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Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium

Ken Matsumoto [1], Rika Miki, Mizuho Nakayama, Norifumi Tatsumi, Yuji Yokouchi *

Division of Pattern Formation, Department of Organogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto, 860-0811, Japan
The 21st Century COE 'Cell Fate Regulation Research and Education Unit', Kumamoto University, Japan

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ABSTRACT

Hepatic epithelial morphogenesis, including hepatoblast migration and proliferation in the septum transversum, requires the interaction of hepatic epithelium with the embryonic sinusoidal wall. No factors that mediate this interaction have yet been identified. As the β-catenin pathway is active in hepatoblast proliferation, then Wnt ligands might activate the canonical Wnt pathway during liver development. Here, we investigated the role of Wnts in mediating epithelial vessel interactions in the developing chick liver. We found that Wnt9a was specifically expressed in both endothelial and stellate cells of the embryonic sinusoidal wall. Induced overexpression of Wnt9a resulted in hepatomegaly with hyperplasia of the hepatocellular cords, and in hyperproliferation of hepatocytes. Knockdown of Wnt9a caused a reduction in liver size, with hypoplasia of hepatocellular cord branching, and hypoproliferation of hepatoblasts, and also inhibited glycogen accumulation at later developmental stages. Wnt9a promoted in vivo stabilization of β-catenin through binding with Frizzled 4, 7, and 9, and activated TOPflash reporter expression in vitro via Frizzled 7 and 9. Our results demonstrate that Wnt9a from the embryonic sinusoidal wall is required for the proper morphogenesis of chick hepatocellular cords, proliferation of hepatoblasts/hepatocytes, and glycogen accumulation in hepatocytes. Wnt9a signaling appears to be mediated by an Fzd7/9-β-catenin pathway.

Introduction

The liver is the center of metabolism for carbohydrates, fats, and proteins in vertebrates. To perform these metabolic functions effectively, the liver possesses a microstructure composed of hepatocellular cords, single cell thick epithelial sheets of hepatocytes. The hepatocellular cords are lined by a wall of sinusoids (capillaries characteristic of liver), that is mainly composed of endothelial and stellate cells (DeRuiter et al., 1993; Gouysse et al., 2002). From the portal–central vein, portal blood is transported to the hepatocytes of the hepatocellular cords via the sinusoids (Wisse et al., 1985). The blood plasma can pass into the subendothelial space of Disse through the fenestrated endothelium of the sinusoids and substances from the blood can then diffuse into the hepatocytes where they are metabolized. Finally, the portal blood plasma containing the metabolites drains into the central vein via the sinusoids (Cooper, 1997; Havel and Hamilton, 2004; Mahley and Ji, 1999). Thus, the hepatic sinusoids are essential for the metabolic function of the liver.

In the liver development, hepatic epithelium and the blood vessels appear to be mutually interdependent. In the interaction between hepatic epithelium and blood vessels, especially, the blood vessels or their precursors have important roles for liver development in mammalian embryos. At E8.5 of mouse embryo development, hepatic endoderm, induced from the foregut by fibroblast growth factors (FGFs) from the cardiac mesoderm, invaginates into the adjacent septum transversum mesenchyme (STM) to form a liver bud composed of a multi-layered epithelium (Jung et al., 1999; Rossi et al., 2001). Next, at E9.5, hepatoblasts from the liver bud migrate into the septum transversum where they proliferate to form the embryonic hepatocellular cords. Formation of the hepatocellular cords is accompanied by formation of the embryonic sinusoids that line the cords (Hentsch et al., 1996; Matsumoto et al., 2001; Sosa-Pineda et al., 2000; Zhao and Duncan, 2005). The intimate relationship between the embryonic hepatic epithelium and the embryonic sinusoids suggests

* Corresponding author. Fax: +81 96 373 6625.
E-mail address: yokouchi@kumamoto-u.ac.jp (Y. Yokouchi).
[1] JSPS Fellow.

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that the sinusoids are important for hepatocellular cord formation. Recently, the VEGFR2 null mouse embryo, which lacks endothelial cells around the liver bud, was reported to exhibit complete arrest of migration of hepatoblasts from the liver bud and of hepatocellular cord formation in the septum transversum (Matsumoto et al., 2001). These data strongly suggest that some factor secreted from the embryonic sinusoidal wall, or its precursors, controls the migration and proliferation of hepatoblasts.

In the chick embryo, blood vessels are also crucial for liver bud morphogenesis during liver development. The initial phase of embryonic hepatic histogenesis involves a species-specific morphogenetic process at the ductus venosus (Yokouchi, 2005). Subsequent development involves morphogenetic processes that show conservation across species. During the early developmental processes (Hamburger–Hamilton stages (HH)14–17, E2–2.5), the liver buds (anterior hepatic diverticulum and posterior hepatic diverticulum on the dorsal and ventral sides, respectively, of the ductus venosus, a vein in the septum transversum unique to the avian embryo) elongate along the ductus venosus in both anterior and lateral directions to envelop the ductus venosus (Le Douarin, 1975; Romanoff, 1960; Yokouchi, 2005). This process is regulated by Neurturin, a chemoattractant expressed in the ductus venosus (Tatsumi et al., 2007). At subsequent stages of development (HH17+-, E2.5-), hepatoblasts from the liver buds migrate in a radial direction in the STM to form embryonic hepatocellular cords, with the accompanying formation of embryonic sinusoids. This conserved developmental process suggests that the embryonic sinusoids also play an important role in hepatocellular cord formation in the developing chick liver. To date, however, the factor(s) derived from the embryonic sinusoidal wall that promotes hepatocellular cord formation has not been identified.

Wnts are secreted signaling molecules that play various roles in cellular proliferation, differentiation, patterning and cell polarity in ligand- or context-dependent manners during development. Wnt signaling is mediated by canonical or non-canonical pathways (Gordon and Nusse, 2006; Logan and Nusse, 2004). In the canonical pathway, β-catenin plays a crucial role in signaling. Wnt binds to receptors on Frizzled (Fzd) proteins to inactivate the β-catenin degradation complex (Axin/APC/GSK3-β) through Dishevelled (Dvl). In this situation, cytoplasmic β-catenin that is not phosphorylated dissociates from the complex (stabilized), binds with Tcf/Lef families, translocates into the nucleus, and finally activates transcription of Wnt target genes.

Recently, β-catenin was reported to be important for hepatoblast proliferation in mouse and chick embryos. In E10–14 mouse livers, β-catenin is stabilized in the hepatic cells and localized in the nuclei of hepatoblasts (Micsenyi et al., 2004). Morpholino-induced knockdown of β-catenin in cultured mouse liver slices inhibited hepatoblast proliferation and increased apoptosis (Sodhi et al., 2005). In chick embryos, induced overexpression of the constitutively active form of β-catenin caused hepatomegaly and hyperproliferation of hepatoblasts (Suksaweang et al., 2004). Moreover, induced expression of Dkk-1, a strong Wnt antagonist, caused liver hypoplasia and hypoproliferation of hepatoblasts (Suksaweang et al., 2004). These observations suggest the existence of a Wnt ligand that activates the canonical Wnt pathway in the developing liver and is involved in hepatoblast proliferation.

In our previous study, we searched for Wnt ligand(s) that mediate epithelial–mesenchymal interactions in the developing chick liver (Yokouchi, 2005) and found that Wnt9a was expressed in the hepatic vein of the HH17 (E2.5) chick liver.

Here, we investigated the expression pattern of Wnt9a in the developing chick liver, and performed functional analyses using gain-of-function and loss-of-function strategies to elucidate the signal transduction mechanism. Our data demonstrate that Wnt9a emanating from the embryonic sinusoidal wall is required for proper morphogenesis, proliferation, and glycogen accumulation of the chick hepatic epithelium and that Wnt9a signaling appears to be mediated by the Fzd7/9-β-catenin pathway.

Materials and methods

Embryos

SPF chick embryos were obtained by incubation of fertilized White Leghorn eggs (Takeuchi hatchery, Nara, Japan) and were staged according to Hamburger and Hamilton (1951) and by day of incubation (embryonic day, E).

In situ hybridization

Embryos were fixed in 4% paraformaldehyde/PBS and processed for cryosectioning or paraffin wax sectioning as described previously (Wilkinson, 1993) and sectioned at 10 μm, except for the sections in Figs. 5G, H (5 μm). Paraffin wax embedded embryos were sectioned at 8 μm. Single- and double-colored in situ hybridizations were performed as described previously (Wilkinson, 1993) with minor modifications (see Supplementary methods). The probes for *hhex*, *Albumin* and *Env* were prepared as described previously (Yanai et al., 2005) (Takeuchi et al., 2003). The *Wnt9a* probe was transcribed from ChEST530d5 (MRC Geneservice). Other probes were transcribed from the appropriate templates by RT-PCR using the primers listed in Table 1.

Knockdown of gene expression

Details of the following protocols are given in the Supplementary methods: designing templates for small hairpin RNAs (shRNAs) against Wnt9a; construction of an shRNA expressing vector in pEGFPH1 (a kind gift from Dr. Chi-chung Hui, this plasmid transcribes EGFP mRNA under a CMV promoter and can transcribe a shRNA composed of two complements of 19 nt sequences separated by a 4 nt spacer, under a human H1 promoter); the gene silencing assay for evaluating shRNAs; construction of the GFP-Wnt9a expressing vector; and, construction of RCAS(BP)A carrying an H1 promoter and shRNAs templates.

Immunoprecipitation assay and immunoblotting

For the binding assay with Frizzled, cWnt9a-Fc and GFP-Fc were constructed by PCR from the full-length cDNAs of Wnt9a and GFP using the following primers and pSlax12-Wnt9a and pEGFPN1 as templates, respectively: for Wnt9a-Fc, (5′-CGACTAGTACTCACTCCCGTCTTTACAGGTGTAAAC-3′, 5′-ATTACCCCTCAC TAAAGG-3′ (T3 primer sequence of pSlax*Wnt9a*)); for GFP-Fc, (5′-CGACTAGTACTCACTCTTGTACAGCTCGTCCATGC-3′, 5′-TAGTGAACCGTCAGATCCG-3′). The PCR products were inserted into the pEF–Fc vector that encodes the Fc domain of human IgG (kind gift of Dr. Tanaka H, Kumamoto University). cFzd1 was obtained by RT-PCR using the primers listed in Table 1. mFzd4 was a gift from Dr. S. Nakagawa, Riken Wako. Full-length mFzd7 and hFzd9 were purchased from Invitrogen. To produce the cysteine rich domain (CRD) at the N-terminus of Frizzled proteins (Fzd-CRD), partial cDNAs for cFzd1-CRD (1–228), mFzd7-CRD (1–241) and

Table 1
PCR oligonucleotides

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Frizzled-1	CTTCATGAGTCGCAACTTCG	TCCACCAGGTAGGTGAGGAC
Frizzled-2	TCAACGAGGACGAGATCC	GCGTGTAGAACTTCCTCC
Frizzled-3	GCAGACAGCAGCATTAGC	AACCAACAGTGGCACGAG
Frizzled-4	AACAGGAACAGAGGCAGG	AACCAGAACCTGGATGCG
Frizzled-5	AGCTATGCGCAGTACTTCC	CAAGACTCGAGAGTTTTGCC
Frizzled-6	TCGATCAGTGTGCACCAC	TTGTTGTGCCACCTGTGC
Frizzled-7	CCTCAACCAGACCATCC	CGTGCTTCATGATGGTGC
Frizzled-8	GGTGTGATGACTGGACGTC	GCATGAAGACGGCGTAGTC
Frizzled-9	GACAACCCCGAGAAGTTCC	CCATCCAGACGAAGGCAAAGTCC
Frizzled-10	TCAGTGTGAGCAGGCGAG	TCAGCTCATCTCCAGCCAC
Tcf-1	TCAAGTCCTCGTTGGTG	AGGATCTGGTTGATGG
Tcf-3	ATGTCCAGCCTGATGTCCAG	GTCTGTGGTGCACAGC
Tcf-4	GGATGGTTAGTACCAC	CTTCCATCTGAAGAGG
B-catenin	GAAGGCCTTCTAGGAACTC	GCAACTCTACAGGCCAATC
Wnt9a[a]	ACGAAGCCCTGACCATCCT	CGTCTCCTTGAAACCTCTC
Gapdh[a]	ACG CCATCACTATCTTCCAG	CAGCCT TCACTACCCTCTTG
Pepck[a]	TGAGTCGACAGATTCTCC	ACATCCAACCGATGAAG
G6pase[a]	TGAGACCTTCCAGCACATCC	TCTGTGCCTTCTCCAGTGTC
Gys[a]	ATCATGCAACCAGCTGACTC	GGAGCACATGGTTCCATCTC
Glycogen phosphorylase[a]	GCACCTGGACCATATTGCGT	TTGGCCACTCCGTTGACAG
Env[b]	AAACGAAGCACCTCACACC	AAGGCAGGCACACTACTAG
Flk-1[b]	CTCTCCCAACTTCACCTGC	CACTTGTCTGGTTGGATGC

Primer sequences used for PCR amplification.
[a] Used for qReal-time and semi-quantitative RT-PCR.
[b] Used for semi-quantitative RT-PCR.