Characterization and Isolation of Promoter-Defined Nestin-Positive Cells from the Human Fetal Pancreas

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Studies using adult human islets and mouse embryonic stem cells have suggested that the neurepithelial precursor cell marker nestin also identifies and can be used to purify β-cell precursors. To determine whether nestin can be used to identify β-cell progenitors in the developing human pancreas, we characterized nestin expression from 12 to 24 gestational weeks, purified nestin$^+$ cells using an enhancer/promoter-driven selection plasmid, and determined whether nestin$^+$ cells can differentiate into β-cells. Nestin was visualized in the platelet endothelial cell adhesion molecule and α smooth muscle actin–positive blood vessels and colocalized with vimentin in the interstitium. Nestin was not observed in pan cytokeratin (pCK)-positive ductal epithelium or insulin cells. Purified nestin$^+$ cells also coexpressed vimentin and lacked pCK immunoreactivity. Purified adult and fetal pancreatic fibroblasts also expressed nestin. The nestin enhancer/promoter used in the selection plasmid was sufficient to drive reporter gene expression, green fluorescent protein, in human fetal pancreatic tissue. Exposure of selected nestin$^+$ cells to nicotinamide, hepatocyte growth factor/scatter factor, betacellulin, activin A, or exendin-4 failed to induce pancreatic and duodenal homeobox gene-1 or insulin message as determined by RT-PCR. Transplantation of nestin$^+$ cells and fetal pancreatic fibroblasts into athymic mice also failed to result in the development of β-cells, whereas nestin$^-$ fetal pancreatic epithelial cells gave rise to functional insulin-secreting β-cells. We conclude that nestin is not a specific marker of β-cell precursors in the developing human pancreas.
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EGF, epidermal growth factor; FBS, fetal bovine serum; GFP, green fluorescent protein; HAF, human adult fibroblast; hCMV, human cytomegalovirus; HFF, human fetal fibroblast; HGF/SF, hepatocyte growth factor/scatter factor; ICC, islet-like cell cluster; neoR, neomycin resistance; pCK, pan cytokeratin; PDX-1, pancreatic and duodenal homeobox gene-1; PECAM, platelet endothelial cell adhesion molecule.

The overall goal in the treatment of type 1 diabetes is the maintenance of normoglycemia in patients with diabetes. Replacement of β-cell mass offers an alternative to standard insulin replacement and may overcome the long-term side effects associated with current therapies. However, an abundant source of tissue that will satisfy the demand for β-cells has yet to be found. Recently, a potential source of cells was described in and isolated from the adult rat and human islets of Langerhans (1,2). These cells were characterized by the expression of the neurepithelial marker nestin. With the use of selective adhesion in tissue culture conditions, nestin$^+$ cells were enriched and expanded. Addition of growth factors to these cultures induced the expression of β-cell markers. Insulin expression has also been induced from nestin$^+$ mouse embryonic stem cells in vitro (3–5). These in vitro observations have led to the hypothesis that nestin is a marker of pancreatic stem cells; however, little is known about the role of nestin in human pancreatic development.

Nestin is an intermediate filament protein originally described in the developing central nervous system (6). Human and rodent neurepithelial stem cells colocalize nestin and vimentin before cell cycle exit and neuronal or glial differentiation upon which nestin expression is lost (6,7). Because of the similarities between β-cells and neurepithelial development (8), a similar transient expression of nestin was proposed to occur in the human insulin-producing β-cell precursors (1,2). However, nestin expression is not restricted to human neurepithelial stem cells. Not surprisingly, nestin is also observed in human glioblastomas and neurectodermal tumors (9). In addition, angiogenesis occurring in neoplastic tissue is associated with an increase in nestin$^+$ endothelial cells compared with the subpopulation of nestin$^+$ endothelial cells in the normal brain tissue. Developing human myocytes also express nestin, which persists in the adult skeletal muscle and is upregulated during regeneration and in myosarcomas (10). Nestin is also widely expressed in the human gastrointestinal submucosa and in gastrointestinal stromal cell tumors of mesenchymal origin (11). Therefore, nestin is expressed in a variety of human cell types, observed during development, terminal differentiation, and oncogenic transformation.

Here, we describe the characterization of nestin expression in the developing human pancreas and determine the ability of nestin$^+$ cells, isolated using a promoter-defined

selection plasmid, to differentiate into the β-cell lineage in vitro and in vivo after transplantation. We show that nestin is specifically localized to the fetal pancreatic vasculature and fibroblasts. In addition, proven models of β-cell development fail to induce β-cell differentiation from a pure population of nestin$^+$ cells derived from the human fetal pancreas.

RESEARCH DESIGN AND METHODS

Tissue procurement. Human fetal pancreata used in these experiments were provided by Advanced Biosciences Resources (Oakland, CA) and Central Laboratory for Human Embryology, University of Washington (Seattle, WA) after the termination of pregnancy by dilation and extraction between 12 and 24 weeks' gestation. Informed consent for tissue donation was obtained by the procurement center. In addition, our institutional review board reviewed and approved use of fetal tissue for these studies.

Tissue processing and cell lines. Fetal pancreata were enzymatically digested for the generation of islet-like cell clusters (ICCs) as previously described (12). After 4 days in suspension, ICCs were collected for protein analysis, transplantation, or in vitro expansion, where cell clusters were transferred to dishes coated with the HTB-9 matrix and cultured in the presence of 10 ng/ml hepatocyte growth factor/scatter factor (HGF/SF; donation from Genentech, South San Francisco, CA) to facilitate the outgrowth and expansion in monolayer (13). Human fetal and adult pancreatic fibroblast cell lines (HFF and HAF, respectively) were previously generated in our laboratory and were cultured as described (14). A human neonatal foreskin fibroblast (Hs 168.Fs; American *Type Culture* Collection, Manassas, VA) also served as a control.

pCI-nes-neo vector construction. The pCI-nes-neo vector was generated by replacing the SV-40 enhancer/early promoter of the pCI-neo vector (Promega, Madison, WI) by the second intron of the human nestin gene followed by 160 bp of the basic HSV TK promoter. The second intron and TK promoter are sufficient to drive nestin gene expression in neurepithelial precursors (15). Briefly, the pCI-neo vector was cleaved with *Dra*III and *Stu*I, liberating the SV-40 enhancer/promoter. The nestin enhancer TK promoter insert was excised from the vector Nes 1852/TK Lac Z (provided by Dr. Urban Lendahl, Medical Nobel Institute, Stockholm, Sweden) by *Hin*dIII and *Not*I digestion. Vector and insert were ligated, and the orientation of the insert was confirmed by restriction digests.

For confirming that the second intron of the human nestin gene and TK promoter are also sufficient to drive reporter gene expression in human pancreatic precursors, the nestin enhancer TK promoter insert was subcloned into the *Sma*I site of the *pEGFP* promoterless vector (Clontech, Palo Alto, CA; the *Hin*dIII and *Not*I sites were not recreated after fill-in and ligation into the *Sma*I site). The cassette containing the nestin enhancer TK promoter driving the green fluorescent protein (GFP) coding sequence was excised with *Hin*dIII and *Not*I.

Adeno-shuttle plasmid pΔE1Z was constructed by inserting the human cytomegalovirus (hCMV) promoter-enhancer, intron, multicloning site, and bovine growth hormone poly-A signal sequence into the adenovirus E1 region in the plasmid pΔE1sp1A (Microbix Biosystems, Toronto, Ontario, Canada) (16). The hCMV promoter was removed by *Hin*dIII and *Not*I digestion and replaced with the nestin-GFP insert. The adenovirus backbone pJM17 was provided by Dr. Frank Graham. Viral lysates from 293 cells were used to plaque-purify a clonal isolate, and viral titers were checked by plaque formation assay on 293 cells as previously described in our laboratory (17).

Infection, transfection, and selection of nestin$^+$ cells. Fetal pancreata were processed for the generation of ICCs, except that the cell clusters were dispersed into single cells using warm 0.25% Trypsin 24 h after the initial digestion. The single-cell suspension was plated overnight on HTB-9–coated dishes in RPMI 1640 (Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS; Gibco) without antibiotics and transfected with the pCI-nes-neo vector using Lipofectamine Plus reagent as suggested by the manufacturer (Invitrogen, Carlsbad, CA). Selection was in the presence of 400 μg/ml G418 (Gibco) for 10–14 days and surviving colonies were grown to confluence and exposed to a variety of differentiation factors.

Virus infection was performed as previously described in our laboratory, achieving infectivity of 95% (17). Briefly, monolayers of ICCs were incubated with adenoviral particles (MOI 80) in RPMI 1640 with 10% FBS for 2 h at 37°C. Cells were washed twice in PBS, cultured for 4 days, and fixed in 4% paraformaldehyde for immunofluorescent analysis.

Cell culture. The selected nestin$^+$ cells from the same pancreas were split and expanded in RPMI 1640 with 11 mmol/l glucose containing 10% FBS supplemented with either 10 ng/ml HGF/SF grown on HTB-9 matrix or 20 ng/ml epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF2; both Invitrogen) grown on tissue culture–treated plates (Nunc, Rochester, NY). For differentiation, early passage cells from both expansion protocols were split into serum (10% FBS) or serum-free media (RPMI 1640) and grown on either HTB-9 matrix or tissue culture–treated six-well plates. Serum-free medium was supplemented with ITS (BD Biosciences, San Jose, CA) and 400 μmol/l $MnCl_2$ (Sigma, St. Louis, MO). Both serum and serum-free media contained 4 nmol/l betacellulin (R&D Systems, Minneapolis, MN), 10 nmol/l exendin-4 (GLP-1 analogue; Sigma), 10 ng/ml HGF/SF, 4 nmol/l activin A (CalBiochem, San Diego, CA), or 10 mmol/l nicotinamide (Sigma). An untreated control was performed in parallel. Cells were exposed to the growth factors for 6 days and collected for RT-PCR analysis.

In addition to monolayers, early passage nestin$^+$ cells were reaggregated as previously described (13) and grown in RPMI 1640 containing 10% Human Serum (BioWhittaker, Walkersville, MD) either with or without 10 mmol/l nicotinamide for 4 days. HFFs were treated in parallel with the nestin monolayers and reaggregates as a negative control. Control ICCs were also plated on HTB-9 in 10 ng/ml HGF/SF, transfected with the pCIneo parent vector, expanded for 5 days, reaggregated, and cultured in the presence of 10 mmol/l nicotinamide for 4 days to demonstrate that both transfection and expression of neomycin resistance do not impair β-cell differentiation.

RT-PCR. RNA was purified using the RNeasy minikit (Qiagen, Valencia, CA) and reverse transcribed using Superscript II with 250 ng of random primer (both Invitrogen) and 1 μg of total RNA in a reaction volume of 20 μl. For detection of insulin and pancreatic and duodenal homeobox gene-1 (PDX-1) in the nestin$^+$ cells, 1.5 μl of cDNA was used for each PCR (35 cycles) in a total volume of 30 μl. Oligonucleotide primers used for the PCR were insulin (18), PDX-1 (19), and β-actin (Stragene, La Jolla, CA). For semiquantitative analysis of insulin and neomycin resistance expression after reaggregation of the fetal monolayers, the PCR was carried out using 1 μl of cDNA in a total volume of 30 μl over 22 cycles, and β-actin was used as a control. The forward and reverse primers for neomycin resistance are 5′-AGGCTATTCGGCTATGACTGG-3′ and 5′-GATACTTTCTCGGCAGGAGC-3′, respectively. The whole PCR product was loaded onto a 1.2% Agarose gel and stained with ethidium bromide.

Western blotting. SDS-PAGE immunoblotting was performed as previously described (7) with some alterations. Briefly, cultured cells were lysed in TBS (pH 7.8) containing 5 mmol/l EDTA, 1% TritonX100, 0.2% SDS, and a cocktail of proteinase inhibitors. Total cell lysates (10 μg) were resuspended in reducing NuPage sample buffer (Invitrogen) and electrophoresed on a 10% SDS–polyacrylamide gel and transferred onto Opti-tran nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Membranes were blocked with TBS containing 5% nonfat milk and 0.05% Tween and incubated in mouse anti-human nestin (1:1,000; provided by Conrad Messam, National Institutes of Health, Bethesda, MD) for 2 h at room temperature. Blots were then incubated for 1 h with peroxidase-conjugated anti-mouse antibodies (1:5,000; Jackson ImmunoResearch, West Grove, PA), followed by chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ).

Transplantation. Nestin$^+$ cells isolated from pancreata of gestational ages 18–24 weeks were reaggregated and cultured in RPMI 1640 with 11 mmol/l glucose, 10% human serum, and either with or without 10 mmol/l nicotinamide for 2–4 days before transplantation. ICCs and reaggregated monolayers of expanded ICCs pretreated with nicotinamide were used as positive controls, and reaggregated HFFs were used as negative controls. The cell clusters were transplanted into athymic nude mice under the kidney capsule as previously described (20). After 3 months, the animals were challenged with 3 g/kg glucose i.p., and human C-peptide was measured by radioimmunoassay as previously described (20).

Immunofluorescence. Fetal pancreata ranging from gestational ages 12 to 24 weeks were collected and processed for cryosectioning as previously described (21). The expanded ICCs in monolayer and selected nestin$^+$ cells were grown on coverslips coated with HTB-9 and processed as previously described (13). Primary antibodies used were sheep anti-human insulin (The Binding Site, San Diego, CA), mouse anti-human nestin and rabbit anti-human nestin 331B (provided by Conrad Messam), mouse anti-pan cytokeratin and mouse anti-vimentin (both from Immunotech, Villepinte, France), mouse anti-platelet endothelial cell adhesion molecule (PECAM; Caltag, Burlingame, CA), mouse anti–α smooth muscle actin conjugated to FITC (Sigma), and rabbit anti–Ki-67 (Dako, Carpinteria, CA). Affinity-purified FITC-donkey anti-rabbit IgG, Rhodamine-donkey anti-mouse IgG, and Cy5-donkey anti-sheep IgG (5 μg/ml; all from Jackson ImmunoResearch) were directed against unconjugated primary antibodies. Control sections for all experiments were incubated with a cocktail of normal sheep, rabbit, and mouse IgGs (Jackson ImmunoResearch), followed by the appropriate secondary antibodies. Sections and coverslips were mounted in antifade medium (Biomeda, Foster City, CA) and viewed on Zeiss Axiovert 35 mol/l microscope equipped with a laser

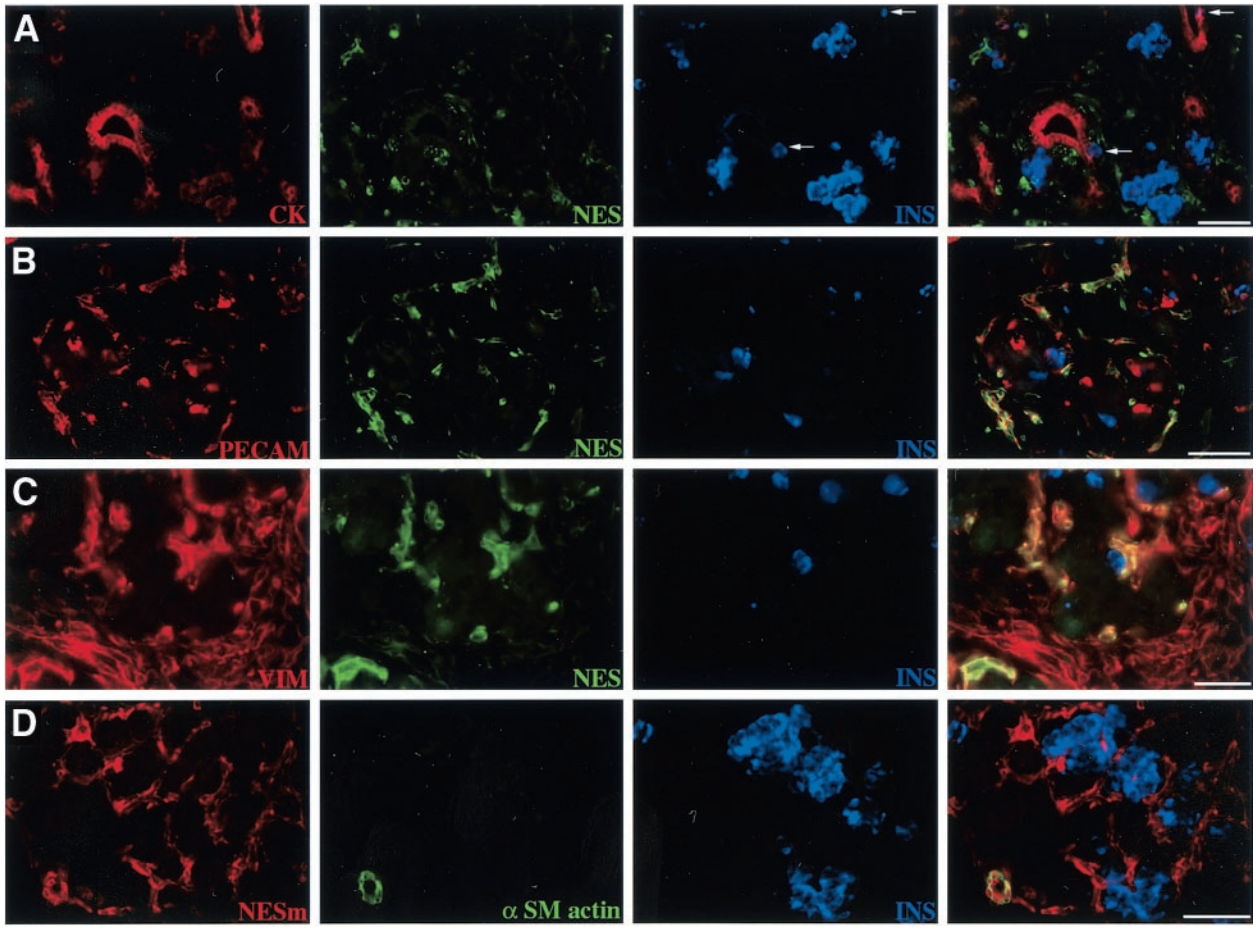

FIG. 1. Localization of nestin, insulin, and pCK (*A*), PECAM (*B*), vimentin (*C*), or α smooth muscle actin (*D*) in the human fetal pancreas. No colocalization of nestin and insulin was observed using either polyclonal (*A–C*) or monoclonal nestin (NESm) antibody (*D*). *A*: No colocalization of nestin and pCK was observed during pancreas development. Immature β-cells associated with the pCK-positive ductal epithelium were nestin negative (arrows). *B*: Colocalization of PECAM and nestin was observed in the blood vessels supplying the developing pancreas. *C*: Vimentin and nestin were also colocalized in the developing pancreatic mesenchyme. *D*: Nestin was also observed in α smooth muscle actin–positive blood vessels. Bar = 50 μm.

scanning confocal attachment (MRC-1024; Bio-Rad Laboratories, Hercules, CA). Color composite pictures were processed using Adobe Photoshop 6.0 (Adobe Systems, Mountainview, CA).

RESULTS

Nestin expression in the fetal human pancreas. The localization of nestin in the fetal human pancreas was unchanged from gestational ages 12 to 24 weeks. Nestin was not seen in pan cytokeratin (pCK)-positive epithelium (Fig. 1*A*). Colocalization was observed in the interstitium with the mesenchymal marker vimentin (Fig. 1*B*). Nestin was also localized to PECAM-positive microvasculature and to the α smooth muscle actin–positive larger blood vessels (Fig. 1*C* and *D*). The monoclonal and polyclonal nestin antibodies showed the same distribution (Fig. 1*D*). Insulin and nestin colocalization was not observed using either the monoclonal or the polyclonal nestin antibody (Fig. 1). The pattern of nestin immunoreactivity was the same in expanded monolayers of fetal pancreatic tissue (ICCs) in vitro (data not shown).

Nestin promoter activity in human fetal pancreatic cells. The second intron of the human nestin gene (nestin enhancer) followed by 160 bp of the basic HSV TK promoter has been shown to be sufficient to drive nestin gene expression in neurepithelial precursors in vivo (15). For demonstrating that the same regulatory elements were sufficient to drive gene expression in human fetal pancreatic cells, GFP was placed under the control of the nestin enhancer TK promoter and introduced to the primary cells via adenoviral infection. The same regulatory elements driving GFP expression were successfully used to identify neurepithelial precursors in embryonic stem cells (16,22).

GFP expression was observed in a subpopulation of fetal pancreatic cells in monolayer, demonstrating that the nestin enhancer TK promoter was sufficient to drive gene expression in human fetal pancreatic cells. Colocalization with the nestin protein and promoter activity was observed (Fig. 2*A*), and in a similar pattern to in vivo, robust GFP expression was associated with vimentin-positive cells and was absent in pCK-positive cells (Fig. 2*A* and *B*). In some monolayer preparations, a rare population of pCK-positive cells also contained a very low level of GFP expression. However, these cells were never observed to coexpress the nestin protein. Insulin was never observed in GFP-positive cells (Fig. 2*B*).

Selection of nestin$^+$ cells. Selection of nestin$^+$ cells using the nestin enhancer/promoter driving neomycin resistance resulted in a pure population of nestin$^+$ cells as detected using both the polyclonal and the monoclonal nestin antibody (Fig. 2*C*). In the same pattern that was observed in the intact pancreas, nestin and vimentin

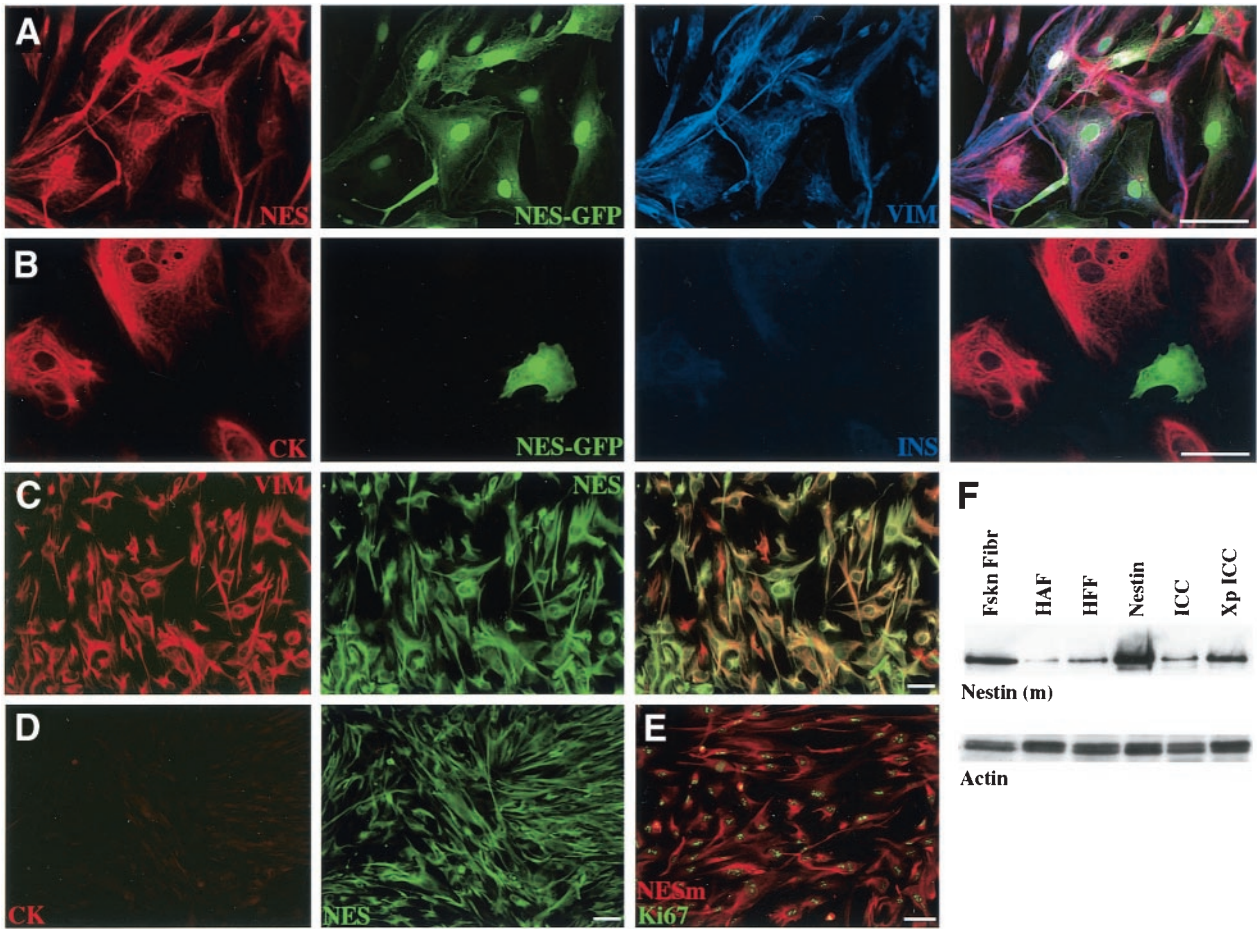

FIG. 2. Nestin promoter activity (*A* and *B*) and characterization of selected nestin$^+$ cells (*C* and *D*). The expression of GFP driven by the nestin promoter in human fetal pancreatic cells in monolayer (*A* and *B*). *A*: GFP-positive cells also colocalize nestin and vimentin. *B*: Robust GFP expression was absent in pCK-positive cells, and insulin was never observed in GFP-positive cells. Both the polyclonal (*C* and *D*) and monoclonal (*E*) nestin antibody detected a pure population of nestin$^+$ cells after selection. *C*: Vimentin and nestin colocalization was observed. *D*: No colocalization of nestin and pCK was evident. *E*: Ki67 was observed in virtually all of the nestin$^+$ cells. Bar (*A–E*) = 50 μm. *F*: Western blot detecting nestin expression using the monoclonal antibody. Nestin selection resulted in a purified population of nestin$^+$ cells compared with ICCs, expanded ICCs (Xp ICCs), HFFs, HAFs, and neonatal human foreskin fibroblasts (Fskn Fibr).

colocalized, whereas no colocalization with pCK was detected (Fig. 2*C* and *D*). The majority of the cells were also positive for the cell cycle marker Ki67 (Fig. 2*E*). Further proof for the effective selection of nestin$^+$ cells was provided by the increase in the amount of nestin protein detected in whole-cell lysates of nestin$^+$ cells when compared with free-floating ICCs, expanded ICCs in monolayer, human fetal pancreatic fibroblasts, adult human pancreatic fibroblasts, and neonatal human foreskin fibroblasts (Fig. 2*F*).

In vitro differentiation of nestin$^+$ cells. The transfection procedure and expression of neomycin resistance (neoR) in human fetal pancreatic cells may be detrimental to the cells' ability to further differentiate. Transfection of the PCIneo parent vector into monolayers of expanded ICCs resulted in a high level of neoR expression (Fig. 3*A*). Both transfection of plasmid DNA and expression of neoR did not impair β-cell differentiation and the induction of insulin message after the reaggregation of the monolayer into a three-dimensional islet-like structure (Fig. 3*A*).

Early passage nestin$^+$ cells from each pancreas were subjected to a variety of conditions to stimulate β-cell development. Both serum and serum-free conditions were tested in conjunction with either tissue culture–treated plastic as a substrate or the laminin-rich HTB-9 basement membrane matrix. Growth factors known to stimulate β-cell development, HGF/SF, nicotinamide, activin A, betacellulin, and the GLP-1 analogue exendin-4 (12,14,18,23) were also added to the serum and serum-free media. The transcription factor PDX-1 is expressed in pancreatic precursor cells and becomes restricted to β-cells in the mature islet, where it controls insulin transcription (8). Induction of PDX-1 or insulin message could not be detected using RT-PCR in any of the culture conditions tested, regardless of the gestational age (Fig. 3*B* and *C*). Expansion of nestin$^+$ cells in either HGF/SF or EGF/FGF2 had no effect on differentiation potential.

Reaggregation into an islet-like three-dimensional cell cluster is known to stimulate insulin gene transcription in fetal pancreatic cells compared with cells grown in monolayer (13) (Fig. 3*A*). Reaggregated nestin$^+$ cells cultured in human serum in the presence or absence of nicotinamide showed neither PDX-1 nor insulin message (Fig. 3*D*). A similar result was found using reaggregated HFFs (Fig. 3*D*).

Transplantation of nestin$^+$ cells. An effective model to examine human β-cell development is transplantation of fetal pancreatic cells into an in vivo environment provided

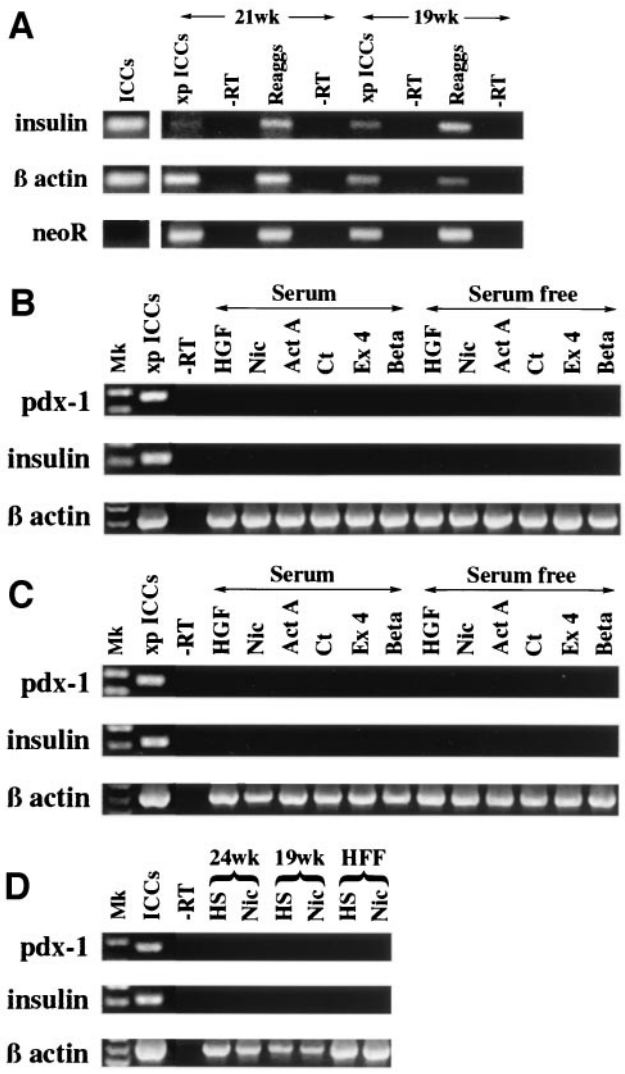

FIG. 3. RT-PCR analysis of nestin$^+$ cells after β-cell differentiation protocols. *A*: Semiquantitative analysis of insulin expression after transfection of PCIneo and expression of neoR. Neither transfection nor expression of neoR affected β-cell differentiation and the increase in insulin expression observed upon reaggregation (Reaggs) of expanded ICCs in monolayer (xp ICC). NeoR expression was not detected in untransfected ICCs. Nestin$^+$ cells were grown on tissue culture–treated plastic (*B*) or HTB-9 basement membrane matrix (*C*) or were reaggregated and grown in suspension (*D*) and compared with expanded ICCs grown in monolayer (Xp ICCs) or ICCs grown in suspension. Cells were grown in the presence or absence of serum (Ct) and challenged with HGF, nicotinamide (Nic), activin A (Act A), exendin 4 (Ex 4), or betacellulin (Beta). Figures are representative of all ages studied. *A* and *B*: Nestin$^+$ cells failed to express pdx-1 or insulin on either substrate studied. *C*: Both nestin$^+$ cells and reaggregated HFFs failed to express pdx-1 or insulin when grown in suspension in human serum (HS) with or without nicotinamide (Nic). Mk, DNA ladder; -RT, reverse transcriptase control.

under the renal capsule of athymic mice. Differentiation of β-cells in the graft tissue can be monitored with the detection of human C-peptide after glucose stimulation (13). Three months after engraftment, positive control grafts containing either ICCs or reaggregated monolayers of expanded ICCs released high levels of human C-peptide after glucose stimulation (Fig. 4). This level of release corresponds to a graft that contains 50% of insulin-positive cells (24). In contrast, the levels of C-peptide detected in the selected nestin$^+$ cells were not significantly different from the HFF-negative control graft and the background

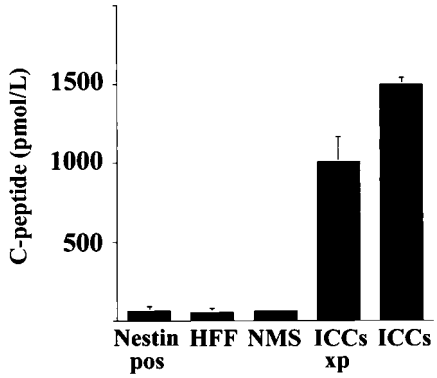

FIG. 4. Circulating C-peptide levels in nude mice 3 months after transplantation with nestin$^+$ cells (n = 7), HFFs (n = 4), expanded (xp) ICCs, and ICCs grown in suspension (n = 5). NMS, nude mouse serum. Bars = SE.

levels seen in the sham-operated animal (Fig. 4). No evidence of insulin-positive cells was seen in the nestin graft. Pretreatment with nicotinamide did not affect the outcome of the reaggregated nestin$^+$ graft; therefore, the results for treated and untreated aggregates were combined.

DISCUSSION

Recent studies using mouse embryonic stem cells and expanded adult human islets suggested that nestin is a marker of β-cell progenitors (1–5). Nestin$^+$ cells therefore have been proposed as a potential source of β-cells for the treatment of type 1 diabetes.

Here, we characterized and isolated a population of nestin$^+$ cells from the developing human pancreas and investigated its ability to differentiate into β-cells both in vitro and after transplantation. We found that nestin is localized specifically to the mesenchyme of the developing human pancreas but not to any epithelial cell population. Furthermore, we showed that after isolation, nestin$^+$ cells do not differentiate into β-cells, neither in vitro nor in vivo.

The mesenchymal expression of nestin was localized to the vasculature of the developing pancreas and vimentin-positive stromal cells. Localization of nestin to the blood vessels is consistent with previous reports describing nestin expression in a diverse group of human organs, including the CNS (9), the gastrointestinal tract (11), and the majority of the vasculature supplying developing organs in the fetal mouse (25). Nestin expression was also observed in the blood vessels during adult rat pancreatic regeneration and fetal pancreatic development (26). More recently, nestin expression was shown to be confined to the endothelium in the adult human pancreas (27). Although a recent study demonstrated the requirement of blood vessels for β-cell development (28), our studies demonstrate that the β-cell lineage itself has no developmental relationships with a putative pool of nestin$^+$ precursors.

Vimentin-positive stromal cells have been well characterized as pancreatic fibroblasts. Colocalization of nestin and vimentin within pancreatic fibroblasts is also consistent with the observations of fibroblast-like cells expressing nestin in the human gastrointestinal tract (11). Nestin expression was also solely localized to the developing

mouse pancreatic stroma; however, cell types in the interstitial tissue were not fully characterized (29).

The mesenchymal localization of nestin is not consistent with the currently accepted model of pancreatic β-cells arising from the ductal epithelium. An alternative to vimentin and nestin colocalization representing a population of fibroblasts is a population of cells similar to the neurepithelial precursors. The pure population of nestin cells selected on the basis of nestin enhancer/promoter activity also expressed a high level of vimentin, and virtually all of the cells were mitotically active. Neurepithelial precursors also colocalize vimentin and nestin (6,7); this expression pattern in the pancreas may represent migrating neural crest cells as suggested in past models of islet development (30). However, compelling evidence by LeDouarin (31) excluded the contribution of neurectodermal cells to pancreatic lineages.

Generation of insulin-secreting cells from nestin$^+$ mouse embryonic stem cells has led to further models of β-cell differentiation. Preferential growth of nestin$^+$ embryonic stem cells ("nestin selection") has recently been shown to increase the yield of insulin$^+$ cells (5). This increase was attributed to the possible selection of endocrine precursors because nestin may have a role in neuroendocrine migration (5). Therefore, in the human fetal pancreas, ductal cells may lose their epithelial phenotype upon a migratory stimulus and transiently express nestin and other intermediate filament proteins such as vimentin before insulin expression. This would explain their stromal localization in the fetal pancreas. However, there was no loss of epithelial phenotype before the expression of insulin in the human fetal pancreas, because colocalization of epithelial cytokeratins and insulin was observed in the current study and by others (32). Furthermore, neither nestin and cytokeratin nor nestin and insulin colocalization was observed, suggesting that a transient stage of nestin expression is not required before hormone production and loss of the epithelial markers. Isolation of the of nestin$^+$ pancreatic cells supported the in vivo observations because β-cell differentiation was not achieved in vitro or in vivo. The results from the current study, however, do suggest that the nestin$^+$ mouse embryonic stem cells that generate insulin-producing cells (3–5) are a different population from that seen in the fetal pancreas.

The nestin selection approach clearly suggests that nestin is not a specific marker of β-cell precursors during normal development. However, the potential shortfalls of this approach must be noted. Although the second intron of the human nestin gene is sufficient to drive gene expression in neurepithelial precursors (15,16,22), additional elements of the nestin promoter may be required for gene expression in human fetal pancreatic cells. The current study demonstrated that the second intron of the nestin gene was sufficient to drive GFP expression in human fetal pancreatic cells. Furthermore, GFP colocalization with nestin protein was observed in the same cell types as seen in vivo.

The developmental stage at which the selection plasmid is introduced is also of critical importance. It is possible that during 12–24 gestational weeks, transient expression of nestin is not observed in β-cell precursors, therefore explaining the lack of immunofluorescent evidence and the isolation of a nestin$^+$ β-cell precursor. It seems unlikely that during the period of 12–24 weeks' gestation, when ICCs contain a large proportion of β-cell precursors capable of differentiating into a population of 50% insulin-positive cells after transplantation (24), that our approach would miss the detection of nestin$^+$ β-cell precursors.

The efficiency of transfection raises the concern that the selection plasmid did not transfect a small subpopulation of nestin$^+$ cells capable of undergoing β-cell differentiation. An adenoviral vector containing the same regulatory elements driving GFP expression was used to transduce 95% of the fetal pancreatic cells. Robust GFP expression was observed in the same population of cells that were selected on the basis of neoR introduced by transfection.

From the data presented, we conclude that nestin$^+$ cells in the fetal pancreas are unlikely to generate β-cells and do not represent a candidate population from the fetal pancreas to be used for transplantation therapy. This conclusion is based on characterizing the population of nestin$^+$ cells during human pancreatic development, isolating a pure population of nestin$^+$ cells, and challenging the cells both in vitro and in vivo with conditions known to stimulate β-cell differentiation.

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REFERENCES

1. Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF: Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 50:521–533, 2001
2. Abraham EJ, Leech CA, Lin JC, Zulewski H, Habener JF: Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells. *Endocrinology* 143: 3152–3161, 2002
3. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R: Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* 292:1389–1394, 2001
4. Hori Y, Rulifson IC, Tsai BC, Heit JJ, Cahoy JD, Kim SK: Growth inhibitors promote differentiation of insulin-producing tissue from embryonic stem cells. *Proc Natl Acad Sci U S A* 99:16105–16110, 2002
5. Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM: Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc Natl Acad Sci U S A* 100:998–1003, 2003
6. Lendahl U, Zimmerman LB, McKay RD: CNS stem cells express a new class of intermediate filament protein. *Cell* 60:585–595, 1990
7. Messam CA, Hou J, Major EO: Coexpression of nestin in neural and glial cells in the developing human CNS defined by a human-specific anti-nestin antibody. *Exp Neurol* 161:585–596, 2000
8. Edlund H: Developmental biology of the pancreas. *Diabetes* 50 (Suppl. 1):S5–S9, 2001
9. Dahlstrand J, Collins VP, Lendahl U: Expression of the class VI intermediate filament nestin in human central nervous system tumors. *Cancer Res* 52:5334–5341, 1992
10. Kobayashi M, Sjoberg G, Soderhall S, Lendahl U, Sandstedt B, Sejersen T: Pediatric rhabdomyosarcomas express the intermediate filament nestin. *Pediatr Res* 43:386–392, 1998
11. Vanderwinden JM, Gillard K, De Laet MH, Messam CA, Schiffmann SN: Distribution of the intermediate filament nestin in the muscularis propria of the human gastrointestinal tract. *Cell Tissue Res* 309:261–268, 2002
12. Otonkoski T, Beattie G, Mally M, Ricordi C, Hayek A: Nicotinamide is a

potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. *J Clin Invest* 92:1459–1466, 1993
13. Beattie GM, Rubin JS, Mally MI, Otonkoski T, Hayek A: Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes* 45:1223–1228, 1996
14. Otonkoski T, Cirulli V, Beattie M, Mally MI, Soto G, Rubin JS, Hayek A: A role for hepatocyte growth factor/scatter factor in fetal mesenchyme-induced pancreatic beta-cell growth. *Endocrinology* 137:3131–3139, 1996
15. Lothian C, Lendahl U: An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. *Eur J Neurosci* 9:452–462, 1997
16. Lenka N, Lu ZJ, Sasse P, Hescheler J, Fleischmann BK: Quantitation and functional characterization of neural cells derived from ES cells using nestin enhancer-mediated targeting in vitro. *J Cell Sci* 115:1471–1485, 2002
17. Leibowitz G, Beattie GM, Kafri T, Cirulli V, Lopez AD, Hayek A, Levine F: Gene transfer to human pancreatic endocrine cells using viral vectors. *Diabetes* 48:745–753, 1999
18. Hui H, Wright C, Perfetti R: Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. *Diabetes* 50:785–796, 2001
19. Assady S, Maor G, Amit M, Itskovitz-Eldor J, Skorecki KL, Tzukerman M: Insulin production by human embryonic stem cells. *Diabetes* 50:1691–1697, 2001
20. Hayek A, Beattie GM: Experimental transplantation of human fetal and adult pancreatic islets. *J Clin Endocrinol Metab* 82:2471–2475, 1997
21. Cirulli V, Beattie GM, Klier G, Ellisman M, Ricordi C, Quaranta V, Frasier F, Ishii JK, Hayek A, Salomon DR: Expression and function of alpha(v) beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells. *J Cell Biol* 150:1445–1460, 2000
22. Andressen C, Stocker E, Klinz FJ, Lenka N, Hescheler J, Fleischmann B, Arnhold S, Addicks K: Nestin-specific green fluorescent protein expression in embryonic stem cell-derived neural precursor cells used for transplantation. *Stem Cells* 19:419–424, 2001
23. Demeterco C, Beattie GM, Dib SA, Lopez AD, Hayek A: A role for activin A and betacellulin in human fetal pancreatic cell differentiation and growth. *J Clin Endocrinol Metab* 85:3892–3897, 2000
24. Beattie GM, Lopez AD, Otonkoski T, Hayek A: Transplantation of human fetal pancreas: fresh vs. cultured fetal islets or ICCS. *J Mol Med* 77:70–73, 1999
25. Mokry J, Nemecek S: Angiogenesis of extra- and intraembryonic blood vessels is associated with expression of nestin in endothelial cells. *Folia Biol (Praha)* 44:155–161, 1998
26. Lardon J, Rooman I, Bouwens L: Nestin expression in pancreatic stellate cells and angiogenic endothelial cells. *Histochem Cell Biol* 117:535–540, 2002
27. Klein T, Ling Z, Heimberg H, Madsen OD, Heller RS, Serup P: Nestin is expressed in vascular endothelial cells in the adult human pancreas. *J Histochem Cytochem* 51:697–706, 2003
28. Lammert E, Cleaver O, Melton D: Induction of pancreatic differentiation by signals from blood vessels. *Science* 294:564–567, 2001
29. Selander L, Edlund H: Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas. *Mech Dev* 113:189–192, 2002
30. Dubois PM: Ontogeny of the endocrine pancreas. *Horm Res* 32:53–60, 1989
31. Le Douarin NM: On the origin of pancreatic endocrine cells. *Cell* 53:169–171, 1988
32. Bouwens L, Wang RN, De Blay E, Pipeleers DG, Kloppel G: Cytokeratins as markers of ductal cell differentiation and islet neogenesis in the neonatal rat pancreas. *Diabetes* 43:1279–1283, 1994