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LGR5 and Nanog identify stem cell signature of pancreas beta cells which initiate pancreatic cancer

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ABSTRACT

Pancreas cancer, is the fourth leading cause of cancer death but its cell of origin is controversial. We compared the localization of stem cells in normal and cancerous pancreas using antibodies to the stem cell markers Nanog and LGR5. Here we show, for the first time, that LGR5 is expressed in normal pancreas, exclusively in the islets of Langerhans and it is co-localized, surprisingly, with Nanog and insulin in clusters of beta cells. In cancerous pancreas Nanog and LGR5 are expressed in the remaining islets and in all ductal cancer cells. We observed insulin staining among the ductal cancer cells, but not in metastases. This indicates that the islet's beta cells, expressing LGR5 and Nanog markers are the initiating cells of pancreas cancer, which migrated from the islets to form the ductal cancerous tissue, probably after mutation and de-differentiation. This discovery may facilitate treatment of this devastating cancer.

1. Introduction

Pancreas cancer, mainly Pancreatic Ductal Adeno Carcinomas (PDAC), is a lethal cancer with a 5-year survival rate of only 3% and a median survival of less than 6 months [1]. Despite experimental effort, the cell-of-origin of PDAC was not identified yet [2–4].

The pancreas is composed of a branching network of duct (10%) and acini (85%), involved in exocrine function and islets of cells involved in endocrine secretion of hormones (2%). The common mutations identified in PDAC were found to be in *KRAS, SMAD4 CDKN2A (p16), TP53,* and *CyclinD1* and several lines of analysis were attempted to identify the cancer stem cell (CSC) of PDAC. In one methodology, pancreatic CSC have been identified by a functional test and characterized as expressing specific surface markers. It was found that CD44, CD24, EpCAM, CD133, CXCR4 and c-Met were specifically expressed in the PDAC stem cells [5]. This method, however, is open for artifacts due to cell handling and the result reflects the markers expressed at a particular time point, which is not necessarily correlated with the time of activation of the CSC. In another line of experiments various pancreatic cells were transfected with mutant KRas followed by transfer to recipient animals and analysis of the type of tumor formed. These experiments resulted in suggestions for various potential CSC in the pancreas. Some identified either the acinar cells or the ductal cells as CSC and few suggested the islets cells or even the absence of TSC as a source for CSC [6–9].

We took a different approach based on the CSC concept. This concept [10,11] states that tumors contain a small subpopulation of CSC that differs in its properties from the bulk of the tumor. The CSCs are more resistant to chemotherapy, possess self-renewal property, and preserve the initial tumor property that drives tumorigenicity. When the tumor is treated with radiation, chemotherapy or targeted therapy the bulk of the tumor may shrink but the resistant CSCs are selected to survive. Later on the tumors relapse to generate a resistant mutant of the tumors as well as metastases [12]. Striking analogy exists between CSC and TSC; both possess self renewal capability. The TSC are responsible for renewal of the normal tissue but if cancer develops it is very often by mutation of the TSC. A mutation in the TSC may transform this cell and endow it with oncogenic properties so that it becomes the cancer initiating cell. This was verified in various tissues including solid tumors (e.g. skin, intestine, breast, blood, etc.) [13].

Our methodology compares, the localization of unique stem cells markers in normal and cancerous pancreas. As representative markers we used the embryonic stem cell (ESC) marker Nanog [14] and the adult intestine stem cell marker LGR5 [15] (Leucine-rich repeat-containing G-protein coupled receptor 5). Nanog, a transcription factor, is known to be associated with pluripotency and self-renewal and although its expression is silenced upon

Abbreviations: EP, exocrine pancreas; ESC, embryonic stem cell; CSC, cancer stem cell; LGR5, Leucine-rich repeat-containing Gprotein coupled receptor; PDAC, Pancreatic Ductal Adeno Carcinomas; TSC, tissue stem cell.

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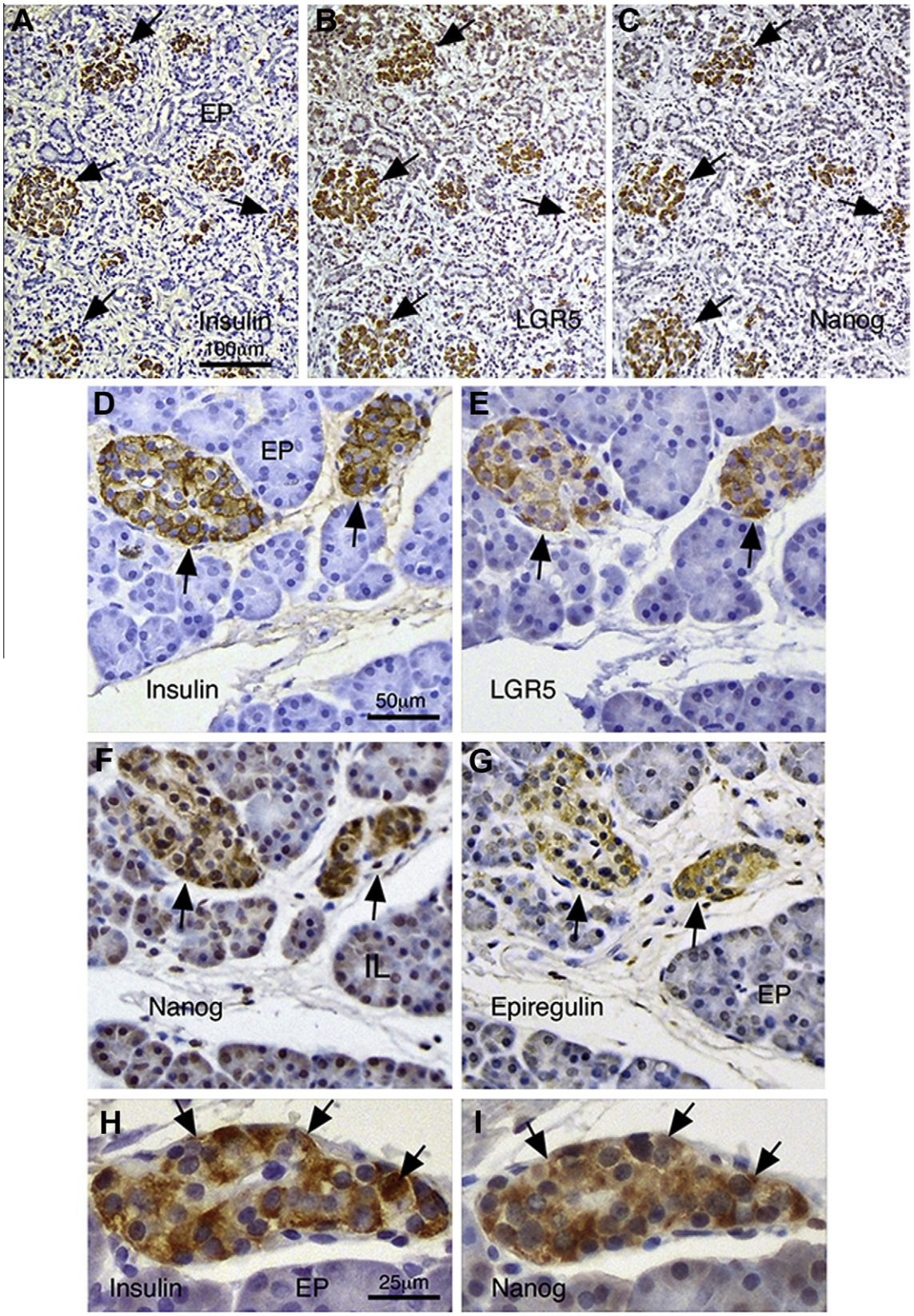

Fig. 1. Immunocytochemical staining of normal pancreas for insulin, LGR5 Nanog and epiregulin. (A–C) Comparison of staining with antibodies to insulin (A) LGR5 (B) or Nanog (C) in adjacent sections. The staining revealed clusters of beta cells in Langerhans islets stained with antibodies to insulin and overlapping pattern following staining for LGR5 and Nanog (arrows) No staining is evident in the exocrine pancreas (EP). Original magnification ×100. (D–G). Comparison of staining of couple of Langerhans Islets (arrows) with antibodies to insulin (D) LGR5 (E) Nanog (F) and epiregulin (G) in adjacent sections. The patterns of labeling for LGR5, Nanog, and epiregulin overlap the pattern of staining for insulin. No staining is evident in the acini of the exocrine pancreas (EP). Original magnification ×200. (H and I) Comparison the staining of insulin expressing cells and Nanog expressing cells in islets of Langerhans (arrows) at the edge of the exocrine pancreas (EP) in serial sections at high magnification (×400). The pattern of labeling is almost identical for both markers.

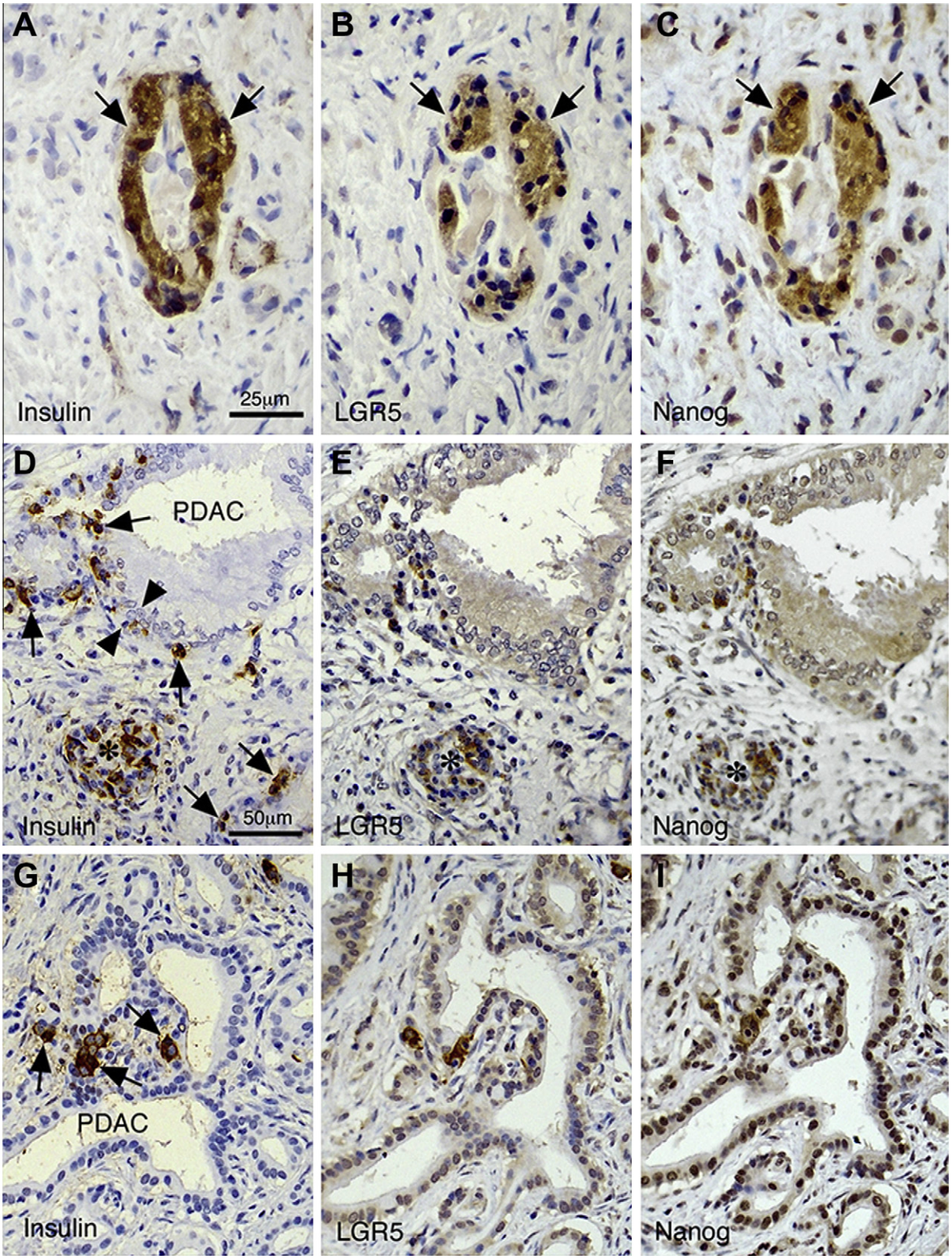

Fig. 2. Immunocytochemical labeling of cancerous pancreas for insulin, LGR5 and Nanog. (A–C) Formation of a duct-like structure in the pancreatic stroma. (A) Cells stained for insulin forming a duct- like structure (arrows). (B) Similar structure stained for LGR5. (C) A more complete structure stained for Nanog. (A–C) Adjacent sections. Original magnification ×400. (D–F) Adjacent sections showing association of insulin containing cells in the development of PDAC. (D) clustered cells of decomposed islet (asterisk), a small duct and a large duct labeled by staining to either insulin or LGR5 (E) and to Nanog (F). The Upper left (arrows) and upper right show formation of a small duct labeled occasionally with insulin containing cells (arrows) (D); weak labeling of the same ducts for LGR5 (E). More intensive label with antibodies to Nanog (F). The wall of the ducts very often contains more than one cell layer (opposing arrows). Original magnification ×200. (G–I) Disappearance of insulin staining in developing tubular structure of PDAC. (G) Only few insulin containing cells are visible in between, or associated with walls of ducts (arrows). (H) Moderate labeling with antibodies to LGR5, mainly cytoplasmic, is associated with part of the cells. (I) Intensive labeling with Nanog is evident in the nuclei of most cells of the entire microscopic field (k). (G–I) Adjacent sections. Original magnification ×200.

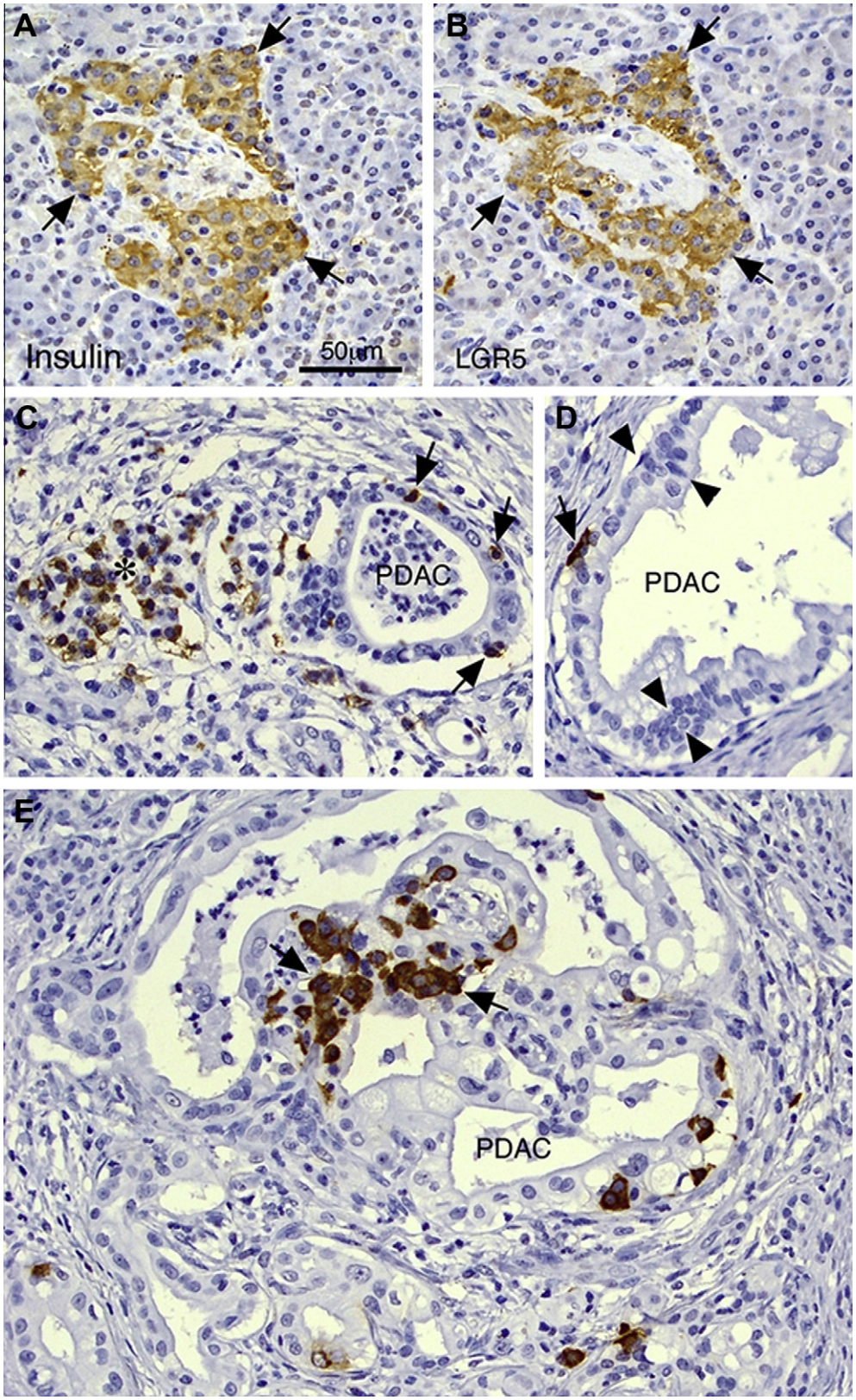

Fig. 3. Association of insulin containing cells with the formation of PDAC. (A–E) Gradual initial disintegration of islet of Langerhans in a pancreatic cancer, still embedded in the exocrine tissue a, Staining with antibodies to insulin (arrows) (B). Staining with antibodies to LGR5.Note the overlapping pattern of insulin and LGR5 staining (arrows). Serial sections. Original magnifications ×200. (C) Partially decomposed islet of Langerhans associated with PDAC (asterisks). Note few insulin containing cells are integrated in the duct wall (arrows). (D) Part of a duct containing couple of insulin containing cells (arrows). The duct wall contains multilayered cells (opposing double arrow head). (E) PDAC associated with insulin containing cells (arrows). Original magnification ×200.

differentiation, it was found to be re-expressed in many tumors. The other marker is the tissue stem cell marker (TSC) LGR5, which marks stem cells in the small intestine, colon, and hair follicle and maintains tissue renewal.

LGR5 positive cells are also highly expressed in colon cancer, indicating that the TSC are the cell-of-origin of cancer in intestine and colon cancers [15,16]. The discovery of this marker has already greatly improved our understanding of stem cell biology and has major implications for the identification and isolation of human adult stem cell populations. Here we describe LGR5 as a marker for potential cancer stem cells in the pancreas.

2. Materials and methods

We obtained five specimens of biopsies of PDAC, eight specimens that included both portions of intact pancreatic and PDAC and six specimens of normal pancreatic tissues. In addition, we got samples of three PDAC of liver metastatic tumors. The age of the patients was 53–67 years old. Tissues were fixed with formalin embedded in paraffin and sections of 4 μm were stained with specific antibodies, using the indirect immunocytochemistry [18,24] as follows: anti insulin were polyclonal in guinea pig (Sigma–Aldrich, Israel). Anti LGR5, affinity purified polyclonal in rabbit (Acris Herford, Germany). Anti Nanog, Mouse monoclonal (Sigma–Aldrich, Israel). Anti epiregulin; polyclonal affinity purified (R&D, USA). Second antibodies: simple stain (mouse or rabbit) conjugated to peroxidase (Nickiru Bioscience, Japan). Pre-incubation with appropriate nonspecific IgG yields no staining. Antibody dilution was as provided by the suppliers.

Twenty fold excess of the appropriate antigen abolished the staining. Positive control to LGR5; staining of nerve cells in the brain (provided by Acris) and specific staining of ovarian and colon cancer [18,23]. Additional negative control to anti insulin was absence of staining of exocrine tissue and normal ducts. Positive control for anti Nanog was staining of colon and ovarian cancers. [18,24]. Stained sections were inspected in each patient at Nikon light microscope (Nikon Eclipse E800, Tokyo, Japan) using a digital Nikon camera (Model DS-Ri1) at magnification × 10– ×400. All paraffin sections of normal and cancerous pancreases and metastases were obtained from Wolfson Hospital with the permission of Helsinki Committee after receiving informed consent.

3. Results

To analyze the TSC and the potential cancer initiating cells in the pancreas we compared by Immunocytochemistry the localization of LGR5 and Nanog in normal and in cancerous pancreas. Fig. 1 shows the staining of adjacent sections of normal adult pancreas with antibodies to either LGR5 or Nanog (Fig. 1B and C). The results showed almost complete overlap in the localization of the two markers in the islets of Langerhans. Surprisingly, the staining of these markers overlapped also with the localization of Insulin (Fig. 1A). Furthermore, no localization of LGR5 or Nanog was observed elsewhere in the pancreas, neither in the acinar nor in the ductal cells of the pancreas. Higher magnification showed clearly the co-localization of the LGR5, Nanog and insulin (Fig. 1D–F). In addition, epiregulin, a member of the EGF family, which is a ligand for EGFR and known to be an insulinotropic factor in beta cells, also overlaps the LGR5 and Nanog localization in the islets (Fig. 1G). Activation of EGFR by epiregulin may promote EGFR related transformation [17]. The labeled markers, particularly LGR5, suggest that the islets' beta cells contain the TSC of the pancreas. TSC contain cells that, upon mutation, may become cancer stem cells. This suggests that the beta cells are potential cells-of-origin of PDAC. We therefore looked at cells expressing the above markers in cancerous pancreas.

Immunocytochemistry of pancreatic cancer with antibodies to insulin, LGR5 and Nanog showed that insulin expressing cells exists also outside the islets and overlaps LGR5 and Nanog expression (Fig. 2A–C). It is also shown that dismantled islets are present in close vicinity to ducts and may provide cancer cells by migration (Fig. 2D–F). Furthermore, insulin labeling in cancerous pancreas is down regulated whereas LGR5 labeled moderately most of the cancer cells and Nanog also labeled intensively most of the cells (Fig. 2D–F). This is shown in Fig. 3 where the number of insulin labeled cells is diminishing (Fig. 3C–E) and the LGR5 and Nanog are expresses in most cells (not shown).

This result indicates that insulin production gradually ceases when beta cells leave the islets. If this is the case, it is likely that in metastases of PDAC we should not expect to see expression of insulin. To verify this we observed sections from liver metastases of pancreatic cancer after staining with antibodies to Nanog, LGR5 and insulin. Indeed, it is clear that no insulin staining was observed, whereas LGR5 showed wide-spread expression and Nanog showed intense expression in all cells of the tumor (Fig. 4A–C). This high level expression of Nanog is a striking new observation and similar to our observation on the high expression of Nanog in ovarian cancer [18]. The gradual loss of insulin expression in PDAC and its absence in liver metastases, in spite of strong expression of Nanog and LGR5, is a strong support to our contention that the cells-of-origin of the PDAC are the islets' beta cells, since they are the only ones labeled by insulin, Nanog and LGR5 in the normal

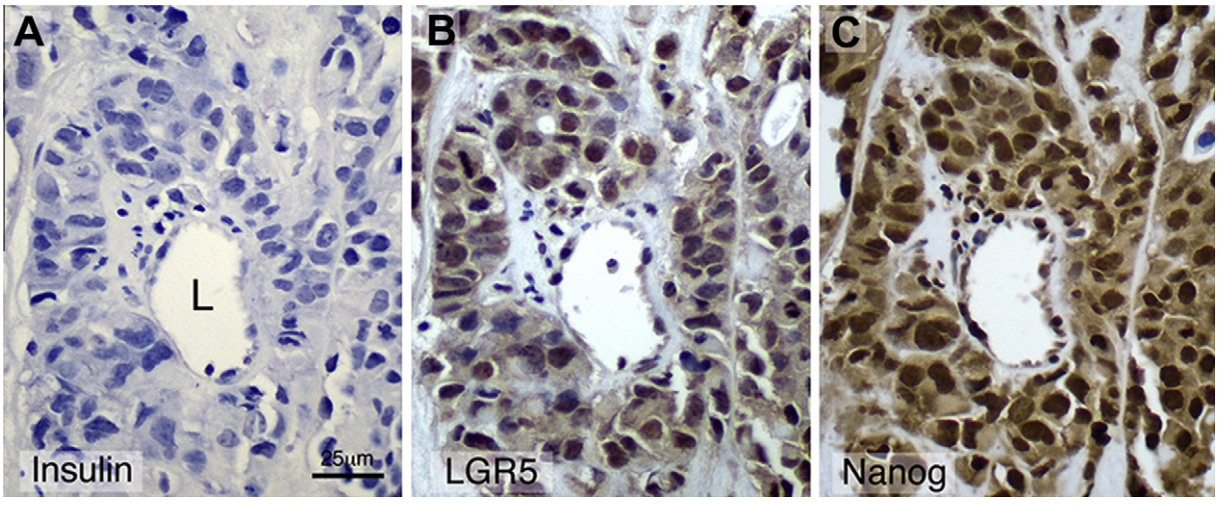

Fig. 4. Staining of PDAC metastases in liver with antibodies to insulin, LGR5 or Nanog. (A) Incubating with antibodies to insulin yield no staining. A lumen of the duct is evident at the center (L). (B) Moderate staining with antibodies to LGR5 is evident both in the nuclei and in the cytoplasm leaving the cells at the wall of the duct unstained. (C) Heavy staining with anti Nanog antibodies in all the cells in the field. (A–C) Adjacent section. Original magnification ×400.

pancreas. It is also a verification of the concept that TSC are the origin of cancer cells in many cases. During tumor development, Nanog expression takes place in all the tumor cells, LGR5 is expressed in part of them and insulin is not expressed at all. It is also possible that the high expression of Nanog is partly responsible for the aggressiveness of PDAC, as well as of ovarian cancer. Interestingly, all cells of the same duct were labeled with antibodies to epiregulin (data not shown), indicating growth or cancer promoting signaling through EGFR in PDAC.

4. Discussion

The identity of the tumor initiating cell in PDAC is still controversial. Many experiments were performed in vitro and in vivo by transplanting KRas-transformed pancreas cells to recipient in an attempt to see the type of tumor generated in vivo, in order to elucidate this question. We changed the methodology by attempts to localize the stem cells directly in the normal pancreas using known stem cell markers. Our approach is based on the hypothesis that cancer is initiating mainly by mutation in the TSC [13]. These adult tissue stem cells are endowed intrinsically with self renewal capability (like CSC) and need only some mutations to undergo malignant transformation. By comparing the immunocytochemical localization of the stem cell markers Nanog and LGR5 in normal and cancerous pancreas we find that the islets of Langerhans are the only location in normal pancreas that was stained by antibodies to both Nanog and LGR5 and therefore form a niche of TSC that contain the potential cell-of-origin of PDAC. The localization of the stem cell markers to the islets is a novel finding, since it shows that the endocrine cells may contribute to the exocrine-like tumor. It also implies that the malignant cells have to migrate out from the islet and contribute to the formation of cancerous acini and ducts in order to generate PDAC. We also observed decomposed and disorganized islets stained for LGR5 and Nanog near ducts (Fig. 2D–F), indicating that they originate in islets since LGR5 and Nanog were co-localized only at the islets of the normal pancreas.

Although the origin of pancreatic cancer is controversial, our results find some support in previous work. It was reported that tumors derived from culture of the islets of Hamster and contain mutation in KRas and p16 led to PDAC formation when transplanted into the submandibular gland of hamster [19]. Recently experiments with genetically engineered mice showed that insulin positive cells in Langerhans islets can serve as PDAC progenitors in the context of KRas mutation and inflammation [20]. This result supports our direct identification of the pancreas stem cells niche in the islets of the normal pancreas that express two well known stem cells markers; one of them, LGR5, was established as intestine, colon and hair stem cells [15,21].

It is also of interest that PDX-1, a transcription factor for insulin expression in beta cells, was shown recently to be an oncogene for pancreatic cancer [22]. Furthermore, knock-down of PDX-1, with shRNA Specific to PDX-1, resulted in improved survival and ablation of human pancreatic tumor in a xenograft mouse model [22]. It is also well known that the causal correlation between diabetes and pancreas cancer is complex. The protective effect of the anti-diabetic drug metformin against pancreatic cancer is another hint for a possible causal relationship that involves the beta cells [23]. Our study should enhance search of the connection between PDAC and diabetes.

In conclusion, we showed that pancreatic islets beta cells contain cells-of-origin of PDAC that express their unique markers in the PDAC tumor cells. The identity of the markers in the beta cells and PDAC implies that cancer cells migrate from the islets to proliferate and contribute to the formation of cancerous ducts. During this process they cease insulin expression and activate Nanog and LGR5, which become major biomarker for PDAC. Our results are essential for developing new methods for PDAC diagnosis and may help finding new ways to treat this lethal cancer by targeting Nanog or LGR5. It may also help finding the functional connection between pancreas cancer and diabetes.

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