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The left-right asymmetry of liver lobation is generated by Pitx2c-mediated asymmetries in the hepatic diverticulum

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ABSTRACT

Internal organs exhibit left-right asymmetric sizes, shapes and anatomical positions, but how these different lateralities develop is poorly understood. Here we use the experimentally tractable *Xenopus* model to uncover the morphogenetic events that drive the left-right asymmetrical lobation of the liver. On the right side of the early hepatic diverticulum, endoderm cells become columnar and apically constricted, forming an expanded epithelial surface and, ultimately, an enlarged right liver lobe. In contrast, the cells on the left side become rounder, and rearrange into a compact, stratified architecture that produces a smaller left lobe. Side-specific gain- and loss-of-function studies reveal that asymmetric expression of the left-right determinant Pitx2c elicits distinct epithelial morphogenesis events in the left side of the diverticulum. Surprisingly, the cellular events induced by Pitx2c during liver development are opposite those induced in other digestive organs, suggesting divergent cellular mechanisms underlie the formation of different lateralities.

1. Introduction

Most vertebrates acquire a left-right (LR) asymmetrical internal anatomy during development. For example, some organs are found exclusively on one side of the body (e.g., spleen), or adopt an asymmetrical configuration within the visceral cavity (e.g., intestine). In addition, the left and right sides of individual organs (e.g., heart), or left and right counterparts of paired organs (e.g., lungs), manifest LR laterality in their dissimilar sizes and shapes. Abnormal embryonic LR patterning results not only in reversal or elimination of such lateralities, but is also associated with common and severe birth defects in these organs (Aylsworth, 2001; Zhu et al., 2006). Thus, understanding the cellular and molecular events that underlie the development of organ-specific asymmetries may be vital for ascertaining the etiology of numerous congenital defects. Yet, for most organs, the morphogenetic events that generate their particular positional or structural laterality remain among the least understood phases of organogenesis.

The liver is an essential metabolic organ that originates as a single midline evagination of the ventral foregut, the hepatic diverticulum (Bort et al., 2006; Tremblay and Zaret, 2005; Zorn and Wells, 2009). The endoderm cells of the diverticulum (nascent hepatocytes) delaminate from the gut and mingle with the surrounding septum transversum mesenchyme and angioblasts, where they are induced to differentiate into bile canaliculi and form liver cords (Tremblay and Zaret, 2005). During its morphogenesis, the liver not only becomes positioned asymmetrically in the body cavity, but also displays intrinsic morphological asymmetry in the disparate sizes and shapes of its left and right lobes. For example, in humans, the right side of the organ grows to be 5–6 times larger than the left, with a distinct morphology and more complex lobation pattern (Abdel-Misih and Bloomston, 2010; Gray, 1918). Although the exact morphologies vary, similar asymmetries in liver laterality exist across vertebrate species (Smallwood, 2010).

In humans and animal models with defective LR asymmetry, the liver displays abnormal or indeterminate lobation, indicating that this feature is dependent on global embryonic LR patterning cues (Lin et al., 2000; Brueckner, 2007; Shiraishi and Ichikawa, 2012). Such cues are established early in development by the expression of Nodal, a TGFβ growth factor, exclusively in the left lateral plate mesoderm (LPM) (Basu and Brueckner, 2008; Blum et al., 2014; Brennan et al., 2002; Kawasumi et al., 2011; Yoshiba and Hamada, 2014). Nodal signaling then activates expression of the homeobox transcription factor, *Pitx2,* which is subsequently retained on the left side of developing organs (Faucourt et al., 2001; Raya et al., 2006), where it elicits changes in cell properties and behaviors that ultimately generate tissue-level asymmetries (Davis et al., 2008, 2017; Kurpios et al., 2008). During liver morphogenesis, the expression of *Pitx2* is limited to the left LPM-derived septum transversum mesenchyme (STM) that surrounds the hepatic diverticulum (Shiratori et al., 2006; Meno et al.,

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1998). Bilateral expression of *Pitx2* in the surrounding mesenchyme is associated with abnormal liver lobation (Meno et al., 1998; Hamada et al., 2002), a correlation that suggests liver lobe morphology may be influenced by Pitx2 activity. However, the mechanisms by which Pitx2 might regulate the morphogenesis of the developing lobes is unknown.

As for other asymmetrical organs, the proximate morphogenetic mechanisms which actually engender asymmetry in the liver are poorly understood. Classical embryologists suggested that the laterality of liver lobation is merely a physiological consequence of the asymmetrical remodeling of the fetal vasculature, which creates unequal blood flow and trophic cues in the left versus right lobes (Hutchins and Moore, 1988). However, retrospective studies of human embryos indicate that, shortly after its initial budding, a greater proportion of the liver's volume already lies on the right, suggesting the process that generates liver asymmetry precedes vascularization (Heisler, 1907; Hutchins and Moore, 1988). Moreover, a recent fate mapping study concludes that mouse hepatocytes become segregated to left vs. right lobes soon after progenitors are restricted to the liver fate, suggesting that liver laterality may have “a cellular basis” i.e., it is not merely the result of LR asymmetrical growth constraints (Weiss et al., 2016). Nonetheless, the timing of when liver LR asymmetry is specified, or how left versus right morphogenetic events diverge to yield the differential sizes and/or shapes of the contralateral lobes, remains unknown.

Here, we describe the morphogenetic events that underlie the development of asymmetrical lobation in the vertebrate liver. Using the highly accessible embryos of the frog, *Xenopus laevis*, which develop anatomically simple liver lobes, we show that liver laterality arises early in hepatic morphogenesis, commencing with LR asymmetric remodeling of the endoderm-derived epithelium of the hepatic diverticulum. Side- and tissue-specific modulations of gene function show that these early cellular differences yield disparate lobe size and morphology, and are dependent on left-sided expression of *Pitx2c* in the surrounding mesoderm. Our results thus define cellular and molecular events that underlie the development of morphological laterality within the liver and, as such, broaden the emerging view of the variable morphogenetic mechanisms that shape different LR asymmetries throughout the body.

2. Results

2.1. The early liver diverticulum is left-right asymmetric

To determine when LR asymmetry is first established during liver morphogenesis, we characterized the size and shape of the organ in the *Xenopus* embryo at successive stages of development (Fig. 1). Consistent with classical observations in humans (Hutchins and Moore, 1988), morphometric measurements of the area occupied by the right and left halves (relative to the ventral midline; see Methods) of the *Hhex*-expressing (Zorn and Mason, 2001) tissue of the *Xenopus* liver bud revealed that, soon after formation of the hepatic diverticulum (by NF 33), the right side of the organ becomes larger than the left ($p < 0.0001$; Fig. 1A-D). During subsequent development (NF 40–42), the nascent right lobe of the liver continues to enlarge and extend while the left lobe remains the same size and retains a more spherical shape (Fig. 1E-F). By tadpole stages (e.g., NF42–44), the right lobe has acquired an irregular border (Fig. 1G) and elongated along the dorsoventral axis, exhibiting a greater gross length to width ratio than the left lobe ($p < 0.0001$; Fig. 1H). These results demonstrate that morphological liver asymmetry arises early in development, evident at the gross level as unequal expansion and lengthening of the left and right sides of the initial liver bud.

2.2. Cellular morphogenetic asymmetries in the early liver diverticulum

To ascertain the morphogenetic differences that underlie the unequal growth of the contralateral halves of the early liver, we first defined the fates of left and right cells during early liver morphogenesis. In these experiments, we took advantage of the practical amenability of the *Xenopus* model in which reagents may be easily targeted to the left or right sides of developing organs by standard microinjection (Davis et al., 2017). Using this approach, we delivered mRNA encoding membrane-tethered versions of green fluorescent protein (GFP) or mCherry lineage tracers into the left versus right dorsovegetal cells of the 8-cell blastula (Fig. 2A), thus differentially labeling the progenitors contributing to each side of the prospective foregut organs, including the liver (Fig. 2B-D).

We found that, at the gross anatomical level, cells from the right or left side of the embryo will contribute primarily to the right or left lobe of the mature liver, respectively (Fig. 2D), consistent with previous studies (Weiss et al., 2016; Muller et al., 2003). Although the left and right sides of the embryo initially contribute equivalently to the budding liver diverticulum (NF 32; Fig. 2B, E), at subsequent stages, right cells come to occupy a slightly greater proportion of the diverticulum, as indicated by the distribution of mCherry- versus mGFP-labeled cells in section (Fig. 2C-C”). This differential proportion is evident in both transverse (e.g., Fig. 2F-H) and frontal sections (data not shown), and suggests that perhaps the right side of the early diverticulum proliferates more than the left side. However, we found no LR differences in total nuclei (Fig. 3A-E), mitotic indices (Fig. 3F), or apoptosis (data not shown) at these stages to support the idea that there are different numbers of cells on the two sides. Therefore, we hypothesized that the broader distribution of right side cells is more likely due to LR differences in morphogenetic remodeling of the hepatic diverticulum.

To investigate this idea directly, we compared epithelial architecture in the left versus right endoderm of the early diverticulum. Prior to the appearance of overt LR asymmetry (NF 32), the entire diverticulum is composed of a pseudostratified epithelium (Fig. 2E), with no LR differences in cell shape (e.g., apical constriction, length to width ratio; Fig. 2K-L) or in the number of nuclei layers evident along the apical-basal axis (Fig. 2M). However, at subsequent stages (NF 33–35; Fig. 2F-G), cells in the right side of the diverticulum (Fig. 2I) become apically constricted ($p < 0.01$; Fig. 2K) and acquire a greater length-to-width ratio ($p < 0.01$; Fig. 2L). In contrast, the cells on the left side of the diverticulum (Fig. 2J) become less apically constricted and less columnar ($p < 0.01$; Fig. 2K-L), with the nuclei of the left epithelium arranged in more layers ($p < 0.01$; Fig. 2M). Consequently (NF 37), while the right epithelium begins to expand and form involutions, the left side forms a thicker epithelium with a more compact tissue architecture (Fig. 2H).

Notably, the appearance of these cellular asymmetries correlates with the emergence of gross anatomical differences between the left and right halves of the early liver bud (see Fig. 1), suggesting that the asymmetry of liver lobation is generated by this early LR disparity in endoderm epithelial morphogenesis in the hepatic diverticulum.

2.3. Pitx2c is required for left lobe morphogenesis

As observed in other vertebrates, *pitx2c* is expressed asymmetrically in the mesoderm surrounding the *Xenopus* liver diverticulum (Fig. 4). As the expression pattern is left-sided at stages prior to and during the development of the asymmetries described above, we hypothesized that this factor plays a role in distinguishing left versus right side diverticular morphogenesis. To determine the requirement for *pitx2c* in this process, we employed a *pitx2c* morpholino previously shown to efficiently knock down Pitx2c translation in the *Xenopus* foregut (Davis et al., 2017), targeting this reagent to only the left side of