



Published in final edited form as:

J Mol Endocrinol. 2016 April ; 56(3): R115–R125. doi:10.1530/JME-15-0313.

Regulation of Liver Development: Implications for Liver Biology across the Lifespan

Philip A. Gruppuso^{1,2} and Jennifer A. Sanders^{1,3}

¹Division of Pediatric Endocrinology, Rhode Island Hospital and Brown University, Providence, RI 02903

²Molecular Biology, Cell Biology and Biochemistry, Brown University, Providence, RI 02905

³Department of Pathology and Laboratory Medicine, Brown University, Providence, RI 02905

Abstract

The liver serves a spectrum of essential metabolic and synthetic functions that are required for the transition from fetal to postnatal life. Processes essential to the attainment of adequate liver mass and function during fetal life include cell lineage specification early in development, enzymic and other functional modes of differentiation throughout gestation, and ongoing cell proliferation to achieve adequate liver mass. Available data in laboratory rodents indicate that the signaling networks governing these processes in the fetus differ from those that can sustain liver function and mass in the adult. More specifically, fetal hepatocytes may develop independent of key mitogenic signaling pathways, including those involving the Erk mitogen-activated protein (MAP) kinases and the mechanistic target of rapamycin (mTOR). In addition, the fetal liver is subject to environmental influences that, through epigenetic mechanisms, can have sustained effects on function and, by extension, contribute to the developmental origin of adult metabolic disease. Finally, the mitogen-independent phenotype of rat fetal hepatocytes in late gestation makes these cells suitable for cell-based therapy of liver injury. In the aggregate, studies on mechanisms governing fetal liver development have implications not only for the perinatal metabolic transition but also for the prevention and treatment of liver disorders throughout the lifespan.

Introduction

The liver is essential to a panoply of metabolic, synthetic and other physiologic functions that are required for the viability of the fetus and, in turn, the offspring during the perinatal transition (Cowett 2011). Liver development from mid-gestation to term may be thought of as comprising two interrelated but distinct processes, growth in liver mass and functional differentiation. The net result of these two processes is sufficient capacity for the liver to ensure metabolic homeostasis during and beyond parturition, an effective transition to the

Corresponding Author: Philip A. Gruppuso, M.D., Division of Pediatric Endocrinology, Rhode Island Hospital, 593 Eddy Street, Providence, RI 02903, ; Email: Philip_Gruppuso@brown.edu

Declaration of Interest

The authors have no conflicts of interest to report.

enteral acquisition of nutrients, and adequate synthetic function to ensure the physiologic well-being of the newborn.

The present review will focus on several areas: early liver development, including cell lineage specification; basic mechanisms regulating functional differentiation gleaned from studies in the late gestation fetal rat; signaling pathways involved in the regulation of liver mass during rodent development; non-human primate studies on the role of insulin in liver development; maternal nutrition and fetal programming as they affect liver physiology, and; the application of the fetal hepatocyte phenotype to cell-based therapy for liver disease. We will focus, in part, on our own work on late gestation liver development in the rodent. However, we will also focus on a well-established literature on the role of hormones in liver enzymic differentiation as well as contemporary studies on liver epigenetics and cell signaling.

Early liver development and cell lineage specification

The temporal sequence of events that comprise normal liver development can be aligned in the laboratory rat and human based on a number of specific characteristics and changes (Figure 1). This allows one to extrapolate from often employed rodent models to humans and non-human primates. In this review, we will focus on data derived from the laboratory rat and from the Rhesus monkey.

The adult human liver is composed of four lobes with hepatocytes, the major parenchymal cell type, constituting approximately 80% of the liver mass. Hepatocytes and cholangiocytes (biliary epithelial cells) are derived from the definitive endoderm which is formed during the third week of human gestation (Bort, et al. 2006; Wilson, et al. 1963). Transforming growth factor β , Wnt, fibroblast growth factor, notch, and bone morphogenic protein control the key signaling pathways for hepatogenesis (Si-Tayeb, et al. 2010). The spatial and temporal regulation as well as the mechanisms by which these pathways contribute to liver development have been elucidated [reviewed in (Gordillo, et al. 2015)]. The convergence of the ventral portion of the endoderm adjacent to the cardiac mesoderm and septum transversum results in hepatic specification. The liver bud is formed when hepatic endodermal cells, termed hepatoblasts, proliferate and migrate into the septum transversum around gestational day 28 in the human and day 12 in the rat (Bort et al. 2006; Hutchins and Moore 1988; Severn 1971; Shiojiri, et al. 1991; Wilson et al. 1963). Bipotential hepatoblasts express alpha-fetoprotein (AFP) as well as markers for both hepatocytes (albumin, hepatocyte nuclear factor [HNF] 4, keratin 8 and keratin 18) and cholangiocytes (keratin 19) (Cascio and Zaret 1991; Nava, et al. 2005; Shiojiri et al. 1991).

Hepatoblasts located near the portal vein give rise to cholangiocyte precursors while those located away from the portal vein become hepatocytes. Hepatocytic differentiation begins during the eighth week of gestation in humans and on day 15 in the rat. The process continues throughout the remainder of embryonic and postnatal development. Hepatocytes are heterogeneous, with their function largely determined by their localization within the liver lobule. This results in the compartmentalization of key enzymes and transporters, which in turn accounts for metabolic zonation of liver. The differentiation of hepatocytes is

controlled by a complex network of transcription factors including HNF1a, HNF4, HNF1b, and FoxA2 (Kyrnizi, et al. 2006). The fetal liver is the major site for hematopoiesis during embryonic development, with hematopoietic progenitors colonizing the liver bud soon after the cells invade the surrounding mesenchyme (Collardeau-Frachon and Scoazec 2008). As hepatoblasts mature along a hepatic lineage, the cells no longer support hematopoiesis, consistent with the movement of hematopoietic cells to the bone marrow near the end of the second trimester in humans (Collardeau-Frachon and Scoazec 2008; Cumano and Godin 2007). Like hepatocytes, the cholangiocytes gradually mature, forming tubules at 14 weeks with remodeling of the ducts and termination of cholangiocyte differentiation occurring at approximately 30 weeks (Haruna, et al. 1996). In the rat, the process begins at day 15.5 and terminates during the neonatal period (Shiojiri et al. 1991). Liver development and adult liver function are dependent on the coordinated interaction of hepatocytes, cholangiocytes, sinusoidal epithelial cells, Kupffer cells (liver-specific macrophages), stellate cells, pit cells (natural killer cells) and epithelial cells.

Late gestation liver development in the rat

At birth, the fetus makes an abrupt transition from placental nutrient supply to the utilization of endogenous sources to maintain metabolic homeostasis (Mayor and Cuezva 1985). As is the case in the adult, the liver serves as a repository for stored carbohydrate in the form of glycogen and as a distribution center for peripheral nutrient stores, most notably fat in white adipose tissue and skeletal muscle protein. The utilization of these two peripheral sources of stored energy requires hepatic fatty acid oxidation, ketogenesis and gluconeogenesis. The enzymatic machinery essential for these processes is induced through complex mechanisms that involve regulation of gene expression at the levels of transcriptional control, RNA processing, RNA translation, and the post-translational processing and modification of proteins. For many years, investigation of this series of complex physiologic events employed the laboratory rat as a model system.

Enzymic differentiation of the liver in late gestation was long ago shown by Greengard and co-workers to be responsive to *in situ* administration of specific hormones to fetal rats (Greengard and Dewey 1967). Those studies, which involved determination of fetal liver enzyme activities, showed that glucose-6-phosphatase could be induced by cAMP-mediated hormones and by administration of thyroxine, but not by growth hormone. Similar results were obtained for tyrosine aminotransferase, a critical enzyme in amino acid metabolism. These studies were interpreted at the time as indicating that cAMP-mediated hormones are responsible for the induction of essential hepatic metabolic pathways, including gluconeogenesis, immediately following birth (Greengard 1969).

Girard and co-workers, examining enzymic liver differentiation, noted that induction of the key rate-limiting enzymes for gluconeogenesis, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase among others, occurs during the immediate postnatal period in the rat (Girard, et al. 1973). These investigators attributed this enzyme induction to a sharp decrease in the insulin-to-glucagon ratio that coincided with a postnatal fall in serum glucose.

With regard to a direct role for insulin, work by Porterfield (Porterfield 1979) indicated that insulin plays a limited prenatal role. While insulin could suppress the induction of glucose-6-phosphatase, it could do so only during the immediate preterm period. These data are consistent with our own observations regarding the expression and activity of the insulin signaling network in the late gestation fetal rat (Figure 2). Insulin receptor abundance and insulin-induced receptor self-phosphorylation are reduced at the end of gestation relative to in the adult animal (Anand, et al. 2002; Gruppuso, et al. 1991; Khamzina, et al. 2003). More impressive is the very low level with which the primary insulin receptor substrate, IRS-1, is expressed at the protein level in late gestation liver. This low level of IRS-1 content is reflected in impaired IRS-1 tyrosine phosphorylation and IRS-1-mediated signaling to phosphatidylinositol-3 kinase (PI3K). IRS-2, a paralog of IRS-1, is preferentially expressed in fetal liver. Its tyrosine phosphorylation and ability to mediate activation of PI3K is high in the late gestation fetus relative to the adult. However, signaling to two downstream kinases, Akt and S6 kinase 1, is markedly attenuated in the fetus, indicating an uncoupling of the pathway downstream from PI3K in late gestation.

The older literature on enzymic differentiation, along with our studies on insulin signaling, supports the conclusion that liver growth and functional differentiation late in gestation in the fetal rat are largely independent of the growth promoting effects of insulin. However, studies on enzymic differentiation suggest that cAMP-mediated counterregulatory hormones are biologically active during fetal life and are critical for enzymic differentiation.

This conclusion is further supported by studies on hepatic glycogen metabolism performed more than forty years ago. During the last third of gestation in the rat, there is a marked increase in hepatic glycogen content. This occurs in concert with induction of the key enzymes of glycogen metabolism, glycogen synthase, phosphorylase and phosphorylase kinase (Gruppuso and Brautigan 1989). While constituting an anabolic process that is insulin-mediated in the adult (Miller, et al. 1973; Miller and Lerner 1973), the induction of glycogen synthase and accumulation of glycogen precedes the late gestation rise in fetal plasma insulin concentrations (Mayor and Cuezva 1985).

There is an established role for glucocorticoids in the induction of hepatic glycogenesis (Moses, et al. 1981). Accumulation of hepatic glycogen in the late gestation fetal rat is markedly reduced if both maternal and fetal glucocorticoid synthesis is limited by the combination of maternal adrenalectomy and fetal hypophysectomy. Liver glycogen content is restored by the direct administration of glucocorticoid to the fetus (Jost 1961). This effect is likely mediated by the induction of glycogen synthase (Moses et al. 1981). In unperturbed fetal rats, administration of glucocorticoid can induce premature glycogen deposition (Greengard and Dewey 1967).

With regard to the relevance of studies in the rat to human physiology, liver glycogen accumulation in the rat begins on day 18 of gestation (Devos and Hers 1974) whereas, in the human, liver glycogen accumulation is initiated during the first trimester on or about week 8 (Schwartz, et al. 1975). It then continues throughout gestation. In both species, glycogen accumulation accompanies the appearance of glycogen synthase (Devos and Hers 1974; Schwartz et al. 1975). These and other data indicate a limited role for insulin in liver

differentiation during mid-gestation in the human, similar to the situation that pertains in the rat. In contrast, glucocorticoids appear essential.

Late gestation liver growth in the rat: Clues to the physiologic regulation of liver development

During the last five days of gestation in the rat, there is a tripling of liver mass, total liver DNA and total liver protein. Owing to a corresponding decline in the liver's hematopoietic element, there is an increase in relative hepatocyte mass. A number of years ago, we began to focus on the signaling mechanisms that accounted for this extraordinary rate of somatic growth, and relationship between fetal growth control and the growth of a similar magnitude that occurs in liver regeneration following partial hepatectomy.

To better understand the signaling mechanisms regulating late gestation hepatocyte development in the rat, we employed primary cultures using defined conditions (Curran, et al. 1993). In a comparison of insulin with two factors that are mitogenic for hepatocytes, transforming growth factor α (TGF α) and hepatocyte growth factor (HGF), we found that late gestation fetal hepatocytes did not exhibit synergy between insulin and these two growth factors (Gruppuso, et al. 1994). In addition, TGF α and HGF, but not insulin, induced activation of the Erk mitogen-activated protein kinase pathway in primary fetal hepatocyte cultures (Gruppuso et al. 1994). We interpreted these studies as indicating that insulin, while it might serve a hepatotrophic role *in vivo*, is not a mitogen for late gestation fetal rat hepatocytes.

In our subsequent studies, a caveat to this interpretation emerged. Having examined the response of cultured fetal hepatocytes to insulin and the growth factors mentioned above, we undertook studies on the regulation of mitogenic signaling pathways *in vivo*. Our approach to these studies was to administer growth factors directly to fetuses by intraperitoneal injection *in situ*. As noted above, this approach demonstrated a marked impairment of insulin signaling to PI-3 kinase in the late gestation fetus (Anand et al. 2002). More unexpected was the observation that mitogen-induced activation of Erk signaling was markedly attenuated in the fetus (Boylan and Gruppuso 1994, 1996). Like other so-called mitogen-activated protein kinase pathways, the Erk pathway consists of a cascade of three kinases. We found that the uncoupling of the Erk pathway in late gestation fetal liver was accounted for by an interruption of signaling from the intermediate kinase in the pathway, MEK, to the terminal Erk kinase (Boylan and Gruppuso 1998). This unusual circumstance may be attributable to differences in the expression or function of scaffolding proteins that provide for functional organization of the pathway (Sacks 2006), or to dual specificity phosphatases that can act as negative regulators of this pathway (Keyse 2008). While we have not identified these or other specific mechanisms as accounting for the uncoupling of MEK signaling to Erk, this observation supported the hypothesis that late gestation liver growth, unlike liver regeneration in the adult (Fausto, et al. 1995), is not dependent on the actions of trophic factors acting through this well-characterized signaling pathway.

Further support for this conclusion came from studies on another critical pathway for cellular growth, that involving mTOR, the mechanistic target of rapamycin (Albert and Hall

2015; Laplante and Sabatini 2012). mTOR participates in two signaling complexes, mTORC1 and mTORC2. mTORC1, the canonical target of the immunosuppressive drug rapamycin, is a key regulator of protein synthesis, cell growth and gene expression. mTORC1 is regulated directly by nutrient availability and by growth factor receptor tyrosine kinases. Given the high rate of asynchronous hepatocyte proliferation in the late gestation rodent fetus, we hypothesized that mTORC1 signaling would be constitutively active and that inhibition of mTORC1 by rapamycin would potentially inhibit fetal hepatocyte proliferation in vivo.

In a series of studies aimed at testing this hypothesis, we administered rapamycin to late gestation fetal rats by intraperitoneal injection in situ. DNA synthesis was assessed as the incorporation of bromodeoxyuridine (BrdU) into DNA, which we detected using immunohistochemistry. These experiments (Boylan, et al. 2001) yielded an unexpected result; fetal hepatocyte proliferation in the late gestation (E19) fetus was independent of mTORC1 signaling. In further experiments, we showed that the translation of mRNAs with complex 5' oligopyrimidine tracts, so-called 5'TOP mRNAs, was also insensitive to rapamycin-induced inhibition of mTORC1 signaling (Boylan, et al. 2015; Gruppuso, et al. 2008). These results were in sharp contrast to those obtained in adult rats subjected to refeeding after fasting or to partial hepatectomy. In fact, liver regeneration is exquisitely sensitive to rapamycin-induced inhibition of mTORC1 (Boylan et al. 2001), consistent with the established role of a number of mitogenic signaling pathways in liver regeneration (Fausto et al. 1995).

Although manipulation of mTORC1 activity does not have deleterious effects in utero, mice expressing a constitutively active form of RagA, a GTPase involved in the activation of mTORC1 by amino acids, die shortly after birth due to the inability to induce autophagy and maintain glucose homeostasis (Efeyan, et al. 2013). In contrast to the wealth of data on mTORC1, the pathways upstream and downstream of mTORC2 are less well understood. mTORC2 is responsive to insulin and is involved in the regulation of the actin cytoskeleton, cellular metabolism and aging (Lamming, et al. 2012; Zoncu, et al. 2011). In adult liver, mTORC2 induces glycolysis through activation of Akt and is essential for the expression of genes involved in lipogenesis (Hagiwara, et al. 2012; Yuan, et al. 2012). We performed genomic and phosphoproteomic analyses in mice in which *rictor*, an essential component of mTORC2, was deleted. We did so to better characterize the physiologic role of mTORC2. We showed that mTORC2 regulated the expression of a large set of genes involved in intermediary metabolism and in ribosome and proteasome biogenesis (Lamming, et al. 2013). It is likely that both mTORC1 and mTORC2 signaling play a critical role in fetal metabolism and adaptation of the newborn during the early postnatal period.

From these studies, a picture emerges in which fetal liver growth in the late gestation rat is “mitogen-independent.” That is, the increase in hepatocyte mass that occurs at the end of gestation in the rat is largely independent of the mitogenic signaling pathways that are active in the adult rat (illustrated in Figure 3).

The role of insulin in liver growth and physiology in the non-human primate

With regard to the role of insulin in late gestation liver development in humans, studies performed on non-human primates by Susa, Schwartz and co-workers in the 1970s and 1980s (McCormick, et al. 1979; Susa, et al. 1984a; Susa, et al. 1979; Susa, et al. 1984b; Susa and Schwartz 1985) are particularly informative. These investigators were focused on the role of fetal insulin in the fetopathy of diabetes in pregnancy. They implanted osmotic minipumps in fetal rhesus monkeys to achieve a constant infusion of insulin for the last three weeks of gestation. Like the human infant of a diabetic mother, in utero hyperinsulinemia resulted in newborn rhesus monkeys with soft tissue hyperplasia, elevated erythropoietin levels, commensurate polycythemia, and impaired lung maturation (Susa et al. 1979; Susa et al. 1984b). Supraphysiologic insulin levels, consistent with those seen in the human fetus of a diabetic mother, resulted in an increase in hepatic mass.

Fetal hyperinsulinemia was associated with reduced activity of two hepatic gluconeogenic enzymes, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (McCormick et al. 1979). The activities of key lipogenic enzymes, fatty acid synthase complex, glucose-6-phosphate dehydrogenase and ATP citrate lyase, were increased (McCormick et al. 1979; Susa et al. 1984a). In contrast, glycolytic enzymes and glycogen metabolism were unaffected.

These data indicate that insulin exerts effects on liver enzymic differentiation in the late gestation primate fetus. This may be interpreted as indicating that hepatic insulin signaling couples late in gestation in the primate, a developmental time point beyond that which corresponds to late gestation in the rat. The primate observations are consistent with an evolving role for insulin in hepatic enzymic differentiation in the human as gestation proceeds.

Maternal nutrition and fetal programming

The developmental origins of health and disease hypothesis (DOHaD) states that perturbations of the intrauterine environment can result in long-term effects on gene expression through changes to the epigenome, and that these epigenetic effects can lead to increased risk of developing diseases later in life (Waterland and Michels 2007). Experimental and epidemiologic studies suggest that alterations in maternal nutrition during pregnancy and/or lactation lead to changes in fetal organ development and the programming of metabolic pathways (Barker 2012). A wealth of studies has shown a correlation between infant birth weight and risk of developing metabolic disease. Early studies by Barker and colleagues (Barker, et al. 1989) focused on the effects of maternal undernutrition and low birth weight. As in humans, caloric restriction during mid- to late gestation in the rat results in decreased birth weight and predisposition to glucose intolerance and type 2 diabetes later in life (Yuan, et al. 2011). Liver is a target for fetal programming. Alterations in nutrient supply caused by either uteroplacental insufficiency or protein restriction result in changes in hepatic structure and function, ultimately leading to altered expression of glucose 6-phosphatase, phosphoenolpyruvate carboxykinase, and fructose biphosphatase (Burns, et al. 1997; Desai, et al. 1997a; Desai, et al. 1997b; Lane, et al. 2002). Alterations in histone

posttranslational modifications within the promoter regions of PGC-1, a key regulator of gluconeogenic enzymes and carnitine-palmitoyl transferase I (CPT1), a rate-limiting transporter in fatty acid oxidation, persist postnatally in intrauterine growth retarded animals and are thought to underlie many of the changes in gene expression (Fu, et al. 2004; Lane, et al. 2001; Lane et al. 2002).

There is growing interest in the potential for detrimental effects of maternal obesity and fetal overnutrition on the offspring. A number of epidemiologic studies have shown an association between maternal body mass index and gestational diabetes and increased offspring adiposity, type 2 diabetes and non-alcoholic fatty liver disease (Boney, et al. 2005; Dabelea, et al. 2000; Gademan, et al. 2014; Lake, et al. 1997; Stewart, et al. 2013). A number of animal models have provided insight into the molecular mechanisms underlying altered fetal programming in response to maternal obesity and overnutrition. These include alterations in food intake, increased oxidative stress and dysregulated pancreatic and adipose function (Ozanne, et al. 2011). As in undernutrition, altered epigenetic programming is believed to underlie many of the effects of maternal overnutrition, although the molecular mechanisms resulting in the common phenotype of increased risk of metabolic syndrome are most likely distinct.

The fetal hepatocyte phenotype and cell-based therapy for liver disease

Our studies characterizing fetal hepatocyte proliferation in the late gestation fetal rat as “mitogen-independent” led us to consider the possibility that these cells would have the capacity to repopulate an injured adult liver. Such a capacity had been attributed earlier to mid-gestation fetal rat hepatoblasts (Dabelea, et al. 2000; Oertel, et al. 2008) and to late gestation fetal epithelial cells expressing cholangiocyte markers (Simper-Ronan, et al. 2006). In addition to its implications for fetal metabolism and the perinatal transition, studies on mechanisms of fetal liver development are relevant to a cell-based approach to therapy for liver disease. The only therapy currently available for liver failure associated with acute or chronic liver injury is transplantation. However, the lack of tissue available for transplant has hampered the success of this therapy (Kim, et al. 2015). The concept of hepatocyte transplantation as an alternative to liver organ transplantation holds great potential. However, it also presents numerous challenges and thus far has shown limited efficacy due to the limited replicative potential of the adult cells used for transplantation (Hansel, et al. 2014). The identification of the optimal source of cells for transplant and the elucidation of the effect of the liver microenvironment on these cells are key areas of future research.

As noted above, we hypothesized that the mitogen independence and relatively well differentiated phenotype of late gestation fetal rat hepatocytes would allow these cells to proliferate and restore functional liver mass upon transplantation to an injured adult liver. We utilized the well-established dipeptidyl peptidase IV (DPPIV) transplant model developed by Thompson and Hixson (Thompson, et al. 1991). This model takes advantage of a naturally occurring inactivating mutation in the DPPIV gene present in German Fischer rats. This allows for the syngeneic transplantation of cells isolated from American Fischer rats (DPPIV+) into German Fischer (DPPIV-) hosts without the need to mark cells by transfection or other means. Pretreatment of the DPPIV- hosts with mitomycin C followed

by 2/3 partial hepatectomy at the time of transplantation blocks endogenous adult hepatocyte proliferation thus providing a selective growth advantage to the transplanted cells (Brilliant, et al. 2009). We immunoisolated DPPIV+ late gestation (ED19) fetal rat hepatocytes based on the expression of the hepatic marker leucine aminopeptidase (LAP) (Mowery and Hixson 1991). The LAP+ and LAP- fractions isolated from DPPIV+ American Fischer fetal rats were transplanted via splenic injection into pretreated DPPIV- German Fischer adult hosts. One month following transplantation the LAP+ fetal cells had formed numerous hepatic and endothelial colonies, while the LAP- cells did not repopulate the injured adult liver (Figure 2). These studies indicate that the phenotype of fetal hepatocytes and liver development can contribute to the selection of optimal cell sources for transplantation. Future studies in this area may also provide strategies for expansion of cell sources in vitro and other tissue engineering strategies necessary for successful cell-based therapy for liver disease.

Conclusions and future perspectives

The process of liver development and attainment of differentiated function during the fetal and early postnatal period is an intricate temporal process that relies on a shifting and complex network of signaling pathways. An extensive literature that includes our own studies indicates that the systems governing liver growth and functional differentiation during mid- to late fetal development differ from those that account for the regeneration of adult liver under conditions of liver injury or reduced mass.

Recent research has shown that epigenetic mechanisms including DNA methylation and histone modifications are involved in patterning the liver and regulating the ontogeny of metabolic enzymes and transporters (Moscovitz and Aleksunes 2013; Xu and Zaret 2012). Additional research is needed to understand how epigenetic, transcriptional, translational and post-translational mechanisms intersect to control normal liver development. Future studies will likely involve a combination of global omics-based approaches coupled with human tissue and physiologic animal models. Such an approach offers a more complete understanding of liver development and functional differentiation, which would relate directly to the physiology and pathophysiology of metabolic adaptation in the newborn. As has been seen more recently, ongoing research on liver development may also contribute to the elucidation of mechanisms involved in the developmental origins of adult metabolic dysregulation. All of this work has the potential to contribute to the prevention and treatment of metabolic disorders, liver failure and liver carcinogenesis.

Acknowledgments

Funding

The work reported herein was supported by the National Institutes of Health (grants R01HD024455 and R01DK100301)

We thank Ms. Joan Boylan for her helpful discussions and comments regarding the contents of this paper.

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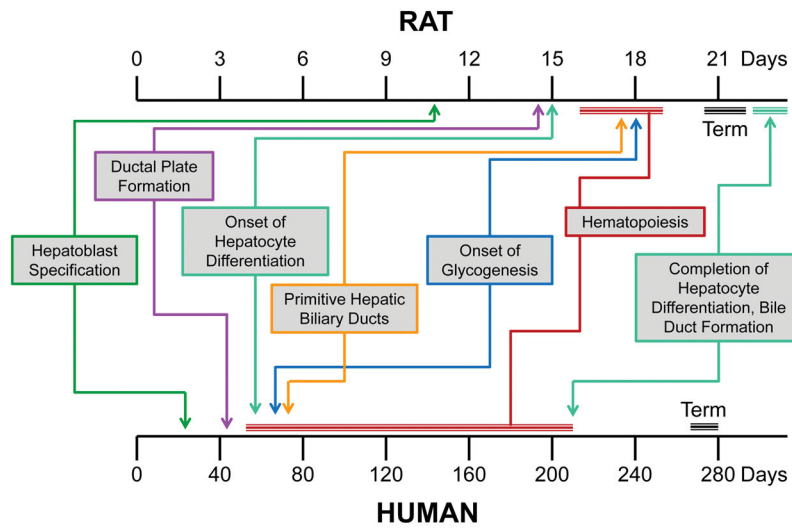


Figure 1.

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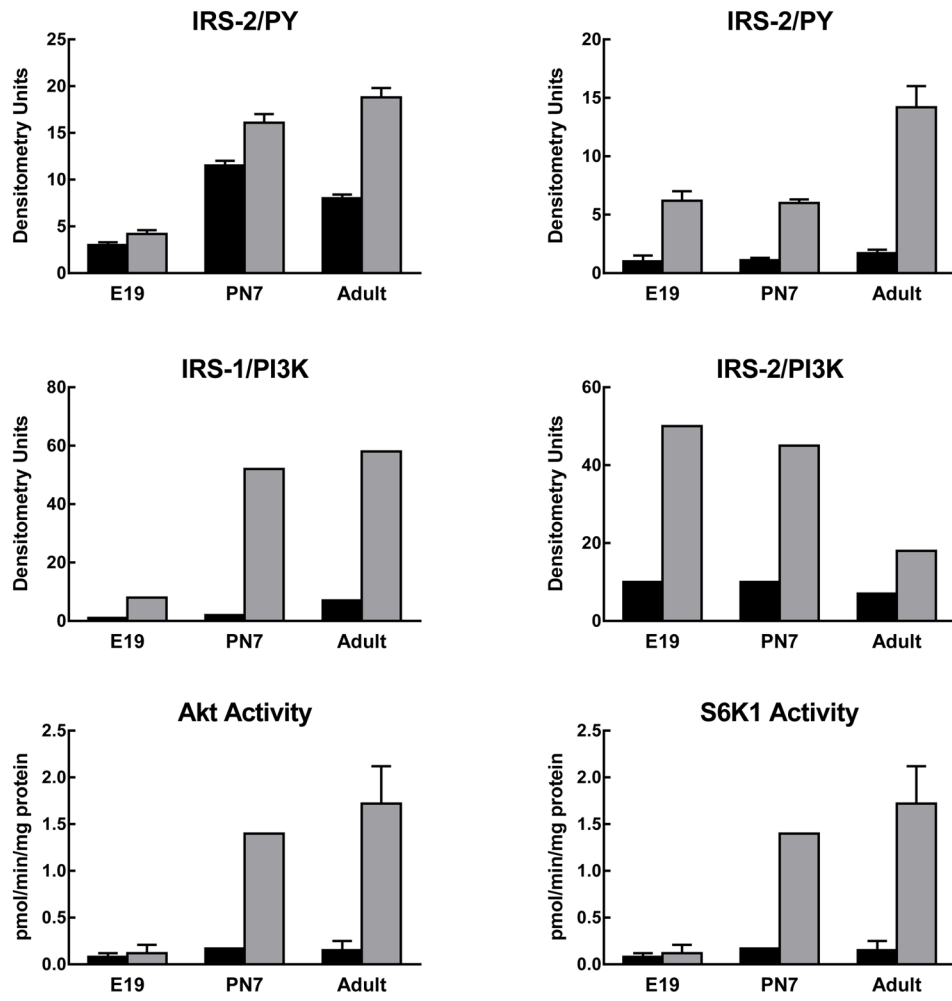


Figure 2.

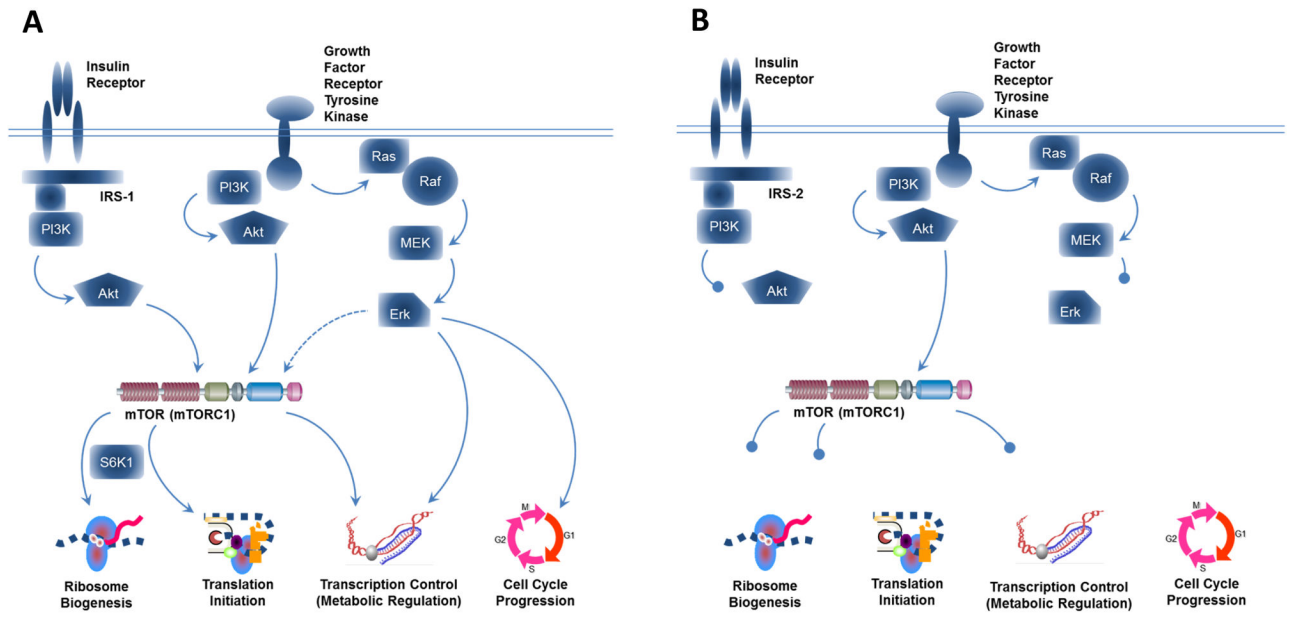


Figure 3.

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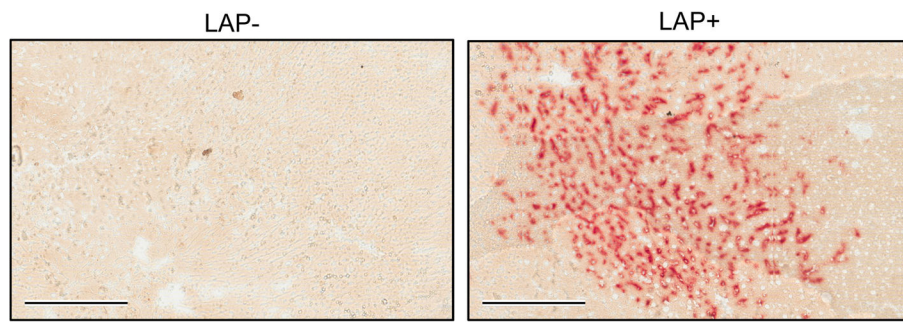


Figure 4.

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