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RESEARCH ARTICLE

Expression Pattern of Stem Cell Markers in Developing Mouse Pancreas

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CD29: Integrin beta-1
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Pdx-1: Pancreatic and duodenal homeobox 1

ABSTRACT

Identification of stem cells in-vivo opens up the possibility of their expansion in-vitro, exploiting their multipotency in treating diabetes type 1 and type 2. Little is known about the relationship between a common pancreatic transcription factor (Pdx-1) and stem cell markers (CK19, CD29, CD56) in mice during pancreatic organogenesis. In this study the focused was on the prenatal immunohistochemical expression of stem cell markers (Pdx-1, CK19, CD29, and CD56) with special reference to their site and degree of expression during the prenatal pancreatic organogenesis. Material and Methods: Whole embryos and pancreatic tail of different prenatal groups (days 13, 15, & 18) were stained by H&E and immunohistochemically stained for Pdx-1 and stem cell markers, CK19, CD29, and CD56. Data was statistically analyzed for the evaluation of changes in the pattern and the degree of expression of stem cell markers in the pancreas. Results: Pdx-1 a transcription factor with role in pancreatic organogenesis was expressed in the duct, acini and islets in all ages. CK19 was expressed in the duct at day 13 and 15 prenatally, but in islet at day 18. Acinus and islets were positive for CK19 at all ages. CD29 on the other hand had positive expression in the duct while acini and islets had it at day 18 only. The acinar and islet cells were positive for CD56 at day 18 only. In conclusion, PDX-1 a transcription factor is vital in early pancreatic organogenesis whereas CK19, CD26 and CD56 are purportedly involved in generation of β-cells.

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INTRODUCTION

Pancreatic development is one of the best examples of organogenesis. Both gain and loss-of-function studies conducted in mice over the last decade have contributed to the understanding of pancreatic development (Pan, 2011).

Stem cells are undifferentiated cells capable of self-renewal and differentiate in specialized cell types. The remarkable ability of stem cells to differentiate towards functional cells makes them suitable modalities for treating diabetes. However, in order for stem cells to be clinically relevant, identification of stem cells in-vivo and their proliferation as well as differentiation in-vitro is important (Cheng *et al.,* 2010). From a clinical perspective, the pancreas is an important focus of stem cell research because it is an attractive target for cell replacement therapy. In type-I diabetes, the insulin-producing β-cells that reside in the pancreatic islets of Langerhans are destroyed by autoimmune attack, self-renewing stem cells could provide an unlimited source of β-cells for transplantation. Such therapeutic efforts require the prospective isolation of stem cells with the potential to produce β-cells and the development of methods to direct their expansion and differentiation (Sell, 2013). Many studies revealed that the pancreatic stem cells can differentiate into functioning endocrine cells under suitable conditions of cultivation (Warnock *et al.,* 2005).

According to the U.S. National Cancer Institute, about 46,420 new pancreatic cancer-related cases were reported with 39,590 deaths in 2014 in the United States only (Siegel *et al.,* 2014). Pancreatic cancer is ranked as fourth leading cause of cancer-related mortalities and predicted to be second leading cause by 2020 (Kadera *et al.,* 2013). It was because of this reason that the current

study was conducted so as to locate stem cell niches and harness the potential of stem cells for in-vivo organogenesis and treating diabetes.

MATERIALS AND METHODS

Animal preparation: Seventeen adult male and 34 adult female swiss albino mice (Balb/c) (20-25 grams), aged 10-12 weeks were obtained from the animal house of the Faculty of Pharmacy - King Abdulaziz University, Jeddah. All the animals were acclimatized for a week. The experiment was conducted in accordance with the ethical principles and guidelines of the Canadian Council of the Animal Care, subsequently approved by the National Ethical Committee of King Abdulaziz University. Mice were maintained at 22-24°C and 55% relative humidity with light and dark periods at 12-h each with free access to food and water. Males and females were kept together overnight to mate. Presence of the vaginal plug was considered day 0 of gestation (Frantz *et al.,* 2011).

Isolation of embryos: Pregnant mice were euthanized by cervical dislocation, a short incision was made in the abdominal skin (peritoneum), uterus was aseptically removed and placed in a petri dish. Embryos extracted at days 13, 15 and 18 were fixed in 10% buffered formalin (pH 7.4).

Growth parameters examination: Body weight and length for all groups were recorded. Measurements for the embryos were recorded before further process.

H&E staining: The specimens were processed and preserved in paraffin blocks. Paraffin sections of 4µm were obtained using a rotatory microtome and stained with Hematoxylin and Eosin (H&E) to observe the general structure of prenatal pancreas under light microscope (Olympus BX51, Japan) and photographed (Olympus DP20 digital camera).

Immunohistochemistry (IHC): For immunohisto-chemical analysis, paraffin-embedded tissues were cut into 5µm sections, placed on poly-L-lysine coated slides and dried for 12h in an incubator at 37°C. Dried sections were rehydrated, pre-treated with 0.01 M citrate buffer solution (pH 7.4) in an oven at 93^{o}C for 20 minutes to unmask the antigenic sites. Thereafter, sections were left to cool at room temperature and washed with PBS (pH 7.4). The slides were later incubated for 10min with 3% H_2O_2 (vol/vol) to quench the endogenous peroxidase activity. Following blocking, the sections were incubated overnight at 4^{o}C with specific primary antibodies according to manufacturer's instructions. Slides were washed and covered by appropriate biotinylated secondary antibodies and incubated in a humidity chamber for 30min. The immunoreactions were visualized by incubating the sections for 2min in a 0.1% 3,3-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako, Denmark). The sections were lightly counterstained with Mayer's Hematoxylin and mounted on DPX, observed under light microscope and photographed.

The intensity of staining (IS) was graded according to the following assessment: negative staining (-), weak staining (+), mild staining (++), and strong staining (+++). Quantitatively, the percent area of immunopositive staining for each maker was independently evaluated. For each age (Days 13, 15 & 18), 6 consecutive fields (x600) of 3 serial sections from each block of 6 animals were taken and results expressed as mean±SD. This was followed by morphometric analysis by digital light microscopic photos. Images were analyzed using image pro-plus program (version 6) (Media Cybernetics® USA), and the percent area was recorded as screen shots (Fig. 1). Finally, changes in the percent area of the positive cells in the pancreatic tissue were summarized (Fig. 7).

Statistical analysis: Statistical analysis was performed using SPSS and results of different groups were evaluated using Student Unpaired One Way ANOVA with $P<0.05$.

RESULTS

H&E stain: Examination at day 13 showed pancreas of mouse fetus appearing as an extension of pancreatic mesenchymal tissue with few tubular structures between the transversely cut sections of duodenum and liver (Fig. 2: A & B). On day 15, sections revealed a progressive formation of acinar and tubular like structures. Appearance of aggregation of islet-like clusters of cells was also observed (Fig. 2: C & D). Sections from day 18 had a thin capsule enclosing partially lobulated pancreatic tissue. Examination at higher resolution displayed typical pancreatic secretory acini with evident eosinophilic granules and tubular like ducts with simple cubical cell lining. Apparently well-developed islets of pancreas were evident in most of the examined sections (Fig. 2: E & F).

Pdx-1 expression: Examination of the Pdx-1 immunostained serial sections of embryos revealed positive Pdx-1 expression starting from the day 13. Expression was observed in mesenchymal cells (MCs) and cells lining the developing tubular like structures (Fig. 3: A & B) which increased by day 15 of pregnancy. Expression was also observed in acinar-like structures (Fig. 3: C & D). Fetal pancreatic tissue examination by day 18 displayed strong positive cells in the interstitial MCs, acinar cells, and some islet cells (Fig. 3: E & F).

CK19 expression: Examination of the CK19 immunostained serial sections revealed positive expression as observed in sections of day 13. Epithelial cells, lining the developing duct, had mild positive expression that was also observed in some cells between the ductal portion **(**Fig. 4: A & B). By day 15, there were few CK19 positive epithelial cells among the cells, lining the developing tubular but none in acinar like structures (Fig. 4: C & D). On day 18, fetal pancreatic tissue displayed few positive cells in the developing islets (Fig. 4: E & F).

CD29 expression: Positive expression was observed at day 13 in the mesenchymal cells (MCs) only as no expression was observed in epithelial cells lining the developing tubular like structures (Fig. 5: A & B). At day

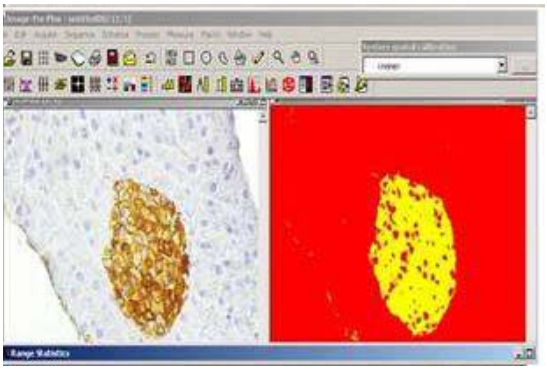

Fig. 1: Screen shots of the image pro plus, showing how the brown immunostained area fraction of positively stained cells was marked as class colour I (yellow) and the remaining unstained area as class colour II (red).

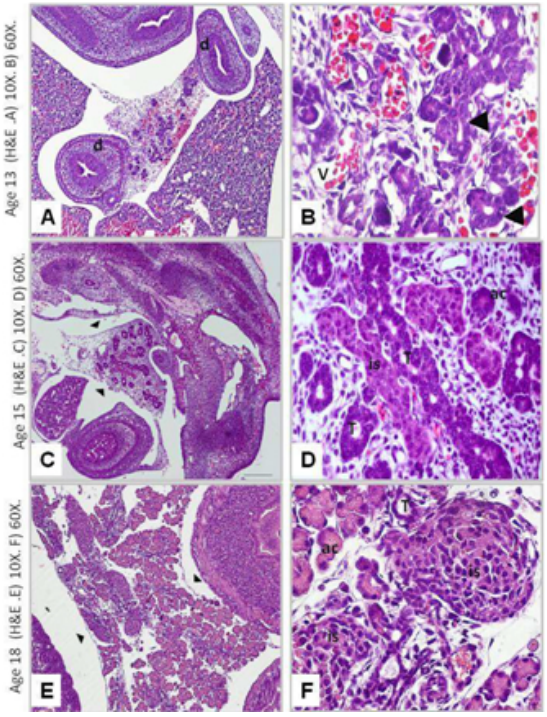

Fig. 2: Photomicrographs of sections from the prenatal mice pancreas. A & B: day 13 of pregnancy, showing the extension of highly vascular pancreatic mesenchymal tissue with few tubular like structures between the transversely cut section of duodenum (d). C & D: day 15 of pregnancy, showing progressive formation of acinar (ac) and tubular like structures (t) and appearance of aggregation of non-tubular clusters of cells (islet like) (is). E & F: day 18 of pregnancy, showing a thin capsule (☐) enclosing partially lobulated pancreatic tissue and higher resolution showing the typical pancreatic secretory acini (ac) and tubular structure with simple cubical cells. Notice the well-developed islets (is) (A, C & E at 10x magnification while B, D & F at 60x magnification).

15 no cells positive for CD29 were observed while some mild positive interstitial MCs around acinar and developing islets appeared at day 18 (Fig. 5: C, D, E & F).

CD56 expression: Early expression of CD56 was only observed in mesenchymal cells (MCs) at day 13 **(**Fig. 6: A & B) which gradually increased by day 15 (Fig. 6: C & D). At day 18 some mild positive cells in the center of the developing islets and few mesenchymal cells around the developing acini were observed (Fig. 6: E & F).

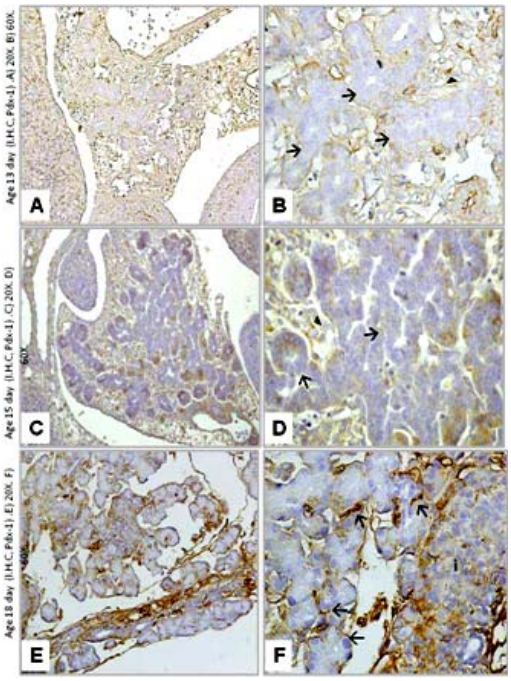

Fig. 3: Photomicrographs of sections from the prenatal mice pancreas immunostained by anti PDX-1. A & B: day 13 of pregnancy, showing the positive expression of Pdx-1 in the mesenchymal cells (MCs) (☐) and cells of developing tubular like structures (☐). C & D: day 15, there is an apparent increase in Pdx-1 positive MCs (☐) and cells of developing tubular and acinar like structures (☐). E & F: day 18 of pregnancy, showing strong presence of pdx-1 cells in the MCs and some acinar cells (☐) some cells in the islet (i) (A, C & E at 20x while B, D & F at 60x magnification).

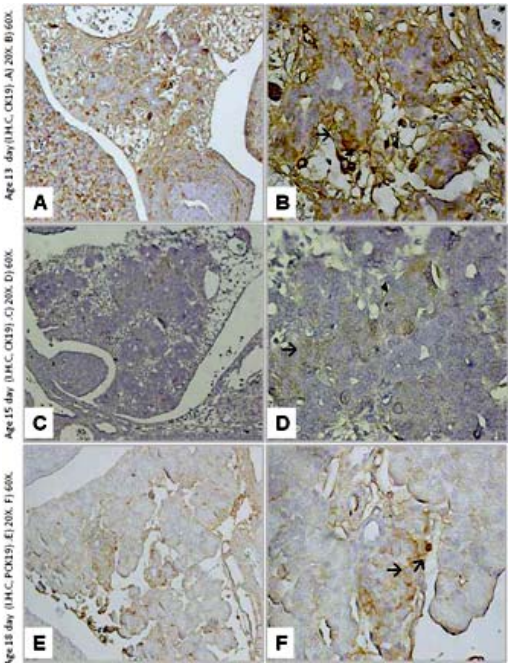

Fig. 4: Photomicrographs of sections from the prenatal mice pancreas immunostained by anti CK19. A & B: day 13 of pregnancy, showing positive expression of CK19 by most of the epithelial cells lining the ducts. Notice few mild positive reactions by some of the cells between the developing ducts (☐). C & D: day 15 showing few cells lining the ducts with positive CK19 expression (☐). E & F: day 18 of pregnancy, showing positive CK19 cells in the islet cells (☐) (A, C & E at 20x while B, D & F at 60x magnification).

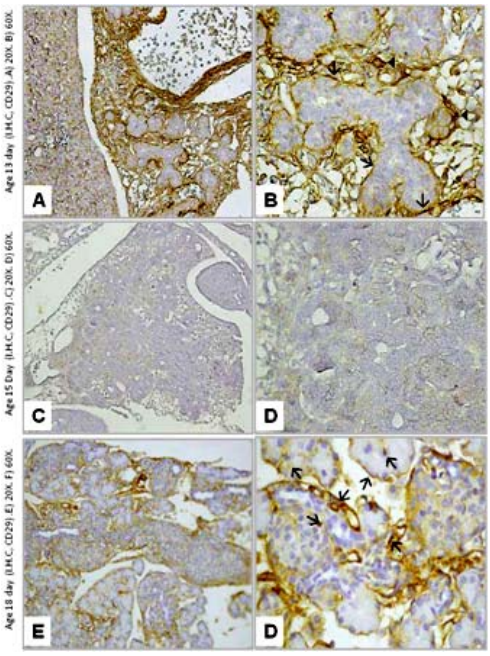

Fig. 5: Photomicrographs of sections from the prenatal mice pancreas immunostained by anti CD29. A & B: day 13 of pregnancy, showing positive expression of CD29 in the mesenchymal cells (MCs) (☐) while negative expression in epithelial cells of developing tubular like structures (☐). C & D: day 15, showing negative expression of CD29 by all pancreatic tissue sections. E & F: day 18 of pregnancy, showing mild positive expression by mesenchymal cells around the acinar and some cells of islets (☐) (A, C & E at 20x while B, D & F at 60x magnification).

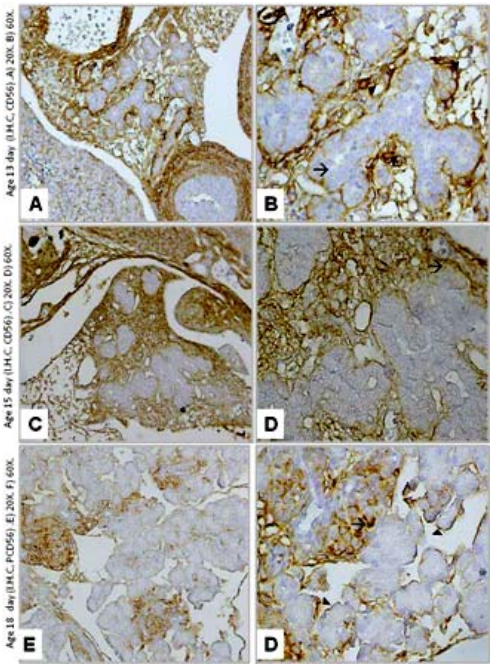

Fig. 6: Photomicrographs of sections from the prenatal mice pancreas immunostained by anti CD56. A & B: day 13 of pregnancy, showing CD56 positive mesenchymal cells (MCs) (☐) with negative expression in epithelial cells of developing tubular like structures (☐). C & D: at day 15, MCs were positive for CD56 with negative expression in duct and acini. E & F: day 18 of pregnancy, showing mild positive expression of CD56 in central islets (☐), some of the cells surrounding acini also displayed positive reaction for CD56 (☐) (A, C & E at 20x while B, D & F at 60x magnification).

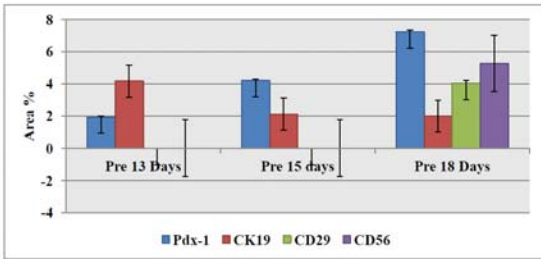

Fig. 7: Percent area of pdx-1, CK19, CD29 and CD56 positive cells in mouse pancreas during the prenatal period (pre).

Table 1: Body weight (gm) and body length (cm) during the prenatal period

Group A	Body weight	Body length
Pre 13 days	0.45±0.08	1.48±0.08
Pre 15 days	0.82±0.10*	1.92±0.10*
Pre 18 days	1.48±0.21*	3.15±0.23

Values (mean±SD) bearing asterisk in a column differ significantly ($P \leq 0.05$).

Growth parameters: Body weight and length significantly increased in embryos, aged 18 days, prenatal compared to those at day 13 (Table 1).

DISCUSSION

The histological and immunhistological study on different types of stem cell markers expressed during pancreatic organogenesis is a classic example of epithelia-mesenchymal interaction. Our histological results are in agreement with the results of a number of earlier studies that stated the 3 developmental stages embryonic pancreas is known to pass. An early, undifferentiated stage followed by epithelial branching and morphogenesis with the concomitant formation of primitive ducts (Gittes *et al.,* 1996). The presence of acinar cells with evident zymogenic granules and islet of Langerhans in H&E stained sections (Fig. 2) is at variance to previous studies (Wang *et al.,* 2013). The organogenesis of islets in mice pancreas was studied by three-dimensional reconstructions from serial section micrographs. On embryonic day (E) 12, an endocrine cluster consisting mainly of glucagon expressing cells maintained connection with the pancreatic endoderm at several regions. On E15-E17, the group of cells enlarged by fusion of newly formed buds. Although the proportion of insulin-expressing cells increased, they were located in the periphery of the cluster (Hara *et al.,* 2007). It is pertinent to mention that the control of pancreatic differentiation in the early embryo is poorly understood. This might be primarily because the previous studies focused on the possible interaction of the pancreatic mesenchyme with the undifferentiated pancreatic epithelium via a 'mesenchymal factor' (Gittes *et al.,* 1996). The current results displayed the cytoplasmic nature of Pdx-1 expression starting from the day 13 in different types of pancreatic cells. On the contrary, findings by other groups suggested expression of Pdx-1 in the nuclei of human embryonic and early fetal pancreas cells. Those studies support the idea of transcription factor's suggested role in human pancreas formation (Wang *et al.,* 2013). One study stated that early embryonic expression of PDX-1 is restricted to the

developing pancreatic buds, a reason why we were unable to detect the expression at an earlier stage (Xuan *et al.,* 2012). Several transcription factors are required for pancreas specification, the pancreatic primordium expresses the homeodomain protein Pdx-1 before and during budding (Vaz *et al.,* 2014; Bailey *et al.,* 2014; Greggio *et al.,* 2015). Our findings suggest variable expression of Pdx-1 in the cells of ducts, acinus and islet up to 90 days of age. Though the expression in the developing ductal epithelium remains uniform, nevertheless it is lost in the ductal cells, being primarily restricted to the islet cells, with low levels detectable in some acinar cells. Under certain conditions, Pdx-1 expression again becomes detectable in individual pancreatic ductal epithelial cells, raising the possibility of multipotent stem cells within the mature ductal epithelium. In one such study, Area III in pdx-1, and the larger XhoI-BglII fragment (Pdx1XB) were incapable of directing cell selective reporter gene activation (Vaz *et al.,* 2014; Bailey *et al.,* 2014), suggesting that these sequences might participate in other aspects of Pdx-1 gene expression. However, one of the studies provided the evidence for Area III facilitating the early and broad expression of Pdx-1 in pancreatic buds (Weibe, 2007). Variable effects of Pdx-1 in the context of different cell types point to the limited understanding of the mechanisms and pathways encompassing Pdx-1 action (Babu *et al.,* 2007). CK19 positive epithelial cells are progenitors for islet cells and ductal cells. This cytokeratin forming intermediate filaments that appeared in the cytoplasm is observed in the epithelial lining of ducts, acini and islets starting from the day 15. The suggested stem cells that express CK19 were mainly observed in acinar and islet cells. These results are in agreement with an earlier study, stating that CK19 is widely expressed within the ventral half of the early embryo including the pancreatic epithelial cells. CK19 demarcates all of the pancreatic epithelial cells, a small proportion of which co-express insulin in the early fetal pancreas. The detection of CK19 is sequentially diminished in insulin-positive cells, as the primitive islet structures become distinct from the CK19-positive branched epithelia (Piper *et al.,* 2004). A study focusing on stem/progenitor cells in pancreatic islets from rats using CK19 and Pdx1 as markers in islet samples suggested the presence of stem cells in all islet samples (Gao *et al.,* 2003; Banerjee and Bhonde, 2003; Yang, 2007). Islet cells are derived from duct-like precursor cells that arise from the ductal embryonic buds of islet cells (Noguchi *et al.,* 2003). CK19 positive ductal cell colonies emerge in early embryonic stage during the development of the pancreas to form the functional units of the endocrine pancreas (Yang, 2007; Roost *et al.,* 2014). The pancreatic mesenchymal stem cells (MSCs) express CD29 as observed in this study (Seeberger *et al.,* 2006). It has been established that pancreatic MSCs have the ability to differentiate into mesoderm (osteocytes, adipocytes and chondrocytes) and endoderm (hepatocytes and beta-cells) linages. The pancreatic MSCs express Pdx1, Nkx2.2 (Homeobox protein Nkx-2.2), Isl1 (Insulin gene enhancer protein ISL-1), NeuroD (Neurogenic differentiation), Ngn3 (Neurogenin 3), and Nkx6.1 (Homeobox protein Nkx-6.1), key transcription factors implicated in the development of endocrine islet cells (May and Kaestner, 2010). The cytoplasmic expression of CD56, another stem cell marker is markedly observed in the cells lining the acinar and islet cells in late gestation. The CD56+ luminal cells might represent developmental and regenerative changes of pancreatic ducts (Fujisawa *et al.,* 2003; Fattah, 2008).

Conclusions: Immunohistochemically, the variable degrees of expression of all the markers (Pdx-1, CK19, CD29, and CD56) ranged from weak, medium and strong, depending on embryonic stage. The markers were expressed in the cytoplasm and membrane of the stem cells. The intensity of their expression is however different in the three cell lineages. The islets are the sites with abundant stem cells where different markers can be expressed especially in latter stages. Results of the current study accentuate that the positive expression of Pdx1 in the three cell lineages of pancreas from the earlier gestational age suggest its vital role in pancreatic neogenesis. However, late expression of CK19, CD29, and CD56 especially in the islets, suggest their role in the generation of new β-cells. Further studying these developmental stages by immunoelectron microscopy and confocal microscopy would enhance and pinpoint sites of expression with more precision.

Author's contribution: *SAMK* was the principal investigator, under whose guidance the whole study was carried out. TAN executed the experiments with the guidance of SKA, *SAMK* and FAQ. WI with the help of *S*AMK critically reviewed and analyzed the data.

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