A vHNF1/TCF2-HNF6 Cascade Regulates the Transcription Factor Network That Controls Generation of Pancreatic Precursor Cells

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Generation of pancreatic precursor cells in the endoderm is controlled by a network of transcription factors. Hepatocyte nuclear factor-6 (HNF6) is a key player in this network, because it controls the initiation of the expression of pancreatic and duodenal homeobox 1 (Pdx1), the earliest marker of pancreatic precursor cells. To further characterize this network, we have investigated how the expression of HNF6 is controlled in mouse endoderm, by using in vitro and in vivo protein-DNA interaction techniques combined with endoderm electroporation, transgenesis, and gene inactivation in embryos. We delineated Hnf6 regulatory regions that confer expression of a reporter gene in the embryonic endoderm but not in extraembryonic visceral endoderm. HNF6 expression in the embryonic endoderm was found to depend on an intronic enhancer. This enhancer contains functional binding sites for the tissue-specific factors of the forkhead box A and HNF1 families. Among the latter, variant HNF1 (vHNF1)/TCF2, which is expressed before HNF6 in the endoderm, was found to be critical for HNF6 expression. Therefore, the sequential activation of vHNF1, HNF6, and Pdx1 in the endoderm appears to control the generation of pancreatic precursors. This cascade may be used to benchmark in vitro differentiation of pancreatic precursor cells from embryonic stem cells, for cell therapy of diabetes. Diabetes 55: 61–69, 2006

Understanding the mechanisms that govern pancreatic cell differentiation is a prerequisite to guide in vitro stem cell differentiation toward a β-cell phenotype for cell therapy of diabetes. Many efforts have been devoted to understanding the transcription network that controls β-cell differentiation from endodermal pancreatic precursor cells (1,2), but less is known about the transcription factors that control the generation of the pancreatic precursors in the endoderm. Signaling from the mesoderm and ectoderm around embryonic day (e) 7–7.5 (mouse embryo) renders the endoderm competent to respond to signals of the activin and fibroblast growth factor families, which specify the pancreatic precursor cells. The latter signals are produced by the notochord, the aortic endothelium, and mesenchymal cells, and they specify pancreatic precursor cells in ventral and dorsal endodermal regions. Around e9–9.5, the specified regions give rise to a dorsal and a ventral pancreatic bud, which later fuse to form a single organ in which endocrine cells differentiate (3–5).

Acquisition of competence and specification of the pancreatic precursors result from regionalized expression of transcription factors. This constitutes a dynamic transcriptional network that directs differentiation of the endodermal cells to a pancreatic fate (1,2). Several transcription factors are broadly expressed in the endoderm, including the variant homeodomain hepatocyte nuclear factor vHNF1 (also called TCF2 or HNF1β) (6), the forkhead factors forkhead box A 1 (FoxA1) and FoxA2 (7), and the zinc-finger transcription factors GATA4, -5, and -6 (8). Some of these factors contribute to render the endoderm responsive to signals from adjacent tissues (9). Other factors, such as the pancreas transcription factor 1a (Ptf1a) and the pancreatic and duodenal homeobox factor 1 (Pdx1), are expressed in a more restricted domain of the endoderm that contains the pancreatic precursors (10,11). Lineage-tracing experiments have shown that the three pancreatic tissues (exocrine acini, endocrine islets, and ducts) are derived from Pdx1-expressing progenitors (12). Therefore, knowing how the expression of Pdx1 is initiated in the endoderm provides critical information on the transcription factor network that regulates the generation of pancreatic precursors.

The homeodomain factor homeobox 9 is required for initiation of Pdx1 in the dorsal endoderm but not in ventral endoderm (13,14). In contrast, the Onecut transcription factor HNF6 (also called Onecut 1) is an essential regulator of Pdx1 in both the dorsal and ventral endoderm (15). HNF6 expression precedes that of Pdx1, being first expressed at e8.5 (8-somite stage) in the foregut-midgut region of the endoderm. Later, HNF6 becomes restricted to the liver and the pancreas (15–18). Inactivation of the HNF6 gene in mice results in a delayed onset of Pdx1 expression in the endoderm leading to pancreatic hypoplasia (15). At later stages of pancreas development, the
absence of HNF6 leads to defective pancreatic endocrine cell differentiation, resulting from reduced expression of the proendocrine transcription factor Ngn3 (19).

Because Pdx1+ endodermal cells are an obligate intermediate population in the generation of β-cells and because HNF6 is an essential regulator of Pdx1 in the endoderm, characterizing how HNF6 is regulated in the endoderm would help understand how the transcription factor network directs differentiation of endodermal cells into pancreatic precursors. In this work, we characterized the regulatory regions of the mouse Hnf6 gene that drive expression of HNF6 in the endoderm. Moreover, we identified vHNF1 as a regulator of HNF6 expression in the endoderm and thus define a vHNF1 → HNF6 cascade as an important component of the endodermal transcription factor network.

RESEARCH DESIGN AND METHODS

RNase protection. RNase protection was performed with 25 μg total RNA from adult mouse liver (20). Probe A was synthesized from the Asp718 (−60)–linearized plasmid pBS.Stul/Clal (−756/+160) containing the StuI/Clal (−756/+160) fragment of the mouse Hnf6 promoter. Probe B was synthesized from the AvaII (−393)–linearized plasmid pBS.ApaLI/XhoI (−415/−183) containing the ApaLI/XhoI (−415/−183) fragment.

DNA-protein interactions. DNase I footprinting and electromobility shift assays (EMSA) with nuclear extracts from adult mouse liver and from AR42J cells (21,22) were as described previously (23,24), except that the protein–DNA incubation buffers contained 1% (v/v) glycerol. For supershifts, extracts were incubated with 1 μl antibody for 20 min on ice before adding the probe. The antibodies were: anti–specific protein (Sp) 1 and anti–Sp-3 (sc-20X and sc-644X, Santa Cruz Biotechnology, Santa Cruz, CA), anti-HNF1α (Sp1; gift from J. Ferrer), anti-vHNF1/vHNF1β (sc-7411; Santa Cruz Biotechnology), anti-FoxA1 (gift from W. Schmid), and anti-FoxA2 (gift from T. Jessell).

The double-stranded oligonucleotides used as probes in EMSAs were Hnf6 promoters (I−I3), 5′-TTTGTGTTAATATTTATCCCGACCGGTG-3′ (footprint 1), 5′-CTGGGTATAGGGGCGAAAGAC-3′ and 5′-CACTTGAAATGTCACCGGTC-3′ (footprint b), and 5′-CATTTTGTGTTGTTGATTATTTCACTCCGCGTGT-3′ (footprint e). Double-stranded competitor oligonucleotides were 5′-GATCGTGTTGTTGGGGGGGGGGTTGTAAGACTC3′ (Sp1), 5′-GATGGTTGTTGATCTGCAGTTGTAAGACTC3′ (HNF1), and 5′-GATGGTTGTTGATCTGCAGTTGTAAGACTC3′ (FoxA). Competitor oligonucleotides (50 ng) were added to the reaction before probe addition.

Plasmids. The transfected constructs contained mouse Hnf6 promoter fragments cloned upstream of the luciferase gene in pGL3 (Promega). Region E3 was cloned downstream of the luciferase gene in pGL3.Promega. Region E3 was cloned downstream of the luciferase gene in the BsaHI and SalI sites of pGL3.Stul/Clal (−756/+160). Mutation of the Sp1 site in the promoter was performed by replacing bases with a GAL4 site. The footprints of region I−I3 were cloned upstream of the luciferase gene in pGL3 (Promega). Region I−I3 was cloned upstream of the luciferase gene in pGL3.Stul/Clal (−756/+160) containing the StuI/Clal (−756/+160) fragment of the mouse Hnf6 promoter. Probe B was synthesized from the AvaII (−393)–linearized plasmid pBS.ApaLI/XhoI (−415/−183) containing the ApaLI/XhoI (−415/−183) fragment.

Generation of transgenic mice. Mice were treated according to the principles of laboratory animal care of the University of Louisville Animal Welfare Committee. Fertilized B6D2F2 eggs were injected with 5 ng/pL purified Hnf6 promoter–LucZ-E3 fragment from pSK.Tvl/Clal/E3 and were transferred at the two-cell stage in CD1 pseudo-pretreated females. β-Galactosidase activity was quantified as described previously (26).

Diploid chimeric embryos. Diploid chimeric embryos with maximal vHnf1−/− embryonic stem cell contribution were generated (28). Because the vHnf1 gene was disrupted by replacing the first exon by the LacZ gene, the contribution of wild-type and vHnf1−/− embryonic stem cell–derived embryos was evaluated by either X-gal staining or immunohistochemistry using anti–β-galactosidase antibody (Cappel) on frozen sections. The embryos were characterized by the presence of β-galactosidase + (vHnf1−/−) cells in the vHnf1-expressing tissues. Control embryos, which were derived from morulae not aggregated with embryonic stem cells, and embryonic stem cell–aggregated embryos were implanted together, and analyses were performed on embryos from the same somite stage. Frozen sections were analyzed by an in situ hybridization using vHnf1 and Hnf6 probes, as described previously (28), and by immunohistochemistry using anti-Hnf6 antibody (sc-10509; Santa Cruz Biotechnology) and Vectastain (ABC kit, rabbit; Vector Laboratories).

Results

Structure of the mouse Hnf6 gene. Our previous work showed the location of the promoter and exons in the rat Hnf6 gene (30) and of the exons in the mouse Hnf6 gene (19) but did not investigate how HNF6 expression is regulated during development. To address this question, we characterized the structure of the mouse gene by searching for regions that are conserved between mouse and human. Using the BLAST 2 sequences program (31), we mapped regions within 38 kb of the mouse Hnf6 locus (Fig. 1A). A conserved 460-bp region, homologous to the rat gene promoter, was found upstream of the translation initiation codon. Another conserved region, 802-bp long, was found 7 kb upstream of the initiator ATG. We identified eight conserved intronic regions of 100–500 bp (11–18).

We then located the transcription initiation site of the mouse Hnf6 gene by RNase protection. Antisense RNA probes were incubated with liver RNA, and analysis of the RNase-digested products revealed the presence of two transcription initiation sites separated by ~200 bp (Fig. 1B). The site located 192 bp upstream of the translation initiation site corresponds to the rat transcription initiation site (30) and was considered as nucleotide +1. The second initiation site was at the limit of detection by RNase protection, but was confirmed by RT-PCR (data not shown). The two transcription initiation sites were detected in liver and in pancreas of embryos and adult mice and in the pancreatic and hepatic cell lines AR42J and FTO2B (data not shown). Because of the lack of splice sequence between these two sites, initiation from them is...
expected to give rise to mRNAs that contain the same coding exons.

**Regulation of the Hnf6 promoter.** To delineate the mouse Hnf6 promoter, we transiently transfected luciferase reporter plasmids containing 5′ deletion fragments of the sequence located upstream of the transcription initiation site. The activity of the constructs was compared in the pancreatic cell line AR42J. As shown in Fig. 2A, the region from −756 to −303 was dispensable for promoter activity. In contrast, a deletion from −303 to −183 bp abolished activity, thereby delineating the promoter within 300 bp upstream of the transcription initiation site.

We then looked for transcription factor–binding sites, and DNase I footprinting analysis was performed on two contiguous fragments spanning nucleotides −397 to +160. Nuclear extracts from liver or from AR42J cells were used as a source of proteins. Three footprints were detected (Fig. 2B). They were easier to delineate with liver extracts, whose protein concentration was higher than that of AR42J cell extracts. Candidate transcription factors were identified using the Match (32) program (Fig. 2C). Footprint 1 contains a STAT5 site, which is conserved between the rat and mouse promoters (33).

Footprint 2 is within the −303/−183 region, which is critical for promoter activity, and the corresponding sequence contains a consensus site for the Sp1 family of transcription factors. In EMSA, incubation of an oligonucleotide corresponding to footprint 2 with nuclear extracts from liver or from AR42J cells generated retarded complexes that were supershifted in the presence of antibodies directed against Sp1 or Sp3 (Fig. 2D). Binding of Sp1 was predominant with AR42J extracts, whereas Sp3 binding was predominant with liver extracts. To investigate the role of this Sp1/Sp3 site, a mutation preventing Sp1/Sp3 binding was introduced in the promoter, and the construct was tested for its activity in AR42J cells (Fig. 2E). Mutation of the Sp1/Sp3 site inhibited promoter by 75%. Footprint 3 also binds Sp1 and Sp3 (data not shown), but deletion of the corresponding sequence did not affect promoter activity.

We concluded from these experiments that 1) the activity of the mouse Hnf6 gene promoter is located within 300 bp upstream of the major transcription initiation site, and 2) the main regulators of this activity are the ubiquitous Sp1 and/or Sp3 transcription factors.

**Identification and in vivo activity of an intronic enhancer.** Because the Hnf6 promoter is regulated by ubiquitous transcription factors, it seemed unlikely that its activity would be the sole determinant of the tissuespecific expression of HNF6 during development. We therefore looked at the other conserved Hnf6 regions and focused on those that could confer expression in the pancreatic lineage. The conserved region located at −7 kb or each of the eight conserved intronic regions were cloned in a luciferase reporter construct containing the Hnf6 gene promoter, and the activity of the constructs was tested by transient transfection of AR42J cells. We found that none of the regions enhanced the activity of the Hnf6 promoter, except region I3, which induced a fourfold stimulation (Fig. 3A).

To verify whether the promoter and the intronic I3 region were able to drive transcription in developing embryos in a tissue- and stage-specific way, we generated transgenic embryos harboring a LacZ reporter gene under the control of these two regulatory regions (Fig. 3B). Transgenic embryos expressed β-galactosidase at e9 (one
FIG. 2. Regulation of the Hnf6 promoter. A: Hnf6 promoter–luciferase reporter plasmids were transfected in AR42J cells. Luciferase activities (means ± SE, n ≥ 3) were normalized for the activity of the empty vector. B: Transcription factor–binding sites in the Hnf6 promoter. Probes were incubated with nuclear extracts from AR42J cells (Ar) or from mouse liver (Li) before digestion with DNase I. C: Hnf6 promoter sequence with regions footprinted in B (boxes) and potential binding sites for transcription factors (underlined). D: EMSAs show that Sp1 and Sp3 bind to footprint 2. E: Transfection of Hnf6 promoter–luciferase reporter constructs shows that the Sp1/Sp3 site is necessary for full Hnf6 promoter activity (means ± SE, n ≥ 3).
endoderm. These regions correspond to the presumptive territories of the pancreas and liver in the definitive endoderm (34,35). At later stages, β-galactosidase expression in this line was similar to that in the other transgenic embryos. β-Galactosidase expression was also studied in the extraembryonic visceral endoderm of the embryo collected at e9 and of e8 and e8.75 embryos from the transgenic line. No β-galactosidase activity was found in the visceral endoderm, suggesting that the construct was specifically active in the definitive endoderm.

**Identification of cis sequences and trans-acting factors in intronic region I3.** To identify the cis-acting sequences and transcription factors that regulate the activity of the I3 region, we performed DNase I footprinting experiments. Five footprints were detected (Fig. 4A). Using the Match program, putative binding sites were found for CAAT/enhancer-binding protein (C/EBP) (footprint a), FoxA (HNF3; footprint b), HNF6 (footprint c), nuclear factor 1 (footprint d), and HNF1 and Pdx1 (footprint e) (Fig. 4B). We then mutated each consensus sequence and tested the effect of the mutation by transflecting the prehepatic and prepancreatic endoderm of cultured embryos (Fig. 4C). Whole e8.5 embryos (six to eight somites) were electroporated and were cultured for 24 h before measuring the relative luciferase activities. The results showed that the promoter induces only a marginal luciferase activity. However, this activity was strongly increased when the promoter was associated with the I3 region. Mutations in the putative C/EBP-, HNF6-, or Pdx1-binding sites of region I3 had no significant effect. In contrast, mutation of the nuclear factor 1 site reduced transcriptional activity, and the strongest reductions were measured when the consensus sites for FoxA or for HNF1 were abolished.

To verify whether FoxA can bind to the footprint b region and HNF1 to the footprint e region, we performed EMSAs using liver nuclear extracts (Fig. 4D). Three retarded complexes were observed with a probe corresponding to footprint b. They were inhibited in the presence of an excess unlabeled FoxA-binding oligonucleotide. Moreover, the upper band was inhibited by an anti-FoxA1 antibody, and the middle band was supershifted by an anti-FoxA2 antibody. The lower band corresponds most likely to FoxA3, which is known to migrate faster than FoxA1 and FoxA2 (24). When EMSAs were performed with an oligonucleotide corresponding to footprint e, a DNA-protein complex was detected. It was supershifted by anti-HNF1α and anti-vHNF1 antibodies, and it was inhibited in the presence of an excess unlabeled HNF1-binding oligonucleotide. From these data, we concluded that FoxA and HNF1 factors bind to the I3 region of the mouse Hnf6 gene in vitro and are critical for Hnf6 gene expression in the endoderm.

**In vivo regulation of HNF6 expression by vHNF1 in the endoderm.** The expression of the transgene driven by the I3 region, at e10.5 (Fig. 3B), not only recapitulates the endogenous Hnf6 expression in endoderm-derived organs (15–18), but is remarkably similar to that exhibited by vHnf1α mRNA at that stage (6,28). The above electroporation data showed that HNF6 expression in the endoderm depends on a gene region that can bind HNF1. In addition, our real-time RT-PCR experiments have shown that HNF1α mRNA is barely expressed in the endoderm (data not shown), reinforcing the notion that vHNF1 could be the major regulatory factor of the I3 region. Moreover, the lack of vHNF1 disrupts pancreas development from early embryo, e9.5 (two embryos), and e10.5 (two embryos), in the endodermal, hepatic, and pancreatic regions known to express endogenous HNF6 (15–18). In addition, one transgenic line was established to analyze β-galactosidase activity at earlier stages. In this line, β-galactosidase became detectable at e8.75 (10–11 somite stage) in two lateral regions of the ventral endoderm and in the dorsal...
stages (28). These observations prompted us to examine whether vHNF1 does interact with the I3 region in vivo. We performed chromatin immunoprecipitation experiments with a vHNF1 antibody and nuclear extracts from microdissected endoderm of e9.5 embryos (15–25 somites). The results (Fig. 5A) showed that the I3 region is enriched in the immunoprecipitated fraction, compared with the DNA regions coding for the HNF6 homeodomain or for 28S RNA, which served as negative controls. Using real-time PCR, we calculated an eightfold enrichment of region I3 after immunoprecipitation with the vHNF1 antibody. We concluded from these experiments that vHNF1 binds in vivo to the Hnf6 region I3 in the endoderm.

To further confirm the functional importance of vHNF1, we analyzed HNF6 expression in vHnf1/H11002/H11002 endoderm. To overcome the early embryonic lethality of vHnf1 knockout mice, we generated diploid chimeric embryos by aggregating vHnf1−/− embryonic stem cells with wild-type morulae (28). In chimeric embryos at e9.5, vHnf1−/− cells, which express β-galactosidase, were identified by immunohistochemistry using an anti-β-galactosidase antibody, whereas HNF6 expression was examined by in situ hybridization and immunohistochemistry. As shown in Fig. 5B, HNF6 expression in control embryos was found in the gut, dorsal and ventral pancreatic buds, and liver primordium, as expected. In contrast, HNF6 transcripts or protein were not detected in vHnf1−/− cells of chimeric embryos, which also exhibited reduced dorsal pancreatic and liver buds and no ventral pancreas, as previously reported (28).

From these results, we concluded that vHNF1 is a critical regulator of HNF6 expression in the endoderm and that the vHNF1→HNF6 cascade is an important component of the transcription network that regulates the onset of pancreas differentiation.

DISCUSSION

HNF6 is a regulator of pancreatic specification of endodermal cells because in its absence, the expression of the
pancreatic specification marker Pdx1 is delayed (15). The aim of the present work was to identify regulators of HNF6 expression in the endoderm to further characterize the network of factors that controls development of the pancreas precursors.

Our endoderm electroporation data and analysis of chimeric embryos show that vHNF1 is a critical regulator of HNF6 expression in the developing endoderm. In wild-type mice, the expression of vHNF1 becomes detectable after implantation in the extraembryonic visceral endoderm, and in the embryonic endoderm, this expression starts at the two-somite stage, i.e., before HNF6 expression (6,15,36). This places vHNF1 upstream of HNF6 in a cascade that regulates endoderm and early pancreas differentiation (Fig. 5C). In Hnf6−/− embryos, the lack of HNF6 results in transient lack of activation of the Pdx1 gene, with Pdx1 being detected only around e9 in the dorsal endoderm and around e9.5 in the ventral endoderm. This delay in specification leads to dorsal and ventral pancreatic hypoplasia, the ventral pancreas being more severely affected (15). In vHnf1−/− embryos, the dorsal pancreatic bud is hypoplastic and expresses Pdx1, like in Hnf6−/− embryos, but no ventral pancreas is found (28). Together with the regulation of HNF6 by vHNF1 described in this study, these data are compatible with a requirement for a vHNF1→HNF6→Pdx1 cascade to generate pancreas precursors. After e9.5, Pdx1 is expressed in the dorsal pancreas of either vHnf1−/− or Hnf6−/− embryos, supporting the role of other regulators for dorsal pancreas specification. Previous work has shown that homeobox 9 (13,14) and Ptf1a (10) also control pancreas specification. Because vHNF1 is required for Ptf1a expression in the developing pancreatic bud (28), Ptf1a may also be downstream of vHNF1 when generating pancreatic precursors in the endoderm.

Our endoderm electroporation data show that FoxA factors control HNF6 expression in the endoderm. The timing and location of FoxA1 and FoxA2 expression in the endoderm (7,37) are compatible with a role of both factors in the control of the onset and maintenance of HNF6 expression in the endoderm. FoxA3 may contribute to the initiation of HNF6 expression in the ventral pancreas domain. FoxA3 mRNA was detected in the ventral endoderm near the cardiac mesoderm but not in the dorsal prehepatic domain (7). FoxA factors contribute to the prehepatic endoderm competent to acquire a hepatic fate through binding to a number of genes that become expressed when the endoderm becomes specified (38). Binding of FoxA2 to its target genes induces modifications in the chromatin structure that make it accessible

FIG. 5. vHNF1 controls HNF6 expression in the endoderm. A: vHNF1 binds to region 13 in the endoderm. PCR analysis after chromatin immunoprecipitation of e9.5 endoderm with a vHNF1 antibody. Amount of chromatin in the samples was normalized by PCR amplification of DNA sequences coding for the Hnf6 homeodomain (exon 3) or for 28 S RNA. B: Sections from e9.5 control or chimeric embryos derived from vHnf1−/− embryonic stem cells were analyzed by in situ hybridization (Hnf6) and immunohistochemistry (HNF6, β-gal). In chimeric embryos, the dorsal pancreas was identified by staining the adjacent section for Pdx1 (data not shown). dp, dorsal pancreas; g, gut; gb, gall bladder; li, liver; vp, ventral pancreas. C: Network of transcription factors that controls development of pancreatic precursors in the endoderm.
for other transcription factors (39). We postulate that FoxA factors play similar roles in pancreas development and HNF6 expression in the endoderm.

Transgenic embryos harboring a LacZ reporter gene under the control of the Hnf6 promoter and I3 region did not express β-galactosidase in the extraembryonic visceral endoderm or in the liver and pancreas beyond e10.5 (not shown). Therefore, these regulatory regions only control Hnf6 gene expression in the prepancreatic and prehepatic regions of the definitive endoderm and in the early liver and pancreatic buds. This was unexpected, because the I3 region contains binding sites for C/EBP, FoxA, and HNF1, which are tissue-specific factors active in the visceral endoderm, liver, and pancreas. Therefore we propose that the promoter and I3 region cooperate with other regulatory regions to confer the full expression pattern of HNF6. The conserved regions identified in Fig. 1 are good candidates.

In vitro differentiation of insulin-producing cells has been proposed as a source of tissue for transplantation therapy of type 1 diabetes. Protocols were described to differentiate embryonic stem cells into insulin-containing cells via the formation of embryoid bodies and selection of nestin-producing cells (40–44). However, the validity of insulin-positive cells obtained by these methods remains controversial (45,46). Moreover, differentiation of embryonic stem cells into embryonic bodies results in the generation of derivatives of the extraembryonic visceral endoderm. In the embryo, pancreatic cells originate from the definitive endoderm. Efficient differentiation of insulin-producing cells from embryonic stem cells may therefore require the optimization of a series of steps corresponding to the sequential stages of pancreatic development from the definitive endoderm, and not from visceral endoderm (5). Our present work shows that the sequential activation of vHNF1, HNF6, and Pdx1 characterizes the initial phases of pancreas development from the definitive endoderm and may be used for bench-marking in vitro pancreatic differentiation. Finally, our construct driving in vivo expression of a reporter gene in the definitive endoderm, without expression in the visceral endoderm, may constitute a useful tool when one differentiates embryonic stem cells in vitro toward the definitive endoderm and pancreatic lineages.

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