The Transcription Factor Hepatocyte Nuclear Factor-6/Onecut-1 Controls the Expression of Its Paralog Onecut-3 in Developing Mouse Endoderm

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During development, the endoderm gives rise to several organs, including the pancreas and liver. This differentiation process requires spatial and temporal regulation of gene expression in the endoderm by a network of tissue-specific transcription factors whose elucidation is far from complete. These factors include the Onecut protein hepatocyte nuclear factor-6 (HNF-6), which controls pancreas and liver development as shown in our previous work on Hnf6 knock-out embryos. In mammals, HNF-6 has two paralogs, Onecut-2 (OC-2) and OC-3, whose patterns of expression in the adult overlap with that of HNF-6. In the present work, we examine the expression profile of the three Onecut factors in the developing mouse endoderm. We show that HNF-6, OC-2, and OC-3 are expressed sequentially, which defines new steps in endoderm differentiation. By analyzing Hnf6 knock-out embryos we find that HNF-6 is required for expression of the Oc3 gene in the endoderm. We show that OC-3 colocalizes with HNF-6 in the endoderm and in embryonic pancreas and liver. Based on transfection, chromatin immunoprecipitation, and whole embryo electroporation experiments, we demonstrate that HNF-6 can bind to and stimulate the expression of the Oc3 gene. This study identifies a regulatory cascade between two paralogous transcription factors, sheds new light on the interpretation of the Hnf6 knock-out phenotype, and broadens the transcription factors network operating during development of the endoderm, liver, and pancreas.

In the mouse, cells in the definitive endoderm differentiate from embryonic day 8 (4–6-somite stage) to form the epithelial lining of the primitive gut and associated organs (reviewed in Refs. 1 and 2). These organs arise from the gut by budding into the surrounding mesenchyme. This process requires precise signaling, in time and space, from the mesoderm and the regionalized expression of tissue-specific transcription factors in the endoderm. This unique combination of transcription factors in the endoderm delineates a specific transcriptional network that specifies the presumptive organ territories. For instance, during development of the liver and pancreas, two organs that derive from adjacent endodermal regions, a core endodermal program is constituted by the expression of transcription factors such as the FoxA factors, the GATA factors, Hb9, Pdx-1, Hex, HNF-1, HNF-4, Nr5a2, and HNF-6 (reviewed in Refs. 3 and 4). Not all the components of this endodermal program have been identified and the auto- and cross-regulatory loops that control their function are not well understood. Elucidation of this network is required for full understanding of pancreas and liver development and for implementing such knowledge in the controlled differentiation of cells for cell therapy.

Hepatocyte nuclear factor-6 (HNF-6) belongs to the Onecut (OC) family of transcription factors (5, 6). These factors possess a single cut domain, hence their name, and a divergent homeodomain, which are both involved in DNA binding. Onecut proteins have been identified not only in humans and rodents, but also in model organisms such as Caenorhabditis elegans (6), Drosophila melanogaster (7), and Danio rerio (8). In addition to Hnf6, mammalian genomes contain two other Onecut genes called Oc2 and Oc3 (9, 10). The sequence conservation in their cut and homeodomains suggests that these paralogs may bind the same genes, and indeed many HNF-6 binding sites on DNA are recognized by OC-2 and OC-3 (9, 10). During mouse development, HNF-6 is first detected at e8.5 in the foregut-midgut region. As development proceeds, HNF-6 becomes restricted to the liver and the pancreas (11, 12). Expression of Oc2 in the endoderm is superimposable to that of Hnf6 (13), and later on it is restricted to the liver, pancreas, gut, and stomach. Nothing is known on the endodermal expression of OC-3, but at later stages Oc3 is found in the gut and stomach (10). Thus, the three Onecut factors share partially overlapping expression domains and may be functionally redundant.

Inactivation of the mouse Hnf6 gene revealed that it is important for development of the pancreas at the precursor stage in the endoderm (14). Thus, Hnf6 knock-out embryos display a pancreatic hypoplasia that results from defects in early pancreas specification. Indeed, the onset of Pdx-1 expression is delayed in the endoderm of Hnf6 knock-out embryos, leading to a reduced number of specified cells in the pancreatic territory.

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at the time of budding. Consistent with this, HNF-6 can bind to and stimulate transcription of the Pdx1 gene (14).

In the present work, we wished to further characterize the transcription factor network in the endoderm. We have examined the time course of expression of the three Oct factors in the developing endoderm of normal mouse embryos and have investigated whether inactivation of Hnf6 influences the expression of Oc2 and Oc3. We show here that, in the endoderm of Hnf6 knock-out embryos, Oc2 expression is unaffected, but Oc3 expression is abolished. We demonstrate that HNF-6 can induce Oc3 expression and we provide evidence that this is a direct effect. Our work defines new stages in endoderm differentiation and identifies a paralogous regulatory cascade in the Octen transcription factors, which operates during differentiation of the endoderm, liver, and pancreas.

EXPERIMENTAL PROCEDURES

Animals—The Hnf6 knock-out mice, raised in our animal facility and treated according to the principles of laboratory animal care of the University Animal Welfare Committee, were obtained as described (15). The Hnf6+/− mice were backcrossed to C57Bl/6J to produce Hnf6+/−, Hnf6−/− (Jackson) or using a goat anti-guinea pig antibody coupled to Alexa molecular Probes), using a goat anti-mouse antibody coupled to Texas Red (Santa Cruz), rabbit anti-Pdx-1 at 1:1000 (a kind gift from C. V. Wright), rabbit anti-Prox-1 at 1:1000 (Covance), and guinea pig anti-Prox1 at 1:100 (BD Transduction Laboratories), rabbit anti-HNF-6 at 1:25 (Upstate) for 16 h with 5 μg of the CMV promoter-driven pcDNA3-EGFP (Invitrogen), pCMV-Flag/HNF-6, or pCMV-Flag/HNF-6-VP16 expression vectors. To perform chromatin immunoprecipitation experiments were transcribed in 10-cm dishes for 20 h with 15 μg of the pCMV-Flag/HNF-6 expression vector.

Protein Extraction and Western Blotting—Protein extraction was performed according to the manufacturer’s instructions on the same samples from which RNA was isolated with the Tripure reagent. Equal volumes were loaded on SDS-PAGE gels, as protein concentration could not be measured. Western blotting was performed as described (21) with anti-GFP (Molecular Probes) or anti-FLAG (M2, Sigma) antibodies.

Chromatin Immunoprecipitation—This was performed essentially as described (16). On 106 BMEL cells transfected with the Flag/HNF-6 expression vector. DNA-protein complexes were cross-linked by addition of formaldehyde to the culture medium (final concentration 1%). After 10 min at room temperature, the cross-linking reaction was stopped by addition of glycerol (final concentration 0.125 M) for 5 min. Cells were washed and harvested by scraping into phosphate-buffered saline containing 0.5 mM phenylmethylsulfonyl fluoride. The cells were lysed in a buffer containing 10 mM Tris–Cl, pH 8.0, 0.5 mM KCl, 0.5 mM IGGPAL CA-630, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitors, and nuclei were collected. Nuclei were lysed in a buffer containing 50 mM Tris–Cl, pH 8.0, 10 mM EDTA, 1% SDS, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitors, and were sonicated to produce DNA fragments of 200 to 1000 bp in length. The samples were centrifuged to eliminate cell debris, and DNA-protein complexes were precleared with salmon sperm DNA/protein A-agarose (Upstate) for 15 min at 4 °C with rotation. Each sample was then diluted with 0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–Cl, pH 8.0, 167 mM NaCl, an aliquot was removed as “input” control, and the remaining sample was divided into two aliquots. M2 (anti-FLAG, Sigma) antibody was added to one aliquot, and anti-tubulin (Sigma) antibody was added to the second aliquot as a nonspecific antibody control. Samples were incubated with antibody overnight with rotation at 4 °C. The following day, salmon sperm DNA/protein A-agarose beads were added to the samples and incubated for 1–2 h at 4 °C with rotation to collect DNA-protein antibody complexes. Beads were washed and DNA-protein complexes were eluted by two successive incubations at 65 °C for 10 min with freshly prepared elution buffer (1% SDS, 0.5–1% sodium dodecyl sulfate). DNA samples were added to the eluted and input samples and incubated at 65 °C for 4–6 h to revert cross-links. Samples were precipitated overnight at −20 °C by addition of 2.5 volumes of 100% ethanol, resuspended in proteinase K buffer (10 mM Tris–Cl, pH 7.5, 5 mM EDTA, 0.25% SDS), and treated with proteinase K for 2 h at 45 °C. After this, DNA was purified by phenol/chloroform and chloroform extraction followed by ethanol precipitation. PCR was performed on 1/10th of the immunoprecipitated chromatin.

Whole Embryo Culture and Electroporation—Whole embryo culture and electroporation have been described in detail. Briefly, intact e8.5 embryos were incubated in a DNA solution and placed between the electrodes. Electroporation was performed using a square-wave pulse generator (ElecTro Square Porator; ECM 830, BTX). After electroporation, embryos were cultured for 24 h at 37 °C in a roller culture system (31 rpm). Electroporated embryos developed normally and were further dissected for RNA extraction or processed for paraffin embedding, as for normal e9.5 embryos.

RESULTS

Hnf6, Oc2, and Oc3 Are Expressed Sequentially in the Endoderm—Work from our laboratory has shown that HNF-6 is expressed in the mouse endoderm starting around the
have revealed expression of Oc2 in the endoderm of 14-somite (e9) embryos (13). No data are available on Oc-3 expression in the endoderm. To determine the level of expression of the three Onecut factors in the endoderm, we resorted to real time PCR experiments on RNA extracted from endoderm isolated by dispase treatment of the embryos at the 5-somite to the 14-somite stage. We also measured Pdx-1 mRNA as an endodermal marker. As shown in Table I, the three Onecut factors were expressed in the endoderm. HNF-6 mRNA was the most abundant and was clearly detectable at the 5-somite stage. The onset of Oc2 expression was delayed as compared with that of HNF6, being detectable at the 7-somite stage. Oc3 expression became significant only at the 14-somite stage. On e9.5, expression of Oc3 had increased to the point of reaching about 25% of that of Hnf6 at that stage (Fig. 1A). We conclude from these data that the three Onecut factors become expressed sequentially in the developing endoderm, in the order HNF-6, OC-2, OC-3.

**OC3 Expression Is Down-regulated in Hnf6 Knock-out Embryos**—As HNF-6 is the first Onecut factor to be expressed in the endoderm, we investigated whether it could regulate the expression of the two other Onecut factors. To this end, we examined the consequences of inactivating the Hnf6 gene. We compared control and Hnf6 knock-out embryos and determined the expression of Oc2 and Oc3 at e9.5 (22 somites) in the primitive gut region that will give rise to the liver, pancreas, stomach, and duodenum, and which corresponds to the HNF-6 expression territory. Whereas the expression of Oc2 was unaffected in the absence of HNF-6, the expression of Oc3 was undetectable as compared with controls (Fig. 1A). Expression of Oc3 in the primitive gut of Hnf6 knock-out embryos was also abolished at e10.5 (35 somites, Fig. 1B) and this persisted in the pancreas and liver at e12.5 (Fig. 1, C and D). We conclude that HNF-6 controls Oc3 gene expression in the endoderm and the developing pancreas and liver.

| Somites | Transcription factor mRNA copy number (transcription factor mRNA copy number/actin mRNA copy number) × 10^6. |
|---------|--------------------------------------------------------------------------------------------------|
| 5       | HNF-6: 37, OC-2: ND, OC-3: ND, Pdx-1: 10                                                                 |
| 6       | HNF-6: 81, OC-2: ND, OC-3: ND, Pdx-1: 40                                                                 |
| 7       | HNF-6: 1279, OC-2: 5, OC-3: 48, Pdx-1: 48                                                           |
| 9       | HNF-6: 1764, OC-2: 1, OC-3: 52, Pdx-1: 52                                                          |
| 14      | HNF-6: 1680, OC-2: 9, OC-3: 93, Pdx-1: 93                                                            |
| 16      | HNF-6: 2407, OC-2: 21, OC-3: 2, Pdx-1: 128                                                          |

* ND, not detectable.

**Fig. 1. Extinction of Oc3 expression in the endoderm and in developing pancreas and liver of Hnf6 knock-out mouse embryos.** Onecut expression levels were assessed by real time PCR on cDNA from individual control (wt, black bars) or Hnf6 knock-out (Ko, white bars) embryos. RNA was extracted from the primitive gut at e9.5 (A), e10.5 (B), or from the pancreas (C), or liver (D) at e12.5. The results are expressed as (One- cut mRNA copy number/actin mRNA copy number) × 10^6. Data are mean ± S.E. for at least 3 embryos.
indicates that OC-3 is expressed in discrete regions of the endoderm were HNF-6 is present.

HNF-6 Binds to and Stimulates Transcription of the Oc3 Gene—To determine whether HNF-6 can bind to, and directly stimulate, the transcription of the Oc3 gene, we resorted to cultures of BMEL cells isolated from e14.5 liver as described in Strick-Marchand and colleagues (19, 20). These cells have properties of hepatoblasts, as they can differentiate toward the hepatocytic or biliary lineages (19, 20). They express the three Onecut transcription factors and can be transfected with high (close to 80%) efficiency. Moreover, we found that Hnf6 knockout BMEL cells express five times less Oc3 than wild type cells (data not shown). We first examined if HNF-6 could stimulate endogenous Oc3 gene transcription. Cells were transfected with a GFP expression vector, as a control, or with Flag/HNF-6 or Flag/HNF-6/VP16 expression vectors. Total RNA was extracted 24 h later and reverse transcribed to measure endogenous Oc2 (top panel) and Oc3 (bottom panel) expression levels by real time PCR. Data are expressed as described in the legend to Fig. 1. B, proteins were extracted from the samples (pool of the duplicates) shown in A and expression from the transfected vectors was assessed by Western blotting (WB) with antibodies (α) against GFP or the FLAG epitope. C, BMEL cells were transiently transfected with the Flag/HNF-6 expression vector and cross-linked chromatin was immunoprecipitated (IP) with an anti-tubulin (Tub) or anti-Flag/HNF-6 (HNF-6) antibody. PCR was performed on total chromatin (Input) and on immunoprecipitated chromatin using pairs (a, b, and c) of specific primers (arrows) for the conserved region of the Oc3 gene, as schematized. Black boxes represent the HNF-6 binding sites.

HNF-6 Controls OC-3
We then searched for HNF-6 binding sites in regulatory regions of the Oc3 gene. Comparison of the human and mouse genes revealed sequence conservation over about 1 kb upstream (−2880 to −1950 from the ATG) of the coding sequence. This region contains three conserved HNF-6 binding consensus sequences (Fig. 3C). To test if HNF-6 binds to the Oc3 gene, we performed chromatin immunoprecipitation experiments. The chromatin from Flag/HNF-6-transfected BMEL cells was immunoprecipitated using an anti-FLAG antibody and the immunoprecipitated fraction was tested by PCR for the presence of Oc3 gene regions containing the HNF-6 binding sites. As shown in Fig. 3C, the three regions were amplified from chromatin that had been immunoprecipitated with the anti-FLAG antibody. No amplification was observed with an unrelated antibody. Altogether, these results indicate that HNF-6 binds to a conserved region of the Oc3 gene in native chromatin and stimulates transcription of this gene.

HNF-6 Induces Oc3 Gene Expression in the Developing Endoderm—We then designed experiments to confirm that HNF-6 can stimulate Oc3 gene transcription in the mouse endoderm. We recently developed a system that allows delivery of DNA to discrete regions of the mouse endoderm in situ such as the prepancreatic or prehepatic territories.2 Electroporation of precisely oriented 6- to 8-somite embryos is followed by a 24-h culture of the whole embryos such that they reach the 18–22-somite stage (Fig. 4). The orientation of the embryo is such that the DNA molecules will move toward the anode and target a region of the endoderm (dashed line). The electroporation is followed by a 24-h culture in a rolling bottle; white ovals represent individual embryos. B, embryos were electroporated with a GFP expression vector, without or with the Flag/HNF-6 expression vector. After 24 h in culture, RNA was extracted from the embryos, reverse-transcribed, and Gfp or Hnf6 expression was assessed by PCR. C, expression levels of the endogenous Onecut genes was assessed by real time PCR on the same cDNAs. Data are expressed as described in the legend to Fig. 1.

We next addressed the question as to whether HNF-6 can induce Oc3 expression in cells that are not fated to normally express Oc3. By orientating the embryo between the electrodes so that DNA is delivered in the anterior part of the foregut pocket, we targeted the esophageal region (boxed in Fig. 5D), as demonstrated by the expression of Hnf6 in some cells of this region (Fig. 5E). We observed cells that were positive for Oc3 in this electroporated esophageal region (Fig. 5F), indicating that HNF-6 is able to turn on the transcription of the Oc3 gene ectopically.

DISCUSSION

Development of the liver and pancreas from the endoderm involves the hepatic or pancreatic commitment of endodermal precursors, followed by differentiation of these precursors into
the corresponding tissue-specific cell types. These processes depend on a network of transcription factors, whose onset of expression and mutual control is far from being completely elucidated. Our earlier work has demonstrated that HNF-6 is an important member of this network, by showing that inactivation of the $Hnf6$ gene perturbs liver and pancreas development and by identifying transcription factors as HNF-6 target genes. Thus, in the mouse embryo, HNF-6 stimulates $Hnf1$ expression in liver, thereby controlling intrahepatic bile duct morphogenesis (16), and in the pancreas HNF-6 stimulates expression of $Hnf1\beta$ (24) and $Ngn3$ (15), thereby controlling endocrine differentiation. HNF-6 also plays a role at an earlier stage in the endoderm where it is involved in the onset of $Pdx1$ expression (14). In this paper, we identify a second transcription factor as an HNF-6 target in developing endoderm, namely its paralog OC-3. Indeed, we show here that HNF-6 can bind to the $Oc3$ gene in native chromatin and that it can stimulate $Oc3$ expression not only in transfected cells, but also in the endoderm in situ. Moreover, this regulatory cascade is developmentally relevant, as inactivation of the $Hnf6$ gene leads to a down-regulation of OC-3 both in the endoderm and in the endoderm-derived organs that express HNF-6, namely the liver and pancreas.

It is noteworthy that induction of $Oc3$ expression by HNF-6 is limited in time and space. At e9, expression of OC3 in the endoderm is detected only in a subpopulation of HNF-6-expressing cells, whereas at e10.5 OC-3 expression extends to almost all the HNF-6 territory, which corresponds to the liver, pancreas, and part of the stomach and gut. Later on, HNF-6 becomes restricted to the liver and the pancreas, whereas OC-3 disappears from these tissues but remains expressed in the stomach and in the intestine (10–12). The induction of $Oc3$ by HNF-6 may require time and space-restricted cooperation of

**FIG. 5.** HNF-6 can induce $Oc3$ gene transcription in the gut endoderm as well as ectopically. A–C, immunofluorescence staining, on adjacent sections of an embryo electroporated at e8.5 in the gut endoderm with the Flag/HNF-6 expression vector and cultured for 24 h up to the 20-somite stage, for E-cadherin (red), HNF-6 (green), Prox1 (green), OC-3 (red), and Pdx-1 (green) proteins, as indicated. hb, hepatic bud. D, Hoechst staining of a section of a whole embryo electroporated at e8.5 in the foregut pocket with the Flag/HNF-6 expression vector and cultured for 24 h up to the 18-somite stage. E, and F, analysis of the area delineated in D, by immunofluorescence for E-cadherin (red), HNF-6 (green), and OC-3 (red) proteins, as indicated.

**FIG. 6.** Expression of the One-cut factors and their control of other transcription factors in developing endoderm, liver, and pancreas. A, time course and relative levels of expression of the three One-cut transcription factors in the mouse endoderm. e, embryonic day. B, One-cut target genes. Gene promoters are represented by a rectangle and the transcripts by a thick arrow. Thinner arrows indicate a direct stimulatory effect, the dashed line an indirect effect, and the dotted lines an involvement of the transcription factor in a differentiation event. See text for details.
HNF-6 with other Oc3 regulatory factors and/or posttranslational regulation of HNF-6 activity (25).

We also show here that all three Onecut factors are expressed in a region of the endoderm that will eventually form the stomach, duodenum, liver, and pancreas. Interestingly, they are expressed in the sequential order Hnf6, Oc2, Oc3, so that one Cut factor expression defines three successive steps in endoderm maturation (Fig. 6A). This is relevant for understanding pancreas and liver development and for implementing this information in the context of cell therapy. Indeed, transcription factors alone or in combination are used to promote controlled cell differentiation and are useful markers to determine the precise differentiation stages of cells that are cultured in well defined conditions.

Our gene expression analysis of Hnf6 knockout embryos extends our knowledge of the transcription factor network in the endoderm (Fig. 6B). First, OC-2 induction is independent from HNF-6 because we show here that OC-2 is expressed in the Hnf6 knockout embryos and that electroporation of HNF-6 in cultured cells or in the endoderm does not induce Oc2. Second, our data would rule out that it is OC-2 that triggers Oc3 gene transcription in the endoderm. Indeed, in the Hnf6 knockout, OC-3 is not expressed despite the normal expression of OC-2. Moreover, ectopic expression of HNF-6 in the esophagus induced Oc3 expression, in the absence of OC-2.

Another implication of the present work bears on the interpretation of the endodermal phenotype of Hnf6 knockout embryos. Pdx1 expression in their endoderm is retarded from the 8–12-somite stage until the 19–22-somite stage (14), i.e. Pdx1 appears at the time when OC-3 becomes significantly expressed in the normal embryo. This could have suggested that it is OC-3 that triggers Pdx1 expression in the absence of HNF-6. Our data show that this is unlikely, as in the Hnf6 knockout, OC-3 eventually appears despite the absence of OC-2. The question of the possible role of OC-2 in inducing Pdx1 in the Hnf6 knockout embryos remains open, because OC-2 levels are unaffected in these knock-out embryos. On the other hand, the fact that OC-3 is down-regulated in the Hnf6 knockout embryos may account for some developmental defects of these embryos. These defects might result from failed expression of genes that are targeted specifically by OC-3 alone or by both HNF-6 and OC-3. Because, in normal embryos, OC-3 is first detected on the ventral side (prehepatic and pancreatogenic territories) of the endoderm, one would expect that the consequences of this combined HNF-6/Oc3 deficiency appear first in the ventral prepancreatic and/or prehepatic endoderm. This is indeed the case. In Hnf6 knockout embryos, development is more severely affected on the ventral side than on the dorsal side, with an absence of gallbladder, a longer delay in the expression of Pdx1, and an extremely reduced ventral pancreatic bud (14, 16).

Earlier experiments with cultured cells and on Hnf6 knockout embryos have shown that HNF-6 can bind to and stimulate the transcription of HNF-6 targets, such as Hnf4, Hnf1β, and Ngn3. The present work extends our knowledge of the control exerted on these factors, not only by HNF-6, but also by the other Onecut proteins (Fig. 6B). In the endoderm, HNF-6 is required for normal expression of Pdx1 (14), Oc3 (this paper), and Hnf1β, but not for that of Hnf3β (14) or Hnf4. In the liver, HNF-6 is critical for expression of Oc3 (this paper) and Hnf1β, and this HNF-6 → Hnf1β cascade controls bile duct development (16). Whereas independent inactivation of Hnf6 or Oc2 does not affect Hnf4 expression in liver, the combined inactivation of Hnf6 and Oc2 strongly reduces expression of the α7 isoform of HNF-4 (26). The expression of Hnf3α is also controlled by HNF-6 in the liver, but via an inhibitory, transforming growth factor β-dependent mechanism (27). In the pancreas, HNF-6 is required for Oc3 expression (this paper) and HNF-6 controls Hnf1β (24) and Ngn3 (15) expression during endocrine differentiation. Although no OC-3 target genes have been identified to date, this factor may be involved in the differentiation of the endoderm and endoderm-derived tissues, downstream of HNF-6.

In conclusion, the data presented here broaden our understanding of the network of transcription factors that are expressed in the endoderm. They define stages in the embryonic expression pattern of the Onecut transcription factors. They identify OC-3 as a new target of HNF-6 in the endoderm as well as in developing pancreas and liver, pointing to a new mechanism by which HNF-6 can control developmental events.

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