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Review

Gene regulatory networks in differentiation and direct reprogramming of hepatic cells

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ABSTRACT

Liver development proceeds by sequential steps during which gene regulatory networks (GRNs) determine differentiation and maturation of hepatic cells. Characterizing the architecture and dynamics of these networks is essential for understanding how cell fate decisions are made during development, and for recapitulating these processes during *in vitro* production of liver cells for toxicology studies, disease modelling and regenerative therapy. Here we review the GRNs that control key steps of liver development and lead to differentiation of hepatocytes and cholangiocytes in mammals. We focus on GRNs determining cell fate decisions and analyse subcircuitry motifs that may confer specific dynamic properties to the networks. Finally, we put our analysis in the perspective of recent attempts to directly reprogram cells to hepatocytes by forced expression of transcription factors.

Contents

1. Liver development: selection of gene regulatory network representation

The sequential steps of liver development are coordinated by intercellular signaling effectors that modulate the activity of intracellular transcription factor (TF) networks [1]. The combination of cell-extrinsic and cell-intrinsic cues constitutes gene regulatory networks (GRN) in which TFs determine spatial and temporal expression of genes and eventually drive hepatic cell differentiation and liver morphogenesis. GRNs acting in liver development can be reconstructed through the analysis of genome-wide studies and by assembling subnetworks identified in experiments addressing the function of small sets of genes. Therefore the multiple sources of data and the inherent complexity of the GRNs led to various

Abbreviations: BMP, Bone Morphogenic Protein; Cdx2, caudal type homeobox 2; C/EBP, CCAAT/Enhancer Binding Protein; ES, embryonic stem; FGF, Fibroblast Growth Factor; FoxA, Forkhead box factor A; GRN, gene regulatory network; Grg3, groucho-related gene 3; HNF, Hepatocyte Nuclear Factor; OC2, Onecut2; Prox1, Prospero homeobox 1; Sox, SRY-related high mobility group box transcription factor; TβRII, TGFβ type II receptor; Tbx3, T-box 3; TGF, Transforming Growth Factor; TF, transcription factor; YAP, Yes-Associated Protein; Wnt, wingless-type MMTV integration site.

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modes of network representations. Particularly convenient for concise representations of GRNs in liver development are activity flow diagrams representing epistatic relationships. While not providing detailed mechanistic insight, such maps are easily transposable when designing cell culture protocols for production of hepatic cells for regenerative therapy. They also convey essential information and provide a strong framework for qualitative and quantitative dynamic modelling.

Here we review the key cell fate decisions made during liver development. At each step, we attempt to define GRNs that are represented as directed, sequential but non-mechanistic activity flow diagrams. To avoid designing GRNs that inappropriately integrate components from distinct species, our analysis focuses on mammalian systems. We then discuss the subcircuitry motifs and the implementation of GRNs for TF-mediated reprogramming of cells to hepatocytes.

2. Gene regulatory network operating during liver specification

The liver precursor cells are located in a midline domain and in two lateral and more posterior domains of the ventral foregut endoderm [2,3]. Liver specification, *i.e.* the initiation of hepatic gene expression, occurs when these domains merge at the ventral midline. The pioneer TFs Forkhead box (Fox)A1/A2 and GATA4/6 initially open the chromatin of liver genes which become primed ("competent") for subsequent occupancy by additional transcriptional regulators, eventually leading to transcriptional activation [4]. FoxA1 and FoxA2 function redundantly and are required for initiation of liver development [5]. Similar functional redundancy was suggested for GATA4 and GATA6 [6], but recent studies identified unique functions for each factor [7].

These observations raised questions on the mechanisms that trigger transition from competency to specification. Fibroblast Growth Factors (FGFs), Bone Morphogenic Proteins (BMPs) and Wingless-type MMTV integration site (Wnt) proteins are secreted by mesodermal tissue adjacent to prehepatic endoderm and promote early hepatogenesis [8–10]. The FGF, BMP and Wnt signaling pathways are conserved across species during liver specification [11,12].

In mammals, the involvement of Wnt is not demonstrated *in vivo* but is suggested from the presence of non-canonical Wnt signaling components in liver progenitors, and from the need to repress canonical Wnt signaling when specifying stem cell-derived endoderm to a hepatic fate [13–16]. Which FGF ligand is responsible for hepatogenesis in mice remains unclear, but the ERK1/2 pathway was shown to be necessary for hepatic gene induction downstream of FGFR1/2/4 [17]. FGFs also cooperate with BMP4; downstream of FGFR1/2/4 they activate the RAS-RAF-ERK and PI3K-Akt pathways, and the Wnt signaling inhibitor NKD1, as well as several TFs [15,17]. BMP activity is mediated by SMAD1/5/8 which forms a complex with SMAD4. The latter recruits the histone acetyltransferase P300 to hepatic genes and stimulates GATA4 expression, indicating that BMP has direct effects on liver genes via SMAD4 and indirect effects via enhanced expression of GATA4 [10,18]. Therefore, hepatic specification is controlled by feedforward loops: a first loop is formed within the BMP cascade by the direct and indirect effects of SMAD4, and a second loop is constituted within the FGF pathway by the ERK/Hepatocyte Nuclear Factor (HNF) 4 and NKD1/β-catenin cascades.

Transcriptional components of the GRN driving hepatic specification further include Klf6, which enhances the expression of GATA4 and FoxA2 in cultured embryonic stem (ES) cells [19]. FoxA2 is inhibited by groucho-related gene 3 (Grg3), a co-repressor that silences FoxA-bound hepatic genes in undifferentiated endoderm, and which becomes extinguished during specification to enable FoxA factors to stimulate transcription [20]. At the specification stage HNF4 is required for expression of several other liver-specific TFs [21]. Hhex, whose expression is only marginally controlled by HNF4, indirectly regulates the response to extracellular signals by determining the position of the endoderm with respect to adjacent sources of FGF and BMP [22]. Finally, HNF1β has little or no effect on endodermal competence as evidenced by near normal expression of *FoxA2* at the 6–8 somite stage in HNF1β-deficient endoderm. However it is critically required for FGF-induced specification of the endoderm in mammals [23].

Fig. 1A proposes an epistasis-based GRN for hepatic specification. This GRN does not take quantitative, spatial and dynamic aspects of signaling into consideration [24–26], despite that induction of liver genes requires well-defined levels of FGFs, and that FGFs are not required for all liver precursor domains: specification of the ventral midline precursor domain is indeed dependent on FGF signaling, whereas the lateral precursor domains develop normally in the presence of FGFR inhibitors [27]. In addition, the midline and lateral precursor domains are subjected to distinct temporal responses to BMPs and FGFs, with induction of BMP signaling preceding FGF signaling in the midline precursors and *vice versa* in the lateral precursors [26]. Together these data indicate that distinct thresholds of FGF signaling determine organ specification along the antero-posterior axis of the endoderm, and also that the requirements and dynamics of FGF- and BMP-mediated induction of hepatic gene expression differ among subsets of liver precursor cells.

3. Liver bud outgrowth and hepatoblast migration

The specified ventral endoderm forms a multilayered pseudostratified epithelium composed of hepatoblasts, which then proliferate, delaminate from the endoderm, and invade the septum transversum. Endothelial cells are dispensable for endoderm specification *in vivo*, but *in vitro* they promote hepatic specification of cultured ES cell-derived endoderm by inhibiting Wnt and Notch signaling [14]. Beyond the stage of specification, endothelial cells are essential for liver bud outgrowth [28].

Liver budding follows shortly after specification, and regulators of specification, like FGFs and BMPs, continue to play a role at the budding stage. Specific functions at the budding stage were identified for a number of TFs: pseudostratification of endoderm cells requires Hhex, and migration of the hepatoblasts into the septum transversum is coordinately controlled by T-box 3 (Tbx3), Prospero homeobox 1 (Prox1), HNF6 and Onecut2 (OC2) [29–32]. The analysis of mice knockout for these TFs revealed that Prox1, HNF6 and OC2 repress E-cadherin and so allow the hepatoblasts to dissociate from each other during migration in the surrounding mesenchyme. Epistatic relations between TFs are shown in Fig. 1B.

4. Proliferation *versus* growth arrest: role in hepatobiliary lineage segregation

Hepatoblasts proliferate and are protected against apoptosis to enable liver growth. Secreted factors controlling proliferation and apoptosis have been reviewed elsewhere [1,33]. Here we focus on mechanisms that control the balance between maintenance of an immature hepatoblast phenotype and differentiation towards the hepatocyte or cholangiocyte lineages in relation with the cells' proliferative state (Fig. 2A).

Hepatoblasts express hepatocyte-specific genes and proliferate while differentiating to hepatocytes. Instead, cells differentiating to cholangiocytes repress hepatocyte genes and undergo growth arrest: cholangiocytes organised as a ductal plate around the

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