Activation of Proneuronal Transcription Factor Ascl1 in Maternal Liver Ensures a Healthy Pregnancy

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SUMMARY

How the maternal liver adapts to pregnancy remains elusive. We found that maternal liver activates the expression of Ascl1, a gene encoding a proneuronal transcription factor, to coordinate the adaptations of maternal organs and the growth of the placenta, enabling a healthy pregnancy and normal postnatal growth of the offspring.

BACKGROUND & AIMS: Maternal liver shows robust adaptations to pregnancy to accommodate the metabolic needs of the developing and growing placenta and fetus by largely unknown mechanisms. We found that Ascl1, a gene encoding a basic helix-loop-helix transcription factor essential for neuronal development, is highly activated in maternal hepatocytes during the second half of gestation in mice.

METHODS: To investigate whether and how Ascl1 plays a pregnancy-dependent role, we deleted the Ascl1 gene specifically in maternal hepatocytes from midgestation until term.

RESULTS: As a result, we identified multiple Ascl1-dependent phenotypes. Maternal livers lacking Ascl1 showed aberrant hepatocyte structure, increased hepatocyte proliferation, enlarged hepatocyte size, reduced albumin production, and increased release of liver enzymes, indicating maternal liver dysfunction. Simultaneously, maternal pancreas and spleen and the placenta showed marked overgrowth; and the maternal ceca microbiome showed alterations in relative abundance of several bacterial subpopulations. Moreover, litters born from maternal hepatic Ascl1-deficient dams experienced abnormal postnatal growth after weaning, implying an adverse pregnancy outcome. Mechanistically, we found that maternal hepatocytes deficient for Ascl1 showed robust activation of insulin-like growth factor 2 expression, which may contribute to the Ascl1-dependent phenotypes widespread in maternal and uteroplacental compartments.

CONCLUSIONS: In summary, we show that maternal liver, via activating Ascl1 expression, modulates the adaptations of maternal organs and the growth of the placenta to maintain a healthy pregnancy. Our studies show that Ascl1 is a novel and critical regulator of the physiology of pregnancy. (Cell Mol Gastroenterol Hepatol 2022;13:35–55; https://doi.org/10.1016/j.jcmgh.2021.08.009)

Keywords: Hepatocyte; Gestation; Insulin-Like Growth Factor 2.

Abbreviations used in this paper: 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AAV8, adeno-associated virus 8; AKT, protein kinase B; Ascl1, achaete-scute homolog-like 1; cDNA, complementary DNA; ERK, extracellular signal-regulated kinase; GD, gestation day; IGF2, insulin-like growth factor 2; IGF2R, IGF2 receptor; ISH, in situ hybridization; mRNA, messenger RNA; P, promoter; PL, placental lactogen; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RNA-seq, RNA sequencing; TBG-Cre, Cre recombinase under the control of hepatocyte-specific thyroxine-binding globulin promoter.

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The establishment and maintenance of pregnancy requires highly coordinated adaptations in maternal, uteroplacental, and fetal compartments. As pregnancy progresses, in the maternal compartment, the liver grows to expand its metabolic capacity; the pancreas proliferates its β-cell population to increase insulin production; the gut alters its microbiome, contributing to immunologic adjustments; the spleen undergoes development and growth of the erythroid lineage; and the kidney expands its volume, allowing for increases in blood flow and fluid retention. These adaptations of maternal nonreproductive organs to pregnancy must be required to meet the demands of the developing and growing placenta and fetus. However, the physiological significance and the regulatory mechanisms of these processes are poorly understood, representing an emerging research field.

We and others have shown that the maternal liver undergoes hyperplasia and hypertrophy, changes its gene expression profile, and thereby markedly expands during gestation in rodents. However, how this is controlled remains elusive. We previously showed that the expression of achaete-scute homolog-like 1 (Ascl1), a gene encoding a basic helix-loop-helix transcription factor essential for neurogenesis, is highly up-regulated in maternal livers of pregnant rats. During development, Ascl1 controls proliferation, cell-cycle exit, and full neuronal differentiation and specification of neural progenitor cells in both the central and peripheral nervous systems. Ascl1 inhibits its own expression by negative autoregulation in the developing nervous system, possibly explaining the lack of overt abnormalities in Ascl1+/− mice, whereas Ascl1−/− pups die within hours after birth as a result of defects in brain development. In the adult, Ascl1 is expressed only in the brain and spinal cord, where there is ongoing neurogenesis, and in developing neuroendocrine cells in multiple organs including the cerebellum, thyroid, and thymus. In this study, we evaluated the activation, and explored the function, of Ascl1 in maternal livers of pregnant mice. We found that Ascl1 not only modulates maternal hepatic adaptation, but also mediates the communication of maternal liver with other maternal organs and the placenta, eventually affecting pregnancy outcomes.

Results

Ascl1 Is Highly Activated in Maternal Hepatocytes

We first evaluated the expression of Ascl1 in maternal livers throughout the course of gestation in mice. As pregnancy advanced, its transcript levels in maternal livers were increased progressively, reaching up to 26,000-fold on gestation days 13 and 15, relative to the prepregnancy state (Figure 1A). Maternal hepatic Ascl1 protein was expressed abundantly as its messenger RNA (mRNA) levels peaked on these 2 gestation days (Figure 1B). Ascl1 transcript was detected exclusively in maternal hepatocytes (Figure 1C). In human beings, Ascl1 mRNA was expressed abundantly in a maternal liver of a pregnant woman, and in livers with diseases such as hepatocellular carcinoma, hepatocellular adenoma, and hepatitis (Figure 1D). Ascl1 transcript was not detectable by in situ hybridization (ISH) in maternal pancreas, spleen, kidney, and the placenta in gestation day 15 mice (Figure 2A). Ascl1 mRNA expression was marginally detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), but did not show significant gestation-dependent changes in maternal kidney on gestation days 8 (early pregnancy), 13 (midgestation), and 18 (late pregnancy) (Figure 2B). Ascl1 transcript levels were below detection by qRT-PCR in maternal pancreas and spleen on those gestation days. The data indicate that the activation of Ascl1 expression is not a generalized phenomenon in the maternal compartment. Thus, it is truly astonishing that pregnancy activates a proneuronal transcription factor in an epithelial cell type (maternal hepatocytes) in such a striking magnitude in both animals and human beings, strongly suggesting a gestation-dependent role for this transcription factor.

Adeno-Associated Virus 8 Containing the Gene for Cre Recombinase Under the Control of Hepatocyte-Specific Thyroxine-Binding Globulin Promoter Efficiently Deletes Ascl1 in Maternal Hepatocytes

Ascl1 is activated in maternal liver during the second half of pregnancy, suggesting a role in the maintenance of pregnancy. To determine this, we took a loss-of-function approach to delete the Ascl1 gene specifically in maternal hepatocytes from midgestation to term and evaluated how pregnancy was affected. We generated timed pregnancies in Ascl1+/− female mice by mating them with Ascl1+/− males. This led to a homogeneous Ascl1+/β fetal genotype. Adeno-associated virus 8 (AAV8)–Cre recombinase under the control of hepatocyte-specific thyroxine-binding globulin promoter (TBG-Cre) virus or AAV8 virus with a null vector (control) virus was injected into Ascl1+/β mice on gestation day 8. Mice were killed on gestation days 15 and 18 for phenotypic assessment. This AAV8-TBG virus has been shown to specifically infect hepatocytes with more than 99% efficiency in mice. qRT-PCR analysis showed that Ascl1 mRNA was almost completely lost in maternal livers of pregnant animals treated with the AAV8–TBG-Cre virus (Figure 3A), indicating highly efficient Ascl1 gene deletion in this organ. As such, mice deficient for Ascl1 in maternal hepatocytes are referred to as hep-Ascl1−/− mice hereafter. We observed massive AAV8–TBG-Cre virus-mediated recombination in maternal hepatocytes in gestation day 18 hep-Ascl1−/− mice. We detected the recombination in the islets of maternal pancreas, in few cells in maternal kidneys, but not in maternal spleen and the placenta (Figure 3B). In a pretest study, we detected green fluorescent protein (GFP) expression in maternal livers but not in fetal livers after injecting AAV8-TBG-GFP reporter virus into pregnant mice. This indicates that the AAV8 virus does not effectively pass through the placenta to infect the fetuses.
Figure 1. Ascl1 activation in maternal livers of pregnant mice and human beings. Timed pregnancies were generated in 3-month-old C57BL/6 female mice. Livers were collected from nonpregnant (NP) mice and maternal livers from pregnant mice at the indicated GDs. (A–C) Ascl1 expression in maternal livers. (A) Hepatic Ascl1 mRNA levels were measured using qRT-PCR and presented as the mean fold changes relative to NP controls ± SD (n = 3). **P < .01, ***P < .001, and ****P < .0001, compared with NP controls. (B) Western blot was performed using liver nuclear lysates with antibodies against ASCL1. Lamin B1 was used as a loading control. (C) Liver sections were subjected to Ascl1 in situ hybridization. Ascl1 mRNA is stained dark brown. (D) Liver sections were prepared from archived paraffin blocks of human liver tissues. Ascl1 mRNA was visualized on liver sections. Representative results are shown with liver sections from a NP woman, a 6 months’ pregnant mother, a hepatocellular carcinoma (HCC) patient, a hepatocellular adenoma (HCA) patient, and a 4.5-month-old child with hepatitis.
RNA-Sequencing Analysis Shows Ascl1-Dependent Transcriptome in Maternal Liver

We next compared the transcriptomes of gestation day 15 maternal livers between the 2 genotype groups of mice by RNA sequencing (RNA-seq) to profile potential Ascl1 target genes in this context. This showed 1274 differentially expressed genes. They were either up-regulated or down-regulated by at least 2-fold with a false-discovery rate of less than 0.05 when maternal hepatic Ascl1 was lacking (Figure 3C). Pathway analysis suggests that these genes are associated with many biological processes including the metabolism of hormones (eg, melatonin), neurotransmitters (eg, serotonin and dopamine), fatty acids, alcohol, carbohydrates, nucleic acids, and amino acids, cell-cycle control, cell function and maintenance, and vitamin D–retinoid X–receptor activation (Figure 3D). These data imply that Ascl1 directly or indirectly regulates a broad spectrum of genes and thereby possesses multiple functions in maternal liver in this physiological state (pregnancy).

Hepatocyte-Specific Ascl1 Knockout Results in Maternal Liver Abnormalities

We subsequently assessed whether maternal livers showed Ascl1-dependent phenotypes. Compared with maternal hepatocytes sufficient for Ascl1, maternal hepatocytes lacking Ascl1 showed an aberrant structure (eosin staining–negative around the nuclei) (Figure 4A), enhanced hepatocyte proliferation (Figure 4B and F), and increased size (Figure 4C and G), hence causing further enlargement of the maternal liver (Figure 4D and E). These abnormalities were accompanied by reduced albumin mRNA expression and protein production and increased circulating alanine aminotransferase (Figure 4H). These observations indicate that Ascl1 loss of function in maternal hepatocytes impairs their structure and function.

Hepatocyte-Specific Ascl1 Knockout Causes Overgrowth of Maternal Pancreas, Spleen, and Kidney

We additionally examined several other maternal organs to estimate whether there were Ascl1-dependent systemic effects in the maternal compartment. Surprisingly, without maternal hepatic Ascl1, the maternal pancreas nearly doubled in size (Figure 5A and B), had a reduced proportion of insulin-positive areas (Figure 5C and D), and unchanged total β-cell mass (Figure 5E), while the concentrations of circulating insulin and nonfasting blood glucose were unaltered (Figure 5F and G). These data suggest that Ascl1 inactivation in maternal liver stimulates the expansion of the exocrine component without interfering with the pregnancy-dependent growth of the endocrine component in the maternal pancreas. Similarly, the maternal spleen also almost doubled in volume with overtly expanded red and white pulps (Figure 6A–C), while the maternal kidney showed enlargement without an obvious histologic alteration (Figure 6D and E). Collectively, we conclude that the loss of function of Ascl1 in maternal hepatocytes imposes systematic effects, causing overgrowth of at least a subset of maternal organs.
Hepatocyte-Specific Ascl1 Knockout Leads to Abnormal Maternal Cecal Microbiota

It is known that the maternal gut microbiome undergoes pregnancy-dependent changes, which are associated with the health of both the mother and the fetus. To determine whether maternal hepatic Ascl1 is relevant to the maternal microbiome, we collected maternal cecal contents from gestation day 18 hep-Ascl1\(^{-/-}\) mice and Ascl1\(^{+/+}\) (control) mice, and compared their microbiome profiles via 16S sequencing (Figure 7A). Most notably, Ascl1 loss in maternal hepatocytes resulted in the following: (1) a complete depletion of Pseudobutyribrio–Roseburia intestinalis, which protects colonic mucosa against inflammation, (2) the appearance of Desulfovibrio axanomicus–vulgaris, which metabolizes a variety of chemicals, and (3) alterations in the relative abundance of 6 other bacteria species. Furthermore, RNA-seq analysis showed that Hamp2, a gene encoding hepcidin antimicrobial peptide 2, which has a strong anti-microbial activity against certain bacteria, depends on Ascl1 for expression. This finding was confirmed by qRT-PCR (Figure 7B). Together, we show that maternal hepatic Ascl1 modulates the adaptation of the maternal microbiota to pregnancy, potentially via regulating Hamp2.

Hepatocyte-Specific Ascl1 Knockout Causes Placental Overgrowth and Aberrant Postnatal Growth of Offspring

We examined the uteroplacental and fetal compartments and postnatal growth of pups to determine whether Ascl1 deficiency in maternal hepatocytes ultimately affects pregnancy outcomes. Compared with the placentas of control mice, the placentas of hep-Ascl1\(^{-/-}\) mice were enlarged markedly, manifested by a 26.9% increase in weight on gestation day 15 and a 33% increase on gestation day 18, with the expansion of both the junctional and labyrinth zones (Figure 8A and B). The distribution of glycogen trophoblast cells visualized by glycogen staining did not appear to be Ascl1-dependent (Figure 8C). By in situ hybridization, we probed the mRNAs of placental lactogen (PL)-I, a marker gene for parietal trophoblast giant cells, and PL-II, a marker gene for parietal trophoblast giant cells, and spongiotrophoblast giant cells, and did not observe an overt Ascl1-dependent distribution of these trophoblast cell populations (Figure 8D). These results of placental structural evaluations suggest that the loss of function of Ascl1 in maternal hepatocytes causes placental overgrowth without disrupting placental structure. Moreover, when comparing hep-Ascl1\(^{-/-}\) pregnant mice with their control mice, the placental levels of insulin-like growth factor (IGF2), a potent placental and fetal growth factor, were reduced by 32% on gestation day 15, but were equivalent on gestation day 18, whereas placental concentration of PL-II, a major hormone produced by the placenta during the second half of gestation, was unchanged (Figures 8E and 9). However, when maternal hepatic Ascl1 was deficient, maternal blood concentration of alkaline phosphatase, an enzyme elaborated primarily from the placenta and a marker for trophoblast differentiation, was increased significantly on gestation day 15 and more than 2-fold on gestation day 18 (Figure 8F). The data of these functional assessments suggest that Ascl1 inactivation in maternal hepatocytes partially influences placental function.

Furthermore, it is known that protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) signaling critically regulates placental development and growth. We found that, without Ascl1 in maternal hepatocytes, placental AKT1 phosphorylation at S473, but not at T308, was increased mildly; in contrast, ERK1 and ERK2 activities were inhibited dramatically, reduced by as much as 80% and 76%, respectively, before parturition (gestation day 18) (Figures 8G and 9). Thus, the loss of maternal hepatic Ascl1 results in strong inhibition of placental ERK signaling.

In addition, we did not observe a difference in fetal weight and number on both gestation days 15 and 18 between the 2 genotype groups of mice (Figure 10A and B). However, compared with those male and female pups born from control dams, male pups born from hep-Ascl1\(^{-/-}\) dams showed a 16% increase in weekly body weight gain at week 1 after weaning, but a 41% decrease at week 2, while female pups born from hep-Ascl1\(^{-/-}\) dams showed a 32% increase at week 1, a 35% decrease at week 2, a 38% decrease at week 3, and a 43% increase at week 5 (Figure 10C and D). Apparently, pups born from the 2 genotype groups of dams grew quite differently after weaning. Taken together, we show that the maternal hepatic Ascl1 null mutation causes placental overgrowth, partial changes in placental function, severe suppression of placental ERK activity, and an abnormal postnatal growth pattern of offspring.

Figure 3. (See previous page). Differentially expressed genes and canonical pathways affected by hepatocyte-specific deletion of Ascl1 in the maternal liver. AAV8-TBG-Cre virus or AAV8 virus with a null vector (AAV8-TBG-null) control virus was injected via tail vein at a dose of $1 \times 10^{12}$ genomic copies per mouse on GD8 Ascl1\(^{+/+}\) mice. (A) Total RNA was isolated from livers of nonpregnant (NP) and GD15 and GD18 Ascl1\(^{+/+}\) and hepatocyte-specific Ascl1 knockout (hep-Ascl1\(^{-/-}\)) mice. Hepatic Ascl1 mRNA levels were measured using qRT-PCR and presented as the mean fold changes relative to NP controls $\pm$ SD ($n = 4$–$5$). $**P < .0001$. (B) AAV8-TBG-Cre virus–mediated recombination in maternal organs and the placenta. The sections of maternal organs and the placenta collected from GD18 hep-Ascl1\(^{-/-}\) mice were subjected to immunostaining with a GFP antibody. (C and D) Ascl1-dependent transcriptome. Total RNA was isolated from livers of Ascl1\(^{+/+}\) and hep-Ascl1\(^{-/-}\) mice on GD15 and was subjected to RNA-seq ($n = 4$–$5$). (C) Differentially expressed genes are presented by the volcano plot. Red: significantly up-regulated or down-regulated genes; black, green, and blue: nonsignificant genes. Differentially expressed genes with at least 2-fold and $P < .05$ were analyzed using the Ingenuity Pathway Analysis. (D) The top enriched canonical pathways targeted by hepatic Ascl1 are presented. Orange, up-regulated; blue, down-regulated. FDR, false-discovery rate; GFP, green fluorescent protein; VDR/RXR, vitamin D receptor/retinoid X receptor.
Figure 4. Maternal liver phenotypes in maternal hepatocyte-specific Ascl1 ablated mice. Livers were collected and weighed from nonpregnant (NP) and GD15 and GD18 Ascl1floflo and hep-Ascl1-/ mice. (A) Liver sections were subjected to H&E staining. (B) Ki67 and β-catenin staining. (D) Morphology of the pancreas. (E) Liver-to-body-weight ratios are presented as means ± SD (n = 4–10). (F) β-cell mass is presented as the means ± SD (n = 3–4). Serum were collected from NP and GD15 and GD18 Ascl1floflo and hep-Ascl1-/ mice. Data from the serum biochemical profile are expressed as means ± SD (n = 5). Hepatic albumin mRNA levels were measured using qRT-PCR and presented as the mean fold changes relative to NP controls ± SD (n = 4–5).

Figure 5. Maternal pancreas phenotypes in maternal hepatocyte-specific Ascl1 ablated mice. Maternal pancreases were collected and weighed from nonpregnant (NP) and GD18 or GD19 Ascl1floflo and hep-Ascl1-/ mice. (A) Morphology of the pancreas. (B) Maternal pancreas-to-total-body-weight ratios are presented as means ± SD (n = 4–5). (C) Maternal pancreatic sections were subjected to insulin immunostaining. (D) The quantification of the insulin-positive islets to the pancreatic section area are presented as means ± SD (n = 3–4). (E) β-cell mass is presented as the means ± SD (n = 3–4). Serum were collected from NP and GD15 and GD18 Ascl1floflo and hep-Ascl1-/ mice. Serum (F) insulin and (G) glucose are presented as the means ± SD (n = 3–6). **P < .01, and ***P < .001.
Hepatocyte-Specific Ascl1 Knockout Leads to Igf2 Activation in Maternal Hepatocytes

The earlier-described observations that several maternal organs were enlarged significantly in hep-Ascl1−/− pregnant mice relative to controls prompted us to look for growth factors regulated by Ascl1 from our RNA-seq analysis. We found 1 candidate: Igf2, which encodes a potent growth factor28–30 and was induced with Ascl1 loss.

To validate the RNA-seq data, we quantified Igf2 mRNA levels by qRT-PCR in maternal livers of gestation days 15 and 18 with and without loss of Ascl1 in maternal hepatocytes. We found robust Igf2 mRNA expression in maternal livers, most strikingly on gestation day 18 (Figure 11A). Consistently, maternal hepatic IGF2 protein was produced abundantly in hep-Ascl1−/− pregnant mice, but was undetectable in control pregnant mice (Figure 11B). In situ hybridization and immunohistochemistry detected rich Igf2 transcripts and IGF2 protein in maternal hepatocytes deficient for Ascl1, but not in controls (Figure 11C and D). In mice, Igf2 is imprinted maternally and is regulated differentially in the placenta and fetus. It is transcribed from 4 promoters, designated Igf2-P0, P1, P2, and P3. Igf2-P0 directs Igf2 transcription in the placenta, whereas Igf2-P1, P2, and P3 direct its transcription in both the placenta and fetus.29,35 We used a set of qRT-PCR primers to analyze promoter-specific Igf2 transcripts in maternal livers (Figure 11E). We found that P0 remained silent no matter the presence or absence of Ascl1 in maternal hepatocytes. In contrast, the other 3 promoters all were activated as a result of Ascl1 loss in these cells. The concentration of circulating IGF2 protein in gestation day 18 hep-Ascl1−/− mice was approximately 9 times higher than that in control mice (Figure 11F). These data collectively show that, in maternal hepatocytes, Ascl1 deficiency results in Igf2 activation via P1, P2, and P3, creating a maternal environment rich in this growth factor. Thus, here we linked Igf2 to Ascl1-dependent phenotypes in our experimental settings.

To further evaluate IGF2 signaling, we examined the expression and activities of several associated signaling molecules in maternal livers of both genotype groups of mice (Figures 11B and 12). We found that, compared with the prepregnancy state, the lack of Ascl1 in maternal hepatocytes prevented gestation-dependent increases in IGF2.
receptor (IGF2R) expression; did not affect the activities of phospho-AKT and phospho-ERK; and increased the expression levels of both total and phosphorylated eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). The data indicate that Ascl1 controls IGF2 signaling also via regulating IGF2R, and 4E-BP1 may transduce IGF2 signaling in this setting.

**Discussion**

It has been an enigma why maternal liver activates a proneuronal gene (Ascl1) in response to pregnancy. Here, we show that Ascl1 is required for the maternal liver to structurally and functionally adapt to this physiological stimulus (pregnancy). Loss of Ascl1 in maternal hepatocytes impairs their cellular structure, reflected by the formation of a layer of eosin staining–negative substance around their nuclei. This substance was Oil Red O staining–negative and periodic acid–Schiff staining–negative, suggesting that it is not a fatty acid or glycogen. Others reported a similar phenomenon in Mst1/2 double-knockout hepatocytes. The connection between Ascl1 and Mst1/2 signaling in maternal hepatocytes will be interesting to explore in the future. Although the eosin staining–negative substance around the hepatocyte nuclei remains unknown, we believe that it is linked to Ascl1 deficiency–caused dysfunction of maternal hepatocytes, characterized by reduced albumin production and increased alanine aminotransferase and aspartate aminotransferase release. Moreover, Ascl1 loss induced increased proliferation and hypertrophy of maternal hepatocytes, which was associated with increased IGF2 production by these cells. IGF2 is a prototypical growth factor driving cell expansion and organogenesis during development, and adult regenerating livers activate IGF2 to promote hepatocyte replication. In addition, we found that Ascl1 also governs IGF2R expression. IGF2R internalizes and degrades IGF2, eliminating excessive IGF2 in tissues and the
Figure 8. Placental phenotypes in maternal hepatocyte-specific Ascl1 ablated mice. Placentas were collected and weighed from GD15 and GD18 Ascl1<sup>fl/fl</sup> and hep-Ascl1<sup>−/−</sup> mice. (A) Placental weights are presented as means ± SD (n = 4–9 dams). (B) Placental sections were stained with H&E. (C) Frozen placental sections underwent periodic acid–Schiff (PAS) staining. Glycogen is stained red to purple. (D) Frozen placental sections were subjected to PL-I and PL-II in situ hybridization staining using the RNAscope 2.5 HD Assay-BROWN kit. The PL-I and PL-II mRNAs are stained dark brown. (E) Western blot was performed using placental lysates prepared from 1 placenta per dam with antibodies against the proteins indicated. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Serums were collected from nonpregnant (NP) and GD15 and GD18 Ascl1<sup>fl/fl</sup> and hep-Ascl1<sup>−/−</sup> mice. (F) The concentrations of circulating alkaline phosphatase (ALP) are presented as means ± SD (n = 4–6). **P < .01 and ****P < .0001.
circulation. Thus, Ascl1 activation not only ensures the silence of Igf2, but also up-regulates IGF2R expression, strictly controlling IGF2 signaling in the maternal compartment. Furthermore, 4E-BP1 is well known for its function of stimulating protein synthesis and cell growth. Ascl1 deficiency–caused increase in hepatocyte cell size can be explained by increased 4E-BP1 activity in these cells.

Here, we show that pregnancy reprograms maternal hepatocytes to activate Ascl1, thereby controlling the transcriptional output and modulating the expression of at least 1274 genes. Of special note, the top canonical pathway describing these differentially expressed genes is one that represents genes involved in the metabolism of neurotransmitters. Ascl1 is known to be essential for the development of serotonergic and dopaminergic neurons and also regulates their neurotransmitter biosynthesis. Remarkably, Ascl1 alone is sufficient to generate functional neurons from fibroblasts and embryonic stem cells, being a key driver of induced neuronal cell reprogramming in different cell contexts. These findings raise the question of whether Ascl1 activation enables maternal hepatocytes to possess some properties of neurons as an adaptive response to
support a healthy pregnancy. We are very interested in answering this question in our future studies.

It is surprising that inactivating Ascl1 in maternal liver induced robust systemic responses in the maternal compartment. This implies that the maternal liver, via activating Ascl1, communicates with other maternal organs to systematically coordinate maternal adaptations to pregnancy, uncovering a novel function of maternal liver during pregnancy. Moreover, we showed an Ascl1/Igf2 axis in maternal hepatocytes, where Ascl1 normally silences the Igf2 locus and thereby avoids the exposure of other maternal organs to this growth factor. Others reported that Ascl1 knockdown resulted in an increase in Igf2 expression in neuronal cells in vitro.45 Thus, this axis may be operating in both maternal hepatocytes and neurons. We gained further insight into this axis in that IGF2 was derived from 3 placental- and fetal-specific promoters of Igf2 in maternal hepatocytes without Ascl1. How Ascl1 controls the activities of these 3 promoters of Igf2 is another question for the future. We believe that this Ascl1/Igf2 axis, at least in part, underlies Ascl1-dependent phenotypes in the maternal compartment. When maternal hepatocyte Ascl1 is lost, excess IGF2 is produced in, and elaborated from, these cells. Locally, this promotes maternal hepatocyte proliferation and growth. Systemically, it induces overgrowth of maternal pancreas, spleen, and kidney. Through these autocrine and endocrine pathways, IGF2 partially mediates Ascl1-dependent phenotypes. It is highly likely that other maternal organs not examined in this study also respond to the null mutation of maternal hepatic Ascl1. However, we are unable to exclude the possibility that Ascl1 expression also is activated in a maternal organ not examined and AAV8-TBG-Cre virus induces recombination there, contributing to Ascl1-dependent phenotypes in pregnant mice.

Our studies show that Ascl1 activation in maternal hepatocytes is required to maintain pregnancy-dependent homeostasis of maternal gut microbiota. It is known that the microbiome shows adaptive changes to accommodate the physiological and immunologic alterations of the host during pregnancy.22,46,47 Here, we found that 8 bacteria species in maternal ceca responded to the deficiency of maternal hepatic Ascl1. Remarkably, among these bacteria species, Pseudobutyrivibrio-R intestinalis was depleted and D oxamicus-vulgaris appeared aberrantly. The former is known to be associated with the anti-inflammatory activity of colonic mucosa,23 whereas the latter participates in metabolism of various substances such as ammonium, lactate, alcohol, and pyruvate.24 Therefore, maternal liver,
by activating Ascl1, controls these 2 bacteria populations in maternal ceca, showing new roles for Ascl1 in maternal liver. Several lines of evidence suggest that the other 6 bacteria species also are important to the health of the host, however, the gestation-dependent functions of these bacteria species remain unclear. Mechanistically, we identified an Ascl1/Hamp2 axis in maternal hepatocytes as a candidate regulator of maternal gut microbiota. Our data show that Hamp2 mRNA expression almost fully relies on Ascl1 activation in maternal hepatocytes. Hamp2 is produced primarily in the liver, has strong antiinflammatory activity against certain bacteria, and regulates immune responses against bacterial pathogens. Hence, we assume that this Ascl1/Hamp2 axis in maternal hepatocytes may partially govern the adaptations of maternal gut microbiota to gestation.

Our studies show that maternal liver, through activating Ascl1, modulates the placenta. This notion is based on our observations that maternal liver Ascl1 deficiency leads to placenta overgrowth and a change in its function. These phenotypes can be partially interpreted as the consequences of the exposure of the placenta to increased maternal IGF2. We also observed that hep-Ascl1/- placenta transiently reduced its IGF2 production on gestation day 15 and largely diminished its Erk1/2 signaling on gestation day 18 relative to their controls. Thus, the placenta used different mechanisms at distinct stages of pregnancy to defensively respond to increased maternal IGF2, eventually restricting its otherwise further overgrowth. We think that maternal liver activates Ascl1 to maintain Igf2 silencing and, by doing this, allows the placenta to appropriately develop and grow without potential maternal inference. It was surprising that the fetal weight was not affected by increased maternal IGF2. This may suggest that the defense responses of the placenta (reduction in IGF2 production and suppression in Erk1/2 activity) to increased maternal IGF2 is so strong that the potential stimulatory effect of this potent hormone on fetal growth is fully blocked. However, we did observe severely impaired postnatal growth of the offspring born from hep-Ascl1/- dams. It is a well-established concept that maternal problems generate long-term adverse effects on the health of the next generation. Hence, we believe that Ascl1 deficiency–caused dysregulation of maternal adaptations and placental abnormality together impair the postnatal growth of the offspring. This model warrants further investigations.

In summary, we show that, as pregnancy advances, maternal hepatocytes activate the expression of Ascl1 to alter their transcriptomes. Via this mechanism, maternal liver systematically coordinates adaptations in the maternal compartment and allows for optimal placental development and growth, collectively ensuring the health of both the mother and her infant during pregnancy and postnatally.

**Methods**

**Mice**

Ascl1fl/fl;R26EYFP/EYFP mice were a generous gift from Dr Francois Guillemot (The Francis Crick Institute, London, United Kingdom). The mice are referred to hereafter as Ascl1fl/fl for simplicity. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were maintained on a 12-hour light/12-hour dark cycle (7 AM on and 7 PM off) at 22°C ± 1°C and given standard rodent chow and water ad libitum. Animals were randomly allotted to experimental groups by body weight. All of the animal experiments were conducted in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The protocols for the care and use of animals were approved by the Indiana University–Purdue University Indianapolis Animal Care and Use Committee.

**Mouse Genotyping**

Genomic DNA was prepared from mouse ear snips using the modified hot sodium hydroxide and Tris (HotSHOT) method. All mice were genotyped by PCR using KAPA Taq PCR Kits (Kapa Biosystems, Inc, Wilmington, MA). Specific primers purchased from Integrated DNA Technologies (Coralville, IA) were used to detect the wild-type and mutant alleles. Primers Ascl1 forward (5’TCTACTGTCCAAACGCAGTAAG3’) and Ascl1 wild-type reverse (5’GCTCTCAACTCTCGTAAAGA3’) were used to detect the Ascl1 wild-type allele (342 bp), and primers Ascl1 forward and Ascl1 mutant reverse (5’TAGACGTGTGCTTGTTAAGTGTA3’) were used to detect floxed the Ascl1 allele (857 bp). PCR conditions were as follows: 35 cycles at 94°C for 30 seconds, 69°C for 30 seconds, and 72°C for 90 seconds.

**Timed Pregnancy and Virus Injection**

Timed pregnancy was generated by mating 3-month-old virgin Ascl1fl/+ female mice with wild-type male mice to ensure heterozygous fetuses with 1 wild-type Ascl1 allele. The presence of a copulation plug in the vagina was designated as gestation day (GD) 1. AAV8-TBG-Cre (AV-8-PV1091; Addgene, Watertown, MA) was injected via tail vein at a dose of 1 × 10^12 genomic copies per mouse on GD8, and mice were killed on GD15 and GD18. AAV8 virus with a null vector (AV-8-PV0148; Addgene) was used as a control.

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**Figure 11.** (See previous page) Igf2 activation in maternal liver deficient for Ascl1. Maternal livers were collected and weighed from nonpregnant (NP) and GD15 and GD18 Ascl1fl/fl and hep-Ascl1/- mice. (A) Hepatic Igf2 mRNA levels were measured using qRT-PCR and presented as the mean fold changes relative to NP controls ± SD (n = 4–5). (B) Western blot was performed using liver lysates with antibodies against the proteins indicated. (C) Igf2 in situ hybridization on liver sections. (D) IGF2 immunostaining. (E) Levels of hep Igf2 promoter-specific transcript variants were measured using qRT-PCR and presented as the mean fold changes relative to NP controls ± SD (n = 4–5). (F) Levels of IGF2 protein in serum were measured using enzyme-linked immunosorbent assay and presented as the mean fold changes ± SD (n = 3–5). *P < .01, ***P < .001, and ****P < .0001. P0, placental-specific Igf2 promoter; P1-3, placent al- and fetal liver-specific Igf2 promoter. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 12. Quantification of the relative intensity of Western blot signals from Figure 11B. Signal intensity was normalized with glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data are presented as the mean fold changes relative to the nonpregnant (NP) state ± SD (n = 3). *P < .05, **P < .01, and ****P < .0001.
### Tissue Collection and Histology

Mice were killed at various time points. Maternal liver, pancreas, spleen, and kidney organs, and placentas and fetuses were collected and weighed. Part of each tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm for H&E staining and histologic analysis. Meanwhile, part of each tissue was embedded in optimal cutting temperature compound (23-730-571; Fisher Scientific, Hampton, NH) on heptane cooled in dry ice and stored at -80°C until processing. The remaining tissues were snap-frozen in liquid nitrogen and stored at -80°C for protein and RNA extraction.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded maternal liver sections were subjected to standard immunohistochemistry. Primary antibodies against β-catenin (610153, 1:100; BD Transduction, San Jose, CA), IGF2 (AF792, 1:1000; R&D Systems, Minneapolis, MN), and Ki67 (RM-9106-S1, 1:100; BD Transduction Systems, San Jose, CA) were used for immunostaining. The slide images were acquired by the Leica DM2000 microscope using the Leica Application Suite program. After approval from the institutional review boards, the laboratory information systems of our institute were searched. A set of paraffin blocks archived in the Department of Pathology of Indiana University School of Medicine were selected. They represented liver tissues from pregnant patients and patients with hepatocellular carcinoma or hepatocellular adenoma. These paraffin blocks were sectioned for ISH with a human Ascl1 probe (459721; Advanced Cell Diagnostics). qRT-PCR

Total RNA was isolated from snap-frozen liver tissue using TRIzol reagent (15596018; Invitrogen, Waltham, MA) as per the manufacturer’s directions. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the Verso cDNA kit (AB1453B; Thermo Fisher Scientific) and diluted 4 times with water. qRT-PCR was performed using the diluted cDNA with either TaqMan Gene Expression Master Mix (4369016; Applied Biosystems, Carlsbad, CA) or PowerUp SYBR Green Master Mix (A25742; Applied Biosystems) with specific gene probes. qRT-PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems) and analyzed by the 7300 System SDS RQ Study Software (Applied Biosystems). qRT-PCR conditions for probes (Ascl1: Mm03058063_m1, 4453320; Igf2: Mm00439564_m1, 4331182; Hamp2: Mm03928990_g1, 4453320; 18S: Mm03928990_g1, 4453320; Applied

### Western Blot

Liver and placental homogenates (10–30 μg) were separated by polyacrylamide gel electrophoresis under reducing conditions and transferred to polyvinylidene difluoride membranes. The following antibodies were used: phospho-AKT (T308, ab76297, 1:5000; Abcam, Cambridge MA), phospho-AKT (S473, 2118-1, 1:2000; Epitomics, Burlingame, CA), ASCL1 (BAF2567, 1:500; R&D Systems), ERK1/2 (9102, 1:2000; Cell Signaling, Danvers, MA), phospho-ERK1/2 (T202/Y204, 4377, 1:2000; Cell Signaling), glyceraldehyde-3-phosphate dehydrogenase (5174, 1:2000; Cell Signaling), IGF2 (A2086, 1:1000; Abclonal, Woburn, MA), Lamin B1 (9087, 1:1000; Cell Signaling), IGF2R (14364, 1:2000; Cell Signaling), p-4E-BP1 (T37/46) (2855, 1:2000; Cell Signaling), and PL-I (1:2000; a gift from Dr Soares at the University of Kansas Medical Center). Immune complexes were detected by SuperSignal West Pico PLUS Chemiluminescent Substrate (34577; Thermo Fisher Scientific). Signals were detected using ImageQuant LAS 4000 Mini (General Electric Life Sciences, Marlborough, MA) and quantified using ImageJ software.

### Table 1. Primers for Promoter-Specific Igf2 Transcripts

| Primer name | Sequence 5' -> 3' |
|-------------|-------------------|
| Igf2-all_F  | CCGCTTCTACTTICAGAC |
| Igf2-all_R  | GTATCTGGGAAGTCTGTC |
| Igf2-P0_F   | TTTTCCACCCGGCCGGAA |
| Igf2-P0_R   | GAGCTGTCTAGTCTGTC |
| Igf2-P1_F   | CCGCCAGCACAGATTTGGA |
| Igf2-P1_R   | CACCAACATCGACTTC |
| Igf2-P2_F   | CCCGCCCTAAGATACCTAA |
| Igf2-P2_R   | AGCACAACATCGACTTCCC |
| Igf2-P3_F   | CGGCCCTTATCAAACCTTC |
| Igf2-P3_R   | GGGTGTCACAGATGTCTC |
| 18S_F       | CTCACCAGCGGAAACCTCAC |
| 18S_R       | CGCTCACCACCAACTAAGAC |

F, forward; R, reverse.

ISH

ISH was performed using the RNAscope 2.5 HD Assay (322310 and 322360; Advanced Cell Diagnostics, Newark, CA) and appropriate probes and as per the manufacturer’s directions. The following probes were used: Ascl1 (476321; Advanced Cell Diagnostics), Igf2 (437671; Advanced Cell Diagnostics), PL-I (405521; Advanced Cell Diagnostics), and PL-II (423681; Advanced Cell Diagnostics). A positive control probe Ppib (310043; Advanced Cell Diagnostics) and a negative control probe DapB (131911; Advanced Cell Diagnostics) were used to determine the efficacy of the protocol. These probes were applied to formalin-fixed, paraffin-embedded sections of mouse livers and placentas. The slide images were acquired by the Leica DM2000 microscope using the Leica Application Suite program. After approval from the institutional review boards, the laboratory information systems of our institute were searched. A set of paraffin blocks archived in the Department of Pathology of Indiana University School of Medicine were selected. They represented liver tissues from pregnant patients and patients with hepatocellular carcinoma or hepatocellular adenoma. These paraffin blocks were sectioned for ISH with a human Ascl1 probe (459721; Advanced Cell Diagnostics).
Biosystems) using TaqMan Gene Expression Master Mix were uracil N-glycosylase incubation (50°C for 2 minutes), polymerase activation (95°C for 10 minutes), and 40 cycles of PCR (95°C for 15 seconds, 60°C for 1 minute). qRT-PCR conditions using PowerUp SYBR Green Master Mix were uracil-DNA glycosylase activation (50°C for 2 minutes), polymerase activation (95°C for 2 minutes), 40 cycles of PCR (95°C for 15 seconds, 60°C for 15 seconds, 72°C for 1 minute), and dissociation curve (95°C for 15 seconds, 60°C for 30 seconds, 95°C for 15 seconds). Primers for detecting promoter-specific Igf2 transcripts are listed in Table 1. Relative gene expression was calculated by the comparative C_T method (∆∆Ct) and normalized to 18S ribosomal RNA transcript levels.

RNA-seq

Total RNA was isolated from snap-frozen liver tissue using the RNeasy Plus Mini Kit (74134; Qiagen, Germantown, MD) as per the manufacturer’s directions. RNA sequencing was performed and analyzed by the Center for Medical Genomics Core (Indiana University School of Medicine). In brief, total RNA concentration and quality were assessed using the Agilent 2100 Bioanalyzer (Santa Clara, CA). Single-indexed, strand-specific cDNA library from total RNA samples (500 ng input with RIN ≥5) was prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) as per the manufacturer’s directions. The quality and size distribution of the libraries were assessed using the Qubit (New York, NY) and Agilent 2100 Bioanalyzer. Libraries (200 pmol/L) were clustered and amplified on cBot using the HiSeq 3000/4000 PE Cluster Kit and sequenced (2 × 75 bp paired-end reads) on a HiSeq4000 (Illumina, San Diego, CA) using the HiSeq 3000/4000 PE SBS Kit. A Phred quality score (Q score) was used to measure the quality of sequencing. The quality of the sequencing data was assessed using FastQC (Babraham Bioinformatics, Cambridge, UK). All sequenced libraries were mapped to the mouse genome (University of California, Santa Cruz [UCSC] mm10) using the STAR RNA-seq aligner (Illumina), reads distribution across the genome was assessed using bamutils (from ngsutils), and uniquely mapped sequencing reads were assigned to mm10 refGene genes using featureCounts (from subread). Genes with CPM less than 0.5 in more than 5 of the samples were removed. The data were normalized using the Trimmed Mean of M-values (TMM) method. Differential expression analysis was performed using edgeR (Bioconductor) and Ingenuity Pathway Analysis with ±2-fold change and P < .05. The false-discovery rate was computed from P values using the Benjamini–Hochberg procedure.

Serum Biochemistry

Blood from nonpregnant and pregnant mice (GD15 and GD18) was collected and left to clot at room temperature. After 2 centrifugations at 3000 rpm, serum was collected and analyzed by Eli Lilly and Company (Indianapolis, IN). Serum insulin levels were measured by the Translation Core at the Indiana University School of Medicine Center for Diabetes and Metabolic Diseases. Serum IGF2 levels were quantified using a one-sixth dilution with the Mouse IGF2 ELISA Kit (EK0381; Boster Biological Technology, Pleasanton, CA) as per the manufacturer’s directions and read with the SpectraMax M2e spectrophotometer (Molecular Devices, San Jose, CA) using the SoftMax Pro 6 program (Molecular Devices).

Microbiome 16S Sequencing

Total microbial DNA was isolated from the snap-frozen cecal sample using the PureLink Microbiome DNA Purification Kit (A29789; Invitrogen) as per the manufacturer’s directions. Microbiome 16S sequencing was performed and analyzed by the Zymo Research Corporation (Irvine, CA). In brief, bacterial 16S ribosomal RNA gene-targeted sequencing was performed using the Quick-16S NGS Library Preparation Kit (Zymo Research). The bacterial 16S primers, custom-designed by Zymo Research, amplified the V3–V4 region of the 16S ribosomal RNA gene. The sequencing library was prepared using PCR reactions in real-time PCR machines to control cycles and therefore prevent PCR chimera formation. The final PCR products were quantified with qPCR fluorescence readings and pooled together based on equal molarity. The final pooled library was cleaned up with Select-a-Size DNA Clean and Concentrator (Zymo Research), and then quantified with TapeStation (Agilent) and Qubit (Thermo Fisher Scientific). The final library was sequenced on an Illumina MiSeq with a v3 reagent kit (600 cycles). The sequencing was performed with more than 10% PhiX (Illumina) spike-in. Amplicon sequences were inferred from raw reads using the Dada2 (Bioconductor) pipeline. Chimeric sequences also were removed with the Dada2 pipeline. Taxonomy assignment, composition bar charts, α-diversity, and β-diversity analyses were performed with Qiime v.1.9.1 (University of Colorado Boulder, Boulder, CO). Taxa that had an abundance that was significantly different among groups was identified by linear discriminant analysis effect size (LEfSe) with default settings if applicable. Differential expression analysis was assessed using Ingenuity Pathway Analysis with ±2-fold change and P < .05.

β-Cell Mass

Pancreatic tissues were fixed in 4% paraformaldehyde (B18715; Sigma Aldrich, St. Louis, MO), embedded in paraffin, and sectioned at 7 μm for collecting 5 sections at 50-μm apart per pancreatic sample. Pancreatic sections immunostained for insulin (sc-9168, 1:100; Santa Cruz) were used to quantify insulin-positive β-cell mass. β-cell mass was determined by the ratio of the insulin-positive stained area to the tissue area multiplied by the pancreas weight.

Statistical Analysis

Data are shown as means ± SD or means ± SEM. Statistical analyses were performed using 1-way analysis of variance with Sidak multiple comparisons or a 2-sided unpaired Student t test with the means ± 95% CIs. Significant differences were defined when P < .05.
All authors had access to the study data and reviewed and approved the final manuscript.

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Conflicts of interest
The authors disclose no conflicts.

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