Identification of a novel putative pancreatic stem/progenitor cell marker DCAMKL-1 in normal mouse pancreas

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Submitted 31 March 2010; accepted in final form 27 May 2010

May R, Sureban SM, Lightfoot SA, Hoskins AB, Brackett DJ, Postier RG, Ramanujam R, Rao CV, Wyche JH, Anant S, Houchen CW. Identification of a novel putative pancreatic stem/progenitor cell marker DCAMKL-1 in normal mouse pancreas. Am J Physiol Gastrointest Liver Physiol 299: G303–G310, 2010. First published June 3, 2010; doi:10.1152/ajpgi.00146.2010.—Stem cells are critical in maintaining adult homeostasis and have been proposed to be the origin of many solid tumors, including pancreatic cancer. Here we demonstrate the expression patterns of the putative intestinal stem cell marker DCAMKL-1 in the pancreas of uninjured C57BL/6 mice compared with other pancreatic stem/progenitor cell markers. We then determined the viability of isolated pancreatic stem/progenitor cells in isotransplantation assays following DCAMKL-1 antibody-based cell sorting. Sorted cells were grown in suspension culture and injected into the flanks of athymic nude mice. Here we report that DCAMKL-1 is expressed in the main pancreatic duct epithelia and islets, but not within acinar cells. Coexpression was observed with somatostatin, NGN3, and nestin, but not glucagon or insulin. Isolated DCAMKL-1+ cells formed spheroids in suspension culture and induced nodule formation in isotransplantation assays. Analysis of nodules demonstrated markers of early pancreatic development (PDX-1), glandular epithelium (cytokeratin-14 and Ep-CAM), and isletlike structures (somatostatin and secretin). These data taken together suggest that DCAMKL-1 is a novel putative pancreatic stem/progenitor marker that can be used to isolate normal pancreatic stem/progenitors and potentially regenerate pancreatic tissues. This may represent a novel tool for regenerative medicine and a target for anti-stem cell-based therapeutics in pancreatic cancer.

CHARACTERIZATION OF STEM CELLS from the hematopoietic and central nervous system has emphasized the importance of specific cell surface antigens that permit the isolation of stem cells by fluorescence-activated cell sorting (FACS) (17, 29). A specific cell surface antigens that permit the isolation of stem cells. Moreover, NGN3-deficient mice do not develop islet cells and are diabetic. These data taken together suggest that NGN3 and nestin are critical components of the pancreatic stem/progenitor cell compartment. A recent study demonstrated that expansion of the β-cell mass following pancreatic duct ligation resulted in ductal NGN3 gene expression and the ensuing differentiation of endogenous progenitor cells (31).

We have recently determined that doublecortin and Ca2+/calmodulin-dependent kinase-like-1 (DCAMKL-1), a microtubule-associated kinase expressed in postmitotic neurons, is a putative intestinal stem cell marker (14, 15). Here we report that DCAMKL-1 is also expressed in pancreatic islet epithelial cells with a distribution similar to the putative pancreatic stem/progenitor cell markers NGN3 and nestin. Furthermore, following DCAMKL-1-based FACS, isolated cells formed spheroidlike structures in suspension culture. When injected subcutaneously into flanks of nude mice, nodules formed and contained cells expressing markers of early pancreatic development (PDX-1), glandular epithelium (cytokeratin-14 and epithelial cell adhesion molecule (Ep-CAM)), and islets (somatostatin and secretin). These data taken together identify DCAMKL-1 as a novel pancreatic ductal and islet stem/progenitor cell marker.

MATERIALS AND METHODS

Experimental animals. Six- to 8-wk-old C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) athymic nude mice (Ncr-nu) (NCI-Frederick, Frederick, MD) were used for the experiments. Mice were housed under controlled conditions, including a 12-h light-dark cycle, with ad libitum access to diet and water. All animal experiments were performed according to the animal protocols approved by the University Animal Study Committee.

Immunohistochemistry. Heat-induced epitope retrieval was performed on formalin-fixed paraffin-embedded sections by utilizing a pressurized decloaking chamber (Biocare Medical, Concord, CA) in citrate buffer (pH 6.0) at 99°C for 18 min. For brightfield microscopy, slides were incubated in 3% hydrogen peroxide, then in normal serum and BSA at room temperature for 20 min. After incubation with primary antibody [DCAMKL-1 1:100 (rabbit), PDX-1 1:1,000 (rabbit), Ep-CAM 1:100 (rabbit) glucagon 1:100 (goat) (Abcam, Cambridge, MA); Ki67 1:300 (rabbit) (Thermo Scientific/Lab Vision, Fremont, CA); NGN3 1:200 (goat), nestin 1:100 (rabbit) (Abgent, San Diego, CA); insulin 1:250 (goat), somatostatin 1:250 (goat), cytokeratin-14 1:100 (rabbit), and secretin 1:100 (goat) (Santa Cruz Biotechnology, Santa Cruz, CA)], the slides were incubated either in polymer–horseradish peroxidase secondary (Dako, Glostrup, Denmark) for rabbit-derived or goat polymer detection kit (Biocare Medical) for goat-derived antibodies as appropriate. Slides were developed with diamobenzidine (Sigma, St. Louis, MO). Tyramine signal amplification for NGN3 in adult mouse tissues was performed per manufacturer’s instructions (Invitrogen, Carlsbad, CA).
For fluorescence microscopy, slides were first incubated in Image-iT FX signal enhancer (Invitrogen), followed by normal serum and BSA at room temperature for 20 min. After incubation with primary antibody overnight at 4°C, slides were incubated in appropriate donkey anti-goat/rabbit Alexa Fluor-conjugated secondary as appropriate [488 (green) and 568 (red) (Invitrogen)].

Microscopic examination. Slides were examined by utilizing the Nikon 80i microscope and DXM1200C camera for brightfield microscopy. Fluorescent images were taken with PlanFluoro objectives, utilizing CoolSnap ES2 camera (Photometrics, Tucson, AZ). Images were captured with NIS-Elements software (Nikon Instruments, Melville, NY).

Real-time reverse transcription-PCR analyses. Total RNA isolated from FACS sorted pancreatic cells was subjected to reverse transcription with Superscript II RNase H-reverse transcriptase and random hexanucleotide primers (Invitrogen). The cDNA was subsequently used to perform real-time PCR by SYBR chemistry (SYBR Green I; Molecular Probes) for specific transcripts using gene specific primers and Jumpstart Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO). The crossing threshold value assessed by real-time PCR was noted for the transcripts and normalized with β-actin mRNA. The changes in mRNA were expressed as fold change relative to control with ±SE value.

Primers used are as follows. β-actin: forward 5'-GGTGATCCACATCTGTGGAA-3', reverse 5'-ACATTGTCTCTCTGAGG-3'; DCAMKL-1: forward 5'-CAGCCTGACAGCTGTGGG-3', reverse 5'-TGACCAAGTGGGGTCCAT-3'; NGN3: forward 5'-CGACCATGCGCCCTCATCCCCCGG-3', reverse 5'-CAAGGTCCCTCCTGACAG-3'; nestin: forward 5'-CACCTCAAGATGGTGCT-3', reverse 5'-GAGCTAGTCATGCTGTC-3'; somatostatin: forward 5'-GGACCGCCAGCTGCAGAT-3', reverse 5'-GGGCT-3'.

Fig. 1. Pancreatic DCAMKL-1 expression in adult mice. A: DCAMKL-1 expression (brown) in the main pancreatic duct (left) (×200) and in the periphery of pancreatic islets (middle) (×400). No DCAMKL-1 expression is observed in acinar cells or accessory ducts (right) (×400). B: immunofluorescence demonstrating DCAMKL-1 (red) and somatostatin (green) staining of pancreatic islets. Colocalization is demonstrated in merged image. C: DCAMKL-1 (red) and glucagon (green) immunofluorescence staining of pancreatic islets. No colocalization is demonstrated in merged image. D: immunofluorescence demonstrating DCAMKL-1 (red) and insulin (green) staining of pancreatic islets. No colocalization is observed in merged image. Images on the far right in B–D are the magnified portion of the corresponding merged images. In the immunofluorescence staining, nuclei are stained blue with Hoechst dye.
Stem/progenitor cell isolation from mouse pancreas. We isolated and propagated DCAMKL-1/H11001 stem/progenitor cells from mouse pancreas according to the procedures developed in neural (23–25) and breast stem cell biology (5). The pancreas and associated duct were rapidly dissected and perfused with 3 ml of cold HBSS containing 1 mg/ml collagenase XI (Sigma-Aldrich) and 1 mg/ml BSA (Sigma-Aldrich). The pancreatic tissues were minced and incubated in HBSS for 13 min at 37°C. Digestion was arrested with cold HBSS (Cellgro-Mediatech, Manasses, VA) containing 10% serum. The solution was shaken by hand for 1 min, washed three times with serum-free HBSS, and filtered through 400-H9262 mesh (Spectrum Laboratories, Rancho Dominguez, CA). The cells obtained (flow through) were incubated with trypsin (Cellgro) at 37°C, pipetted to create a single cell suspension and subjected to FACS based on cell surface expression of DCAMKL-1.

FACS sorting. The single cell suspension was incubated with 1:100 dilution of Alexa Fluor 568-conjugated DCAMKL-1 antibody targeting the COOH-terminal extracellular domain for 25 min and washed twice with HBSS containing 1% BSA (Sigma-Aldrich). The cells were sorted with Influx-V cell sorter (Cytopeia/BD, Franklin Lakes, NJ) and collected cells were grown in tissue culture media: DMEM (Cellgro) containing EGF (25 ng/ml), basic FGF (20 ng/ml), and insulin (5 ng/ml) (Sigma-Aldrich) without serum on nontreated or ultralow adherent plates (BD Biosciences, San Jose, CA) in a suspension culture.

Isotransplantation assay. Collected cells expressing DCAMKL-1 were allowed to form spheroids in suspension culture for 21 days. Spheroids were disassociated, suspended in Matrigel, and injected subcutaneously into the flanks of athymic nude mice (NCr-nu) (NCI-Frederick) housed in specific pathogen-free conditions. Animals were killed, and nodules were excised, fixed in 10% buffered formalin, and subjected to immunohistochemical analysis.

RESULTS
Pancreatic DCAMKL-1 expression. DCAMKL-1 is expressed in the main pancreatic duct (Fig. 1A, left) and on the periphery of pancreatic islets (Fig. 1A, middle). There was no detectable DCAMKL-1 expression within the acinar cells of uninjured adult mice (Fig. 1A, right). To determine the specific islet cell subtype, we evaluated for coexpression of the endocrine markers somatostatin (β-cell), glucagon (α-cell), and insulin (β-cell). We found that both DCAMKL-1 (Fig. 1B, left) and somatostatin (Fig. 1B, second from left) were expressed in the islet periphery. Merged images revealed coexisting of DCAMKL-1 with somatostatin (Fig. 1B, third and fourth from left). Glucagon was also found in the periphery of the islet (Fig. 1C, second from left) but did not colocalize with DCAMKL-1 (Fig. 1C, third and fourth from left). Insulin-expressing cells
were observed throughout the islet (Fig. 1D, second from left) but we did not observe any coimmunostaining with DCAMKL-1 (Fig. 1D, third and fourth from left). Thus DCAMKL-1 expressing cells do not express the two major endocrine cell markers (insulin and glucagon) but do colocalize with somatostatin-expressing cells.

**Pancreatic stem/progenitor cell markers.** The basic helix-loop-helix transcription factor NGN3 controls endocrine cell...
Fate specification. All the major islet cell types, including insulin-producing β-cells, are derived from NGN3-positive endocrine progenitor cells (10). It is well known that NGN3 protein expression diminishes as mice reach adulthood (9, 21). We employed immunohistochemical analysis to determine the cell-specific expression patterns of DCAMKL-1 in newborn mice and with reference to NGN3 expression (8). We observed distinct expression of DCAMKL-1 (Fig. 2A) and NGN3 (Fig. 2B) in early islet formations. Immunofluorescence staining confirmed the presence of DCAMKL-1 (Fig. 2C) and NGN3 (Fig. 2D), with merged images revealing distinct colocalization within these developing tissues (Fig. 2, E and F).

To confirm these findings in adult uninjured mice, we employed immunohistochemical staining on serial tissue sections. We observed common immunolocalized staining for DCAMKL-1 (Fig. 2G), NGN3 (Fig. 2H), and the pancreatic stem/progenitor cell marker candidate nestin (Fig. 2I) in all three sections. Furthermore, immunofluorescence staining of newborn mouse pancreas demonstrated the presence of DCAMKL-1 (Fig. 2J) and nestin (Fig. 2K), with merged images revealing colocalization within a few cells (Fig. 2, L and M). These data suggest that DCAMKL-1 marks pancreatic islet stem/progenitor cells, based on positional evidence, and coexpression with established markers of pancreatic stem/progenitor cells.

Isolation and propagation of pancreatic stem/progenitor cells. Stem cells within a tissue are capable of self-renewal and differentiation. Dontu et al. (5) isolated human mammary stem/progenitor cells from normal breast tissues. When grown in ultralow attachment plates, they formed spheroid structures termed “mammospheres.” To test the hypothesis that there is a small subpopulation of distinct stem/progenitor cells within a normal uninjured rodent pancreas; we digested the mouse pancreas with ultrapure collagenase XI and performed FACS-based cell sorting for DCAMKL-1. On average, we sorted ~0.4% of total cells using this method (Fig. 3, A and B). To characterize the phenotype of the sorted populations, we performed quantitative RT-PCR analyses of total RNA isolated from the DCAMKL-1+ and DCAMKL-1− cells. DCAMKL-1+ population demonstrated markedly increased (~10-fold) expression of DCAMKL-1 (Fig. 3C), NGN3 (Fig. 3D), nestin (Fig. 3E), and somatostatin (Fig. 3F) compared with DCAMKL-1− cells. We observed a 7-fold increase in insulin (Fig. 3G) and 12-fold increase in glucagon (Fig. 3H) within the DCAMKL-1− cells compared with DCAMKL-1+ cells. We did not detect CD133 in DCAMKL-1+ cells but were able to detect significant CD133 expression in DCAMKL-1− cells (Supplemental Fig. S1).

Three weeks after sorting, the formation of spheroids was observed in growth factor-supplemented serum-free media (5) (Fig. 4, A = days 0 and 4, B and C = day 21). Spheroids were separated, suspended in Matrigel, and injected subcutaneously into the flanks of athymic nude mice. After 4 wk we noted nodular growth at the site of DCAMKL-1 spheroid injection (Fig. 4E) compared with no growth in the Matrigel-alone-injected control (Fig. 4D). Interestingly, we noted tan-gray soft tissue outgrowth that extended beyond the original injection site, which appeared to show new blood vessel formation (Fig. 4F). We performed a total of 10 injections of pancreatic spheroids containing 50–100 cells each [into right and left flanks of nude mice (n = 5)]. After 4 wk, we observed growth in three of five nude mice for a total of six nodular growths. As a control, we performed spheroid formation assays for DCAMKL-1− cells. We did not observe spheroid formation in culture, even after 12 wk.

DCAMKL-1-sorted spheroids induce pancreatic epithelial expression in the flanks of nude mice. Histological analysis of the excised nodules revealed single cells with oval nuclei and large nucleoli, which appeared to be epithelial in nature, as well as islet-like structures (Fig. 5A) and ductlike formations (Fig. 5B). The glandular epithelial origin of these cells was confirmed by cytokeratin-14 immunoreactivity (Fig. 5C) (16, 19) and PDX-1, a marker of early pancreatic development (Fig. 5D). Additionally, many of the cells within the islet

![Fig. 4. DCAMKL-1 sorted cells demonstrate growth in vitro and in vivo. FACS isolated DCAMKL-1+ cells in suspension culture at day 1 (A) and demonstrates spheroid formation at day 21 (B) (×400). C: magnified portion of image B. Athymic nude mice 4 wk after subcutaneous injection with either Matrigel alone (D) or spheroid with Matrigel (E); arrow indicates nodular growth. F: image demonstrates a tan-gray soft tissue outgrowth with blood vessel formation under the skin of the DCAMKL-1 spheroid-injected mouse.](http://ajpgi.physiology.org/ by 10.220.33.1 on June 25, 2017)
structures expressed somatostatin (Fig. 5E) and secretin (Fig. 5F) (18). Some of the cells were also positive for Ep-CAM, a marker of cells of epithelial origin (Fig. 5G) (3). Many cells were positive for Ki67, indicating an active proliferating status (Fig. 5H) (2). Furthermore, we observed cells that continued to express DCAMKL-1 in both the ductlike formations (Fig. 5I) and isletlike structures (Fig. 5J). These data taken together strongly suggest that DCAMKL-1-expressing cells isolated from the pancreas of normal uninjured mice by FACS and utilized in isotransplantation assays are in fact stem/progenitor cells.

DISCUSSION

Purification of stem and progenitor cells from an organ is increasingly becoming the gold standard experimental method.
to determine the mechanisms that regulate organ development and regeneration following injury. Establishment of such method of isolation of pancreatic stem/progenitor cells has been difficult because of the lack of definitive cell surface markers that reliably mark these relatively rare cell types. Identification of cancer stem or cancer initiating cells, however, has been much more successful as demonstrated by FACS-based sorting protocols by using a combination of cell surface markers followed by recapitulation of tumors in xenotransplantation assays (4, 5, 30). Similar studies using normal rodent or normal human pancreas have not yet been reported. Identification, isolation, and characterization of pancreatic stem/progenitor cells will permit genetic, biological, and developmental studies of pancreas during normal homeostasis and in response to inflammatory diseases and cancer.

In the pancreas, NGN3-positive cells coexpress neither insulin nor glucagon, suggesting that NGN3 marks early precursors of pancreatic endocrine cells. Pancreatic islet cells, including insulin-producing β-cells, are thought to arise from pancreatic endocrine progenitors expressing the transcription factor NGN3 (8, 13, 20). Moreover, NGN3-deficient mice do not develop any islet cells and are diabetic. Thus NGN-3 is a critical component of the pancreatic stem/progenitor cell compartment, at least with respect to endocrine fate determination. A recent convincing study demonstrated that the adult mouse pancreas contains islet cell progenitors and that expansion of the β-cell mass following injury induced by ligation of the pancreatic duct resulted in ductal NGN3 gene expression and differentiation of endogenous progenitor cells in a cell-autonomous, fusion-independent manner (31). These data suggest that functional islet progenitor cells can be induced in pancreatic ducts following injury. These data lend support to the notion that the main pancreatic duct may possess rare stem/progenitor cells that at least give rise to endocrine lineages. In our study, we have demonstrated that the putative intestinal stem cell marker DCAMKL-1 colocalizes with NGN3 and nestin in the normal uninjured mouse pancreas.

One major difficulty in this study is assessing immunoreactive NGN3 within adult mice. NGN3 protein is expressed abundantly in the developing mouse pancreatic endocrine progenitor cells and then dramatically reduced before terminal differentiation. Moreover, NGN3 is not a cell surface-expressing protein, thereby precluding NGN3-based FACS. We have demonstrated earlier that DCAMKL-1 is a cell surface protein and can be used to isolate mouse intestinal stem cells (15, 28). We utilized a similar strategy in this study to isolate pancreatic stem/progenitor cells.

Although originally considered to be a cytoplasmic protein (7), analysis of the DCAMKL-1 protein using the TMPred program (http://www.ch.embnet.org/software/TMPRED_form.html) suggested that amino acids 534–560 represent a transmembrane domain, and amino acids 561–729 are outside the cell. Furthermore, it has been reported that DCAMKL-1 is expressed in adult brain with two transmembrane domains (amino acids 534–559 and 568–585), which strongly supports the suggestion that it is a cell surface-expressing protein with both intracellular and extracellular domains (11, 27). We previously demonstrated cell surface DCAMKL-1 expression using the Pierce Cell Surface Protein Isolation Kit followed by Western blot for DCAMKL-1 (15). Subsequently, we generated an Alexa Fluor 568-conjugated anti-DCAMKL-1 antibody (28), which targets the putative extracellular COOH-terminal epitope. In our experiments, stem/progenitor cells isolated from normal mouse pancreas formed spheroids in suspension culture. Furthermore, 50–100 cells isolated from a particular spheroid in vitro formed early epithelial and isletlike structures in nude mice and expressed markers of early pancreatic development. We are fully aware, however, that in our isograft model we do not have evidence of acinar cell development and we do not have three-dimensional evidence of a regenerated pancreas. We predict that the flank injection may not provide the optimal niche signals required for normal pancreatic regeneration. However, optimal in vitro three-dimensional culture systems are under development that may potentially overcome these obstacles. Nevertheless, we are encouraged by the vascularization observed within the isograft nodules, which may suggest a potential role for DCAMKL-1 signaling in growth augmentation. Thus the studies presented suggest that DCAMKL-1, a novel putative stem cell marker expressed primarily in quiescent cells of the gut (7, 14, 15), also marks normal pancreatic stem/progenitor cells.

Identification of stem/progenitor cells using a single marker represents a major advancement over many of the typical cell surface markers that generally have to be used in combination and as such represent markers of purification. Although recent studies using cell surface markers to isolate cancer stem cells from tumors have been described (24), similar studies have not been performed utilizing normal tissues. These studies have broad potential applications in regenerative medicine. Isolation and purification protocols in vitro or in vivo could be employed to replace insulin in diabetic patients as an example. Overall, these studies represent a major step toward understanding pancreatic biology under normal conditions. Further studies are underway in our laboratory to determine whether DCAMKL-1 is expressed in pancreatic cancer.

In conclusion, there has been considerable debate on the origin of the pancreatic stem cell. Several transgenic mouse models and injury models have suggested that ductal, islet and acinar cells all have the capability to act as stem cells in the pancreas (6, 22, 26). Nevertheless, the functional data presented here provide strong evidence that DCAMKL-1 expressing cells have clonogenic capacity in vitro and display some evidence of pancreatic lineage determination in an isograft model. Further studies in transgenic lineage tracing model models will likely be required to fully answer these lingering questions.

ACKNOWLEDGMENTS

We thank Dr. Michael Bronze, Chairman of the Department of Medicine University of Oklahoma Health Sciences Center for support.

GRANTS

This research was supported by National Institutes of Health Grants DK-065887, DR-002822 OCAST-AR 101-030, and CA-137482 to C. W. Houchen, Oklahoma University Advanced Immunohistochemistry & Morphology Core, Veterans Administration Medical Center, Oklahoma City.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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