Brief Report

No Evidence for Mouse Pancreatic β-Cell Epithelial-Mesenchymal Transition In Vitro

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We used cre/loxP-based genetic lineage tracing analysis to test a previously proposed hypothesis that in vitro cultured adult pancreatic β-cells undergo epithelial-mesenchymal transition (EMT) to generate a highly proliferative, differentiation-competent population of mesenchymal islet "progenitor" cells. Our results in the mouse that are likely to be directly relevant to the human system show that adult mouse β-cells do not undergo EMT in vitro and that the mesenchymal cells that arise in cultures of adult pancreas are not derived from β-cells. We argue that these cells most likely originate from expansion of mesenchymal cells integral to the heterogeneous pancreatic islet preparations. As such, these mesenchymal "progenitors" might not represent the best possible source for generation of physiologically competent β-cells for treatment of diabetes. *Diabetes* 56:699–702, 2007

Epithelial-mesenchymal transition (EMT) plays an essential role in embryonic development and tumorigenesis (1). Recently, it has been suggested that human pancreatic β-cells may undergo EMT when cultured in vitro (2–4). According to this model, the EMT is accompanied by the drastic downregulation of β-cell insulin expression and transformation of β-cells into rapidly replicating human islet progenitor cells (hIPCs) that can be generated in virtually unlimited quantities. The investigators hypothesized that in the future, it might be possible to induce efficient differentiation of the hIPCs via mesenchymal-epithelial transition into β-cells producing physiological levels of insulin. Because redifferentiation of hIPCs could provide an abundant source of β-cells for transplantation into patients with type 1 and type 2 diabetes, this would represent a major advance. Moreover, a conclusive demonstration of β-cell EMT would signify a fundamental discovery in islet cell biology, with important basic science implications.

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EGFP; enhanced green fluorescent protein; EMT, epithelial-mesenchymal transition; FACS, fluorescence-activated cell sorting; hIPC, human islet progenitor cell; IEF, islet-enriched fraction.

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Given that islet preparations contain islet and non-islet epithelial and mesenchymal cells, a full substantiation of the EMT hypothesis requires a conclusive demonstration that β-cells, and not the other cell types intrinsic to islet preparations, give rise to mesenchymal "progenitors." This can be best accomplished through a direct lineage tracing of the cellular progeny of in vitro cultured β-cells. To carry out such tracing, we used a well-established transgenic mouse cre/loxP-based system (5). Our results provide compelling evidence against EMT of β-cells and suggest that abundant populations of mesenchymal cells in pancreatic cultures are not derived from β-cells.

RESEARCH DESIGN AND METHODS

Transgenic animals. The rat insulin promoter (RIP)-cre/EG mice were obtained by crossing RIP-cre recombinase mice containing a 668-bp fragment of the rat insulin II gene promoter (stock #0 003573; The Jackson Laboratory, Bar Harbor, ME) with double-reporter Z/EG transgenic mice (60). In the RIP-cre/EG mice, the cre-mediated β-cell–specific recombination results in positioning of enhanced green fluorescent protein (*EGFP*) gene, under control of a strong ubiquitous pCAG promoter (7), thus irreversibly labeling β-cells with EGFP (Fig. 1*A*).

Preparation of islet-enriched fractions and in vitro culture. Islet preparations, or islet-enriched fractions (IEFs) (8), were isolated from 6- to 8-week-old mouse pancreas perfused with collagenase V (Sigma, St. Louis, MO) using described protocols (8) and following National Institutes of Health guidelines for use and care of laboratory animals. Human IEFs were isolated and cultured as described (9). Mouse IEFs were cultured in serum-containing medium as in reference 4 or serum-free medium (10). Briefly, for serum-free media culture, cells were cultured in ITSFn medium (11), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN), and 1,400 unit/ml leukemia inhibitory factor (LIF; Chemicon, Temecula, CA). Bone morphogenetic protein-4 (BMP-4; R&D Systems) was added from day 5 at 10 ng/ml, and the ITSFn medium was replaced with serum-free N2 medium (11) containing B27 supplement (Invitrogen) and 1,400 units/ml LIF.

Flow cytometry. Freshly made IEFs were dissociated into single cells for 5–10 min at 37°C by incubation in Ca^{2+}/Mg^{2+}-free PBS. In vitro cultured IEFs were dissociated by trypsinization. The single cell suspensions were fixed in 4% paraformaldehyde for 30 min at room temperature. Intracytoplasmic insulin was detected using guinea pig polyclonal anti-insulin primary antibody (Dako, Carpinteria, CA) and secondary Cy5-conjugated donkey anti–guinea pig antibody (Jackson Immunoresearch, West Grove, PA). The antibody reactions were carried out for 20 min at 40°C in HBSS (Hank's balanced salt solution)/0.1% saponin/1% BSA/10% normal donkey serum. The cells were washed twice with HBSS/saponin/BSA. Gating by forward and side scatter allowed the exclusion of dead cells from the analysis. Flow cytometry (fluorescence-activated cell sorting [FACS]) was carried out on CyAn ADP flow cytometer (Dako) using Summit Software (Dako).

Immunocytochemistry. The cultured cells or paraffin-embedded adult mouse pancreatic sections (10 μ) were fixed with 4% paraformaldehyde for 30 min at room temperature. The following primary antibodies were used: C-peptide, guinea pig polyclonal, 1:400 (Linco, St. Charles, MO); EGFP, mouse monoclonal, 1:200 (Qbiogene, Carlsbad, CA); nestin, rabbit polyclonal, 1:100 (gift from Ron McKay's laboratory, National Institute of Neurological Disorders and Stroke/National Institutes of Health); vimentin, mouse monoclonal,

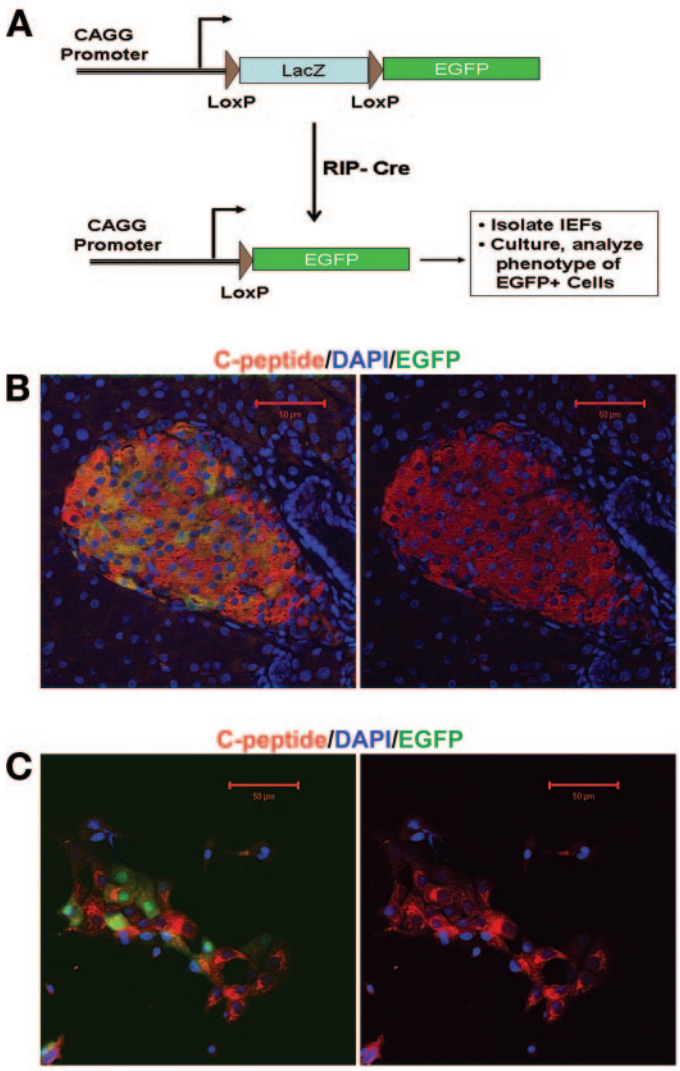

FIG. 1. EGFP-expressing cells coexpress C-peptide before and during the culture. *A*: Diagram of the assay system for β-cell lineage tracing. *B*: Analysis of C-peptide and EGFP expression in pancreatic sections from a 6-week-old RIP-cre/EG mouse by immunocytochemistry. *C*: Analysis of C-peptide and EGFP expression in day-11 RIP-cre/EG IEF mouse cultures. The *left* and *right panels* in *B* and *C* show a split image of the same microscopic fields. Cell nuclei are identified by DAPI staining. The image in *B* was obtained using a confocal laser scanning microscope (Zeiss LSM510). The image in *C* was obtained using Zeiss Axiovert 2 Plus microscope.

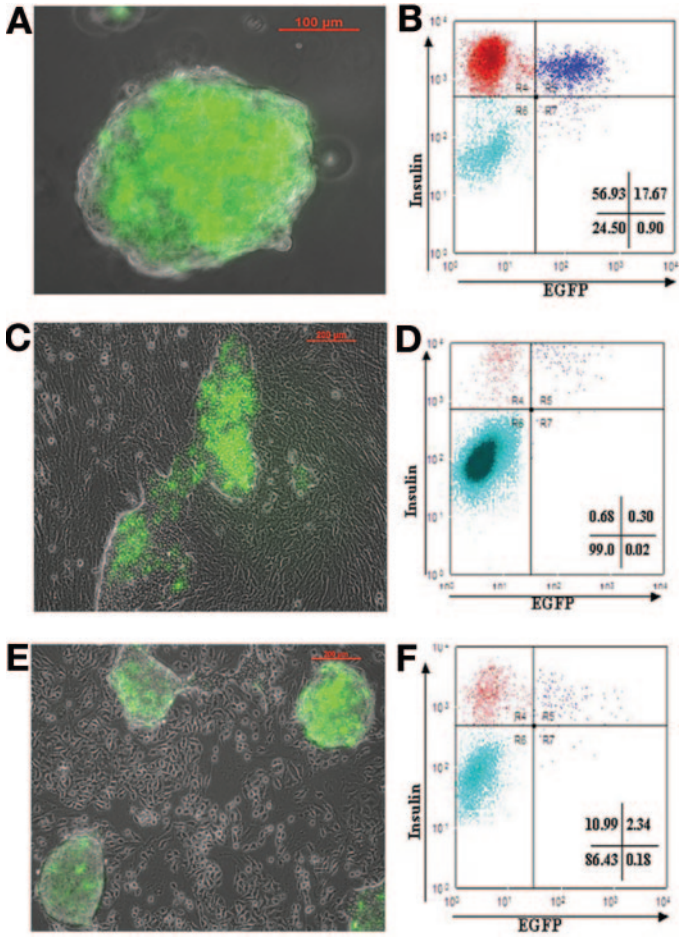

FIG. 2. IEF cultures generate abundant EGFP$^-$ cell populations; β-cells remain insulin$^+$ during the culture. *A*, *C*, and *E*: Microscopic images of live transgenic mouse IEFs before (*A*) and during (*C* and *E*) the culture. *C*: Twelve days of culture in serum-containing medium, protocol as in ref. 4. *E*: Twelve days of culture in serum-free medium, protocol as in ref. 9. Live cell images were obtained using Zeiss Axiovert 200 inverted microscope. *B*, *D*, and *F*: FACS analysis of IEF preparations at time points corresponding with the live cell images shown in *A*, *C*, and *E*. *B* was taken before culture and *D* and *F* following the culture. Percentages of cells expressing insulin, EGFP, and the combinations are indicated on the *bottom right* of each FACS panel. The lower percentage of insulin- and EGFP-expressing cells in *D* relative to *F* reflects a more vigorous cell growth in serum-containing vs. serum-free medium.

1:200 (clone LN-6, IgM); and goat polyclonal, 1:100 (Sigma). Secondary antibodies were: Alexa Fluor 488 (green; Molecular Probes, Eugene, OR) and CY-3 (red; Jackson Immunoresearch). CY-3 IgM (Jackson Immunoresearch) was used in combination with vimentin mouse monoclonal antibody. Reactions with secondary antibodies, omitting primary antibodies, were used as controls.

In situ hybridization/immunocytochemistry. The in situ hybridizations were performed as described (12,13), with digoxigenin-labeled (UTP/DIG; Roche, Indianapolis, IN) insulin 2 riboprobes (212 bases long) or as described in reference 4, with Greenstar oligonucleotide probes (Gene Detect). Antisense and control sense probes were used in parallel in every experiment. The immunocytochemistry was carried out using standard protocols (described above). The immunocytochemistry was carried out after completion of the in situ hybridization. The in situ hybridization protocol using the UTP/DIG probe was calibrated to reduce the concentration of the probe and exposure time to alkaline phosphatase substrate. This calibration allowed us to minimize alkaline phosphatase substrate–mediated quenching of fluorescence in subsequent immunocytochemistry step.

RESULTS

RIP-cre/EG double-transgenic mice whose pancreatic β-cells are specifically and irreversibly marked with EGFP (Fig. 1*A*) allow tracing the fate of β-cell progeny. Thus, if during the culture, EGFP-labeled β-cells undergo EMT and downregulate insulin expression, they would continue to express EGFP. The emergence of EGFP$^+$/insulin$^-$ mesenchymal cells would indicate that the β-cell underwent EMT. Here we used immunocytochemistry, FACS, and in situ hybridization to identify such cells.

To validate our assay system, we examined expression of insulin/C-peptide and EGFP on sections of adult RIP-cre/EG pancreas and in RIP-cre/EG IEFs prior to culture. We found that only ~25% of all insulin-expressing cells coexpressed EGFP (Fig. 1*B* and Fig. 2*A* and *B*). Such incomplete labeling, commonly observed in cre-loxP systems (5), is likely to reflect suboptimal levels of cre-recombinase produced by β-cells. We argue, however, that this level of labeling is sufficient to detect β-cell EMT,

which, as has been suggested (4), occurs at high frequency. Our results show that EGFP$^+$ β-cells are evenly distributed within the body of the islets (Fig. 1*B*) and that insulin$^+$/EGFP$^+$ cell population is composed of cells with the level of insulin expression similar to that in insulin$^+$/EGFP$^-$ population (Fig. 2*B*, compare insulin signal in the *top left* [insulin$^+$/EGFP$^-$] and *top right* [insulin$^+$/EGFP$^+$] quadrants). These results imply that EGFP labeling is not biased toward specific β-cell subpopulations—for instance, those with the highest level of insulin expression and can track an "average" β-cell. Further, we did not detect EGFP$^+$/insulin$^-$ cells in RIP-cre/EG pancreatic sections (Fig. 1*B*) or in RIP-cre/EG IEFs (Fig. 2*B*). We therefore concluded that β-cells are labeled specifically, and no EGFP$^+$/insulin$^-$ cells preexist in these mice.

We cultured RIP-cre/EG mouse IEFs in serum-containing medium as in reference 4 or in serum-free medium as in reference 10. The results described below have been reproduced in six independent experiments; pooled IEFs from two RIP-cre/EG mice were used in each experiment. While we observed active cell growth under both experimental conditions (Fig. 2*C* and *E*), growth in serum-containing medium was more vigorous than under serum-free conditions. After 10–12 days, we obtained confluent cell monolayers mostly consisting of EGFP$^-$ cells. Typically, after 10–12 days in serum-containing medium, we achieved 10- to 15-fold cell expansion and in serum-free medium 5- to 6-fold cell expansion. (Cell expansion is defined as the number of cells at the end/before of the culture.)

Both conditions generated similar results, and we discuss them together. Quantitative analysis by FACS showed drastic reduction of a percentage of EGFP$^+$ cells in the total cell population, specifically, an ~60-fold reduction for serum-containing medium and an ~8-fold reduction for serum-free medium cultures (compare Fig. 2*B* with *D* and *F*). After two to three cell passages (~25–30 days of culture), we completely lost the EGFP signal (results not shown). Despite the reduction in the percentage of EGFP$^+$ cells, the frequency of EGFP$^+$ cells among the insulin$^+$ cells did not change significantly from day 0 to day 12, remaining at ~20% (compare Fig. 2*B* with *D* and *F*), suggesting that EGFP protein did not exert negative effect on β-cell proliferation and/or viability and that pCAG promoter does not become silenced during the course of the culture. Most importantly, under both culture conditions, FACS failed to detect a population of EGFP$^+$/insulin$^-$ cells, indicative of β-cells losing insulin expression and undergoing EMT. Occasionally, we observed EGFP$^+$ cells with mesenchymal-like morphology (Fig. 2*C* and *E* and Fig. S1A [available in an online appendix at http://dx.doi.org/10.2337/db06-1446]). However, these cells remained C-peptide immunoreactive (Fig. 1*C*), implying that they retain β-cell phenotype.

In the next series of experiments we investigated whether any EGFP-expressing β-cells might be acquiring mesenchymal phenotype in culture. Gershengorn et al. (4) used vimentin as a marker of mesenchyme. We found that available anti-vimentin antibodies (RESEARCH DESIGN AND METHODS) perform well on human but not on mouse IEF cultures. However, given that in pancreas both vimentin and nestin mark mesenchymal cells (14,15) and that we (F.A., unpublished observations, and Fig. S1B) and others (4 and Fig. S2B) had shown that a majority of vimentin-expressing cells in human pancreatic cultures coexpress nestin, we reasoned that nestin could serve as a reliable marker for the purposes of our study. Our analysis of coexpression of EGFP and nestin in RIP-cre/EG IEF cultures failed to detect nestin$^+$/EGFP$^+$ cells (Fig. 3*A*); we therefore concluded that EGFP-labeled β-cells are unlikely to be undergoing transformation into mesenchymal cells.

These results are in contrast with those reported in reference 4, where the authors identified proinsulin mRNA in vimentin-expressing cells of human pancreatic cultures. To address this discrepancy, we carried out insulin in situ hybridizations following protocol described in reference 4. To control for a possible nonspecific in situ hybridization signal, we combined proinsulin in situ hybridization with C-peptide immunocytochemistry. In agreement with the results of Gershengorn et al. (4), we observed punctate proinsulin oligonucleotide probe-mediated signal. However, in our experiments, this signal was not associated with the C-peptide immunoreactivity; also, the antisense and the sense probes generate similar patterns, suggesting that a punctate in situ hybridization signal was nonspecific (Fig. S1C).

To further examine expression of proinsulin mRNA in the mesenchyme, we used an alternative in situ hybridization protocol (12,13) utilizing a 212-bp digoxigenin-labeled proinsulin riboprobe instead of Greenstar oligonucleotide proinsulin probe (4). We combined in situ hybridization with C-peptide/nestin immunocytochemistry. Our results (Fig. 3*B*) agree with those in Fig. 3*A*, demonstrating clear signal separation of proinsulin (in situ hybridization)/C-peptide (immunocytochemistry) from nestin (immunocytochemistry) in most of the cells. It is conceivable that cells coexpressing low levels of nestin/proinsulin/C-peptide could have escaped detection in this assay. Also, infrequently we observed nestin$^+$/proinsulin mRNA/C-peptide$^+$ cells (designated by asterisks in Fig. 3*B*). However, given the lack of coexpression of EGFP and nestin in RIP-cre/EG IEF cultures (Fig. 3*A*), such coexpressing cells (Fig. 3*B*) may be generated through mechanisms other than β-cell EMT.

DISCUSSION

Our results demonstrate that β-cell EMT does not take place in mouse pancreatic IEF cultures and that the abundant populations of mesenchymal cells found in these cultures are not derived from β-cells. Although these studies were performed with mouse cells, considering extensive similarities between rodent and human pancreatic development and the fundamental role of EMT in development and tumorigenesis, our findings in the mouse are likely to be directly relevant to the human system. While we cannot completely rule out that rare β-cell EMT would escape detection, given that ~25% of "average" β-cells become labeled with EGFP, it is unlikely that numerous EMT events hypothesized by Gershengorn et al. (4) would remain undetected in this system.

What then is the origin of mesenchymal "progenitor" cells? Human islet preparation used in references 3 and 4, as well as rodent islet preparations used in this work, invariably contain islet and non-islet epithelial and mesenchymal cells (8,9). Mesenchymal cells are known to possess high proliferative capacity in vitro, particularly in serum-containing media. It is thus reasonable to suggest that these mesenchymal cells intrinsic to input IEFs give rise to the abundant populations of these "progenitors."

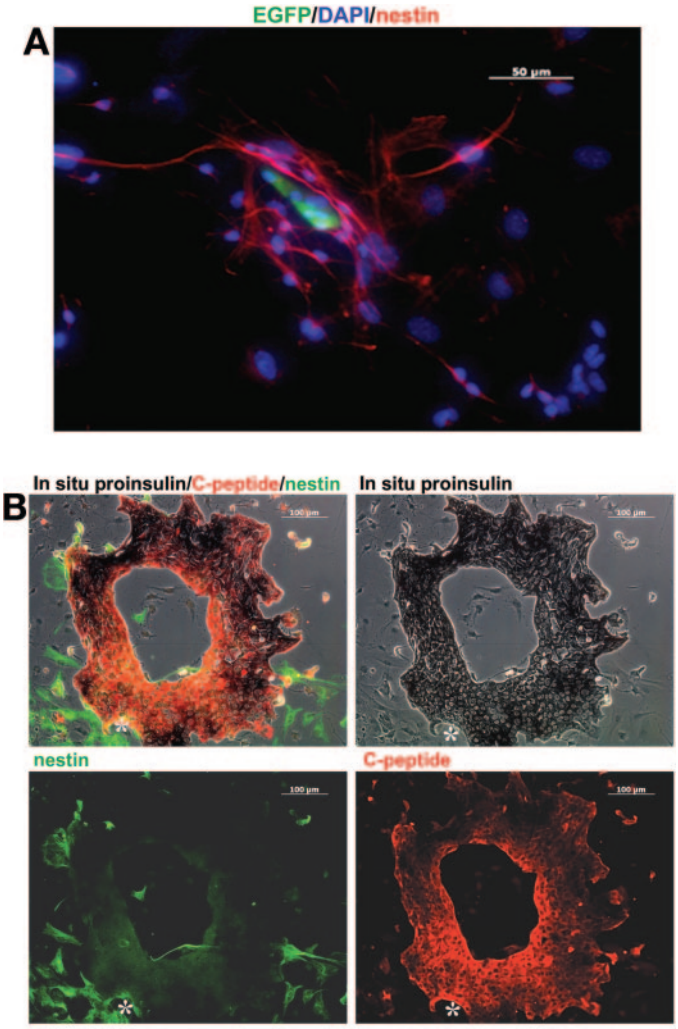

FIG. 3. Mesenchymal cells in IEF cultures are not derived from β-cells. *A*: Analysis of nestin and EGFP expression by immunocytochemistry in day-11 RIP-cre/EG mouse IEF cultures. *B*: Analysis of proinsulin mRNA expression by in situ hybridization and C-peptide and nestin expression by immunocytochemistry on a wild-type mouse in day-8 IEF cultures. The four panels show a split image of the same microscopic field. Cells coexpressing low levels of nestin and proinsulin mRNA are identified by asterisks. The images were obtained using Zeiss Axiovert 2 Plus microscope.

Since they express negligible levels of islet-specific genes (4), hIPCs might not represent optimal candidates for generation of physiologically competent β-cells.

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