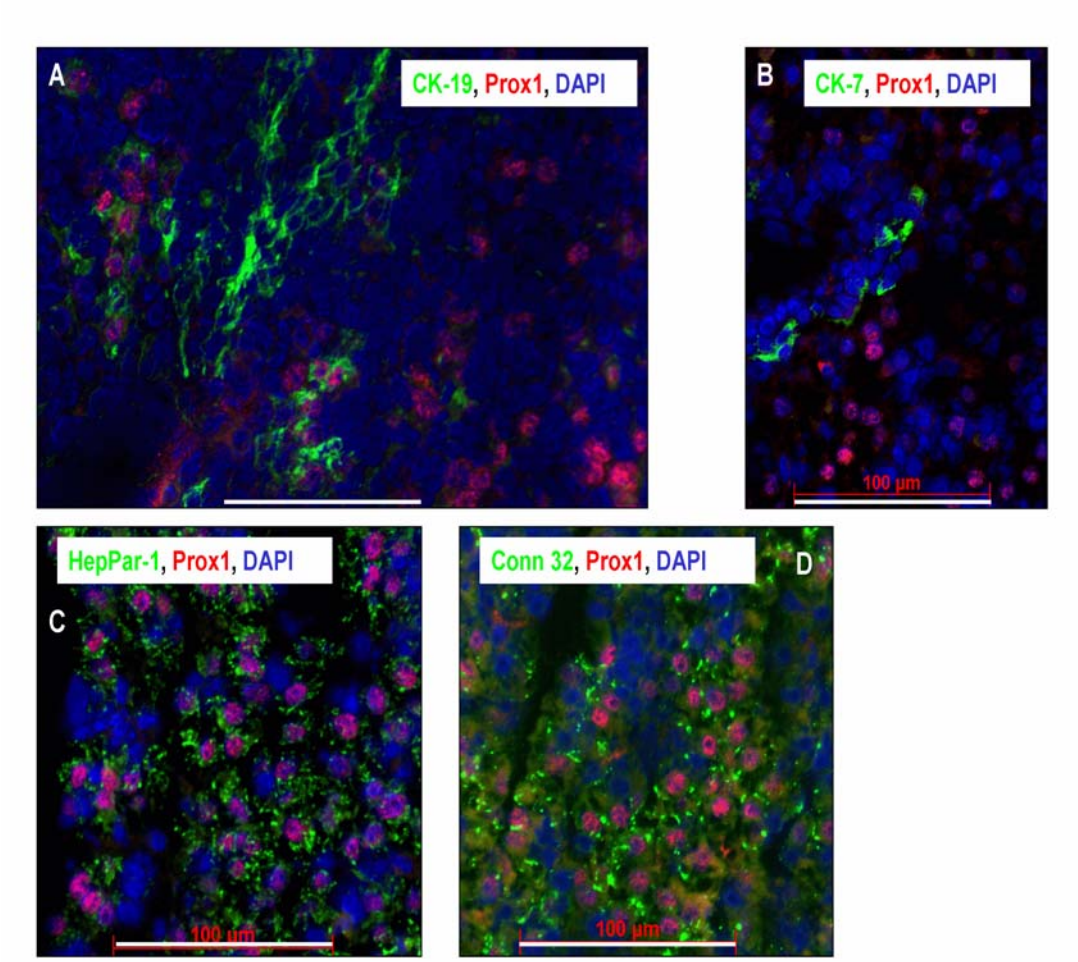


Figure 25: Reactions with anti-Prox1 (*red*) anti-CK-19 (*green*), anti-CK-7 (*green*), anti-HepPar-1 (*green*) and anti-connexin 32 (*green*) antibodies, counterstaining of nuclei with DAPI (*blue*) in the liver of rat embryos at 18 days of gestation. (**A**) Section of rat liver show positivity for CK-19 and Prox1. The Prox1 and CK-19 reactions are separated, but double positive cells are also present (Bar = 100 µm). (**B**) Section of rat liver show positivity for CK-7 and Prox1. The Prox1-positive hepatoblasts and the CK-7 positive bile duct epithelial cells are separated (Bar = 100 µm). (**C**) Section of rat liver show immunofluorescent staining for HepPar1 and Prox1 (Bar = 100 µm). (**D**) Section of rat liver show a positive staining for connexin 32 and Prox1 (Bar = 100 µm). Cell nuclear Prox1 immunostaining co-localises with the cytoplasmatic reaction of HepPar1 and the connexin 32-positive gap junctions.

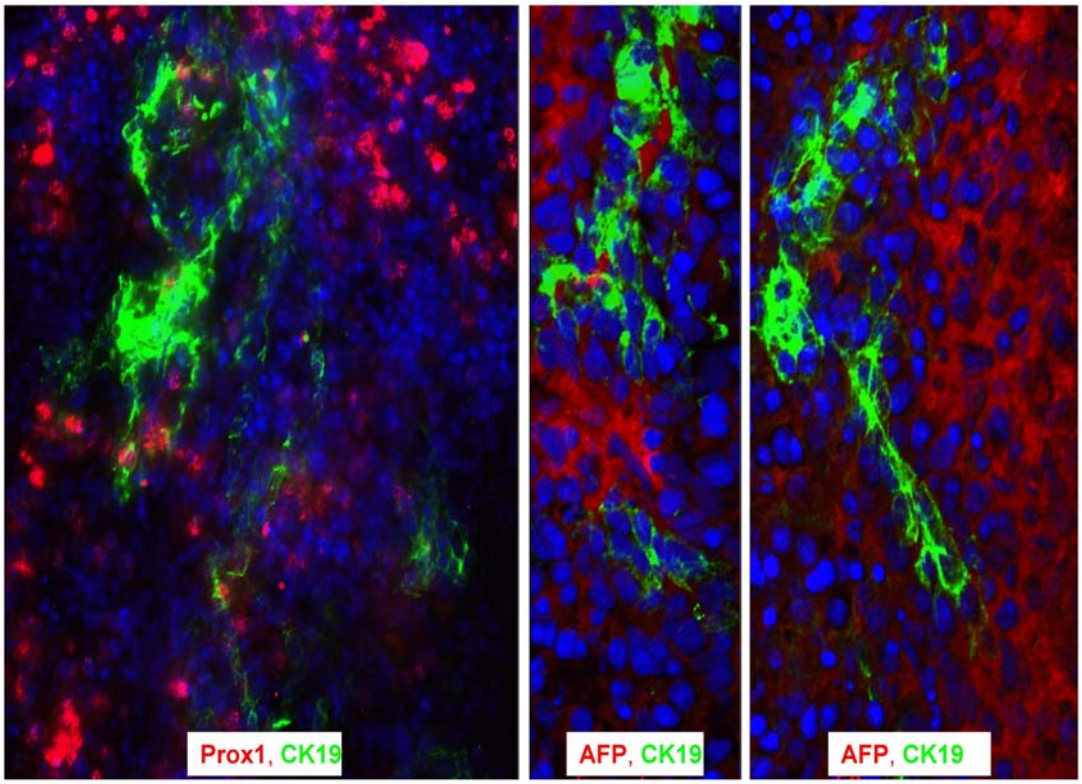


Figure 26: Prox1-, CK-19-, AFP-specific immunohistochemical reactions in embryonic rat liver at E18. CK-19 positive cells (*green*) are mainly Prox1 negative (*red*) (Bar = 50 µm), the CK-19 positive cells are AFP negative (*red*) are also detected in different cells (Bar = 50 µm)

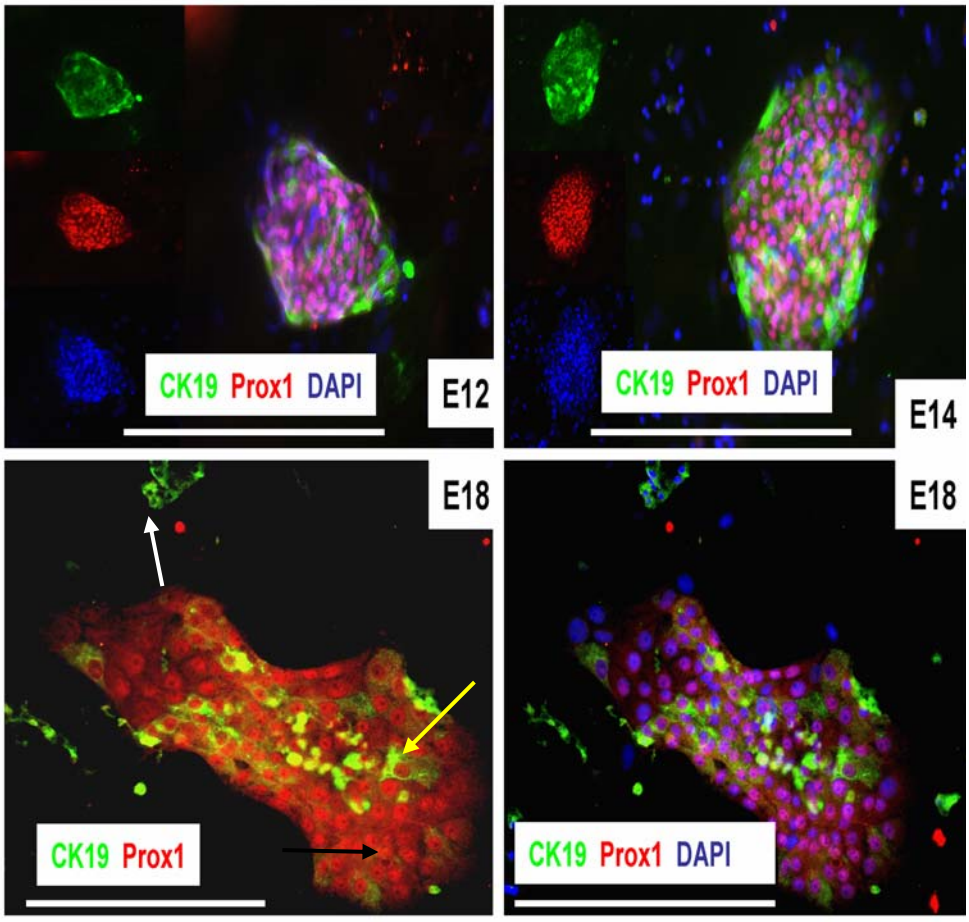


Figure 27: Prox1-, CK-19-specific immunocytochemical reactions in cultured hepatoblasts generated from developmental stages E12, E14 and E18. Hepatoblasts from E12 and E14 are Prox1 (*red*) and CK-19 (*green*) positive. At E18 three cell populations are observed. Prox1 and CK-19 positive cells (yellow arrows), Prox1 positive/ CK-19 negative cells (black arrows) and CK-19 positive/Prox1 negative (white arrows). Counterstaining of nuclei was performed with DAPI (blue) (Bar = 100 µm).

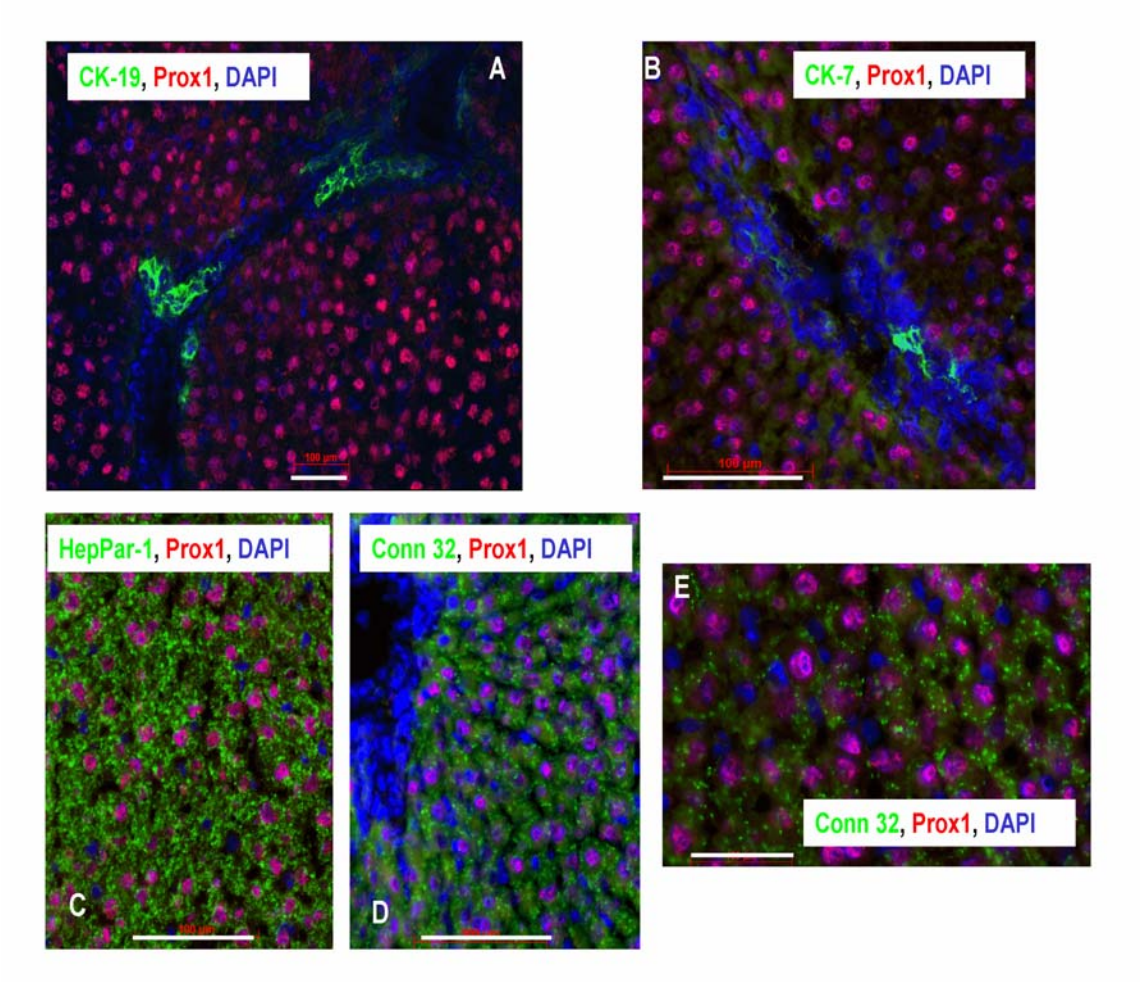


Figure 28: Reactions with anti-Prox1 (*red*) anti-CK-19 (*green*), anti-CK-7 (*green*), anti-HepPar-1 (*green*) and anti-connexin 32 (*green*) antibodies, counterstaining of nuclei with DAPI (blue) in adult rat liver. (**A**) Immunofluorescent staining for CK-19 and Prox1. The Prox1 and CK19 reactions are separated (Bar =50 µm). (**B**) Immunofluorescent staining for CK-7 and Prox1. The Prox1 and CK-7 reactions are separated (Bar = 50 µm). (**C**) Indirect immunofluorescent staining for HepPar1 and Prox1 (Bar = 50 µm). (**D-E**) Immunofluorescent stainings for connexin 32 and Prox1. Cell nuclear Prox1 immunostainings co-localise with the cytoplasmatic reaction of HepPar1 and the connexin 32-positive gap junctions (Bar = 50 µm).

	ED14	ED 18		ED14	ED 18
$Prox1^+/CK19^+$:	28.8± 4.13%	3.66 ± 0.04%	$AFP^+/CK19^+$:	36.69 ± 5.79%	3.8 ± 0.09%
$Prox1^+/CK19^-$:	8.8 ± 0.79%	31.53 ± 13.31%	$AFP^+/CK19^-$:	12.84 ± 2.10%	36.53 ± 3.28%
$Prox1^-/CK19^+$:	5.2 ± 1.38%	13.51 ± 5.90%	$AFP^-/CK19^+$:	8.92% ± 3.03%	14.92 ± 1.67%

Table 3: Quantitative comparison of Prox1- CK-19- and AFP-specific immunohistochemical reactions at embryonic (E14) and foetal stages (E18) of rat liver development. Single- and double-positive cell counts were related to all cell nuclei in the image and expressed in average % + standard error of measurement (SEM) %. In all quantitative immunohistochemical analysis 15 random taken samples from the investigated sections were analyzed

3.8. Effect of interleukin-6 on synthesis and secretion of albumin and AFP

The effect of interleukin-6 (IL-6) on synthesis (intracellular) and secretion (extracellular) of albumin and AFP was investigated during liver development. Hepatoblasts derived from 12, 14 and 18 days of gestation were stimulated with 100 ng/ml IL-6 and radioactive labelled with ^{35}S methionine for 12h. It has been observed that IL-6 has a positive effect on hepatoblasts derived from 14 and 18 days of gestation, whereas synthesis and secretion of albumin and AFP were suppressed. A slight effect of IL-6 on synthesis and secretion of AFP and a high effect of IL-6 on synthesis of albumin was observed in hepatoblasts from 12 days of gestation (Fig. 29A). This finding was confirmed by measurement of mRNA-expression levels of interleukin-6 receptor (IL-6 rec) in hepatoblasts using real time RT-PCR experiment. Hepatoblasts from 14 and 18 days of gestation displayed a high expression of interleukin-6 receptor (IL-6 rec) mRNA than hepatoblasts from E12 (Fig. 29B).

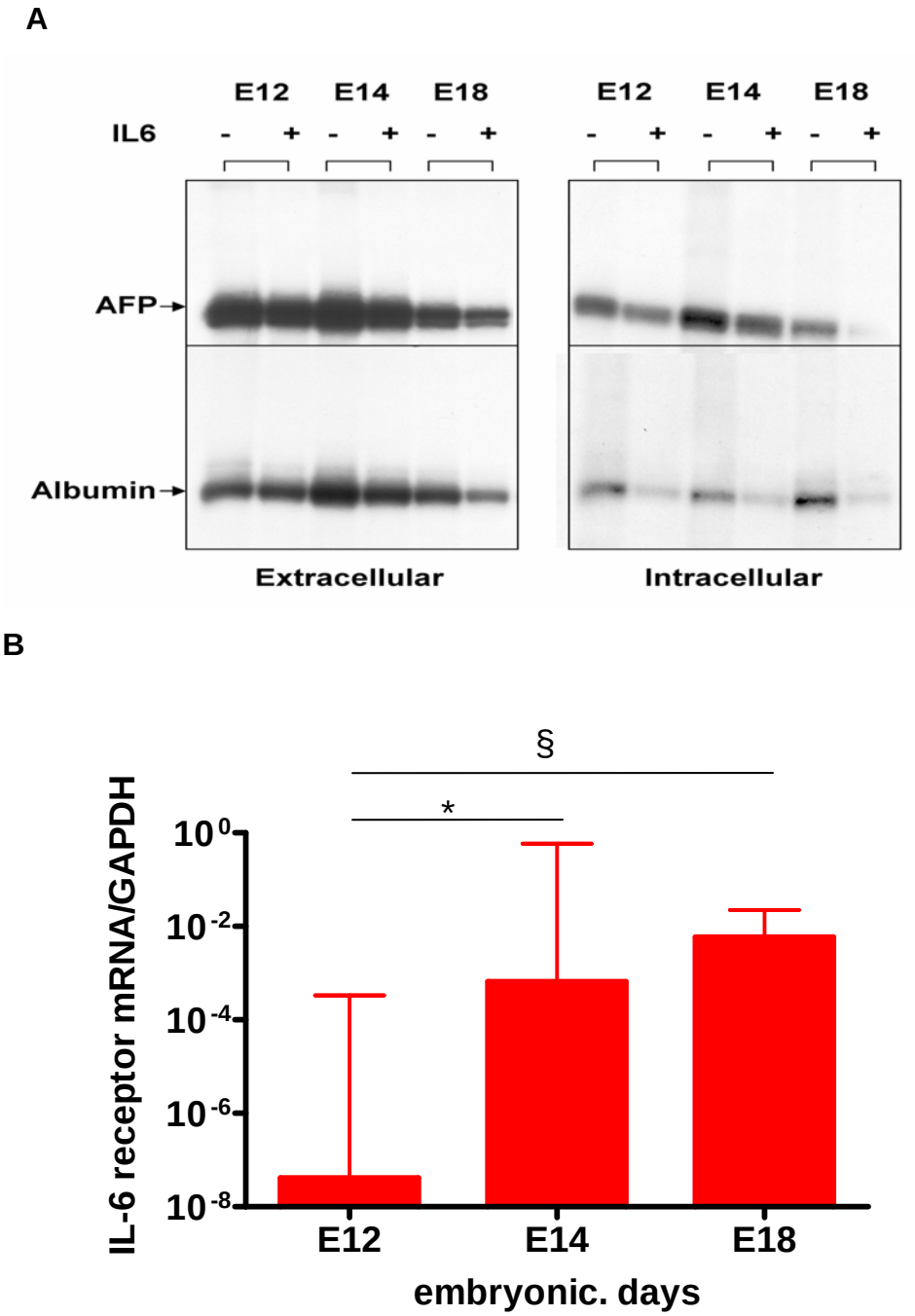


Figure 29: (**A**) Effect of interleukin-6 (IL-6) on synthesis and secretion of albumin and AFP in hepatoblasts at developmental stage E12, E14, E18. The cells were stimulated with 100 ng/ml interleukin-6 and radioactive labelled with 100 µCi ^{35}S-methionine simultaneously overnight. Cell-lysates (intracellular) and supernatants (extracellular) with similar count were used for immunopecipitation of albumin and AFP. (**B**) Real time PCR analysis of interleukin-6 receptor mRNA-expression in unstimulated hepatoblasts. The expression was normalized with the endogenous control of GAPDH. Error bars represent S.E.M, n=3. Statistically significant difference (*P<0.05; §<0.05) analysed by student´s *t*-test

3.9. Characterisation of hepatoblasts after different passages

Hepatoblasts derived from 12 and 14 days of gestation were passaged three times and analysed with a view to find out if hepatoblasts could maintain hepatic phenotype after the passage. Albumin and AFP gene-expression was measured at each passage by using real time PCR and radioactive biosynthetic labelling experiment. We found that after the first passage, hepatoblasts could express, synthesize and secrete albumin and AFP. During the second passage albumin and AFP could not be detected, (Fig. 30 and 31).

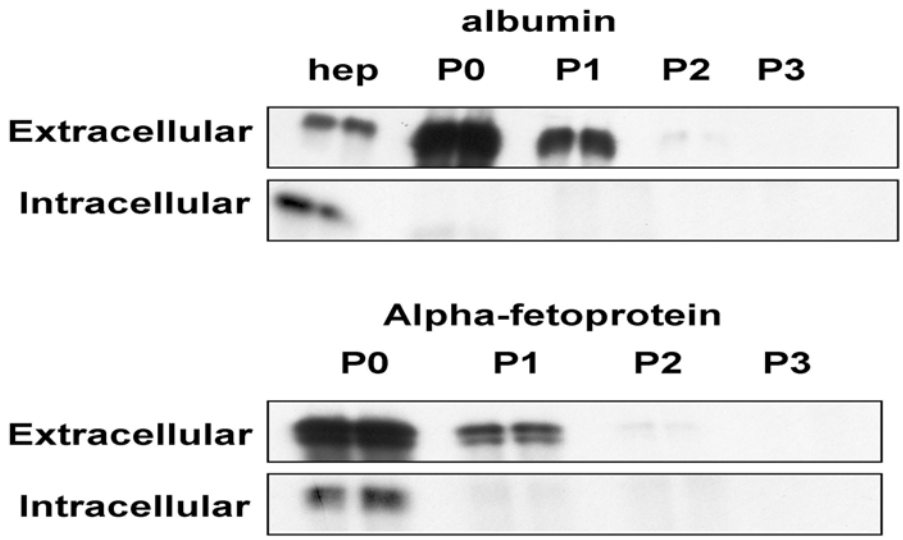


Figure 30: Synthesis and secretion of Albumin and AFP in passaged hepatoblasts derived from 12 and 14 days of gestation. At each passage, cells were labelled with 100 µCi ^{35}S-methionine per well. Albumin and AFP were immunopecipitated from 100 µl cell-lysates (Intracellular) and supernatants (extracellular). As positive control, 25 µl supernatant and cell-lysates of labelled hepatocytes were used.

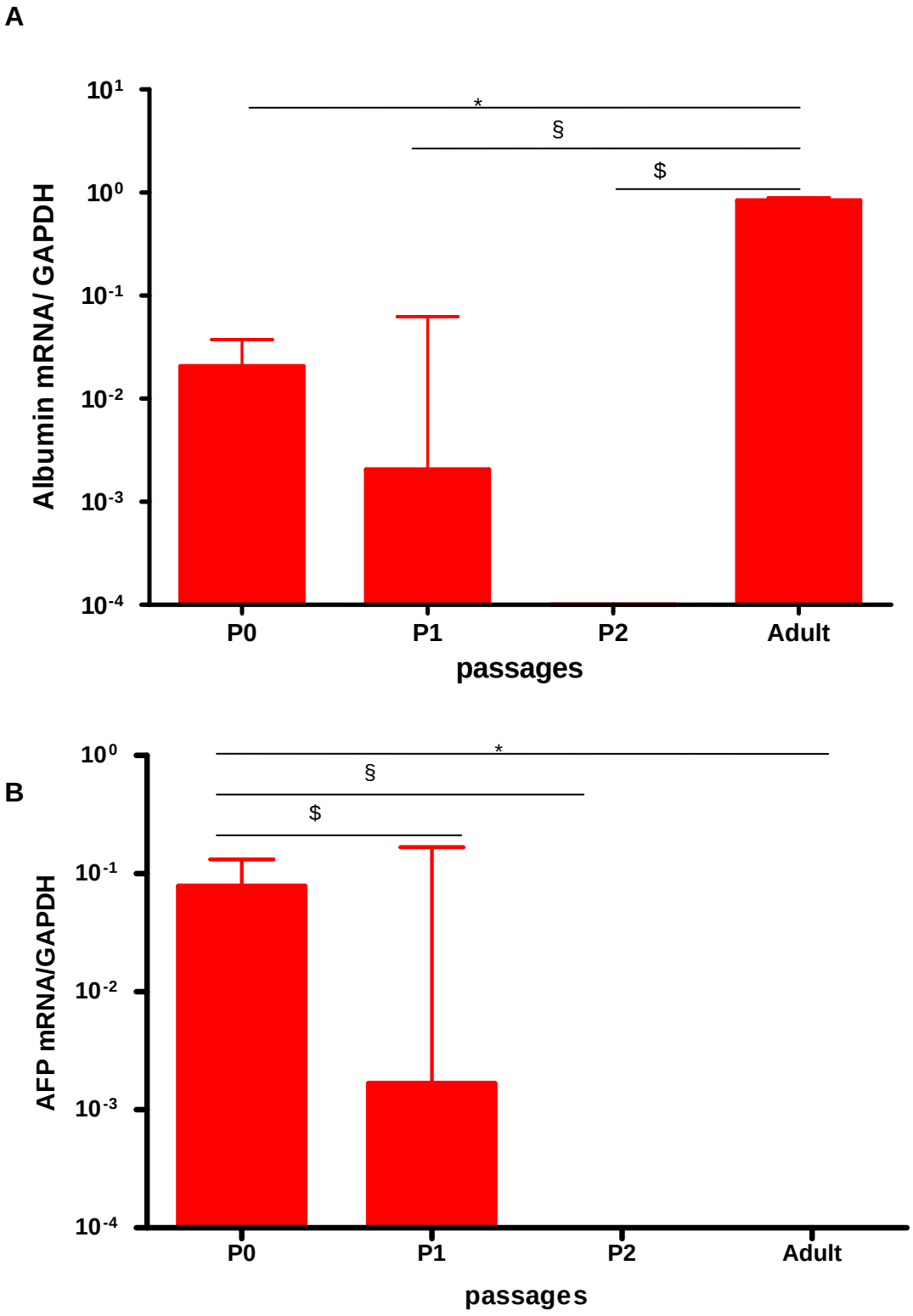


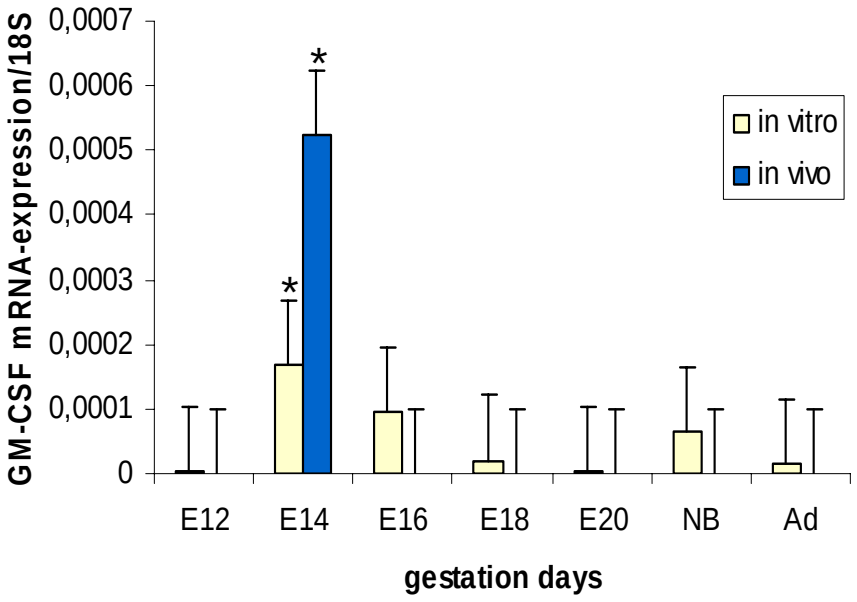
Figure 31: Real-time PCR analysis of albumin (A) and AFP (B) mRNA-expression after passage of hepatoblasts. The expression was normalized with the endogenous control of GAPDH. Error bars represent S.E.M, n=3. Statistically significant difference (*P<0.05; §<0.05; $<0.05.) analysed by student´s *t*-test.

3.10 Changes in gene expression of hematopoietic regulatory cytokines during liver development

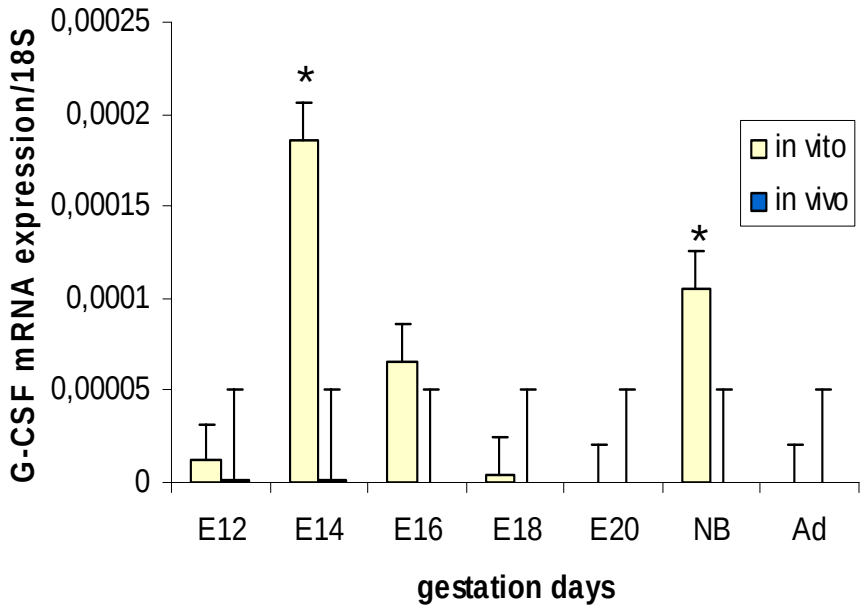
In adult bone marrow, the cytokine controlled hematopoiesis were expressed by nonhematopoietic stromal cells, including fat cells, endothelial cells, fibroblasts and macrophages. In case of inflammation, the cytokines could be synthesized by activated T lymphocyte and macrophage. The embryonic/foetal liver is a mosaic of cells consisting of hepatoblasts, which are mainly represented, hematopoietic stem cells, endothelial cells and fibroblasts. Gene expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and Erythropoietin (Epo) was examined in whole liver (*in vivo*) and cultured embryonic hepatic cells (*in vitro*) during liver development.

At 14 days of gestation, in whole liver (*in vivo*) a strong and statistically significant mRNA expression of the cytokines GM-CSF and Epo was observed (Fig. 32 .A and D). G-CSF was expressed only in cultured hepatic cells (*in vitro*). In contrast, Epo was expressed only in whole liver (in vivo) (Fig. 32 D). G-CSF mRNA was high expressed at 14 days of gestation. It underwent a moderate expression at 16 days of gestation and at new born state (Fig. 32 B). It was observed that the embryonic liver expressed high amount of SCF at 12 day of gestation and slight amount at 14 and 16 days of gestation. During the foetal and postnatal life SCF was down-regulated (Fig. 32 C).

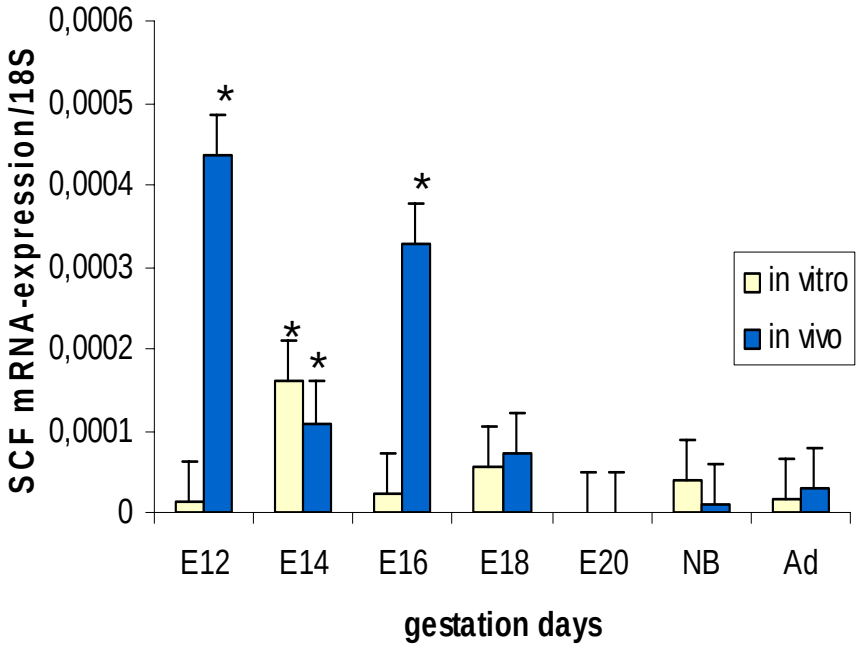
A


GM-CSF mRNA-expression/18S
0,0007
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
*
*
in vitro
in vivo
E12
E14
E16
E18
E20
NB
Ad
gestation days

B


G-CSF mRNA expression/18S
0,00025
0,0002
0,00015
0,0001
0,00005
0
*
*
in vito
in vivo
E12
E14
E16
E18
E20
NB
Ad
gestation days

C


SCF mRNA-expression/18S
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
*
*
*
*
in vitro
in vivo
E12
E14
E16
E18
E20
NB
Ad
gestation days

D

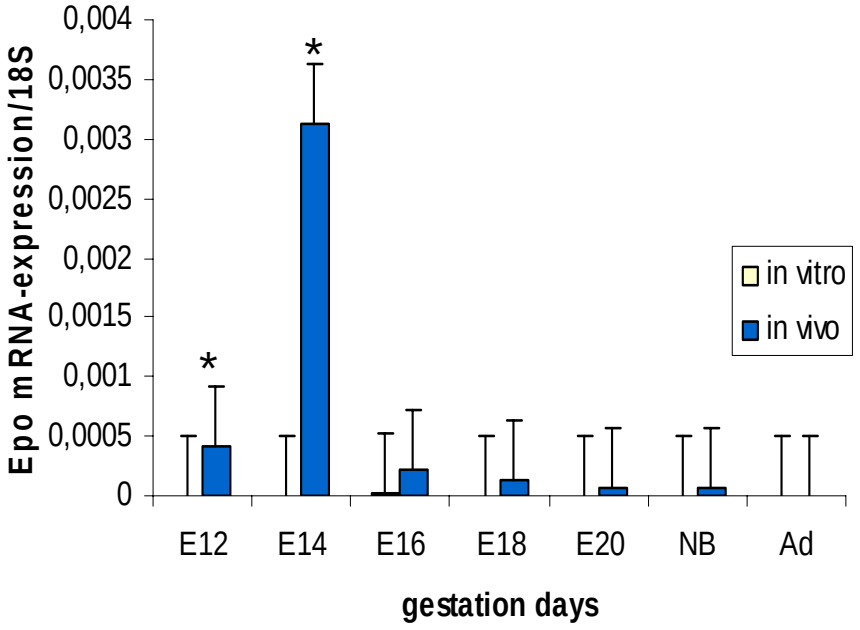

Epo mRNA-expression/18S
0,004
0,0035
0,003
0,0025
0,002
0,0015
0,001
0,0005
0
*
*
in vitro
in vivo
E12
E14
E16
E18
E20
NB
Ad
gestation days

Figure 32: Real-time-PCR -analysis of mRNA from whole liver (*in vivo*) and cultured hepatic cells (*in vitro*). Livers were obtained and the hepatic were cultured at different developmental stage as described in materials and methods. Fold change of granulocyte-macrophage colony-stimulating factor (GM-CSF) (**A**), granulocyte colony-stimulating factor (G-CSF) (**B**), stem cell factor (SCF) (**C**) and Erythropoietin (Epo) (**D**) gene-expression during liver development. Values represent the amount of target mRNA compared to 18S ribosomal mRNA (*P<0.05. Error bars represent S.E.M., n=3)

	Embryonic developmental stage						
Genes	**E12**	**E14**	**E16**	**E18**	**E20**	**New born**	**adult**
GM-CSF in vivo	-	+++	-	-	-	-	-
GM-CSF in vitro	-	+	+	+/-	-	+/-	-
G-CSF in vivo	-	-	-	-	-	-	-
G-CSF in vitro	+/-	+++	+	+/-	-	++	-
SCF in vivo	+++	+	++	+	-	+/-	+/-
SCF in vitro	+/-	++	+/-	+/-	-	+/-	+/-
Epo in vivo	+	+++	+/-	+/-	+/-	+/-	+/-
Epo in vitro	-	-	-	-	-	-	-

+++ : high expression

++ : moderate expression

+ : low expression

-/+ : very low expression

- : no expression

Table 4: Summary of the mRNA expression levels of cytokines GM-CSF, G-CSF, SCF and Erythropoietin (Epo) in whole liver (*in vivo*) and cultured hepatoblasts (*in vitro)* during liver development.

4. DISCUSSION

4.1 Albumin and AFP gene expression at the time of hepatic specification (E10)

The results presented in section 3.2 describe the expression of albumin and AFP mRNA in the ventral foregut region of E10 rat embryos (0-4 somites) as well as in explanted cells generated from ventral foregut endoderm. Previous studies using rat embryos claimed that AFP could be expressed one day before albumin expression (Shiojiri et al., 1991b; Muglia and Locker, 1984)). Muglia and Locker, (1984) used a blot-hybridization followed by densitometric analysis at several exposures. They reported that albumin and AFP mRNA were not detectable in the 10-day RNA preparation taken from the foregut region, even after long exposure times. In 12-day liver RNA, the expression of both genes was observed. Using *In situ* histological experiments neither albumin mRNA nor AFP mRNA could be detected at E9.5-E10. At E10.5 in the ventral endoderm AFP mRNA was clearly detected one day before its coding protein (E11.5). At 11.5 days of gestation albumin mRNA was identified, but its protein appeared one day later (E12.5) (Shiojiri et al., 1991a). This finding is consistent with the studies using 4 to 6 somites mice embryos (E8-E9.5) (corresponding to E9.5-E11 in the rat). It has been shown that AFP mRNA is detected by *in situ* hybridization performed on whole embryos or in explanted cells derived from ventral region of embryos (Gualdi et al., 1996) (Lee et al., 2005). RT-PCR experiment provided a possibility to detect AFP mRNA in the ventral and dorsal endoderm. 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 (Deutsch et al., 2001; Jung et al., 1999; Gualdi et al., 1996; Micsenyi et al., 2004; Serls et al., 2005). In this study, we demonstrated by real time PCR that unstimulated explanted cells derived from ventral foregut region could express albumin mRNA.

Previous histological studies have not examined the synthesis and secretion of albumin and AFP in viable endodermal cells. Previously albumin-expression at protein level was detected by immunofluorescence analysis in sectioned mouse embryos from 9-10 somites stages as well as in single cell cytospin slides generated from dissected ventral foregut endoderm at 8, 10 and 14 somites (Serls et al., 2005). In this study we established, for the first time, a reliable method, using a sensitive radioactive biosynthetic labelling, for analysing the albumin and AFP synthesis and secretion capacity of endodermal cells generated from the ventral foregut region. Our results suggest that the whole program controlling the regulation of gene expression, synthesis and secretion of albumin and AFP already act at the earliest developmental stage, when hepatic specification of endoderm cells begins.

4.2 Characterisation of endodermal cells

The endodermal germ layer gives rise to a number of different organs including the liver. The characterization of endodermal cell populations, which develop into hepatic buds, has not been previously thoroughly investigated. The present study shows that explanted endodermal cells from ventral endoderm could express HNF4-alpha, Prox1, beta-catenin, BMP-4, Foxa-2, and GATA-4. These factors play crucial role in

hepatogenesis. GATA-4 homozygous null mice display defects in ventral foregut development (Molkentin et al., 1997) and GATA-4 is required for endoderm development (Narita et al., 1997; Bossard and Zaret, 1998). BMP-4 signalling transmitted from mesenchymal cells allow the endoderm to induce liver gene expression in response to FGFs (Rossi et al., 2001). Prox1 is expressed in the endoderm (Burke and Oliver, 2002) and its activity is essential for liver development, due to its role in regulation of genes that control proliferation and migration of hepatoblasts (Sosa-Pineda et al., 2000). HNF4-alpha is one of the earliest primary endoderm markers, its expression is restricted during liver development (Duncan et al., 1994). In the adult organism HNF4-alpha is expressed in the liver and is capable to activate the expression of numerous hepato-specific genes (Tian and Schibler, 1991). In this study we demonstrated that HNF4-alpha is expressed by in culture clustered endodermal cells derived from ventral foregut region, which could be the expressing cells for albumin and AFP. Foxa-2 (HNF3beta) is first detected on the seventh day of murine gestation (according to 8.5 day in the rat) in the primitive streak and node (Ang et al., 1993). It has been shown that the Foxa-transcription factors participate in alteration of chromatin structures during activation of liver-specific target genes (Zaret, 2002). Beta-catenin is one of the key proteins in the wnt/beta-catenin pathway. It has been reported that this pathway plays a crucial role during liver development and could activate target gene expression that controls proliferation, adhesion, polarity, apoptosis and lineage specification (Monga et al., 2003); (Willert and Nusse, 1998). The origin, localisation and migration of endodermal cells were examined by endoderm fate maps study in mice embryos from early stage (1 to 6

somites). It has been demonstrated that a cell population at the ventral midline of the endoderm lip (VMEL) express a liver specific gene HNF4-alpha (Tremblay and Zaret, 2005).

4.3. Cellular analysis during liver development

At 12 days of gestation, when the embryonic liver is formed, albumin and AFP genes are mainly expressed by hepatoblasts. In this study we demonstrated that during embryonic and foetal stages about 50% of liver cells are engaged in both albumin and AFP gene expression. In addition the ratio of albumin and AFP producing cells to proliferating cells increase during embryonic stage. At 18 days of gestation the ratio of albumin and AFP producing cells to proliferating cells reaches its maximum. From this stage up to birth, we observed an increase of the number of albumin producing cells. After 18 days of gestation the number of proliferating cells decrease and hepatoblasts produce more albumin than AFP. The volume of the liver expands 84-fold during liver development, and this correspond to 8 doubling of hepatoblasts (Greengard et al., 1972); (Vassy et al., 1988). It is especially noteworthy that, while an increase of liver size and an increase of the ratio of albumin and AFP-producing cells to proliferating cells occurs, about 50% of liver cells are engaged in production of albumin and AFP during liver development. This means that embryonic liver consists of about 50% hepatoblasts, and the other 50% is composed of endothelial and mesenchymal cells.

It has been proposed that the young mammalian liver is composed of a cell mosaic consisting of functionally heterogeneous hepatocytes, some producing AFP, others albumin (Valet et al., 1981); (Kuhlmann, 1979); (Guillouzo et al., 1979). In this study, we show using a double *in situ* hybridization that albumin and AFP genes could be co-expressed earlier by hepatoblasts, at the developmental stage when the liver is formed

(E12) up to new born rats. Previous immunocytochemical analysis in rat and pigs demonstrated that at foetal stage, albumin and AFP genes could be co-expressed by hepatocytes (Carlsson and Ingvarsson, 1979); (Dempo et al., 1983); (Nayak and Mital, 1977). Rat foetuses from seventeen to nineteen days of gestation and newborn animals displayed a simultaneous expression of albumin and alpha-fetoprotein genes by all hepatocytes. However after the first weeks of postnatal life albumin and alpha-fetoprotein genes are expressed respectively by periportal and perivenous hepatocytes (Poliard et al., 1986).

4.4. Albumin and AFP mRNA expression in developing liver

The results presented in section 3.5 indicate that albumin and AFP mRNA levels do not develop strictly in parallel with developing liver. In whole liver (*in vivo*), albumin mRNA remains constant during embryonic stages (between E12 and E16), in contrast, AFP mRNA decreases at 14 days of gestation and maintains until the end of embryonic stage. The highest expression level of both genes was achieved during the foetal stage (E18). Before birth and during the postnatal life, albumin and AFP mRNA expressions are inversely regulated.

Albumin and AFP mRNA levels were measured in explanted hepatoblasts (*in vitro*) at different developmental stage in view to support the data obtained in whole liver (*in vivo*). We observed an increase in albumin and AFP mRNA during 16 days of gestation. Albumin mRNA reaches a peak at 18 days of gestation followed by a slight decrease at 20 days of gestation, and then increase at birth and during the postnatal life. However AFP mRNA reaches a peak at 16 days of gestation and then decrease during the following stages. These results are in agreement with previous

studies performed with northern blot and densitometric analysis of albumin and AFP gene transcripts (Muglia and Locker, 1984). Other studies indicated that from 17 days of gestation up to fifty days after birth, the ratios of AFP synthesis to albumin synthesis and the ratios of AFP mRNA to albumin mRNA decrease gradually (Liao et al., 1980).

4.5. Synthesis and secretion of albumin and AFP during liver development

Previously, synthesis of albumin and AFP was investigated during liver development by immunological techniques (Dempo et al., 1983), and the secretion was examined by direct measurement of serum concentration in yolk sac (Gitlin, 1973). Serum concentration of AFP in foetal liver and yolk sac underwent a decrease followed with a rapid fall after birth to reach a low level in adult state. In contrast, albumin concentration increases from low level early in foetal development to high level after birth and throughout adult life (Abelev, 1974); (Ruoslahti and Terry, 1976). Radioactive biosynthetic labelling method has provided a possibility to assess simultaneously synthesis and release of secreting proteins in viable hepatoblasts. During embryonic stage (E12 up to E16), albumin and AFP proteins were maintained in intracellular and extracellular sites of hepatoblasts. At 18 days of gestation we observed an increase of synthesis and secretion of albumin and AFP, followed by a slight decrease at 20 days of gestation. At birth and during postnatal life albumin and AFP genes were inversely regulated. This finding was supported by measurement of the secretion velocity of albumin and AFP in hepatoblasts at developmental stages E12, E14, E16, E18, and adult hepatocytes using pulse chase experiment. The time needed for synthesis and secretion of albumin and AFP was estimated after a chase period of 15, 30, 45, 60, 90,

120 and 240 min. Previously, it has been examined the residence kinetics for albumin in hepatoma cell line HepG2 using a pulse chase labelling experiment combined with subcellular fractionation. It has been indicated a rapid decrease of albumin in endoplasmatic reticulum and golgi apparatus during a chase period of 40 min (Bostrom et al., 1986). Hepatoblasts generated from embryonic liver at 12 days of gestation needs 45 min to release AFP, in contrast at 14, 16 and 18 days of gestation AFP was secreted rapidly and at the same time (15 min). The secretion velocity of albumin differed from that of AFP. At 14 and 16 days of gestation albumin was secreted after a chase period of 30 min. However, at 18 days of gestation and at adult state a short time (15 min) for albumin secretion was needed. We thought that at embryonic stage, albumin and AFP need different time for synthesis and secretion. This results are in agreement with the reports by other researchers indicating that different secretory proteins leave endoplasmic reticulum (ER) with different kinetics (Lodish et al., 1983); (Fries et al., 1984). We noticed that hepatoblasts from late developmental stage (E18) suggest a secretion velocity, which is comparable to mature hepatocyte. This might be due to the differentiation process affecting the organelles involved in intracellular transfer and secretion during embryonic development. Hepatoblasts derived from 12 days of gestation displayed a high translation activity after 9 days in culture and secreted albumin continuously even after chase period of 8 h. This observation is comparable to mature hepatocyte and hepatoblasts from late gestation stage, which continuously synthesize and secrete albumin and AFP.

4.6. Characterization of intrahepatic bile duct cells during liver development

Porspero-related homeobox 1 (Prox1), a divergent transcription factor is involved in the liver development. Prox 1 is expressed in the endoderm that gives rise to the liver and pancreatic anlagen (Burke and Oliver, 2002). It is not restricted to early embryonic stages, but persists into adulthood (Dudas et al., 2004). In this study we demonstrated the spatial and temporal distribution of Prox1, cytokeratin-7 and -19 in embryonic and foetal rat liver. It was claimed that the hepatoblasts give rise to definitive hepatocytes and cholangiocytes (Lemaigre and Zaret, 2004). The exact mechanisms through which bipotential hepatoblasts decide to become hepatocytes or biliary epithelial cells are still unclear, although several factors that contribute to this cell fate decision have already been identified (e. g. Notch signalling, (Tanimizu and Miyajima, 2004); (Lemaigre and Zaret, 2004). Intrahepatic bile ducts starts to differentiate at E15.5 in the rat liver. CK-19 is detected early in the cells that are committed to the bile duct epithelial lineage, while CK-7 is expressed in the biliary epithelial cells in the late gestational stage (Shiojiri, 1997), (Kiiasov et al., 1997).

In the rat embryonic liver (at E12 and E14), Prox1 and CK-19 were found to be expressed in the same cells (hepatoblasts), but these two markers have shown trends of separated expression at early and late developmental stage. Double-positive cells (CK-19/Prox1) were found in the foetal stage (E18) and CK-7 was found only in Prox1 negative cells. In cultured hepatoblasts derived from different developmental stages, immunoreactions with Prox1 and CK-19 antigens has revealed that foetal liver (E18) contains three cell populations, prox1 positive cells, CK-19

positive cells and prox1/CK-19 positive cells. This data clearly demonstrates that Prox1 expression is lost from cells committed to the bile duct epithelial fate, and remains expressed in hepatocytes. The fact that Prox1 is not expressed in bile duct epithelial cells, which are probably derived from Prox1-positive hepatoblasts, suggests a specific function of the transcription factor for the hepatic lineage. In the adult rat liver, Prox1 was expressed in the hepatocytes, proved by additional markers as HepPar-1 and connexin 32 (Haruna et al., 1996), (Paku et al., 2004), while CK-19 and CK-7 were expressed in the biliary cells. Double expression patterns were not observed.

4.7. Effect of interleukin-6 (IL-6) on albumin and AFP gene expression in developing liver

The results presented in section 3.8 indicated that interleukin-6 (IL-6) has a positive effect on hepatoblasts derived from 14 and 18 days of gestation. After stimulation with IL-6, we observed a decrease in albumin and AFP synthesis and secretion. However in hepatoblasts generated from 12 days of gestation a slight effect on synthesis and secretion of AFP and a high effect on synthesis of albumin were observed. According to this observation, unstimulated hepatoblasts from E12, 14 and 18 days of gestation display differential expression of interleukin-6 receptor (IL-6 rec). Hepatoblasts from E14 and E18 express about 10^5 fold interleukin-6 receptor (IL-6 rec) than hepatoblasts from E12.

Interleukin-6 (IL-6) is known as factor that affects a changing in synthesis of several plasma proteins such as haptoglobin, alpha-fetoprotein, beta 2-microglobulin, and fibronectin in the liver during acute phase reaction (Ramadori and Christ, 1999), (Roncero et al., 1995). It has been

demonstrated that IL-6 causes proliferation and differentiation of rat hepatoblasts derived from 14 days of gestation. On the presence of IL6, albumin producing cells decreased after 8 days in culture. When hepatoblasts were stimulated with IL-6 for 3 days and grown for 5 days without IL-6, a high percentage of albumin producing cells were observed. Besides, albumin and AFP synthesis were suppressed after 8 days and 5 days in culture respectively (Zvibel et al., 2004). The role of IL-6 in liver regeneration is well documented. Mice that were IL-$6^{-/-}$, as well as knockout mice for Tumour Necrosis Factors- receptor 1 (TNF-R1), had impaired liver regeneration after partial hepatectomy. Administration of exogenous IL-6 to IL-6-deficient mice restored hepatocytes proliferation (Cressman et al., 1996). Oncostatin M (OSM), an interleukin-6 family cytokine, in combination with glucocorticoid, induce maturation of hepatocytes, a paracrine mechanism of hepatogenesis; blood cells, transiently expanding in the foetal liver, produce OSM to promote development of hepatocytes in vivo (Miyajima et al., 2000).

4.8. Identification of hepatic phenotype in passaged hepatoblasts

Maintenance of hepatic phenotype for long time in cultured hepatoblasts remains a challenge. Various studies have examined foetal hepatic as well as hepatic stem/progenitor cells and kept them for long-term in culture. However most of these studies were interested in the differentiation process stimulated by different growth factors (Suzuki et al., 2003); (Qin et al., 2004); (Kamiya et al., 2006). Human foetal hepatocytes were kept in primary culture for 2 to 4 months without apparent loss of hepatocytic traits. They displayed high proliferation and the culture can be maintained in serum- free medium in the presence of epidermal growth factor and can be passaged at least twice (Lazaro et al., 2003). It has been demonstrated

that long-term culture of murine hepatoblasts on laminin produces multi-potential hepatic progenitors, which possess a strong proliferative capability, differentiate into both hepatocytes and cholangiocytes, and potentially give rise to pancreatic cells (Tanimizu et al., 2004).

In this study, rat hepatoblasts derived from 12 and 14 days of gestation were passaged three times and mRNA expression as well as synthesis and secretion of the main hepatic markers (albumin and AFP) were examined at each passage using the sensitive methods (real time PCR and radioactive biosynthetic labelling). We observed that hepatoblasts could maintain a hepatic phenotype after one passage. During the second passage the hepatoblasts lost their hepatic phenotype.

4.9. Regulation of hematopoiesis during liver development

In this study we examined mRNA-expression of genes coding for factors regulating hematopoiesis such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and Erythropoietin (Epo) at different embryonic developmental stage in rat liver. A high expression of genes coding for factors which stimulate erythropoiesis was observed at 12 and 14 days of gestation. These factors were down-regulated during the following developmental stages. The developmental stages E12 and E14 were designated as the embryonic stage at which the primitive (at E12) and definitive (at E14) erythropoiesis occur. It was previously reported that erythropoietin promotes the proliferation and differentiation of erythroid progenitor cells and regulates the number of erythrocytes in peripheral blood (Yoshimura and Arai, 1996; Yoshimura and Misawa, 1998). It acts late in the course of erythropoiesis in combination with other factors such as stem cell factor (SCF), interleukin (IL)-3, IL-4, and granulocyte macrophage colony-stimulating factor (GM-CSF) (Yoshimura and Misawa,

1998). The proliferation and differentiation of primitive and definitive erythroid cells could be stimulated by GM-CSF independently of erythropoietin receptor (EPOR) and the activity is comparable to that of erythropoietin in definitive, but not primitive erythropoiesis (Hisakawa et al., 2001). In fact, during liver development the change from primitive hematopoiesis into definitive hematopoiesis coincide with a high expression of genes coding for factors stimulating erythropoiesis.

4.10. The outlook

The use of radioactive biosynthetic labeling followed by specific immunoprecipitation and SDS-PAGE analysis allowed us to monitor, for first time, the synthesis and secretion of albumin and alpha-fetoprotein in endodermal cells derived from ventral foregut region at 10 days of gestation. We thought that 10 days of gestation (E10) is the earliest time point for the emergence of hepatoblasts. It seems that at the time of initiation of hepatic specification (E10) the complete program already operates, controlling the regulation of gene expression of albumin and AFP. This study provides a basis for further investigations on endodermal cells derived from ventral foregut and their application in the regenerative medicine.

Our cellular analysis showed the number of albumin and alpha-fetoprotein producing cells develop in a similar way up to E18. During this time there is an increase of the ratio of albumin and alpha-fetoprotein producing cells to proliferating cells as the liver develops and increases in size. Despite this, we found the ratio of albumin and alpha-fetoprotein to the total number of liver cells remained at 50% throughout liver development. After 18 days of gestation the ratio of albumin producing cells to proliferating cells continues to increase until adulthood. In contrast, the ratio of alpha-

fetoprotein producing cells to proliferating cells reaches a maximum and thereafter decreases. The co-expression (co-localisation) of albumin and alpha-fetoprotein is not restricted to the foetal stage. Both plasma proteins were co-expressed by hepatoblasts from 12 days of gestation, the time when the embryonic liver appears, till birth.

Quantitative analysis revealed that at 18 days of gestation, albumin and alpha-fetoprotein mRNA production reaches a maximum and a high level of synthesis and secretion of albumin and alpha-fetoprotin was observed. Additionally, it was found that at the embryonic stage (from E12 up to E16) alpha-fetoprotein was synthesized and secreted at a higher rate than albumin even though the number of albumin and alpha-fetoprotein producing cells is similar. After 18 days of gestation to birth the kinetics for synthesis and secretion of albumin is similar to that in mature hepatocytes.

Three cell populations, Prox1 positive/CK-19 positive cells, Prox1 negative/CK-19 positive cells and Prox1 positive/ CK-19 negative cells we identified. CK -7 is first detected on the 18 day of gestation. It is expressed by Prox1 negative/CK-19 positive cells. Cultured embryonic liver cells could provide a suitable opportunity to study intrahepatic bile ducts development.

Interleukin-6 (IL-6) has a positive effect on hepatoblasts after 14 days of gestation

The hepatoblasts lost their hepatic phenotype during the second passage.

During liver development the change from primitive hematopoiesis (E12) to definitive hematopoiesis (from E14 to adulthood) coincide with a high expression of genes coding for factors regulating erythropoiesis

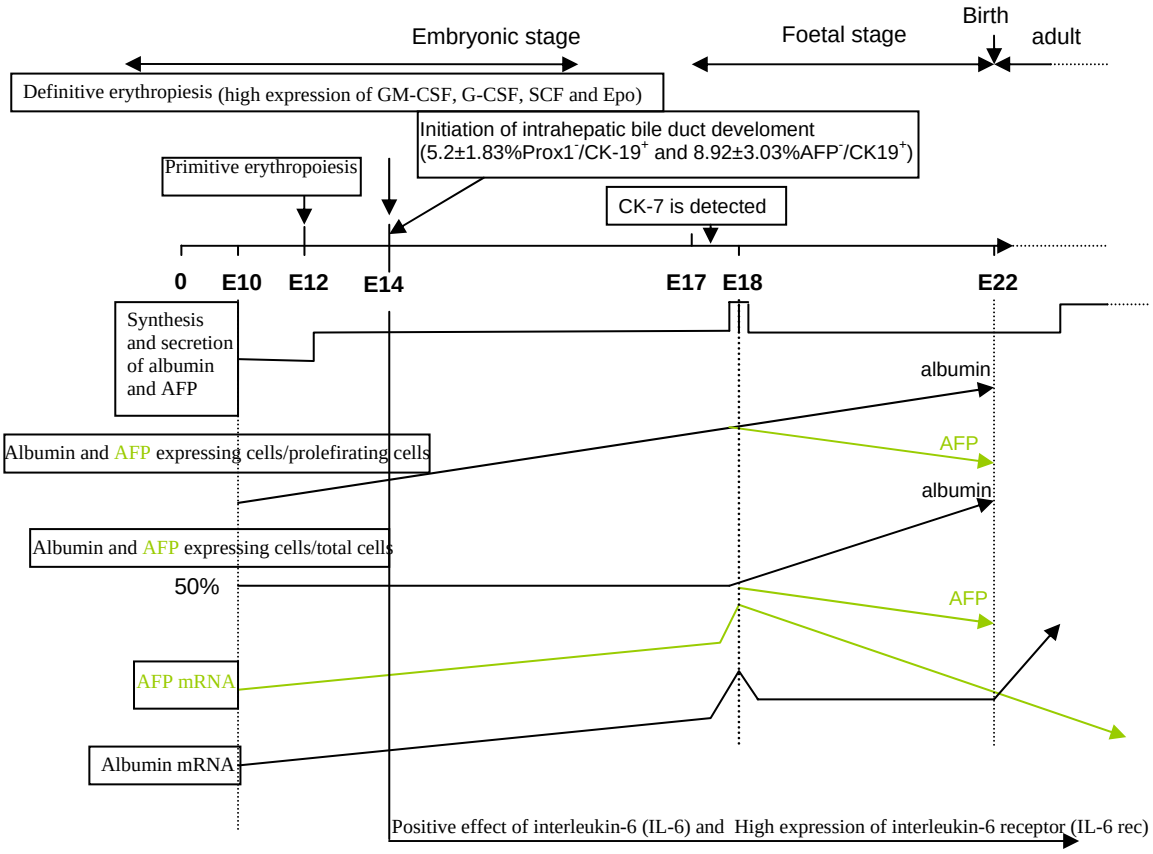


Figure 33: represents the possible changes in characteristics of liver cells during liver development.

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5. ZUSAMMENFASSUNG

Die Leber entwickelt sich als Leberknospe aus dem Entoderm des unteren Abschnittes des Vorderdarmes. Die Leberknospe differenziert sich in 2 Teile: In den Leberteil und in den Galleteil. Während der embryonalen Entwicklung differenzieren sich die entodermalen Zellen zu Hepatoblasten, die sich zu reifen Hepatozyten weiter entwickeln können. Ausgehend vom Mesoderm beginnt die Blutbildung im Dottersack. Zum Zeitpunkt der Entstehung hämatopoetischer Knospen in der fetalen Leber beginnt die erste ortständige Blutbildung. Von dort wandern die Stammzellen in die Milz und später in das Osteoid der Knochenanlage, wo sich das Knochenmark entwickelt. Die embryonalen Leberzellen wurden in einigen Studien charakterisiert. Jedoch ihre Charakterisierung während der Leberentwicklung ist bis jetzt noch nicht analysiert worden. Ziel dieser Arbeit war, in einem Rattenmodell, Leberzellen während der Leberentwicklung *in vitro* und *in vivo* zu charakterisieren. Wir betrachteten den Fortschritt von der Beginn der Leberentwicklung (E10) bis zum Erwachsenen Leber.

Die embryonale Leber ist durch die Expression von Albumin und Alpha-fetoprotein (AFP) charakterisiert. In dieser Studie wurden entodermale zellen aus dem Vorderdarmrohr isoliert. Für die Analyse der Synthese und Sekretion von Albumin und AFP in entodermalen Zellen, es wurde die radioaktive biosynthetische Markierung als sehr empfindliche Methode etabliert. Es könnte gezeigt werden, dass Zeigte, dass die Gen-Expresion, Synthese und Sekretion der Albumin und AFP bereits am frühesten Entwicklungsstadium stattfindet. Außerdem, es wurde gezeigt, dass entodermale Zellen, Entoderm und Leber Marker wie Beta-catenin, HNF4-alpha, Prox1, BMP-4, Foxa2 und GATA-4 expremieren können.

Am zweiten Teil dieser Arbeit waren wir an der Entwicklung der Leber interessiert, nachdem sie als Organ identifizierbar war (von E12 zu Adultstadium). Mit Hilfe morphometrische Analyse wurde es demonstriert, dass ungefähr 50% der gesamten Leberzellen während der embryonalen und fötalen Entwicklung Albumin und Alpha-fetoprotein expremieren können. Zusätzlich wurde es gezeigt, dass während des embryonalen Stadiums das Verhältnis der Albumin- und Alpha-fetoprotein expremierenden Zellen zu den stark vermehrten Zellen sich erhöht. Dieses Verhältnis erreichte sein Maximum am 18 Tag der embryonalen Entwicklung. Die funktionelle Analyse hat gezeigt, dass am 18 Tag der embryonalen Entwicklung Albumin und Alpha-fetoprotein mRNA-expression ein Maximum erreicht hatte und dass eine hohe Rate der Synthese und Ausscheidung von Albumin und Alpha-fetoprotein beobachtet wurde. Zusätzlich konnte gezeigt werden, dass am embryonalen Stadium (von E12 bis zu E16) Albumin und Alpha-fetoprotein mit unterschiedlicher Rate synthetisiert und abgeschieden wurden. Von 18 Tag bis zur Geburt ist die Kinetik der Synthese und der Ausscheidung von Albumin der den reifen Hepatozyten ähnlich.

Drei Zellpopulation wurden in der embryonalen (E12 und E14) und fötalen Leber (E18) identifiziert. Zwei von denen könnten sich entweder zu Hepatozyten (Prox1 positive Zellen/CK19 negative Zellen) oder zur intrahepatischen Gallengang Epithelzellen (Prox1 negative Zellen/CK19 positive Zellen) entwickeln. Die dritte Zellpopulation (positive Zellen für CK19 und prox1) könnte sich zur beiden Hepatozyten und Gallengang Epithelzellen entwickeln. CK-7 positive Zellen wurden erst am 18 Tag der embryonalen Entwicklung identifiziert.

Die Blutbildung wurde in der embryonalen Leber analysiert. Es könnte gezeigt werden, dass die Gene, die die Blutbildung regulieren, wie GM-SCF, G-CSF, SCF und EPO, stark am 12 und 14 Tag der embryonalen Entwicklung (E12 und E14) expremiert sind.

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