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The Onecut transcription factors HNF-6/OC-1 and OC-2 regulate early liver expansion by controlling hepatoblast migration

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Abstract

Liver development in mammals is initiated by the formation of a hepatic bud from the ventral foregut endoderm. The hepatic cells then proliferate and invade the septum transversum mesenchyme, and further differentiate to give rise to hepatocytes and biliary cells. By analyzing mice that are knockout for the transcription factors Hepatocyte Nuclear Factor-6 (HNF-6)/Onecut-1 (OC-1) and OC-2, we show here that these factors redundantly stimulate the degradation of the basal lamina surrounding the liver bud and promote hepatoblast migration in the septum transversum. Gene expression analysis indicates that HNF-6 and OC-2 belong to a gene network comprising E-cadherin, thrombospondin-4 and osteopontin, which regulates liver bud expansion by controlling hepatoblast migration and adhesion. This network operating at the onset of liver development contains candidate genes for investigation of liver carcinogenesis.

Keywords: Onecut; Liver development; Osteopontin; Cell migration; Cell adhesion

Introduction

Liver development in mice starts around embryonic day (E) 8.5 by the formation of an outgrowth of the ventral foregut endoderm. This process is tightly controlled by interactions between the endodermal epithelium and neighboring mesenchymal tissues. In particular, the cardiogenic mesoderm and the septum transversum mesenchyme (STM) secrete Fibroblast Growth Factors (FGF) and Bone Morphogenic Proteins (BMPs), inducing the expression of hepatic genes in the pre-hepatic endoderm (Bossard and Zaret, 1998; Calmont et al., 2006; Gualdi et al., 1996; Jung et al., 1999; Rossi et al., 2001; Zaret, 1999). The expression of Secreted Frizzled Protein 5 in mouse and Xenopus gut endoderm (Finley et al., 2003; Pilcher and Krieg, 2002), and the role of Wnt2b in Zebrafish liver specification (Ober et al., 2006), suggest that Wnt signaling is also required to initiate liver development in mammals. After hepatic specification, hepatoblasts become columnar and proliferate to form a liver bud. This bud, which rapidly undergoes a transition to a pseudostratified epithelium (Bort et al., 2006), is delineated by a laminin- and collagen IV-rich basement membrane and is closely associated with endothelial cells. The latter promote proliferation of the hepatoblasts via an uncharacterized mechanism (Matsumoto et al., 2001). By E9.5, the basement membrane surrounding the bud is degraded, allowing the hepatic cells to invade the surrounding STM. Further development of the liver requires proliferation and differentiation of the hepatoblasts, giving rise to hepatocytic cords associated with sinusoids, and to bile ducts, which are located in the portal spaces (reviewed in Lemaigre, 2003; Lemaigre and Zaret, 2005; Zhao and Duncan, 2005).

Biochemical and genetic approaches have identified several transcription factors that control the onset of liver development. Mice deficient in Forkhead box (Fox) A1 and FoxA2 fail to initiate liver development (Lee et al., 2005), consistent with a

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role of these factors in opening chromatin of hepatic genes following induction by signaling from the cardiogenic mesoderm and STM (Gualdi et al., 1996). A role for GATA transcription factors in early liver development has also been identified. Indeed, in GATA-6 null embryos hepatic specification occurs, but the liver bud fails to expand and hepatic gene expression is not maintained (Zhao et al., 2005). GATA-4, whose expression is stimulated in the endoderm by signaling from neighboring tissues (Rossi et al., 2001), most likely compensates for the absence of GATA-6 in the endoderm of GATA-6 null embryos to allow hepatic specification. In the liver bud, GATA-4 expression abruptly decreases in hepatoblasts (Zhao et al., 2005). The transcription factor Hematopoietically Expressed factor (Hex) is another transcription factor critical for liver bud expansion. The endodermal cells in Hex-null embryos proliferate at a lower rate and the hepatic bud epithelium fails to undergo a transition from a simple columnar to a pseudostratified state (Bort et al., 2004, 2006). In addition, hepatic gene expression is not maintained, and the degradation of the basal lamina surrounding the bud is perturbed (Bort et al., 2004; Keng et al., 2000; Martinez Barbera et al., 2000). Like Hex, the Prospero-related homeobox 1 (Prox1) controls early liver development. In Prox1-null embryos, the liver bud fails to expand, proliferation is decreased and the basal lamina surrounding the bud is not degraded. However, in contrast to Hex-null embryos, Prox1-null embryos form a multilayered liver bud in which hepatoblasts maintain liver gene expression but do not migrate into the STM (Sosa-Pineda et al., 2000). The lack of hepatoblast migration in the Prox1-null embryos may result in part from the abnormally high expression of E-cadherin. Finally, the transcription factor variant Hepatic Nuclear Factor (vHNF)-1 (also called HNF-1β or Tcf2) is also reported to control liver bud expansion, but the mechanisms involved have not yet been investigated (Haumaitre et al., 2005). Taken together, these data indicate that a transcription factor network operating at the onset of liver development exerts a tight control on specification, proliferation, epithelial morphogenesis and cell migration.

The homeodomain transcription factors HNF-6 (also called Onecut (OC)-1) and OC-2 regulate pancreas and liver development. Both factors are expressed in the endoderm, in the developing pancreas, in the liver bud and, at later stages, in the hepatocytes and biliary cells (Clotman et al., 2002; Jacquemin et al., 2003a,b; Pierreux et al., 2004). Their role has essentially been investigated by analyzing the phenotype of HNF-6 and OC-2 knockout mice. They control pancreas specification, endocrine differentiation and duct morphogenesis (Jacquemin et al., 2000, 2003; Maestro et al., 2003; Pierreux et al., 2006; Poll et al., 2006). In liver, HNF-6 and OC-2 control the differentiation of hepatoblasts to hepatocytes or biliary cells, by modulating the response to Transforming Growth Factor-β (Clotman et al., 2005; Clotman and Lemaigre, 2006; Plumb-Rudewiez et al., 2004). HNF-6 also controls hepatocyte maturation as well as B-lymphopoiesis in embryonic liver (Beaudry et al., 2006; Bouzin et al., 2003; Lannoy et al., 2002).

The expression of HNF-6 and OC-2 at the onset of liver development suggests that these factors play a role at this stage. To investigate this possibility, we further analyzed the phenotype of embryos knockout for HNF-6 and OC-2. We show here that HNF-6 and OC-2 control a gene network involved in cell adhesion and migration and that the two factors are required for liver bud expansion by regulating hepatoblast migration.

Methods

Animals

All mice were raised in our animal facilities and were treated according to the principles of laboratory animal care of the local Animal Welfare Committee. *Hnf6* and *Oc2* knockout mice were obtained as described (Clotman et al., 2005; Jacquemin et al., 2000). Osteopontin knockout mice were kindly provided by D. Denhardt, S. Rittling and A.P. Gadeau and have been described (Rittling et al., 1998).

Detection of β-galactosidase activity

For the detection of the β-galactosidase activity, embryos were fixed at room temperature for 15 min in a solution containing glutaraldehyde 0.4% (v/v), $MgCl_2$ 2 mM and EGTA 5 mM in a sodium phosphate buffer (SPP) ($NaHPO_4$ 77 mM/NaH_2PO_4 22 mM), washed three times for 15 min in a solution containing $MgCl_2$ 2 mM, Na-deoxycholate 0.01% and Nonidet P-40 0.02% in SPP, and stained for 2–4 h at 30 °C in the washing solution containing X-gal 1mg/ml, potassium ferricyanide 5 mM and potassium ferrocyanide 5 mM. Embryo were then rinsed in phosphate buffer saline (PBS) and cleared in a benzyl benzoate: benzyl-alcohol (2:1) solution. For section analysis, the stained embryos were incubated overnight in sucrose 20% in PBS at 4 °C, embedded in PBS containing sucrose 15% and gelatin 7.5%, and frozen. Transverse sections were cut at 10 m on a cryostat.

Whole-mount detection of Prox1

For whole-mount immuno-histochemical labeling of Prox1, embryos were fixed for 1 h at 4 °C in paraformaldehyde 4% in PBS. Endogenous peroxydase activity was blocked by immersing the embryos in methanol containing H_2O_2 3% for 30 min. They were then incubated overnight with a rabbit anti-Prox-1 antibody (Covance) at 1:1000, which was detected with the Vectastain Elite ABC kit by using DAB (Vector). After each incubation with antibodies, the embryos were washed in TBST (Tris–Cl 50 mM, pH 7.5, NaCl 150 mM, Triton X-100 0.1%) for 5 h with 5 changes.

Immunofluorescence and TUNEL analysis

For the immunofluorescence labeling, embryos were fixed for 2 h at 4 °C in paraformaldehyde 4% in PBS, incubated overnight in sucrose 20% in PBS at 4 °C, embedded in PBS containing sucrose 15% and gelatin 7.5%, and frozen. Transverse sections were cut at 10 μm on a cryostat. Primary antibodies and dilutions were: goat anti-HNF-4α at 1:100 (Santa Cruz), rabbit anti-laminin at 1:100 (Sigma), rat anti-PECAM-1 at 1:50 (PharMingen), monoclonal mouse anti-E-cadherin at 1:250 (BD Transduction Laboratories), rabbit anti-phosphohistone H3 (Ser10) at 1:50 (Cell Signaling Technology), goat anti-GATA-4 at 1:250 (Santa Cruz), rabbit anti-GATA-6 at 1:50 (Santa Cruz), rabbit anti-Hex at 1:200 (kind gift from C. Bogue) and rabbit anti-Prox1 at 1:500 (Covance). Secondary antibodies and dilutions were donkey anti-goat/AlexaFluor 594 at 1:2000, anti-rabbit/AlexaFluor 594 at 1:3000 or anti-rat/AlexaFluor 594 at 1:1000 (Invitrogen), or biotinylated sheep anti-rabbit and anti-mouse antibody at 1:250 (Chemicon) followed by streptavidin-AlexaFluor 488 at 1:2000 (Invitrogen).

The TUNEL assay was performed by using the Fluorescein in situ Cell Death Detection kit from Roche. The manufacturer's protocol was modified to allow co-labeling with the anti-Prox1 and the anti-GATA4 antibodies. Briefly, sections were unmasked by boiling in sodium citrate buffer 0.1 M, pH 6.0, for 5 min, permeabilized on ice in sodium citrate 0.1%, Triton X-100 0.1% for 2 min and incubated for 1 h at 37 °C with the TUNEL mix. Then, sections were blocked in a solution containing bovine serum albumin 5%, non fat dry milk 3%,

Triton X-100 0.1% in PBS and processed for the immunofluorescent labeling as described above. Fluorescence was detected with a Zeiss Axiovert 200 inverted fluorescence microscope. All pictures were taken using a Coolpix 995 digital camera (Nikon). Quantification of proliferation was done by counting phosphohistone-H3, GATA-4 and HNF-4α positive nuclei.

DNA array analysis

DNA array analyses were performed using an oligo GEArray Mouse Extracellular Matrix and Adhesion Molecules microarray (OMM-013, SuperArray). Total RNA was used as template to synthesize biotinylated cRNA, using the TrueLabeling-AMP Linear RNA Amplification Kit (SuperArray). The cRNA was hybridized overnight at 60 °C with oligo arrays. After washing, arrays were dried and analysed with the Chemiluminescent Detection Kit (SuperArray), according to the manufacturer's instructions. Images were quantified using the data analysis software (GEArray Analyzer).

Reverse-transcriptase polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR

Total RNA was extracted from embryonic liver with the Tripure Isolation Reagent (Roche) and was incubated for 10 min at room temperature and for 2 h at 37 °C in a volume of 25 µl containing Tris–Cl 50 mM (pH 8.3), KCl 5 mM, $MgCl_2$ 3 mM, dithiothreitol 10 mM, 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Life Technologies), 40 units of Rnase OUT (Promega), 3 µg of random hexamers (Life Technologies) and dNTPs 4 mM (Amersham Pharmacia Biotech).

Real-time PCR was performed with SYBR Green Master Mix Reagent (Invitrogen) on a MyiQ cycler (Bio-Rad). Threshold cycles were transformed into copy number according to the standard calibration curve. Absolute copy number for each mRNA was normalized to *β-actin* or *Prox1* mRNA copy number. Primer sequences were 5′-TCCTGAGCGCAAGTACTCTGT-3′ and 5′-CTGATCCACATCTGCTGGAAG-3′ for β-actin, 5′-CCGACATCTCACCTTATTGAG-3′ and 5′-TGCGAGGTAATGCATCTGTTG-3′ for Prox1, 5′-AGGGAGCTGTCTACCAAAGTG-3′ and 5′-CCAGTCTCGTTTCTGTCTTC-3′ for E-cadherin, 5′-GAAAGCAACGGGAGATCCTC-3′ and 5′-GACTGCCCAGGCCCCTGGTTCTGT-3′ for HNF-1β, 5′-TCTGTCAGCCGTGTTTGAGC-3′ and 5′-ATCTGCCCCAGAAGAGGAG-3′ for vitronectin, 5′-GACCGCTTGTAATTGAGC-3′ and 5′-TTGACGTATTTGGGCTGGTA-3′ for thrombospondin-4, 5′-TGAGATTGGCAGTGATTTGC-3′ and 5′-CTGCTTCTGAGATGGGTCAG-3′ for osteopontin, 5′-CTGAGGGGAATGAAGACCAG-3′ and 5′-TCCAGTTCTTGGGCAGTTGG-3′ for bone sialoprotein, 5′-CCACGAACAGTGAGTCATC-3′ and 5′-TCGCCAAAGGTATCATCTCC-3′ for Dentin matrix protein, 5′-AGCCTTCAATGTGGAACGAG-3′ and 5′-CCACCGCTTCATAGGTCATC-3′ for Hyaluronan Synthase-1 (HAS-1) and 5′-GCCTCATCTGTGGAGATGG and 5′-TCCCAGAGGACCGCTTATGC-3′ for HAS-2.

Results

HNF-6 and OC-2 control early liver expansion

To investigate a possible role of HNF-6 and OC-2 in early liver development, we analyzed the phenotype of mice knockout for HNF-6 and/or OC-2. These mice have been described earlier (Clotman et al., 2005; Jacquemin et al., 2000) and were obtained by introducing a neomycine-resistance cassette in the first exon of the *Hnf6* gene ($Hnf6^{-/-}$) and by inserting β-galactosidase coding sequences in-frame into the first exon of the *Oc2* gene ($Oc2^{lacZ/lacZ}$).

OC-2 is expressed in the hepatic epithelial cells throughout liver development, starting in the endoderm. Since β-galactosidase activity mimics OC-2 expression (Clotman et al., 2005), liver morphology can be assessed by β-galactosidase staining of embryos that have an allele with a *lacZ* knockin. Inactivation of one *Oc2* allele ($Hnf6^{+/+};Oc2^{+/lacZ}$) did not perturb the expected normal morphology at E10.5 (Fig. 1A). The inactivation of two alleles of *Oc2*, even in association with one inactivated allele of *Hnf6* ($Hnf6^{+/-};Oc2^{lacZ/lacZ}$), did not produce significant changes in liver morphology (Fig. 1B), indicating that the lack of OC-2 alone does not impact on early liver expansion. In contrast, when embryos had a homozygous inactivation of the *Hnf6* gene combined with inactivation of one or two alleles of *Oc2* ($Hnf6^{-/-};Oc2^{+/lacZ}$ and $Hnf6^{-/-};Oc2^{lacZ/lacZ}$ embryos), the liver was strongly hypoplastic (Figs. 1C, D). The morphology of livers that lack HNF-6 expression ($Hnf6^{-/-};Oc2^{+/+}$) was visualized by whole mount staining with a Prox1 antibody at E10.5. The results showed only a minor reduction in liver size in the absence of HNF-6 as compared to controls (Figs. 1L–N). Since the phenotypes of $Hnf6^{-/-};Oc2^{+/lacZ}$ and $Hnf6^{-/-};Oc2^{lacZ/lacZ}$ embryos were indistinguishable, they are hereafter called *Hnf6/Oc2*-deficient embryos, and wild-type or $Hnf6^{+/+};Oc2^{+/lacZ}$ embryos were used as controls.

To determine at which developmental stage liver expansion becomes impaired in the *Hnf6/Oc2*-deficient embryos, we analyzed earlier stages of development. At E9.5 these embryos showed a hypoplastic and less irregular liver bud as compared to the control embryos (Figs. 1E–H). However, slightly earlier, at E9, no evidence of liver hypoplasia was found (Figs. 1I–K), suggesting that impaired liver expansion starts between E9 and E9.5.

From this set of experiments, we concluded that HNF-6 and OC-2 exert a redundant activity on early liver expansion.

HNF-6 and OC-2 control early hepatoblast migration

To determine how HNF-6 and OC-2 control early liver development, we first envisaged the possibility that liver hypoplasia in *Hnf6/Oc2*-deficient embryos resulted from abnormal hepatic differentiation. At E9 (18–21 somites), *Hnf6/Oc2*-deficient hepatoblasts expressed Hex, Prox1, GATA-6 and HNF-4α (Figs. 2A–H), as in controls. In addition, in control and *Hnf6/Oc2*-deficient embryos, the septum transversum expressed GATA-6 and GATA-4, and GATA-4 was absent from the hepatoblasts (Figs. 2C, D and I, J). Moreover, the expression of HNF-1β which was measured by quantitative RT-PCR, was normal in the mutant embryos (Fig. 5A). Therefore, we found no evidence for abnormal differentiation in *Hnf6/Oc2*-deficient embryos.

Liver hypoplasia in these embryos might also result from reduced proliferation of the hepatoblasts or of the STM. This was measured by double immunofluorescent staining of liver buds with antibodies against the hepatoblast marker HNF-4α or the STM marker GATA-4, and the proliferation marker phosphohistone H3 (Figs. 2O–R). At E9.5, the percentage of proliferating hepatoblasts was 17.8±2.7% (mean±SEM, $n=6$) in control embryos, and 11.9±2.3% (mean±SEM, $n=7$) in *Hnf6/Oc2*-deficient embryos. However, this difference was not statistically significant (p value=0.12, Student's t test). Also, the percentage of proliferating STM cells was 14.6±1.5 (mean±SEM, $n=3$) in control embryos, and 11.3±0.9 (mean±SEM, $n=3$) in *Hnf6/Oc2*-deficient embryos. Again, this difference was not statistically significant (p value=0.13, Student's t test).

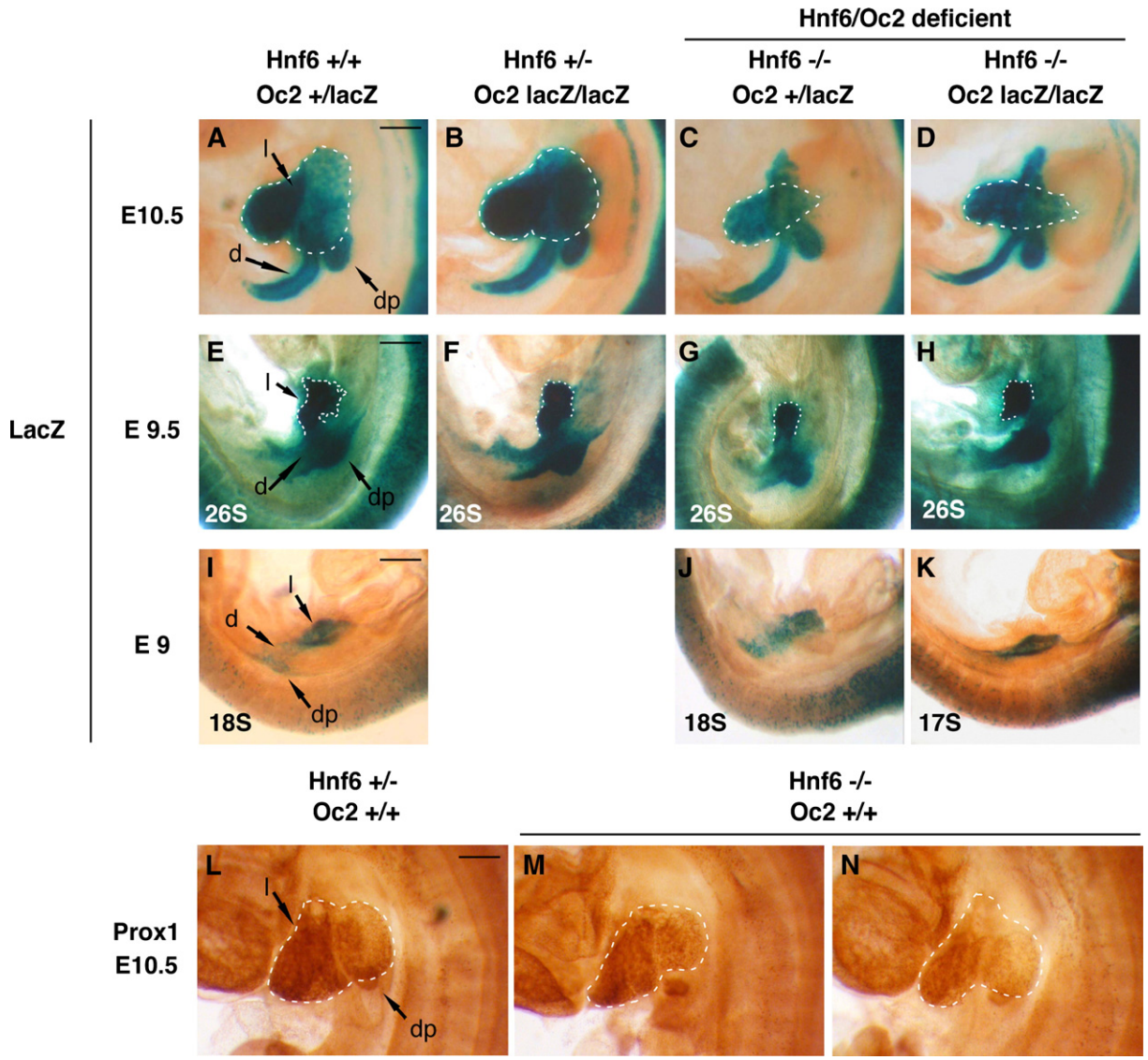

Fig. 1. HNF-6 and OC-2 control early liver expansion. (A–K) Liver morphology from E9 to E10.5 is assessed by staining for β-galactosidase activity in embryos in which at least one *Oc2* allele contains an in-frame knockin of the LacZ gene. (A–D) At E10.5, inactivation of two alleles of *Oc2* in association with one inactivated allele of *Hnf6* does not induce significant changes in liver morphology as compared to control embryos, whereas homozygous inactivation of the *Hnf6* gene combined with inactivation of one or two alleles of *Oc2* induces liver hypoplasia. (E–H) At E9.5, *Hnf6/Oc2*-deficient embryos show liver hypoplasia. (I–K) At E9.0, no evidence for liver hypoplasia is found, suggesting that impaired liver growth starts between E9 and E9.5. (L–N) At E10.5, whole-mount Prox1 labeling shows minor reduction in liver size in the absence of HNF-6 as compared to controls. l: liver bud, dp: dorsal pancreatic bud. The dotted lines surround the liver. Scale bars, 250 μm.

Endothelial cells are known to control liver bud growth (Matsumoto et al., 2001). Therefore, we verified if these cells were present in *Hnf6/Oc2*-deficient embryos by performing PECAM staining on liver bud sections. The results showed that at E9 (18–21 somites), endothelial cells were found in *Hnf6/Oc2*-deficient embryos as in controls (Figs. 2E, F).

Another possible cause of liver hypoplasia in *Hnf6/Oc2*-deficient embryos is apoptosis. This possibility was addressed by performing TUNEL assays on sections co-stained for the hepatoblast marker Prox1 or for the STM marker GATA-4. No significant apoptosis was detected on sections from control or *Hnf6/Oc2*-deficient embryos (Figs. 2K–N). Other regions of the embryo served as positive control for the TUNEL assay (data not shown).

Given the regular shape of liver buds in *Hnf6/Oc2*-deficient embryos as compared to control embryos at E9.5 (Figs. 1E–H), we hypothesized that HNF-6 and OC-2 control hepatoblast migration. We sectioned embryos that had been stained for β-galactosidase activity at E9.5 (25–26 somites) and analyzed the morphology of the liver bud. It appeared multilayered and pseudostratified in all embryos, but delamination of the hepatoblasts and invasion of the STM was impaired in *Hnf6/Oc2*-deficient embryos (Figs. 3A–C). Defective delamination was transient, since at later stages the hepatoblasts have invaded the STM and have colonized the liver lobes (Figs. 4M, N). The lack of liver bud expansion in *Hnf6/Oc2*-deficient embryos is reminiscent of the phenotype of Prox1-null embryos, in which hepatoblasts do not migrate into the STM, possibly as a result

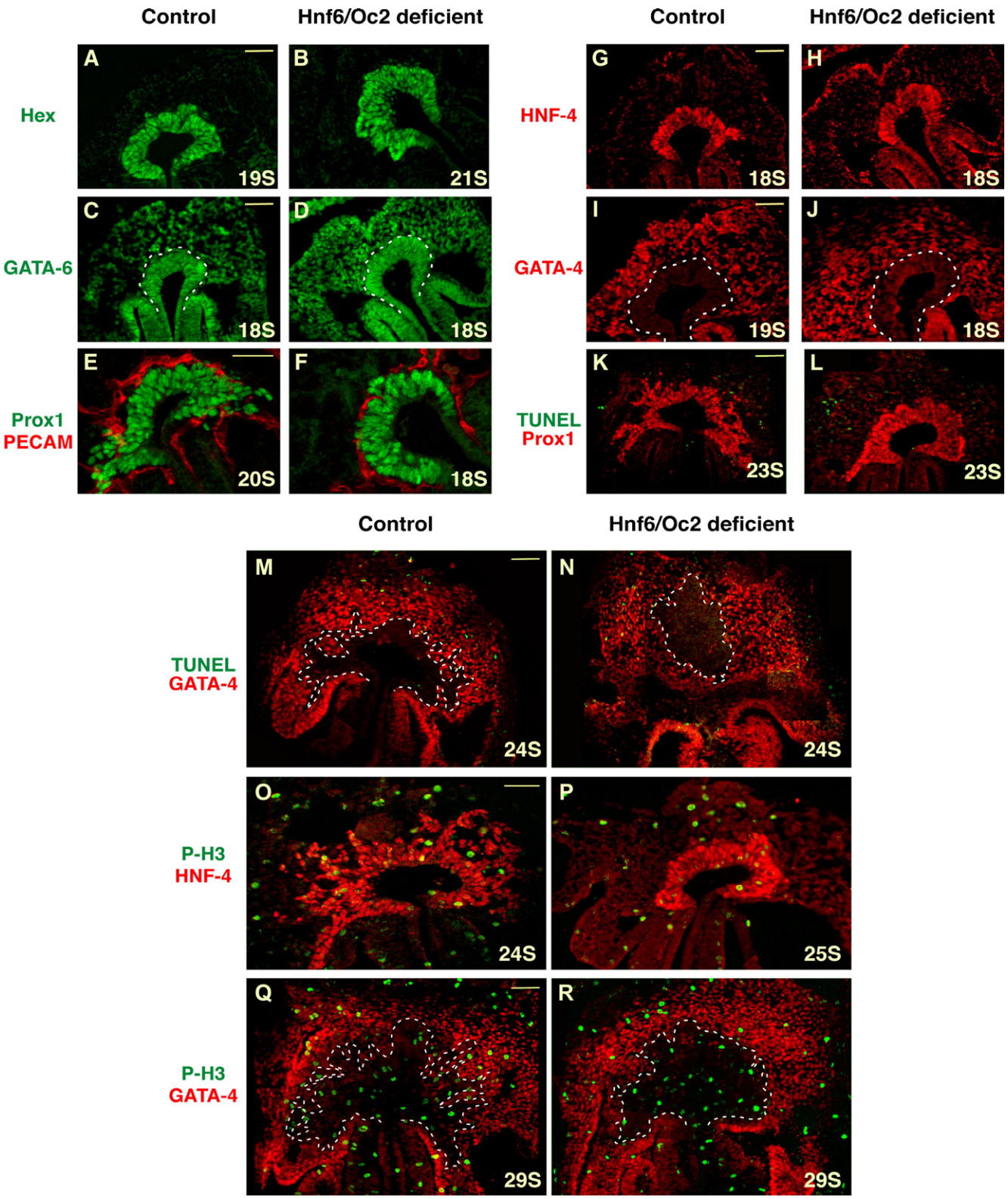

Fig. 2. HNF-6 and OC-2 are not involved in early liver differentiation, apoptosis, proliferation, or in the maintenance of endothelial cells around the liver bud. (A–J) Immunofluorescence analyses were performed on transverse sections of *Hnf6*$^{+/+}$;*Oc2*$^{+/LacZ}$ and *Hnf6*$^{-/-}$;*Oc2*$^{+/LacZ}$ liver buds at E9 (18–21 Somites). The expression of the early hepatic markers Hex, GATA-6, Prox1, HNF-4α and GATA-4 is not affected in the absence of HNF-6 and OC-2. (E, F) Endothelial cells are present around the liver bud of control and *Hnf6/Oc2*-deficient embryos, as detected by PECAM staining. (K–N) The liver hypoplasia in *Hnf6/Oc2*-deficient embryos is not due to apoptosis of hepatoblasts or STM cells. TUNEL assay was performed in parallel with immunofluorescent detection of Prox1 in order to delineate the liver bud or of GATA-4 to label the STM. (O–R) Proliferation is not perturbed in the absence of HNF-6 and OC-2. Phosphohistone H3 positive cells were co-detected with HNF-4α or GATA-4 to label the hepatoblasts and the STM, respectively. The dotted lines delineate the liver bud and migrating hepatoblasts. Scale bars, 50 μm.

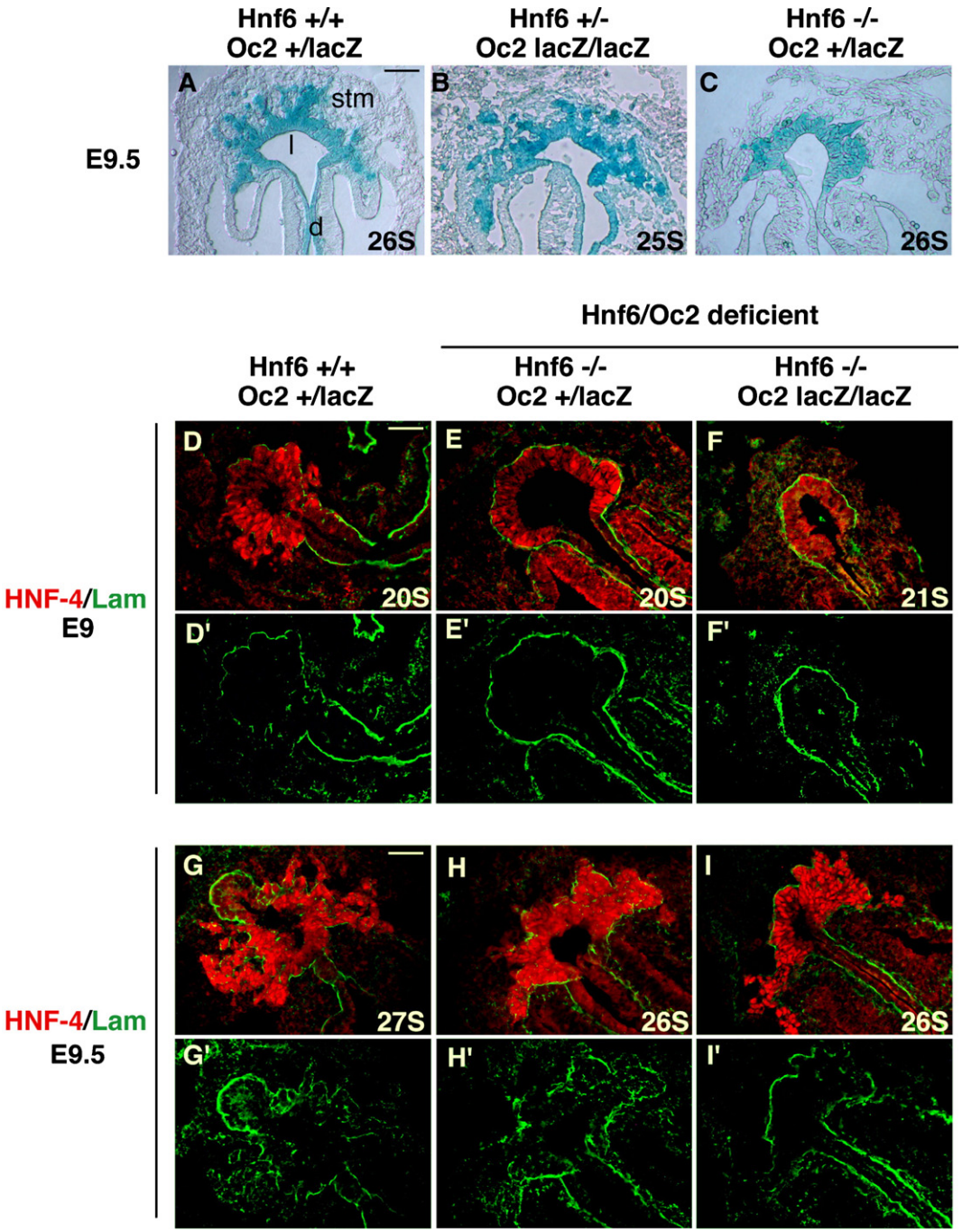

Fig. 3. HNF-6 and OC-2 control hepatoblast migration and basement membrane degradation. (A–C) E9.5− embryos (25–26 somites) were stained for β-galactosidase activity and sectioned. The hepatoblasts of control and $Hnf6^{+/-};Oc2^{LacZ/LacZ}$ embryos migrate out of the liver bud and invade the septum transversum mesenchyme. By contrast, hepatoblasts of *Hnf6/Oc2*-deficient embryos fail to migrate into the surrounding mesenchyme. (D–I) The degradation of the basal lamina is delayed in *Hnf6/Oc2*-deficient embryos. At E9.0, the hepatoblasts, which express HNF-4α, are surrounded by a laminin layer that is already partially degraded in control embryos but not yet in *Hnf6/Oc2*-deficient embryos. At A9.5 (26–27 somites), the laminin layer is degraded in all embryos, but the number of *Hnf6/Oc2*–deficient hepatoblasts that have invaded the STM is reduced as compared to controls. Panels D–I show laminin and HNF-4α staining, panels D'–I' only show the laminin staining. Scale bars, 50 μm.

from impaired breakdown of the laminin layer surrounding the liver bud (Sosa-Pineda et al., 2000). As mentioned above, *Hnf6/Oc2*-deficient embryos have normal expression of Prox1 (Figs. 2E, F, K, L and 5B). However, in these embryos, the degradation of the basal membrane surrounding the liver bud is slightly delayed. Indeed, in *Hnf6/Oc2*-deficient embryos at E9 (20 somites), the liver bud is surrounded by a continuous layer of laminin, whereas the latter is already degraded in controls (Figs. 3D–F and D'–F'). At E9.5 (26–27 somites), the laminin layer is degraded in all embryos, but the number of *Hnf6/Oc2*-deficient hepatoblasts that have invaded the STM is reduced as compared to controls (Figs. 3G–I). In Prox1-null embryos, the

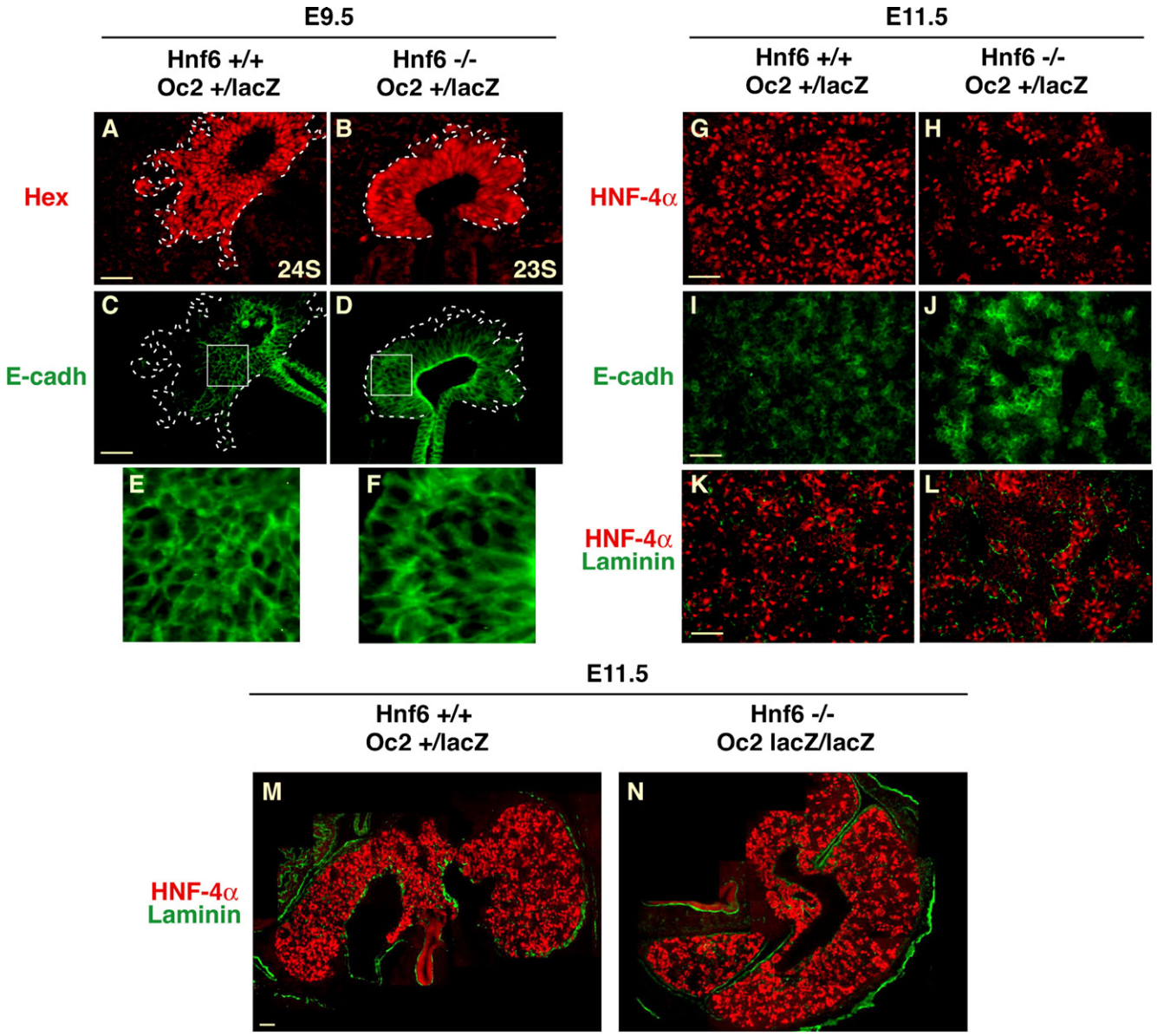

Fig. 4. HNF-6 and OC-2 control E-cadherin expression in early liver development. (A–F) Transverse sections of *Hnf6*$^{+/+}$;*Oc2*$^{+/LacZ}$ and *Hnf6*$^{-/-}$;*Oc2*$^{+/LacZ}$ embryos at E9.5 show that the hepatoblasts, which remain associated with the liver bud, express normal levels of E-cadherin. Embryos were co-stained with a Hex antibody to allow detection of the liver bud. Panels E and F are higher magnification pictures of the boxed area in panels C and D. (G–N) At E11.5, the *Hnf6*$^{-/-}$;*Oc2*$^{+/LacZ}$ hepatoblasts colonize the liver lobes, but form clusters that are surrounded by laminin and show higher E-cadherin levels than in controls. HNF-4α detection is used to identify the hepatoblasts. The dotted lines surround the liver. Panels M and N are low magnification views to illustrate the colonization of liver lobes by the hepatoblasts. Scale bars, 50 μm.

failure to invade the STM is associated with increased expression of E-cadherin (Sosa-Pineda et al., 2000). In *Hnf6/Oc2*-deficient embryos at E9.5 hepatoblasts remained associated with the liver bud but expressed normal levels of E-cadherin (Figs. 4A–F and 5C). In contrast, at E11.5, they had invaded the STM, but formed clusters that expressed higher levels of E-cadherin than in controls (Figs. 4G–L) and which were surrounded by laminin. This was confirmed by quantitative RT-PCR experiments in which the concentration of E-cadherin mRNA, normalized for Prox1 mRNA, was significatively increased in *Hnf6/Oc2*-deficient embryos (Fig. 5D).

Taken together, the data suggest that HNF-6 and OC-2 are not involved in early differentiation, proliferation or apoptosis, but that they control hepatoblast migration, E-cadherin expression and degradation of the basement membrane at the onset of liver development.

HNF-6 and OC-2 control the expression of genes involved in cell migration and adhesion

To further investigate the mechanism by which HNF-6 and OC-2 control early liver expansion, we analyzed the expression of genes that relate to extracellular matrix biology and cell adhesion. We resorted to an array approach allowing to test the expression of 113 genes (oligo GEArray; see Methods). The analysis was performed to compare the RNA of pooled control

($n=3$) and *Hnf6*$^{-/-}$;*Oc2*$^{+/lacZ}$ ($n=4$) liver buds microdissected at E9 (21 somites). Each gene was spotted in quadruplicate on the GE array. The results are shown in Table 1.

Among the matrix degrading enzymes, detectable expression was found for matrix-metalloprotease (MMP)-2 and -14. In *Hnf6/Oc2*-deficient embryos, their expression, as well as that of basal lamina components, was normal. These data suggested that delayed degradation of the basement membrane in *Hnf6/Oc2*-deficient embryos does not result from overproduction of its constituents or from deficient expression of matrix-degrading enzymes.

A number of proteins are involved in the control of cell adhesion and migration. As shown in Table 1, Thrombospondin-4 (Thbs4) was upregulated in *Hnf6/Oc2*-deficient embryos. This was confirmed by quantitative RT-PCR (2.6-fold increase, Fig. 5E). Thbs4 is a glycoprotein of the extracellular matrix with unknown function in the liver. However, it enhances the ability of laminins to promote adhesion and neurite outgrowth of embryonic retinal cells (Arber and Caroni, 1995; Dunkle et al., 2007), raising the possibility that an excess of Thbs4 decreases hepatoblast migration.

Interestingly, the array experiments also indicated that the expression of osteopontin (OPN), also called secreted phosphoprotein-1 (*Spp1*) or early T-cell activation-1 (ETA-1), was downregulated in *Hnf6/Oc2*-deficient embryos. Quantitative RT-PCR experiments confirmed the array data and showed that OPN expression is decreased 47-fold at E9.0 (Fig. 5F). This reduction is stronger than that measured with the GEArray data, because the OPN mRNA levels in *Hnf6/Oc2*-deficient embryos had reached the background for detection on the array. OPN is a secreted protein of the *s*mall *i*ntegrin-*b*inding *li*gand *N*-linked glycoprotein (SIBLING) family, to which a wide range of biological functions have been ascribed (reviewed in Denhardt et al., 2001; Mazzali et al., 2002, Rangaswami et al., 2006). It binds to several receptors including integrins and variants of the hyaluronan receptor CD44. Most relevant to the present work is its proposed role in promoting scattering and migration of cultured embryonic hepatic cells (Medico et al., 2001), suggesting that decreased expression of OPN in *Hnf6/Oc2*-deficient embryos contributes to impaired hepatoblast migration. Therefore, we analyzed OPN knockout embryos (Rittling et al., 1998), but found no evidence for defective hepatoblast migration during early liver development (data not shown).

A number of proteins are considered to have potentially redundant roles with OPN. These include other members of the SIBLING family such as bone sialoprotein (Bsp) and dentin matrix protein (Dmp) (Fisher et al., 2001; Wilson et al., 2005), or ligands, such as vitronectin and hyaluronan that, like OPN, bind to integrin αVβ3 and to CD44, respectively. We measured

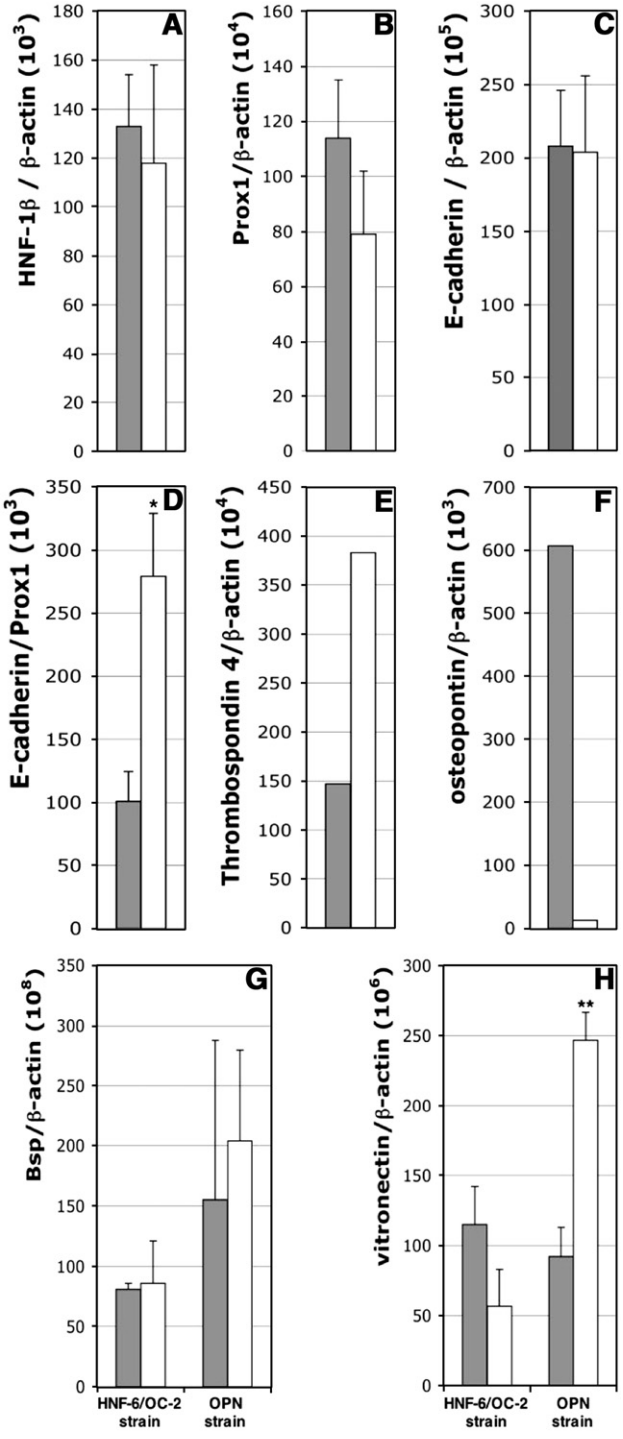

Fig. 5. HNF-6 and OC-2 control the expression of genes in early liver development. Total RNA was extracted from livers at E9.5 or E11.5 and expression of genes was measured by quantitative RT-PCR. Grey bars represent control livers and open bars represent mutant livers. (A–C) The expression of HNF-1β, Prox1 and E-cadherin is normal in the absence of HNF-6 and OC-2 at E9.5 (control: $n=7$; *Hnf6/Oc2*-deficient: $n=5$). (D) At E11.5, the expression of E-cadherin is significantly increased in *Hnf6/Oc2*-deficient hepatoblasts. (*$p=0.015$; control: $n=4$; *Hnf6/Oc2*-deficient: $n=6$). (E, F) At E9, the expression of thrombospondin 4 is 2.6-fold increased whereas those of osteopontin is 47-fold decreased in *Hnf6*$^{-/-}$;*Oc2*$^{+/LacZ}$ liver buds, confirming the GE array analysis. The analysis was performed on pooled control ($n=3$) or *Hnf6*$^{-/-}$;*Oc2*$^{+/LacZ}$ ($n=4$) liver buds. (G) At E9.5, the expression of bone sialoprotein is normal in *Hnf6/Oc2*-deficient and in OPN knockout livers (controls: $n=3$; *Hnf6/Oc2*-deficient $n=4$; OPN knockout: $n=3$). (H) The expression of vitronectin at E9.5 is normal in *Hnf6/Oc2*-deficient liver (control: $n=3$; *Hnf6/Oc2*-deficient $n=4$), but is significantly increased in OPN knockout embryos (**$p=0.006$; control: $n=3$; OPN knockout; $n=3$). Given that *Hnf6/Oc2*-deficient and OPN knockout embryos are not in the same genetic background, the data on the two strains, named HNF-6/OC-2 and OPN strains, are shown separately in panels G and H.

Table 1
RNA from pooled control livers ($n=3$) was compared with that from *Hnf6*$^{-/-}$; *Oc2*$^{+/lacZ}$ ($n=4$) livers microdissected at E9 (21 somites)

	Symbol	Control	*Hnf6*$^{-/-}$; *Oc2*$^{+/lacZ}$	Fold increase
Gene				
Thrombospondin 4	Thbs4	7624	23,214	3.0
Vitronectin	Vtn	18,441	27,257	1.5
Thrombospondin 3	Thbs3	16,687	21,196	1.3
Tissue inhibitor of metalloproteinase 2	Timp2	17,700	20,831	1.2
Tissue inhibitor of metalloproteinase 3	Timp3	18,275	19,290	1.1
Catenin beta	Catnb	53,074	53,480	1.0
Catenin alpha 1	Catna1	52,440	51,331	1.0
Fibronectin 1	Fn1	51,998	49,475	1.0
Elastin microfibril interfacer 1	Emilin1	37,642	35,624	0.9
Fibulin 1	Fbln1	36,908	34,636	0.9
Secreted acidic cysteine rich glycoprotein	Sparc	48,677	45,344	0.9
Procollagen, type VI, alpha 1	Col6a1	32,985	30,189	0.9
Procollagen, type VI, alpha 2	Col6a2	26,193	23,954	0.9
Laminin B1 subunit 1	Lamb1-1	52,070	46,867	0.9
Procollagen, type IV, alpha 3	Col4a3	28,126	25,266	0.9
Procollagen, type XVIII, alpha 1	Col18a1	46,726	40,135	0.9
Periostin, osteoblast specific factor	Postn	50,652	43,342	0.9
Procollagen, type V, alpha 1	Col5a1	45,098	37,612	0.8
Cadherin 2	Cdh2	41,985	34,619	0.8
Sarcoglycan, epsilon	Sgce	39,933	32,729	0.8
Chondroitin sulfate proteoglycan 2	Cspg2	42,753	34,620	0.8
Procollagen, type IV, alpha 6	Col4a6	29,228	23,485	0.8
Extracellular matrix protein 1	Ecm1	41,690	32,909	0.8
Integrin beta 1 (fibronectin receptor beta)	Itgb1	44,179	34,699	0.8
Procollagen, type I, alpha 1	Col1a1	37,537	29,472	0.8
Procollagen, type III, alpha 1	Col3a1	43,141	33,300	0.8
Procollagen, type IV, alpha 2	Col4a2	36,761	28,225	0.8
Procollagen, type IV, alpha 1	Col4a1	42,935	32,740	0.8
Matrix metalloproteinase 2	Mmp2	47,575	36,258	0.8
Matrix metalloproteinase 14	Mmp14	49,341	36,719	0.7
Connective tissue growth factor	Ctgf	37,992	27,809	0.7
A disintegrin-like metalloprotease with thrombospondin 1 motif, 2	Adamts2	29,971	21,679	0.7
Transforming growth factor, beta induced	Tgfbi	45,425	32,303	0.7
Hyaluronan and proteoglycan link protein 1	Hapln1	29,207	20,530	0.7
Secreted phosphoprotein 1 (osteopontin)	Spp1	38,386	14,135	0.4
House keeping gene				
Beta-2 microglobulin	B2m	32,593	40,831	1.3
Heat shock protein 1, beta	Hspcb	53,347	53,949	1.0
Peptidylprolyl isomerase A	Ppia	54,917	54,842	1.0
Heat shock protein 1, beta	Hspcb	54,226	54,134	1.0
Peptidylprolyl isomerase A	Ppia	55,302	54,755	1.0
Ribosomal protein S27a	Rps27a	53,246	52,680	1.0
Biotinylated Artificial Sequence 2	BAS2C	55,187	54,378	1.0
Biotinylated Artificial Sequence 2	BAS2C	54,911	51,399	0.9

Each gene was spotted in quadruplicate on the GE array. Values correspond to arbitrary densitometric units provided by the GEArray Analyzer software.

the expression of the corresponding genes and of Hyaluronan synthases (HAS)-1 and -2 by quantitative RT-PCR in control, *Hnf6/Oc2*-deficient and in OPN knockout livers. We found no detectable expression of Dmp and of HAS-1 and HAS-2, and no significant difference in the levels of Bsp (Fig. 5G). Vitronectin was overexpressed in the livers of OPN knockout mice (Fig. 5H), indicating that the absence of OPN induces potentially compensatory mechanisms. However in *Hnf6/Oc2*-deficient embryos, we detected no significant change in vitronectin expression as compared to controls (Fig. 5H).

In conclusion, the morphological analysis and the gene expression profile of livers deficient in HNF-6 and OC-2 show that these two factors control the expression of a network of genes comprising Thbs4, OPN and E-cadherin, that are involved in cell adhesion and migration, and whose deregulation during early liver development is associated with impaired hepatoblast migration and enhanced cell clustering.

Discussion

In the present paper, we investigated the role of the Onecut transcription factors HNF-6 and OC-2 in early liver development and found that they exert a redundant control on early liver expansion. The absence of both factors in knockout mice is associated with delayed degradation of the basement membrane surrounding the liver bud, impaired hepatoblast migration into the STM, increased hepatoblast clustering, and perturbed expression of a network of genes involved in cell adhesion and migration.

Perturbed degradation of the basal lamina surrounding the liver bud, and impaired migration of hepatoblasts are characteristics of *Hnf6/Oc2*-deficient embryos, but are also hallmarks of embryos deficient in the transcription factor Prox1 (Sosa-Pineda et al., 2000). This suggests that HNF-6, OC-2 and Prox1 are involved in a common gene regulatory network controlling specific aspects of early liver development. While the expression of Prox1 is unaffected in *Hnf6/Oc2*-deficient embryos, it is unknown if HNF-6 and/or OC-2 are regulated by Prox1. The fact that HNF-6, OC-2 and Prox1 may belong to common gene regulatory networks is further underscored by the observation that the pancreatic phenotypes of Prox1-null and HNF-6-null embryos bear significant similarities: both knockout embryos show hypoplastic pancreas and impaired development of endocrine progenitors starting around e12.5 (Jacquemin et al., 2000, 2003; Wang et al., 2005). Furthermore, Prox1 stimulates OPN expression in the pancreas (Wang et al., 2005), as do HNF-6 and OC-2 in the liver.

In *Hnf6/Oc2*-deficient embryos, we detected a delay in the degradation of the basement membrane surrounding the liver bud. Gene expression analysis using oligoGEArrays failed to provide evidence for abnormal expression of basement membrane components or of extracellular matrix-degrading enzymes. However, our data cannot rule out the possibility that HNF-6 and OC-2 indirectly control the post-translational regulation of MMP activity.

Hnf6/Oc2-deficient embryos showed reduced migration of the hepatoblasts from the liver bud into the STM and our analysis demonstrated that HNF-6 and OC-2 control the expression of OPN, Thbs4 and E-cadherin, which are involved in cell migration and adhesion. OPN was downregulated in *Hnf6/Oc2*-deficient embryos. It belongs to the SIBLING family of matri-

cellular proteins, and, among its multiple functions, OPN promotes cell migration and cancer invasiveness (reviewed in Denhardt et al., 2001; Mazzali et al., 2002, Rangaswami et al., 2006). In vitro, OPN promotes scattering and migration of embryonic liver cells (Medico et al., 2001). Downregulation of OPN may contribute to the decreased hepatoblast migration in *Hnf6/Oc2*-deficient embryos, but it is not sufficient given that OPN knockout mice have normal hepatoblast migration. Vitronectin, which, like OPN, binds to integrin αVβ3, is a good candidate to exert redundant functions with OPN. Interestingly, vitronectin expression was upregulated in OPN knockout embryos, suggesting that it may compensate for the absence of OPN and allow normal hepatic development in these embryos. By contrast, there was no vitronectin upregulation in *Hnf6/Oc2*-deficient embryos and we suggest that this may result in the absence of compensation, leading to a deficient hepatoblast migration. Little information is available about the function of Thbs4. Thrombospondins are matricellular proteins that promote disruption of focal adhesions and reorganization of actin stress fibbers, thereby enabling cellular migration (Borstein and Sage, 2002). Thbs4 is known to promote adhesion of embryonic retinal cells to extracellular matrix proteins (Dunkle et al., 2007), raising the possibility that it modulates hepatoblast cell migration. The regulation of the E-cadherin expression is complex and is notably important for the modulation of the migration of different type of cells. Indeed, the repression of E-cadherin decreases cell–cell interactions and thereby favors the migration of epithelial type cells. The upregulation of E-cadherin might contribute to the impaired hepatoblast migration and increased cell clustering at the onset of liver development, as seen in *Hnf6/Oc2*-deficient embryos. Collectively, our data suggest that HNF-6 and OC-2 fine-tune a network of genes involved in cell adhesion and migration, and that the unbalanced expression of those genes in the absence of Onecut factors leads to impaired migration and enhanced cell clustering.

Our data do not imply that HNF-6 and OC-2 exert a direct regulation of the genes, which are up- or downregulated in *Hnf6/Oc2*-deficient embryos. However, Odom et al. (2007) identified E-cadherin and vitronectin as direct targets of HNF-6 in adult hepatocytes, suggesting that at least part of the network of genes regulating cell adhesion and migration is directly controlled by the Onecut factors.

Expression of a number of cell adhesion molecules, including E-cadherin, is co-ordinately stimulated by HNF-4α (Battle et al., 2006), and our results, together with those of Sosa-Pineda and colleagues (2000), show that HNF-6, OC-2 and Prox1 coordinately repress E-cadherin expression. Although the control exerted on E-cadherin by HNF-4α is described at later stages of liver development than its regulation by HNF-6, OC-2 and Prox1, these data collectively suggest that hepatic cell adhesion during development relies on a balance of the effect of all four transcription factors. Cancer invasiveness and metastasis formation are associated with loss of cell–cell interaction, E-cadherin repression, basement membrane degradation and increased cell motility. Also, OPN, which we show here to be a target of HNF-6 and OC-2, is a marker of hepatocarcinoma and promotes angiogenesis, tumor invasion, migration and metastatic spread (Denhardt et al., 2001; Mazzali et al., 2002; Rangaswani et al., 2006). Therefore, our findings in developing liver raise the possibility that a network comprising HNF-6, OC-2, Prox1 and HNF-4α plays a role in hepatic cancer.

In conclusion, the present work uncovers the role of a gene network operating at the onset of liver development to control liver bud expansion, and provides candidate genes for investigation of liver carcinogenesis.

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