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IDENTIFICATION AND LINEAGE TRACING OF ASCL1-EXPRESSING CELLS IN MATERNAL LIVER DURING PREGNANCY

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of

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Sudhanshu Kumar

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LIST OF ABBREVIATIONS

Ascl1	Achaete-Scute Complex Homolog 1
bHLH	Basic Helix-Loop-Helix
β-gal	β-galactosidase
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Cre	Cre-recombinase
CreER	Cre-recombinase Estrogen Receptor
CT	Comparative Cyclic Threshold
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
DAB	3,3-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
ER	Estrogen Receptor
FLP	Flippase
FLP-FRT	Flippase Recognition Target
FFPE	Formalin Fixed Paraffin Embedded
GFP	Green Fluorescent Protein
Hsp	Heat Shock Proteins
i.p	Intraperitoneal
LacZ	Lactose Operon β-galactosidase
LB	Luria Broth
4-OHT	4-Hydroxy-Tamoxifen
OCT	Optimum Cutting Temperature
PCR	Polymerase Chain Reaction
PH	Partial Hepatectomy
PBS	Phosphate Buffered Saline
PEG	Poly Ethylene Glycol
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
SSC	Standard Saline Citrate
SDS	Sodium Dodecyl Sulfate
UV	Ultra Violet
X-gal	X-Galactopyranoside

ABSTRACT

Kumar, Sudhanshu. M.S., Purdue University, August 2013. Identification and lineage tracing of Ascl1-expressing cells in maternal liver during pregnancy. Major Professor: Guoli Dai.

During pregnancy, maternal liver exhibits robust growth to meet the metabolic demands of the developing placenta and fetus. Although hepatocyte hypertrophy and hyperplasia are seen in the maternal liver, the molecular and cellular mechanisms mediating the maternal hepatic adaptations to pregnancy is poorly understood. Previous microarray analysis revealed a most upregulated gene named Ascl1, a transcription factor essential for neural development, in the maternal liver at mid-gestation. The aims of the study were to (1) validate the activation of Ascl1 gene; (2) identify Ascl1-expressing cells; and (3) determine the fate of Ascl1-expressing cells, in the maternal liver during the course of gestation. Timed pregnancy was setup in mice and the maternal livers were collected at various stages of gestation. Maternal hepatic Ascl1 mRNA expression was evaluated by qRT-PCR and northern blotting. The results demonstrated that the transcript level of maternal hepatic Ascl1 is exponentially increased during the second half of pregnancy in comparison with a non-pregnant state. Using a Ascl1-GFP mouse model generated by others to monitor the behavior of neural progenitor cells, we found that maternal hepatic Ascl1-expressing cells are non-parenchymal cells, very small in size,

and expanding during pregnancy. To map the fate of this cell population, we generated an *in vivo* tracing mouse model named Ascl1-CreERT2/ROSA26-LacZ. Using this model, we permanently labeled maternal hepatic Ascl1-expressing cells at midgestation by giving tamoxifen and analyzed the labeled cells in the maternal liver prior to parturition. We observed that the initial small Ascl1-expressing cells undergoing expansion at mid-gestation eventually became hepatocyte-like cells at the end stage of pregnancy. Taken together, our findings strongly suggest that Ascl1-expressing cells represent a novel population of hepatic progenitor cells and they can differentiate along hepatocyte lineage and contribute to pregnancy-induced maternal liver growth. Further studies are needed to firmly establish the nature and property of maternal hepatic Ascl1-expressing cells. At this stage, we have gained significant insights into the cellular mechanism by which the maternal liver adapts to pregnancy.

INTRODUCTION

1.1 Objective

The objectives of the study were to validate the activation of Ascl1 gene, identify the Ascl1-expressing cells, and determine the fate of the Ascl1-expressing cells in the maternal liver during the course of gestation.

1.2 Background

1.2.1 Liver

Liver is an indispensable organ involved in the metabolism and physiological functions and plays an integral role in body homeostasis. It is associated with uptake, storage and metabolism of nutrients, synthesis of plasma proteins, maintaining blood glucose level and removal of toxic compounds and drugs from the body. Liver has two different cell types, parenchymal cells and non-parenchymal cells. Hepatocytes are the parenchymal cells that constitute approximately 80% of hepatic cells. Non-parenchymal cells including endothelial cells, lymphocytes, Kupffer cells and hepatic stellate cells make up the rest 20% of liver cells (Michalopoulos, 2007). The intrahepatic space, called sinusoids, provides large surface area for nutrient absorption and efficient detoxification.

In the sinusoidal space Kupffer cells are present which play important role in phagocytosis of foreign particles. Kupffer cells are also known to secrete cytokines which are indispensable for liver regeneration and growth (Friedman, 2008). Hepatic stellate cells are another residents of sinusoidal space in the liver. Of many functions, storage of vitamin A and the production of the extracellular matrix are the significant ones. Recent studies have indicated that hepatic stellate cells also have the property of liver progenitor cells (Friedman, 2008).

1.2.2 Models to study liver growth and regeneration

Adult hepatocytes don't go through cell division under normal condition although they do retain their innate ability to proliferate in response to physiological demand or injury (Riehle, Dan, Campbell, & Fausto, 2011). Three models widely used to study liver regeneration are: partial hepatectomy, chemical-induced liver injury and chronic liver injury (Michalopoulos, 2007). In partial hepatectomy liver mass is surgically removed and the remaining liver regrows until the original liver mass is restored (Mitchell & Willenbring, 2008). In chemical-induced liver injury model, hepatocyte proliferation occurs in response to cell necrosis instead of loss of liver cell mass (Suk & Kim, 2012). Chronic liver injury is induced by special diets (e.g. choline-deficient, ethionine), long term exposure to toxic xenobiotics (e.g. thioacetamide), or overproduction of endobiotics (e.g. bile acid). In this model, liver progenitor cells are activated after the capacity of mature hepatocyte proliferation is exhausted (Michalopoulos, 2007; Riehle et al., 2011). Another potential model being investigated is pregnancy-induced maternal liver growth.

Pregnancy can be viewed as a natural state akin to parabiosis where organism partly shares blood systems. Here, an adult organism (the pregnant mother) is exposed to an extremely young organism (the fetus). Parabiosis between young and old mice has been used to study systemic factors that can modulate age or injury related decline of progenitor cell activity (Conboy et al., 2005).

1.2.3 Pregnancy-induced maternal liver growth

Pregnancy is associated with widespread maternal changes in the structure and function of almost all the major organs of the mammalian system, including brain (Shingo et al., 2003), pancreas (Huang, Snider, & Cross, 2009; H. Kim et al., 2010; Nielsen, Svensson, Galsgaard, Moldrup, & Billestrup, 1999), immune system (Audus, Soares, & Hunt, 2002) and cardiovascular system (Dai et al., 2011; Rahman & Wendon, 2002). Pregnancy also induces maternal hepatic growth (Dai et al., 2011). Maternal liver mass increases significantly during pregnancy culminating in about 80% liver enlargement (hepatomegaly). But the liver-to-body weight ratio remains same suggesting maternal liver adapts its size in harmony to the body weight. Maternal liver enlargement during pregnancy is a growth response induced by hepatocyte hyperplasia (Dai et al., 2011) and hypertrophy (Milona et al., 2010). This significant physiological change in the maternal liver is also accompanied by the change in its gene expression profile (Bustamante, Copple, Soares, & Dai, 2010). Of all the genes whose expression levels are increased, Ascl1, a transcription factor belonging to bHLH (basic helix-loop-helix) family, is the most up-regulated gene in the list.

1.2.4 bHLH

bHLH transcription factors are the essential cell intrinsic factors controlling multiple mechanisms during neurogenesis and gliogenesis (Bertrand, Castro, & Guillemot, 2002; Ross, Greenberg, & Stiles, 2003). The proneural bHLH factors are necessary and sufficient to initiate the neuronal lineage program in the ectoderm. During neural development the intricate plays between various bHLH transcription factors regulate cell-type commitment, determining the fate of the progenitors cells. Almost all the facets of neural development have been linked to members of the bHLH family. The bHLH transcription factors are defined by a shared structural motif: a basic region and two α-helices connected by a loop. The helical regions are required for dimerization with E-proteins such as Tcf4, E12, E47 or HEB. The basic domain is required for DNA binding to activate or repress downstream gene transcription (Bertrand et al., 2002; Murre et al., 1989). Members of this family bind to DNA either as heterodimer or homodimer. bHLH transcription factors are modified in many ways to confer specificity in DNA binding. This is brought about by central NN position and bases, which are present close to the E-box (Roztocil, Matter-Sadzinski, Gomez, Ballivet, & Matter, 1998). Another level of specificity is provided by the post-translational modification of these transcriptional factors. Some factors are known to auto regulate their own expression (Helms, Gowan, Abney, Savage, & Johnson, 2001). Neural bHLH transcription factors fall into multiple subclasses. Spatially, these bHLH factors are expressed in discrete regions of developing CNS in largely non-overlapping manner (Gowan et al., 2001; Helms et al., 2001). Each bHLH gene functions in distinct temporal and spatial manner

for timely and proper CNS and PNS development. bHLH transcription factors exert their functions in cell differentiation and subtype specification. They are proposed to promote neuronal differentiation program through two mechanisms. First, Ascl1 and Neurog1 have been suggested to couple neuronal differentiation to withdraw from the cell cycle. They do this most likely by activating the expression of p27 (Kip1), a cyclin dependent kinase inhibitor (Farah et al., 2000). Second, Ascl1 and Neurog1 can induce cascade of neuronal differentiation genes such as NeuroD and NeuroM (Fode et al., 2000). Proneural bHLH factors apart from promoting cell differentiation also inhibit glial fates (Cau, Casarosa, & Guillemont, 2002). Thus dual roles of proneural bHLH factors ensure the correct coordination of neurogenesis and gliogenesis without depleting progenitor pools. In case of drosophila, bHLH factors play important role in neural determination as well as subtype specification. But in vertebrates these functions are divided among the different members of the bHLH family. Most of the members of the bHLH family act via notch signaling pathway. Notch pathway aids the progenitor cell to choose a fate based on the presence and distribution of notch receptors (Iso T, Kedes L, Hamamori Y, 2003).

1.2.5 Ascl1

Genetic studies in drosophila discovered ‘proneural’ genes of the achaete-scute complex encoding bHLH transcription factors (Garcia-Bellido, 1979).. The first mammalian homolog of the achaete-scute complex gene was identified in 1990 (Johnson, Birren, & Anderson, 1990). This gene was named Mash1 (mammalian homolog of achaete-scute complex 1), but now is called Ascl1 (achaete-scute complex homolog 1).

Mouse studies have shown that Ascl1 is neuronal differentiation factor which influences neuronal subtype specificity along with the other bHLH members (H. Kim et al., 2010). Apart from neuronal lineages Ascl1 is also known to be present in the progenitors of oligodendrocyte (E. J. Kim, Leung, Reed, & Johnson, 2007) and the dentate gyrus of the forebrain. Forced expression of the Ascl1 alone is sufficient to trans-differentiate mouse fibroblasts to neuronal lineage (Vierbuchen et al., 2010).

Other vertebrate neural bHLH factors including Atoh1 (previously Math1), Neurog1/2 (Neurogenenin1/2), Ptf1a (pancreatic transcription factor 1a) and Olig1/2 were identified later (Ben-Arie et al., 1997; Glasgow, Henke, Macdonald, Wright, & Johnson, 2005; Lu et al., 2000; Ma, Kintner, & Anderson, 1996; Zhou, Wang, & Anderson, 2000). Ascl1 is required for specific subsets of neuron in dorsal spinal cord and mesencephalon, telencephalon, olfactory sensory epithelium, hindbrain, and neurons in the autonomic nervous system (Akagi et al., 2004; Blaugrund et al., 1996; Casarosa, Fode, & Guillemot, 1999; Cau, Casarosa, & Guillemot, 2002; Guillemot et al., 1993; Helms et al., 2005; Hirsch, Tiveron, Guillemot, Brunet, & Goridis, 1998; Miyoshi, Bessho, Yamada, & Kageyama, 2004; Pattyn et al., 2004). Ascl1 is also required for the subpopulations of oligodendrocyte development in telencephalon and spinal cord (Parras et al., 2002; Sugimori et al., 2007; Sugimori et al., 2008).

1.2.6 Lineage tracing

Lineage tracing is a modern genetic approach used to "mark" cells. The mark is transmitted from one generation to the next and all the progenies derived from the marked cell become labeled cells. Lineage tracing has been extensively used for identification of a founder cell, its location and differentiation status. Genetic lineage tracing being an *in vivo* technique enables us to get insight into the behavior of a cell in the intact tissue or organism unlike *in vitro* techniques where the cells are examined in isolation in petridish (Kretzschmar & Watt, 2012). Cre-recombinase is expressed spatially and temporally in cell or tissue specific manner under specific promoter. Cre-recombinase under the control of cell or tissue specific promoter is crossed with a reporter mouse line containing loxP-STOP-loxP sequence. Cre-recombinase excises STOP signal and activates expression of a reporter gene, thus permanently marking the cell and all the progenies derived from marked cell. Two most widely used recombinase systems in combination with different conditional reporter genes are bacteriophage (Cre-LoxP) and *saccharomyces cerevisiae* (FLP-FRT).

The Cre-recombinase activity can be controlled spatially or temporally by inducible recombination. One example is that Cre-recombinase is fused with estrogen receptor to create Cre recombinase –ER fusion protein (CreER). In the absence of estrogen, a natural occurring ligand for the estrogen receptor, or its analog (e.g. tamoxifen), CreER is bound to heat shock proteins and kept in the cytoplasm. In the presence of a ligand, heat shockproteins bound to the estrogen receptor are released and Cre-recombinase estrogen receptor fusion protein diffuses to nucleus where it recombines

with loxP sites (Metzger et al., 1995). To prevent the activation of CreER fusion protein from endogenous estrogen, the native estrogen receptor has been mutated to generate Cre-ERT2 fusion protein which responds only to tamoxifen (Feil et al., 1997). The modified fusion protein is also more responsive to tamoxifen compared to endogenous 17 β-oestradiol, thus requiring lower dosage of analog (Kretzschmar & Watt, 2012). The adverse effect of tamoxifen administration to pregnant mice can be sometimes counteracted by progesterone co-administration.

Of many reporter constructs, β-galactosidase is among the first to be used. β-galactosidase is expressed under the control of ubiquitous promoter, ROSA26. In the ROSA26 lacZ reporter (ROSA26-LacZ) mouse, STOP cassette prevents expression of β-galactosidase. Cre-recombinase recombines with the loxP sites and excises the STOP cassette enabling expression from lacZ reporter, thus permanently marking the cell. All the cells derived from this parental cell are also marked by β-gal expression which can be detected using X-gal. X-gal, an analog of lactose, is hydrolyzed by the β-galactosidase yielding galactose and reduced indole compound. Reduced indole is spontaneously dimerized and oxidized to give an insoluble blue color. β-galactosidase can also be detected by traditional immunohistochemistry by using antibody raised against β-galactosidase.

MATERIALS AND METHODS

2.1 Quantitative realtime polymerase chain reaction

Total RNA was isolated from frozen liver tissue using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbard, CA, USA). The cDNAs were synthesized with total RNA (1 μg) from each sample, diluted 10 times with water and subjected to realtime reverse transcriptase polymerase chain reaction (qRT-PCR) to quantify mRNA levels using TaqMan probe (Applied Biosystems, MA, USA). Primers and probes were designed using Primer Express 2.0 (Applied Biosystems, MA, USA). The probe was labeled with a reporter dye FAM (6-carborxyfluorescein). TaqMan Universal PCR Master Mix (Applied Biosystems, MA, USA) was used to prepare the PCR incubations. Primers and probes were added at a final concentration of 90 and 125 nmol, respectively, in a total volume of 20 μL. The amplification reactions were carried out in the ABI Prism 7900 sequence detection system (Applied Biosystems, MA, USA) with initial hold steps (50^0C for 2 min, followed by 95^0C for 10 min) and 40 cycles of a two-step PCR (92^0C for 15 s, 60^0C for 1 min). The primer (Integrated DNA Technologies, Coralvillem, IA, USA) and probe (Sigma, St Louis, MO, USA) sequences used are listed in Table 1. The comparative cyclic threshold (CT) method was used for relative quantification of the amount of mRNA for each sample normalized to 18s RNA.

2.2 Northern analysis

RNA was extracted using TRIzol reagent from snap frozen tissues (Chomczynski & Sacchi, 1987)(Invitrogen, NY, USA). For filter hybridizations, 20 μg total RNA/lane were separated on 1% formaldehyde-agarose gels, transferred to nylon membranes (Schleicher and Schuell, Keene, NH), and exposed to UV light to cross-link the RNA to the membranes. Radiolabeled probes were synthesized by random primer extension of cDNA inserts that had been separated from the plasmid vector by restriction endonuclease digestion and agarose gel electrophoresis. Radiolabeled products were purified away from unincorporated nucleotides using MicroSpin S-300 HR columns (Pharmacia, Piscataway, NJ). Hybridization was carried out for 12–16 h at 42 C in 50% formamide, 5 × SSC (standard saline citrate), 0.5% SDS, and 5 × Denhardt's solution (1× is 0.02% each BSA, Ficoll, and polyvinylpyrrolidone). After hybridization, membranes were rinsed in 2 × SSC once for 10 min and washed twice in 0.1 × SSC and 0.1% SDS at 50^0C. Blots were probed with ^{32}P-labelled cDNA for Ascl1. G3PDH cDNA was used as an internal control to ensure equal loading and RNA integrity (Lin, Poole, & Linzer, 1997).

2.3 Immunohistochemistry

Formalin-fixed and paraffin-embedded liver sections were sectioned at pathology core. Sections were deparaffinized in tissue clearing agent (Thermo Scientific, MA, USA) for 5 minutes and then passed through decreasing concentration of ethanol (100, 95, 85 and 70%) for 5 minutes each. For epitope retrieval, slides were placed in boiling

citrate buffer (10 mM, pH 6.0) for 10 minutes then were allowed to cool down for 3 hours at room temperature. From this point onwards sections were always kept moist. Sections were blocked using normal serum of the animal in which the secondary antibody was raised for 1 hour at room temperature in humidified chamber. After blocking, sections were incubated with primary antibody diluted in DPBS (dulbecco's phosphate buffered saline) with Ca^{+2} and Mg^{+2} for either 1 hour at room temperature or overnight at 4^0C. Sections were incubated with respective secondary antibodies for 1 hour at room temperature and then developed using DAB (Vector laboratory, CA, USA). The incubation time of DAB was determined empirically depending upon the staining and background. Sections were counterstained with hematoxylin, passed through increasing concentration of ethanol and then lastly mounted with Vector Labs vectamount. Primary antibodies used are listed in Table 2.

2.4 Vapor fixation

To visualize the endogenous fluorescence signal from Ascl1-CreERT2/tDTomato and Ascl1-GFP mice vapor fixation method was used (Jockusch, Voigt, & Eberhard, 2003). 10 μM thick frozen sections were generated. Humidified chamber containing 37% paraformaldehyde was setup with a raised stage to keep slides inverted and exposed to the vapor of the fixative. The chamber was kept at -20^0C and incubated for least 2 hours. After incubation slides were washed in PBS three times for 5 minutes each, mounted with mounting medium containing DAPI and examined under confocal microscope (Olympus FluoView FV1000).

2.5 LacZ staining

Mice were sacrificed by cervical dislocation. Freshly isolated tissue was kept in LacZ fixative solution (2.5% glutraldehyde in 1XPBS/$MgCl_2$) for 4 hours at 4^0C. After fixation tissue was washed in 1X PBS three times for five minutes each. Tissue was incubated in 15% sucrose and incubated for 4 hours at 4^0C. Further, the tissue was incubated overnight in 30% sucrose at 4^0C. Next morning the tissue was frozen in OCT (optimum cutting temperature) over heptane and dry ice and the blocks were stored in -80^0C. Left over tissue was snap frozen in liquid nitrogen and stored at -80^0C. As needed, 8-10 μM frozen tissue sections were cut using cryostat (Leica 3050S, Wetzlar, Germany). For staining, sections were taken out from -80^0C and dried at room temperature for 30 minutes. PAP pen was used to create boundary around the tissue section. 200 μL of freshly prepared LacZ staining solution (Table 3) was added to each slide. The slides were kept in humidified chamber and incubated in cell culture incubator for 2 hours. Slides were washed in 1X PBS three times for 5 minutes each. Sections were counterstained with eosin and mounted with vectamount (Vector Labs, CA, USA).

2.6 Mouse Models

2.6.1 Animal care

Animals were provided food and water *ad libitum*. Animals were placed in a 12-h light and 12-h dark cycle and the temperature and relative humidity were maintained between 20^0C and 24^0C and 40-60% respectively. All the animal experiments were

conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Protocol for the care and use of animals was approved by the Indiana University Purdue-University Indianapolis Animal Care and Use Committee.

2.6.2 Ascl1-GFP mice

Ascl1^{tm1Reed}/J mice were obtained from the Jackson laboratory (stock #012881). In this mouse strain the entire coding region of one allele of the endogenous Ascl1 gene is replaced with green fluorescent protein (GFP) gene, abolishing gene function in one allele of Ascl1 (Leung, Coulombe, & Reed, 2007) (Figure 1). GFP, driven by the Ascl1 promoter sequence, is expressed in all Ascl1-expressing neural progenitor cells in the embryo and the adult brain, including subsets of neurons throughout the central nervous system (CNS) and the peripheral nervous system (PNS), and in neuroendocrine cells in lung and kidney. Homozygotes die within hours of birth due to CNS and PNS disruptions during neural development. Mice heterozygous for the targeted mutation are viable, fertile, and normal in size. These mice are used to study the lineage of distinct Ascl1-expresing cell populations, neuronal turnover, and neuronal replacement upon traumatic injury (E. J. Kim et al., 2007). The mice were genotyped as per the directions from the vendor.

2.6.3 Ascl1-CreERT2/ROSA-LacZ mice

B6.129S4-Gt(ROSA)26Sor $^{tm1Sor/J}$ mice (stock # 012882) were obtained from The Jackson Laboratories (Maine ,USA). The mice were genotyped as per the directions

from the vendor. In Ascl1-CreERT2 mouse, the entire coding region of one allele of the endogenous Ascl1 gene is replaced by a cassette encoding Cre-recombinase and modified estrogen receptor fusion protein (CreERT2). Another mouse line, B6.129S4-Gt(ROSA)26Sor $^{tm1Sor/J}$ mice (stock number 003474) were obtained from The Jackson Laboratories (Maine ,USA). These mice have LoxP-flanked DNA STOP sequence preventing expression of the downstream LacZ gene. Ascl1-CreERT2 mice were crossed with ROSA26-LacZ mice to generate Ascl1-CreERT2/ROSA26-LacZ mice. Cre-recombinase recombines with the LoxP sites and removes the STOP sequence. This results in expression of LacZ thus permanently labeling the cells (Figure 2).

2.7 Timed pregnancy

Breeding cages were setup in late evening. Next day early morning, presence of vaginal secretion (plug) was determined by visual examination of vagina of the female mice (Champlin, Dorr, & Gates, 1973). Presence of plug in the vaginal smear of the mice was considered to the gestational day 1. Plug-positive female mice were housed separately.

2.8 Tissue collection

Animals were sacrificed by cervical dislocation. Body weight, liver weight and number of fetuses were recorded. For formalin-fixed and paraffin-embedded sections, the tissues after dissection were kept in formalin overnight and sent to Indiana University

pathology core for processing. For frozen sections, the tissues were frozen in OCT. The tissue with OCT was allowed to freeze in heptane over dry ice.

2.9 Subcloning

For *in-vitro* studies, the human Ascl1 cDNA was cloned into viral transfer vector pLVX-CMV-IRES-ZsGreen (Figure 9) and pLVX-EF1α-IRES-ZsGreen (Figure 12). The human Ascl1 cDNA was cut out from source vector using restriction enzymes. Target vectors were also cut using same respective restriction enzymes. The digested products were cleaned using the PCR cleaning kit (Promega, Madison, Wisconsin). The digested human Ascl1 insert and digested vector were ligated using T4 ligase (Invitrogen, MA, USA) for 1 hour at room temperature. The ligated product was chemically transformed into SURE 2 competent cells (Agilent technologies, CA, USA). Briefly, the bacterial cells were thawed on ice, 2 μL of plasmid was added and the mix was incubated for 30 minutes on ice. Heat shock was given at 42^0C for 30 seconds and again incubated on ice for 2 minutes. 900 μL of Luria Broth (LB) was added and incubated at 37^0C and 200 rpm for 1 hour. After incubation, the cells were plated on LB plates containing ampicillin as resistant marker. Next morning the plates were checked for transformed colonies. The colonies were randomly picked to check for the clones containing the insert. Mini prep, using Qiagen kit, was performed to isolate the DNA from transformed colonies as per vendor protocol. The DNA was digested with cloning restriction enzymes and the positive clones were sent for sequence verification.

2.10 Generation of lentivirus

HEK 293T cells, host to generate lenti virus, were trypsinized and re-suspended in DMEM with 10% FBS. The cells were counted using hematocytometer and seeded at a density of $5X10^6$ cells in 10 mL of media in 10 cm tissue culture plate. Next morning the media was changed to get rid of unattached cells. Cocktail of transfer vector containing gene of interest (9.0 μg), psPAX2 packaging vector (6.0 μg) and pMD2G envelope vector (3.0 μg) were added in 450 μL of molecular biology grade water in 1.5 mL eppendorf tube. To the previously made cocktail of constructs, 50 μL of 2.5 M $CaCl_2$ was added and mixed. The $CaCl_2$ /DNA mix was added drop by drop into 500 μL of BES (N,N-Bis 2-hydroxyethy-2-aminoethanesulfonic acid) on a vortex and then incubated in dark for 30 minutes for the formation of DNA nanoparticles. After incubation, $CaCl_2$/DNA/BES cocktail was added gently drop by drop to the tissue culture dish containing plated 293T cells. The plate was incubated in CO_2 incubator for 10 hours, and then media was replaced with fresh growth media and further incubated for 48 hours. If the transfer vector has green fluorescent signal then it can be used to check transfection efficiency. After 48 hours, media containing lenti-viruses was collected and centrifuged at 2500*g* for 10 minutes at 4^0C to remove dead cell debris. The supernatant was passed through 0.45 μM low protein binding filter to get rid of cell debris. If high titer of the virus desired, then the collected supernatant can be concentrated. For concentrating virus, 1mL of 50% PEG8000 and 600 μL of 5 M NaCl were added for every 10 mL of collected supernatant. The tube was incubated on a rotating wheel for overnight at 4^0C. After overnight incubation, tube was centrifuged at 4000*g* for 30 min at 4^0C. Supernatant was

discarded without disturbing the virus-PEG pellet. For getting 10X concentration of the virus, 100 μL of PBS was added and the pellet was gently mixed by pipetting. To determine the approximate virus titer, 20 μL of virus was added to Lenti-X-Go stik (Clontech, California) followed by 4 drops of chase buffer and incubated at room temperature for 5 minutes. A red band on the Lenti-X-Go stik of same intensity to that of control indicated the presence of at least 5 X 10^5 IFU/mL. Small aliquots (50 μL) of virus was stored at -80^0C for long term storage. Freeze thaw cycle of the stored virus was avoided as it greatly reduces the transduction efficiency.

RESULTS

3.1 The mRNA expression of Ascl1 in maternal liver during pregnancy

Previous gene expression profile analysis by microarray approach revealed that Ascl1 is the most up-regulated gene in the maternal liver during pregnancy in comparison with a non-pregnant state. To validate the microarray data, mRNA expression of maternal hepatic Ascl1 was quantified at various stages of gestation. Total RNA was extracted from the livers collected from non-pregnant, pregnant (gestation days 4, 8, 10, 11, 13, 15, and 18), and day 10 post-partum mice. Relative quantitative RT-PCR was performed. The result showed that Ascl1 mRNA level was increased exponentially in the maternal liver throughout the second half of pregnancy (Figure 3), in parallel with marked growth of maternal liver during the same period (Bustamante et al., 2010; Dai et al., 2011). The pregnancy-dependent up-regulation in the expression of maternal hepatic Ascl1 gene initiated at mid-gestation (gestation day 10) and peaked during the second half of pregnancy (gestation day 15). The level of maternal hepatic Ascl1 transcript was up to 2,500-fold higher than that in non-pregnant state. Following parturition (post-partum day 10), when the maternal liver regressed to its pre-pregnancy size, hepatic Ascl1 mRNA expression returned to the base level as in non-pregnant animals. To further validate and visualize the striking finding, northern blot analysis was performed using ^{32}P-labeled

Ascl1 cDNA probe and the same set of total RNA samples. In line with the RT-PCR data, Ascl1 mRNA was undetectable in non-pregnant and post-partum livers but was abundantly expressed in the maternal liver from gestation day 11 to 18 (Figure 4). Taken together, we found that pregnancy activates the transcription of a neural gene Ascl1 in the maternal liver in a magnitude that has never been seen in literature to our knowledge for a transcription factor.

3.2 Identification of Ascl1-expressing cells in maternal liver during pregnancy

To determine which cell population in the maternal liver expresses the Ascl1 gene, we performed immunohistochemistry assessments on non-pregnant and pregnant liver sections using eight Ascl1 antibodies commercially available. Unfortunately, no positive staining was detected. Thus, we used Ascl1-GFP mice to identify the Ascl1-expressing cell population instead. In the mouse model, one allele of Ascl1 gene is replaced by GFP coding sequence with preservation of intact Ascl1 regulatory region. Hence, GFP-expressing cells represent Ascl1-expressing cells. Livers were harvested from non-pregnant and gestation day 11 mice. Frozen liver sections were prepared and examined for GFP signal with confocal microscope. Unexpectedly, the accumulation of lipid droplets in hepatic stellate cells was dramatically increased by pregnancy and emitted strong autofluorescence, which adversely interfered with the analysis. Alternatively, we used GFP antibody to achieve our goal immunohistochemically. As shown in Figure 5, GFP-positive cells were seen in pregnant but not non-pregnant livers. Clearly, Ascl1-expressing cells are (1) non-parenchymal cells; (2) very small in size; (3)

adjacent to hepatocytes; and (4) gestation-dependent. The expansion of this cell population correlates with the activation of Ascl1 gene transcription in the maternal liver during the course of gestation.

3.3 Lineage tracing of Ascl1-expressing cells in maternal liver during pregnancy

It has been well known that Ascl1 is expressed in neural progenitor cells which can differentiate into several neural cell types in dorsal spinal cord, telencephalaon, mesencephalon, hind brain and neuron in autonomic nervous system (Blaugrund et al., 1996; Cau et al., 2002; Guillemot et al., 1993; Helms et al., 2005; Hirsch et al., 1998). Thus we hypothesize that hepatic Ascl1-expressing cells represent hepatic progenitor cells that can eventually differentiate into certain liver cells. To test this, we took an *in vivo* tracing approach and used the following two reporter mouse lines.

Our collaborator Dr. Jane Johnson at Southwestern University generated Ascl1-Cre ERT2/ROSA26-tDTomato mouse model and successfully traced the lineage of Ascl1-expressing cells in nervous system (E. J. Kim et al., 2007). Ascl1-CreERT2/ROSA26-tDTomato line was generated by crossing Ascl1-CreERT2 mice to reporter mice containing tDTomato (red florescent protein) cassette preceded by a stop signal flanked by two LoxP sites. Thus, Ascl1-expressing cells can be permanently labeled no matter what cell types they eventually differentiate into. By using this model, we can determine the differentiation lineage of the cells that are expressing Ascl1 when tamoxifen is given. Dr. Johnson's group performed animal treatments and delivered the liver samples to us for analysis. There were three treatment groups including (1) vehicle

treatment at gestation day 10; (2) tamoxifen treatment at gestation day 10, a day before Ascl1 expression peaks; and (3) tamoxifen treatment in non-pregnant state. After treatments, livers were collected from pregnant reporter animals prior to parturition (gestation day 18) or non-pregnant control animals. Frozen liver sections were prepared and subjected to red florescence imaging using confocal microscope. The labeled cells were not seen in liver tissue from vehicle-treated pregnant reporter mice (data not shown), indicating that pregnancy does not activate the Cre in the maternal liver. Similarly, tamoxifen treatment of non-pregnant mice did not label any cells (data not shown). In sharp contrast, numerous labeled cells were observed throughout the liver sections prepared from tamoxifen-treated pregnant reporter mice (Figure 6). Randomly distributed groups of cells were detected upon analysis. We further investigated these groups of cells using an antibody specific for RFP (Figure 7). Labeled cells in the middle of the clusters exhibited strongest RFP expression, while the surrounding cells expressed reduced level of RFP. Morphologically, the labeled cells are hepatocyte-like cells.

To further investigate this phenomenon, a new model, CreERT2/ROSA26-LacZ, was generated. The information regarding this mouse model is provided in the section of Methods (page 22). Tamoxifen was administered on gestation day 10 and day 11. Because Ascl1 expression reaches a peak in the maternal liver on gestation day 11 and the effect of tamoxifen can last for 24 hours, this regimen allows for potentially maximal labeling of Ascl1- expressing cells with minimal tamoxifen administration. Based on the experience of the Dr. Johnson laboratory, repeated administration of the drug causes miscarriage in pregnant mice. Non-pregnant mice receiving tamoxifen were used as control. Maternal livers were collected on gestation day 18, whereas the livers were

isolated from control mice at the corresponding day. Frozen liver sections were prepared for subsequent LacZ staining (Figure 8). As anticipated, we did not see any LacZ-positive cells in control livers. Similar to what we saw in Ascl1-CreERT2/ROSA26-tDTomato pregnant mice, numerous labeled hepatocyte-like cells were randomly distributed in liver parenchyma in the maternal liver in the newly-generated mouse line.

Collectively, by using these two *in vivo* tracing mouse models, we demonstrate that the small Ascl1-expressing cells expanded on gestation day 11 differentiate into hepatocyte-like cells by the end of pregnancy.

3.4 Production of lentivirus expressing Ascl1.

To generate essential tools for future mechanistic studies, two lentivirus vectors expressing Ascl1 were generated.

The first is pLVX-CMV-IRES-ZsGreen-hAscl1. Human Ascl1 cDNA was cloned into pLVX-CMV-IRES-ZsGreen lentiviral vector (Clonetech, CA, USA). Lenti-viruses has advantage over other transfection method in transfecting both dividing and non-dividing cells (Naldini et al., 1996). The presence of green fluorescent gene, ZsGreen, helps monitor the transfection as well as transduction efficiency. Presence of internal ribosome entry site (IRES) allows for simultaneous expression of two proteins separately form the same RNA transcript. The human Ascl1 cDNA was cut out from pTight-N174 vector (Addgene) and then inserted into the multiple cloning site of pLVX-CMV-IRES-ZsGreen (Figure 9), using restriction enzymes XhoI and SpeI. Ligated vector and insert was transformed into competent cells and screened for positive clones (Figure 10). The

selected positive clone was digested using a set of restriction enzymes to confirm the presence of the cloned insert (Figure 11). Further, sequencing primers were designed (Table 5) and DNA sequencing was performed for verification of the insert. The sequencing result was aligned with the expected sequence of the vector and the insert and no sequence errors were found. This vector was named pLVX-CMV-IRES-ZsGreen-hAscl1.

EF1α based viral vector is known to be more efficient in transfecting stem cells and stem cell-like cells over CMV based vectors. Thus, human Ascl1 cDNA was also cloned into another viral transfer vector, pLVX-EF1α-IRES-ZsGreen (Figure 12). The human Ascl1 cDNA was cut from PCMV6-XL5 (Sigma) using restriction enzyme EcoRI and XbaI (Figure 13). The empty vector, pLVX-EF1α-IRES-ZsGreen, digested with EcoRI and XbaI was ligated and screened for positive clones (Figure 14). Diagnostic restriction digestion was performed to confirm the presence of human Ascl1 insert (Figure 15). Further, DNA sequencing analysis confirmed the insert using primers listed in Table 5. Both constructs were used for production of lentivirus as described in the Method section (page 25). The resulted lentiviral particles were later used to transduce HEK293T cells to access their efficiency (Figure 16). Strong GFP signal was observed in almost every cell, indicating high transduction efficiency and Ascl1 expression.

DISCUSSION

We demonstrate here that Ascl1 mRNA expression is markedly up-regulated in the maternal liver in a physiological condition (pregnancy). It is well established that this gene is abundantly expressed in nervous system during development (E. J. Kim et al., 2007; H. Kim et al., 2010). It has also been reported that Ascl1 is expressed in small-cell lung cancer cells, astrocytoma (Somasundaram et al., 2005) and lymphoma (Westerman et al., 2002). Our data revealed, for the first time, the striking activation of Ascl1 gene in the maternal liver during the second half of pregnancy. Importantly, the activation of maternal hepatic Ascl1 is accompanied by robust growth of the maternal liver during the same period (Bustamante et al., 2010; Dai et al., 2011). This observation strongly suggests a vital role of Ascl1 in regulating maternal hepatic growth response to pregnancy. However, a significant flaw of the current study is that we were not able to determine the Ascl1 expression at protein level by western blotting and immunohistochemistry in maternal liver due to the lack of specificity of Ascl1 antibodies in liver tissue. Future work is required to find specific Ascl1 antibodies and alternatively to use mass spectrophotometry approach to determine the presence of Ascl1 protein in maternal liver during pregnancy. Our study has identified the cell population expressing Ascl1 in the maternal liver during pregnancy. Those small Ascl1-expressing cells are drastically expanded and subsequently undergo differentiation giving rise to large

hepatocyte-like cells (Figure 8). At this juncture, we believe that we have potentially found a novel subset of hepatic progenitor cells associated with maternal hepatic adaptations to pregnancy. It has been debating regarding the nature and resource of liver stem/progenitor cells (Michalopoulos, 2007; Riehle et al., 2011). Oval cells, which reside in Canals of Hering of bile duct, have been regarded as the liver stem cell population capable of self-renewal and repopulating the liver (Michalopoulos, 2007). Interestingly, evidence supports the notion that liver stem/progenitor cells originate from the bone marrow (Pertersen, BE et al., 1999). Several other studies indicate that hepatic stellate cells (Asahina, 2012) and hepatocyte-like small cells (Best & Coleman, 2010; Chen et al., 2013; Gynecol et al., 2000) possess the property of progenitor cells. Our *in vivo* lineage tracing study convincingly demonstrates the property of Ascl1-expressing cells as a hepatic stem/progenitor cell population. However, further characterization and identification of Ascl1-expressing cells are needed to firmly establish that this cell population represents a novel group of liver stem/progenitor cells. Future work includes finding other stem/progenitor marker genes expressed by hepatic Ascl1-expressing cells and isolating the cells to test their capability in self-renewal and differentiation.

The current studies have provided a critical clue to determine the gestation-dependent fate of Ascl1-expressing cells in the maternal liver. The hepatocyte-like cells derived from the Ascl1-expressing cells most likely include hepatoblast cells and hepatocytes. The labeled cells in the middle of clusters could be hepatoblasts because of their morphology and high level expression of reporter. The hepatoblasts may give rise to mature hepatocytes that surround the clusters and exhibit lower reporter signal due to their strong metabolic activity. We need to perform more studies to determine whether

they are the two cell types. Hepatoblast markers including CD133, EpCAM, and α-fetal protein can be used for co-immunostaining to tell whether labeled cells contain hepatoblast cells. If labeled cells express mature hepatocyte hallmarks including albumin and hepatocyte nuclear factors without expressing hepatoblast makers, then we can determine that labeled cells are mature hepatocytes. At current stage, we hypothesize that Ascl1-expressing cells are able to differentiate following hepatocyte lineage in the maternal liver in pregnant animals. We predict that, if this hypothesis is tested to be true, we will reveal a novel cellular mechanism driving maternal liver growth in response to the increasing metabolic demand of pregnancy.

In summary, we have demonstrated the activation of Ascl1 gene, the existence of Ascl1-expressing cells, and potential differentiation lineage of the cells in the maternal liver during the course of gestation. Further investigations need to be performed to characterize the nature of the cells expressing Ascl1 and evaluate the role of Ascl1 in the pregnancy-induced maternal liver growth. The current and future studies will further our understanding of liver physiology during pregnancy and pregnancy-associated liver diseases.

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TABLES

Table 1: Primers for real-time polymerase chain reaction

Gene	Symbol	GenBank	Primer	Sequence (5' to 3')
Achaete-scute complex homolog-like 1	*Ascl1*	NM_022384	Forward	TCA GCG CCC AAG CAA GTC
			Reverse	CGT TTG CAG CGC ATC AGT

Table 2: Antibodies

Antibody	Vendor	Catalogue no.	Dilution
Anti-RFP Biotin	Abcam	Ab34771	1:200
Anti-GFP HRP	Invitrogen	A10260	1:200

Table 3: LacZ staining solution

Volume	**10 ml**
0.6 M Potassium Ferricyanide	100 μL
0.6 M Potassium Ferrocyanide	100 μL
1 M $MgCl_2$	20 μL
10% Igepal	20 μL
10% Na deoxycholate	10 μL
X-Gal Solution (40 mg/ml)	250 μL
10X PBS	1 ml
H_20	8.5 ml

Table 4: Sequencing primers

pLVX- CMV-IRES-zSGreen-hAscl1	
FP_Seq_hAscl1_166	5’ GCCAAGATGGAGAGCGGCGG 3'
FP_Seq_hAscl1_167	5' GCGCAACCGCGTCAAGTTGG 3'
RP_Seq_hAscl1_168	5' GCTGACTTGTGACCGCCCCC 3'
pLVX-EF1α-IRES-zSGreen-hAscl1	
FP_Seq_hAscl1_169	5' TCTCAAGCCTCAGACAGTGGT 3'
FP_Seq_hAscl1_170	5' GCCAAGATGGAGAGCGGCGG 3'
RP_Seq_hAscl1_171	5'- TTCCAAGCGGCTTCGGCCAG -3'

FIGURES

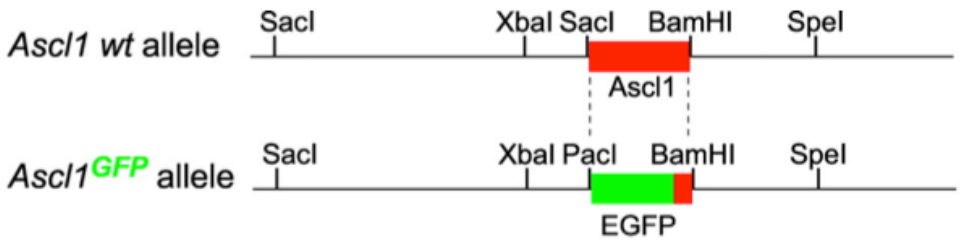

Figure 1. Ascl1-GFP knock-in mice. The coding sequence of one allele of Ascl1 is replaced by nuclear-localized GFP reporter leaving the regulatory region intact. GFP is driven by Ascl1 promoter. GFP-expressing cells thus represent Ascl1-expressing cells.

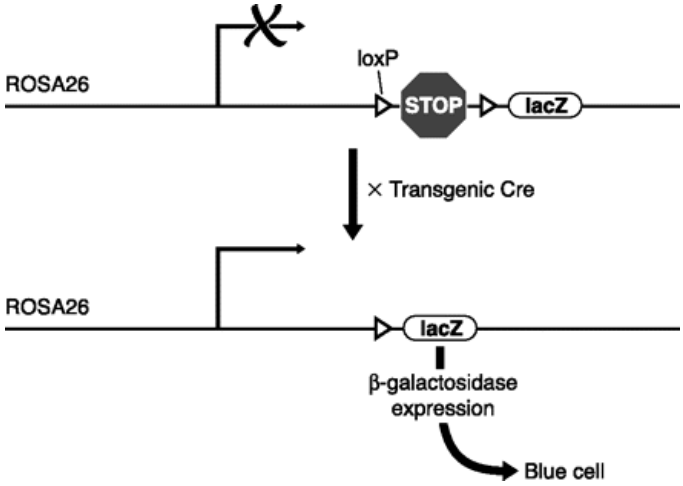

Figure 2. Ascl1-CreERT2/ROSA26-lacZ mice. Ascl1-CreERT2 mice were crossed with ROSA26-LacZ reporter to generate bi-transgenic mice, Ascl1-CreERT2/ROSA26-lacZ. Upon tamoxifen treatment in these mice, Cre-recombinase recombines with loxP sites and the STOP sequence is removed thus resulting in expression of β-galactosidase. β-galactosidase is detected using X-gal giving an insoluble blue color.

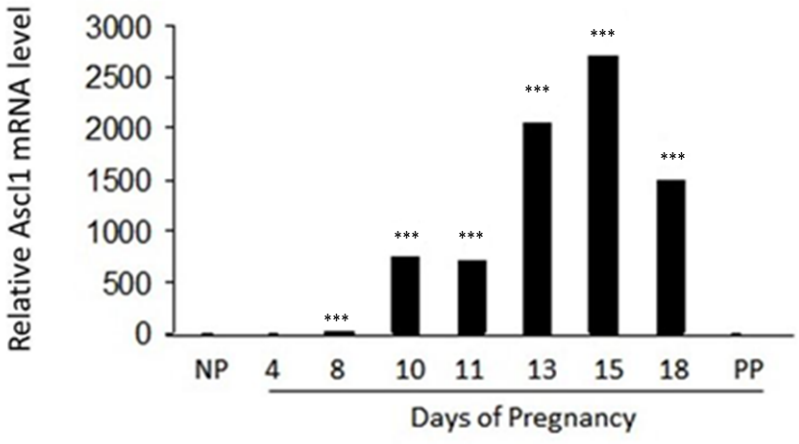

Figure 3. Ascl1 expression in maternal liver during pregnancy (RT-PCR). Total mRNAs were extracted from the livers of non-pregnant (NP), pregnant (gestation days 4, 8, 10, 11, 13, 15, and 18), and post-partum (PP) mice. The mRNA levels of hepatic Ascl1 were quantified by qRT-PCR and are presented as means of fold changes relative to NP control. ***, $P < 0.001$.

(Dr. Dai's unpublished work)

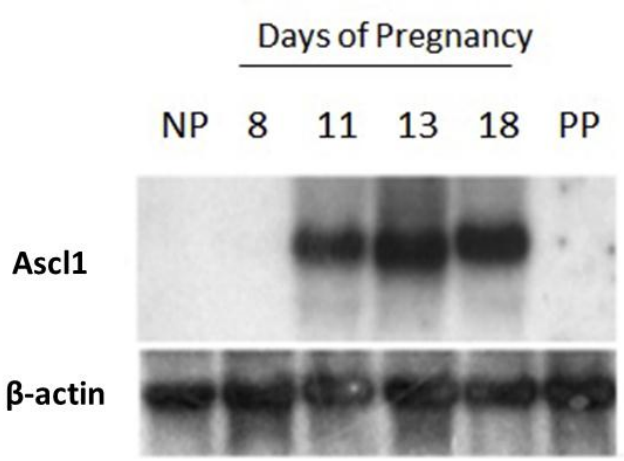

Figure 4. Ascl1 expression in maternal liver during pregnancy (northern blot). Total mRNAs were extracted from the livers of non-pregnant (NP), pregnant (gestation days 8, 11, 13, and 18), and post-partum (PP) mice. Northern blotting was performed with ^{32}P-labeled Ascl1 probe. β-actin mRNA expression was used as loading control.

(Dr. Dai's unpublished work)

A B C

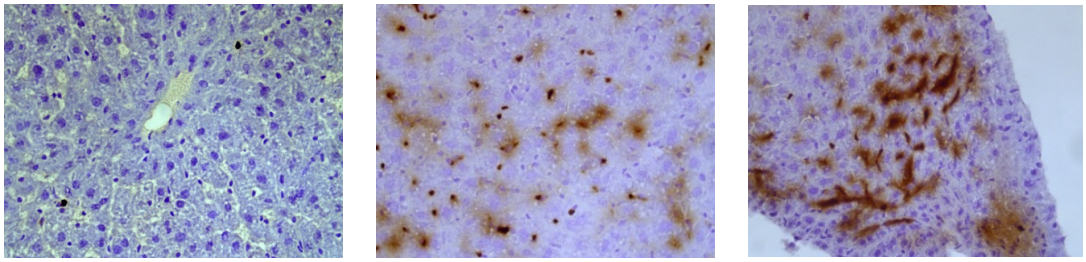

Figure 5. **Identification of Ascl1-expressing cells in the maternal liver during pregnancy.** Livers were collected from non-pregnant (A) and gestation day 11 (B & C) Ascl1-GFP mice. Immuno-histochemistry was performed using anti-GFP antibody. Ascl1-expressing cells were stained dark brown.

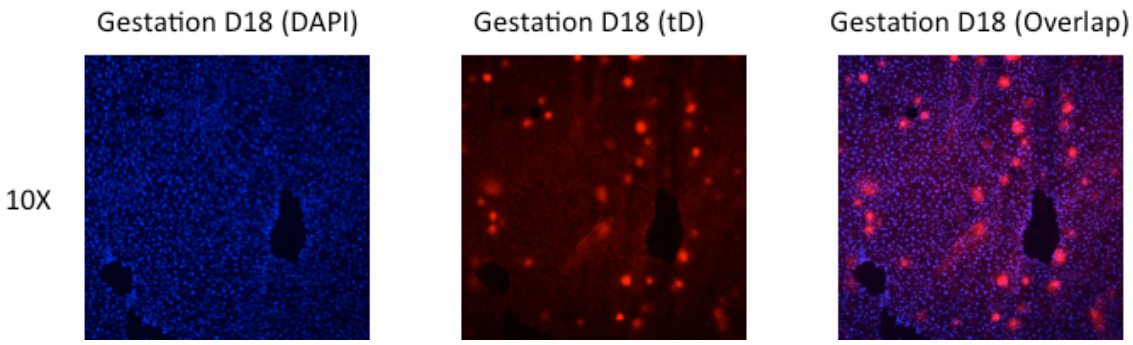

Figure 6. Lineage tracing of Ascl1-expressing cells in maternal liver in pregnant Ascl1-CreERT2/ROSA-tDTomato mice. Tamoxifen was administered (i.p.) to gestation day 10 Ascl1-CreERT2/ROSA26-tDtomato mice. Eight days after the treatment, livers were collected. Red fluorescence was assessed by confocal microscopy in the pregnant liver.

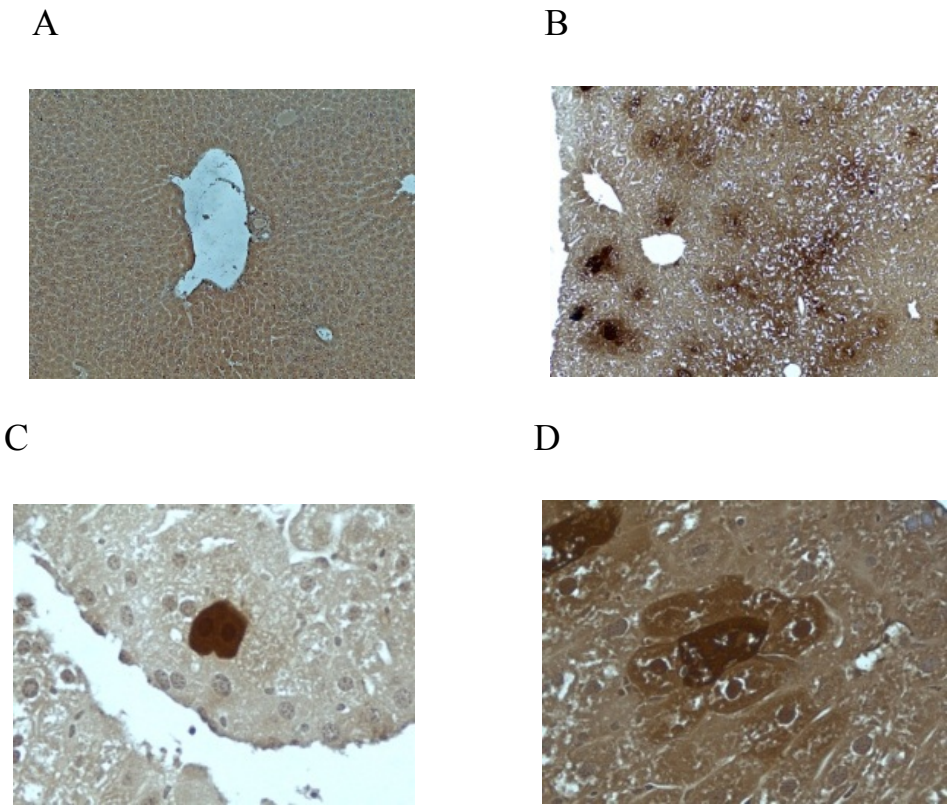

Figure 7: Lineage tracing of Ascl1-expressing cells in maternal liver in pregnant Ascl1-CreERT2/ROSA-tDTomato mice (IHC) Tamoxifen was administered (i.p.) to gestation day 10 Ascl1-CreERT2/ ROSA26-tDtomato mice and non-pregnant control mice. Eight days after the treatment, livers were collected. Immunohistochemistry was performed using anti-red fluorescence protein antibody on liver sections from non-pregnant (A) and pregnant (B, C, & D) mice.

A B

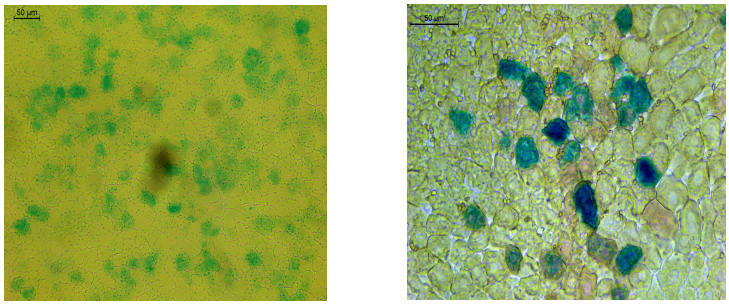

Figure 8. Lineage tracing of Ascl1-expressing cells in maternal liver in pregnant Ascl1-CreERT2/ROSA26-lacZ mice. Tamoxifen was administered (i.p.) on gestation day 10 and day 11 in Ascl1-CreERT2/ ROSA26-LacZ mice. Eight days after the treatment, livers were collected, stained with LacZ staining solution and visualized under 10X (A) and 40X (B) magnification.

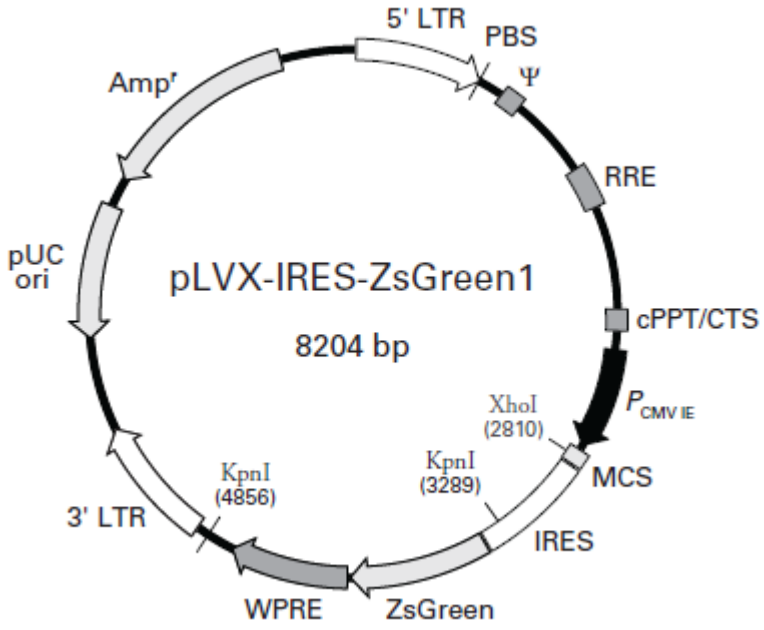

Figure 9. pLVX-CMV-IRES-ZsGreen1 vector map. Backbone with CMV promoter was used to insert hAscl1 cDNA in multiple cloning site using restriction enzymes XhoI and SpeI.

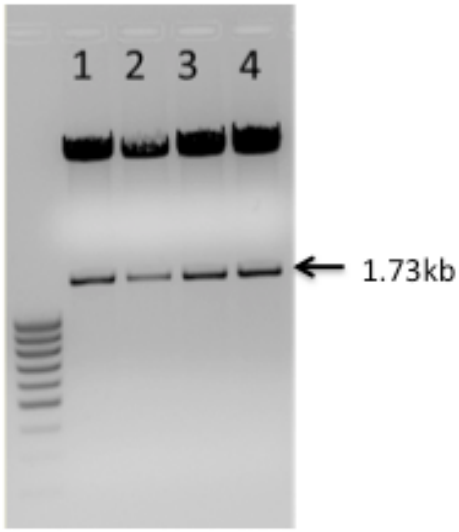

Figure 10. pLVX-CMV-IRES-ZsGreen1 positive colony screening. Digested vector and insert were ligated, transformed into competent cells and screened for positive colonies. Four clones (Lane 1, 2, 3, and 4) were digested with cloning restriction XhoI and SpeI. All the tested clones have hAscl1 insert (1.73 kb).

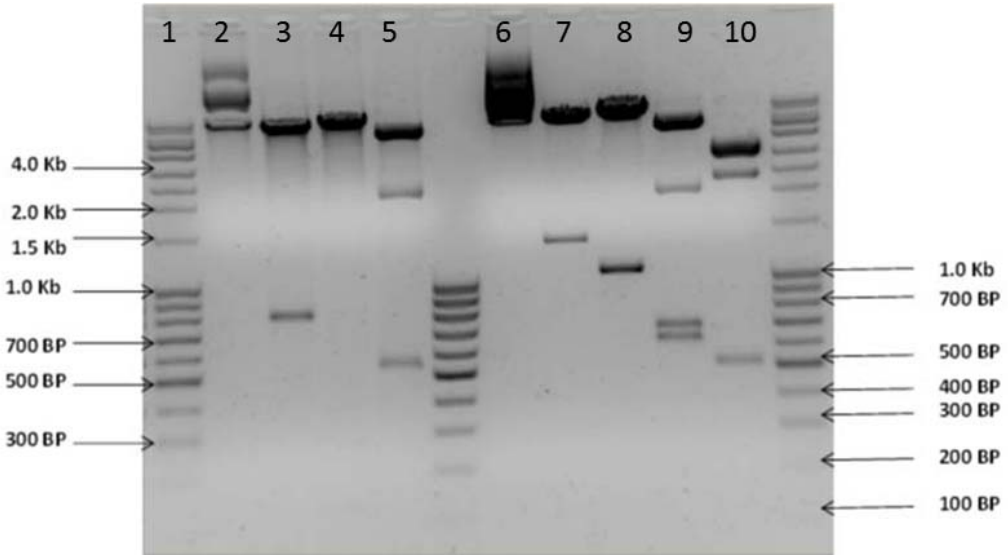

Figure 11. pLVX-CMV-IRES-ZsGreen1 diagnostic restriction digestion. One positive clone was selected and it was digested with different set of restriction enzymes to confirm the presence of hAscl1 cDNA. Lane 1, marker; lane 2, uncut hAscl1; lane 3, XhoI and SpeI digest; lane 4, BamHI digest; lane 5, NcoI digest; lane 6 BglI and XhoI digest; and lane 7, marker.

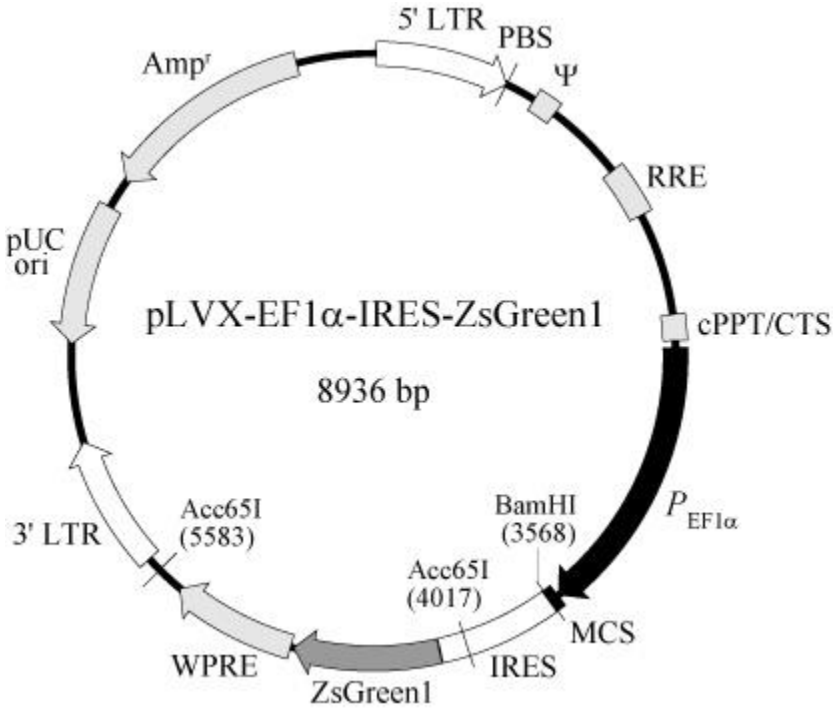

Figure 12. pLVX-EF1α-IRES-ZsGreen1 vector map. Backbone with EF1α promoter was used to insert hAscl1 cDNA in multiple cloning site using restriction enzymes EcoRI and XbaI.

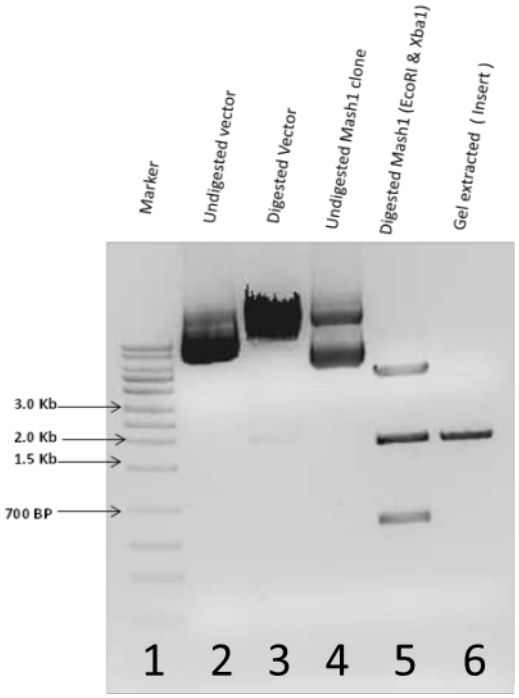

Figure 13. Gel extraction of hAscl1 fragment. PCMV6-XL5 was digested using restriction enzymes EcoRI and XbaI and the Ascl1 band (1.73 kb) was gel extracted and used for cloning. Lane1, marker; lane 2, undigested vector; lane 3, digested vector; lane 4, undigested Ascl1 clone; lane 5, Ascl1 digested with EcoRI and XbaI; and lane 6, gel extracted Ascl1 fragment.

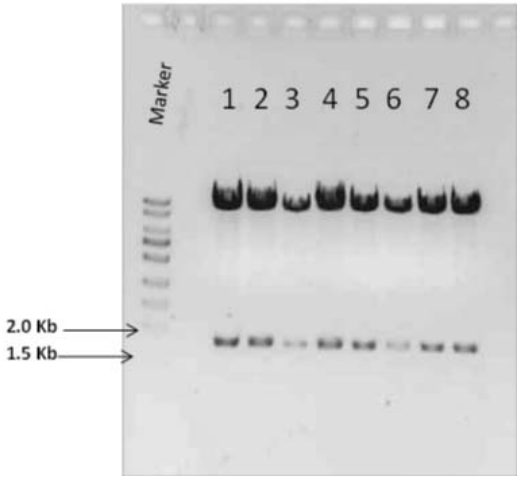

Figure 14. pLVX-EF1α-IRES-ZsGreen1 positive colonies screening. Digested vector and insert were ligated, transformed into competent cells and screened for positive colonies. All the eight clones after digesting with cloning restriction enzymes were positive and had the Ascl1insert (1.73 kb).

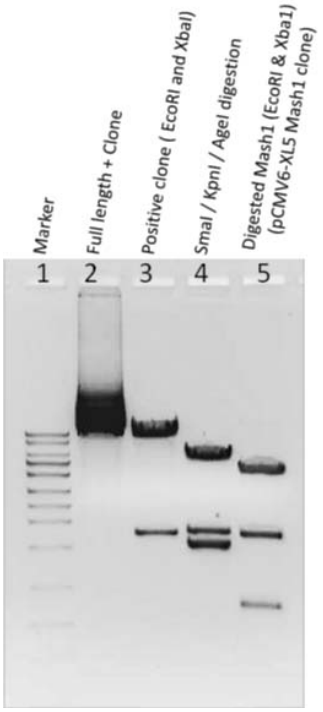

Figure 15. pLVX-EF1α-IRES-ZsGreen1-hAscl1 diagnostic restriction digestion. One positive clone was selected and it was digested with different set of restriction enzymes to confirm the presence of hAscl1 insert. The clone was digested with cloning restriction enzymes EcoRI and XbaI (lane 3), with SmaI, KpnI and AgeI (lane 4) and the original hAscl1 source vector with EcoRI and XbaI (lane 5).

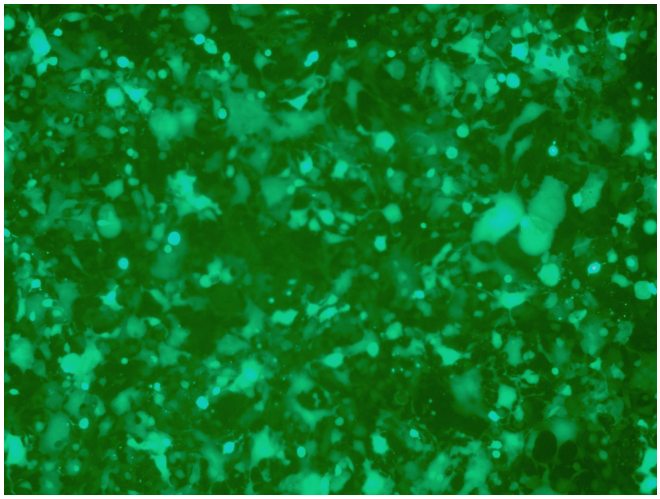

Figure 16. HEK293T cells transduced with Ascl1 expressing lentiviral particles. HEK293T cells were plated with density of 5 X 10^6 cells/10 mL in 10 cm tissue culture plate. Attached cells were transduced next day with 100 µL of lentivirus (5 X 10^5 IFU/mL) containing pLVX-CMV-IRES-ZsGreen-hAscl as transfer vector. After 48 hours most of the cells were transduced.