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Spatiotemporal expression of *D10Wsu52e* in the developing mouse pancreas

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TABLE OF CONTENTS

1. ABSTRACT

D10Wsu52e is a recently discovered and highly conserved mouse gene. FAAP, the protein encoded by *D10Wsu52e,* participates in regulation of integrin-based focal adhesions. To explore the function of FAAP in pancreas development, we assessed the spatiotemporal expression of *D10Wsu52e, paxillin* and *vinculin* in the developing mouse pancreas through quantitative RT-PCR, *in situ* hybridization and histochemistry methods. Our results show that, at e9.5 and e10.5, *D10Wsu52e* mRNA is mainly expressed in the brain, optic vesicles, otic vesicles, limb buds, somites and gut, and also in the pancreatic buds at e10.5. Subsequently, *D10Wsu52e* mRNA is expressed mainly in the epithelial progenitors at e12.5, then decreases in the endocrine precursors and ductal cells, whereas still maintains in the exocrine precursors until e15.5. At e17.5, *D10Wsu52e* mRNA is highly expressed in exocrine acinar cells, but weakly in ductal and endocrine cells. Furthermore, the expression patterns of *paxillin* and *vinculin* mRNAs are similar to *D10Wsu52e* from e12.5 to e15.5, which suggests that *D10Wsu52e* may be related to the acinar development, adhesion and migration of progenitors and endocrine precursors.

2. INTRODUCTION

The pancreas is a mixed function gland with both endocrine and exocrine portion. Endocrine portion is composed of alpha, beta, delta, pp and epsilon cells; the exocrine part is composed of acinar cells secreting digestive enzymes, such as amylase, lipase, protease and nuclease, into the pancreatic ducts, through which they are drained into the duodenum to digest food. All the endocrine, exocrine and ductal cells are developed from the foregut endoderm of early embryos in mammals (1). The mouse embryonic pancreas originates as dorsal and ventral buds which grow out of the endodermal epithelium at embryonic days 8.5 (e8.5) and e9.5, and this event is called the primary transition (2). After this transition, primordial pancreas buds are formed predominantly of undifferentiated duct-like epithelium until e12.5. The second transition occurs from e13.5 to e15.5, when the dorsal and ventral pancreatic anlagens fuse together into a whole organ with the duodenum rotating to the right. During this period, endocrine cells are arrayed as single cells within the pancreatic epithelium, and exocrine acinar cells differentiate from the terminal ductal epithelium (2). From e16.5 to e19, specified endocrine cells migrate from

the ducts and coalesce into islets, after which endocrine hormone or exocrine enzymes increase rapidly, and this period is known as the third transition (3). Cellular signaling molecules and transcriptional factors play crucial roles in different events of pancreas development, including cell fate determination, proliferation, differentiation and migration (4, 5).

D10Wsu52e is a newly discovered mouse gene on chromosome 10, encoding a 55 kD protein named focal adhesion associated protein (FAAP) (6). It is a conserved gene, for which homologous genes have been cloned from human beings (7), rats (Ding, et al. unpublished), C. elegans (8) and cattle (9). The rate of amino acid sequence identity of FAAP with the homologous human protein HSPC117 reached 99.4% (6). It was initially found that D10Wsu52e is expressed in pre-implantation mouse embryos (10) and Sca-1-positive progenitors (11). Later, it was found to be expressed in multiple tissues and organs (http://www.ncbi.nlm.nih.gov/UniGene). A report on HSPC117 had suggested that it is related to cell adhesion and migration, since it is specifically located in the early spreading-initiating center at the time cell adhesion occurs (12). Recently, FAAP was further shown to inhibit cell spreading through regulating vinculin-paxillin association in integrin-based focal adhesions (6). Since integrins were found to be important in mediating adhesion, migration and maintenance of developing pancreatic epithelium (13-16), FAAP might also play roles in these processes. However, the expression of *D10Wsu52e* in the developing pancreas remains unknown. Here, we report the expression patterns of *D10Wsu52e*, and cell adhesion related genes *paxillin* and *vinculin* in the developing mouse pancreas.

3. MATERIALS AND METHODS

3.1. Animals

Outbred strain ICR mice (8 to 10 weeks old) were purchased from a commercial breeder (Jilin University Laboratory Animals Research Centre, ChangChun, China). All the mice were kept in compliance with the protocols established and approved by the Animal Care and Ethics Committee of Northeast Forestry University. One male and four female mice were mated overnight in our facilities, which were maintained at 21-25ºC and 40-60% relative humidity on a 12h light/dark cycle. Pregnancy was confirmed in the following morning by the presence of a vaginal plug, which day was then considered as e0.5. The pregnant mice were sacrificed and the whole embryos were acquired from e8.5 to e10.5, and the embryonic pancreata were acquired from e12.5 to e17.5. The mouse embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, PH7.4) overnight and dehydrated in graded methanol/PBT(1xPBS plus 1% Tween), and then kept at -20ºC until used. The embryonic pancreata were fixed in 4% paraformaldehyde for two hours and dehydrated in 15% sucrose overnight at 4ºC, and then embedded in OCT compound for cryosectioning. 10 um cryostat sections were collected and stored at -80ºC until used.

3.2. RNA isolation and reverse transcription

Total RNA was isolated from e12.5, e15.5 and e17.5 mouse pancreata using Trizol reagent (Invitrogen Corp.) and treated with DNase I (RNase free) (Promega). The total RNA was reverse transcribed using Transcript III (Invitrogen) with oligo-dT primers according to the manufacturer's instructions.

3.3. Real-time quantitative PCR of *D10Wsu52e* mRNA

Real-time quantitative PCR was performed on an Applied Biosystem 7500 HT Sequence Detection System. *Beta-actin* was used to normalize all mRNA quantifications. All reactions were performed in triplicate and included no template controls for each gene. Primers used in these tests included *beta-actin* (5'-CCAACCGTGAAAAGATGACC-3' and 5'-TACGACCAGAGGCATACAGG-3'), *D10Wsu52e* (5'-CAGTTGGCTTGTGCTCGGA-3' and 5'-CCAGGTCATCAGGGGTTGTGT-3'). The relative amount of each mRNA to *beta-actin* was calculated using a quantitation- relative standard curve. The level of significance was determined by one-way analysis of variance (ANOVA) with GraphPad InStat software v3.06. All quantitative data presented are the mean +/- SD from at least three samples per data point.

3.4. Preparation of probe and *in situ* hybridization

The spatial expression patterns were determined by whole mount *in situ* hybridization using a DIG-labeled antisense probe. An antisense probe for the *in situ* hybridization was transcribed with SP6 from the *D10Wsu52e* cDNA full length, *paxillin* fragment (307bp) and *vinculin* fragment (679bp) were cloned into the pGEM-T vector (sense: T7). Briefly, after a graded rehydration, embryos were bleached with 6% hydrogen peroxide in PBT for 1 to 2 hours at room temperature with gently rocking, then treated with 10ug/ml proteinase K at 37ºC, from 6 to 15 min according to their embryonic age. After the embryos were postfixed with 4% paraformaldehyde and 0.2% glutaraldehyde in PBT for 20 minutes, they were pre-hybridized for more than 1 h in hybridization buffer (50% formamide, 5xSSC pH 4.5, 50 ug/ml yeast RNA, 1% SDS, 50 ug/ml heparin) and then the appropriate probe was added at a concentration of 1 ug/ml so as to hybridize overnight at 70ºC. After the embryos were successively washed as a normal procedure, they were blocked in 0.1% blocking reagent (Roche) and 10% normal horse serum (Zemyd) for more than 1h at room temperature, then embryos were incubated overnight at 4°C in alkaline phosphatase conjugated anti-DIG antibody (1:2000, Roche). After being washed in TBST and NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 1% Tween-20, 2 mM Levamisole), embryos were placed in 125 g/ml BCIP and 250 g/ml NBT in NTMT at room temperature with gently rocking. When color had developed to the desired extent, the embryos were washed with PBS and refixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBT for 20 minutes at room temperature. Then, embryos were permeabilized in glycerol for 30 minutes and photographed. *In situ* hybridization for tissue sections was conducted as previously described (17).

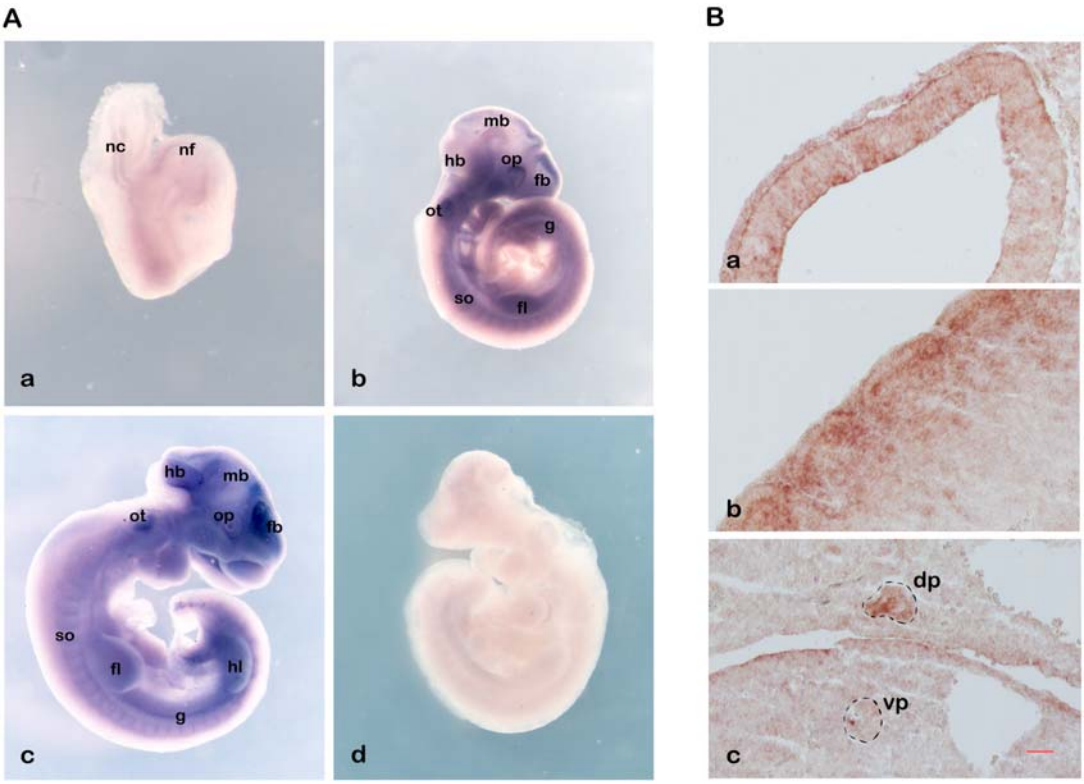

Figure 1. Expression of *D10Wsu52e* mRNA in whole mouse embryos at embryonic days (e) 8.5, e9.5 and e10.5. (A) Embryos were hybridized with a digoxigenin-labeled antisense (a-c, blue) or sense (d) probe for *D10Wsu52e* mRNA. (a) At e8.5, *D10Wsu52e* mRNA is mainly expressed in the notochord (nc) and neural folds (nf). (b) At e9.5, the signal is appeared in the forebrain (fb), midbrain (mb), hindbrain (hb), optic vesicles (op), otic vesicles (ot), forelimb buds (fl), hindlimb buds (hl), gut (g) and somites (so). (c) At e10.5, the signal is detected in fb, mb, hb, op, ot, fl, hl, g and so. (B) The e10.5 embryos were cryostat-sectioned at a thickness of 10 um and performed *in situ* hybridization (brown). *D10Wsu52e* mRNA is strongly expressed in the fb (a) and so (b). (c) The strong signal can be detected in the dorsal pancreatic bud (dp) and a weak signal appears in the ventral pancreatic bud (vp). Scale bar=50 μm.

3.5. Histochemistry

Histochemistry was performed on the sections after *in situ* hybridization, and optimal dilutions and controls were used for each antibody. Affinity-purified rabbit anti-insulin (1:200, Santa Cruz), rabbit anti-amylase (1:500, Sigma), and rabbit anti-pax6 (1:3000, chemicon) were used as the primary antibodies, biotinylated goat anti-rabbit IgG (H+L) (1:200, Jackson Immunoresearch) was used as the second antibody. Otherwise, biotin-conjugated DBA (1:3000, Jackson ImmunoResearch) was used to label pancreatic ducts. Horse radish peroxidase streptavidin (1:200) was used to bind the biotinylated antibodies or DBA. Peroxidase was detected by AEC (Sigma, St. Louis, MO). Images of stained sections were acquired using a microscope (Carl Zeiss, Germany) equipped with a cooled three-chip charge-coupled-device camera (Carl Zeiss, Germany).

4. RESULTS

4.1. Expression of *D10Wsu52e* mRNA in the whole developing embryos

To detect the expression of *D10Wsu52e* mRNA and gain insight into its role in the early developing embryo, we initially performed whole mount *in situ* hybridization at e8.5, e9.5 and e10.5 with a specific cRNA antisense probe. We found that, at e8.5, the expression of *D10Wsu52e* mRNA was limited to the neural folds and notochord, but no apparent signals were detected in the other portions (Figure 1Aa). At e9.5, *D10Wsu52e* appeared in the forebrain, midbrain, hindbrain, forelimb buds, hindlimb buds, somites and gut, prominently in the optic and otic vesicles (Figure 1Ab). This expression was maintained in e10.5 embryos (Figure 1Ac), and strong signals appeared in the brain and somites (Figure 1Ba and b). Furthermore, *D10Wsu52e* mRNA was strongly detected in the dorsal pancreatic bud, and weakly in ventral pancreatic bud on e10.5 embryo sections (Figure 1Bc). However, there were no signals when sense probes were used for *in situ* hybridization analysis (Figure 1Ad).

4.2. Expression of *D10Wsu52e* mRNA in the developing mouse pancreas

To measure the expression of *D10Wsu52e* mRNA at different stages of pancreatic development, we performed real time quantitative RT-PCR experiments to detect the expression levels of *D10Wsu52e* mRNA in pancreatic anlagen on e12.5, e15.5 and e17.5. We found

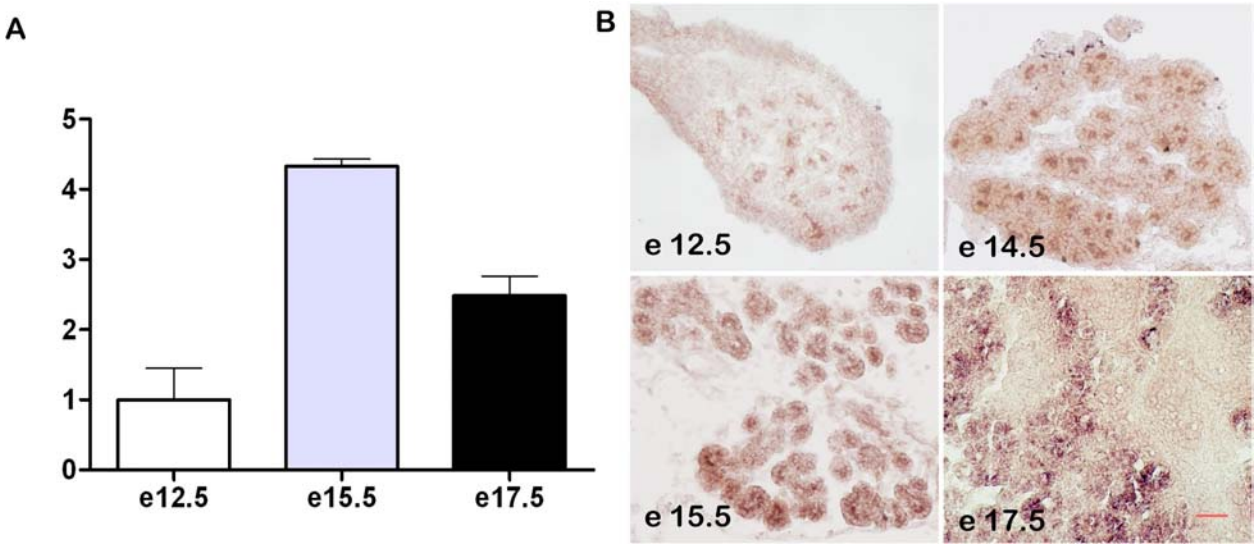

Figure 2. Expression of *D10Wsu52e* mRNA in early mouse embryonic pancreas. (A) The plot shows the qRT-PCR analysis of *D10Wsu52e* expression in the mouse embryonic pancreas at e12.5, e15.5 and e17.5. *D10Wsu52e* levels of e15.5 and e17.5 are indicated relative to that of e12.5, *beta-actin* is used as endogenous control. (B) *In situ* hybridization of *D10Wsu52e* during early mouse pancreas development. The time points are indicated at the top right. Scale bar=50 μm.

that the *D10Wsu52e* mRNA level was 3-fold higher at e15.5 than that at e12.5, and 2-fold higher than that at e17.5 (Figure 2A).

Subsequently, *in situ* hybridization analysis was performed on sections of the embryonic pancreas to obtain more details on the spatio-temporal expression of *D10Wsu52e* mRNA (Figure 2B). We found that, at e12.5, *D10Wsu52e* mRNA was mostly present in duct-like pancreatic epithelial progenitors, also, it was slightly expressed in the surrounding mesenchymal cells. At e14.5 and e15.5, the expression of *D10Wsu52e* mRNA was still strong in the duct-like epithelial structures, but weak in the cells of non-duct-like epithelia and mesenchymal cells. At e17.5, the expression of *D10Wsu52e* mRNA displayed a spatio-specific pattern, in that the hybridization signal was strong in acinar structures, but weak in the other tissues.

To clarify which kind of cells potently expressed *D10Wsu52e* mRNA when the epithelial progenitor cells differentiated after the second transition, we performed histochemical staining on *in situ* hybridization sections at e14.5, e15.5 or e17.5, using antibodies against amylase (acini-specific), pax6 (endocrine precursor specific) and insulin (beta-cell specific), or biotin-conjugated DBA (duct-specific). The cells with high expression of *D10Wsu52e* mRNA were densely stained with the antibody against amylase at e14.5, e15.5 (data not shown) and e17.5 (Figure 3D), whereas the cells with low expression of *D10Wsu52e* mRNA could be stained the antibody against pax6 from e12.5 to e15.5 (Figure 3E and G), the antibody against insulin at e17.5 (Figure 3H) or DBA at e14.5 (Figure 3F), e15.5 and e17.5 (data not shown). These results indicate that *D10Wsu52e* mRNA is highly expressed in the developing and mature acinar cells, but very weakly in endocrine precursors, ductal and insular cells, after the second transition of mouse pancreas development.

4.3. Expression of *paxillin* and *vinculin* mRNAs in the developing mouse pancreas

Paxillin is a scaffold protein that is localized to integrin-based focal adhesions (18). It acts as a substrate of tyrosine kinase FAK, and is involved in the anchorage of the integrin cytoplasmic domain to the actin cytoskeleton via an interaction with another adhesion protein, vinculin (18, 19). According to these findings, we postulated that the expression of both *paxillin* and *vinculin* are related to that of *D10Wsu52e.* Therefore, we performed *in situ* hybridization analysis on sections of the embryonic pancreas with *paxillin* and *vinculin* cRNA probes. Just as in the case with *D10Wsu52e*, the expression of *paxillin* mRNA was maintained in all the epithelial and mesenchymal cells from e12.5 to e15.5 (Figure 4A-D), especially, it was high in the duct-like epithelial cells at e14.5 and e15.5. Histochemical analysis on the *in situ* hybridization sections showed that *paxillin* mRNA was also highly expressed in amylase-positive cells at e15.5 (Figure 4H), but weakly in DBA-positive and pax6-positive cells at e14.5 (Figure 4F and G). However, in contrast with *D10Wsu52e, paxillin* mRNA was expressed ubiquitously in all the pancreatic cells at e17.5 (Figure 4E). In addition, the expression pattern of *vinculin* mRNA was similar to that of *paxillin* mRNA from e12.5 to e17.5 (Figure 5A-E), except that *vinculin* mRNA was strongly expressed in the DBA-positive ductal cells of the pancreas at e14.5 (Figure 5F).

5. DISCUSSION

Pancreas development has been demonstrated to be regulated by a complex gene expression network, in

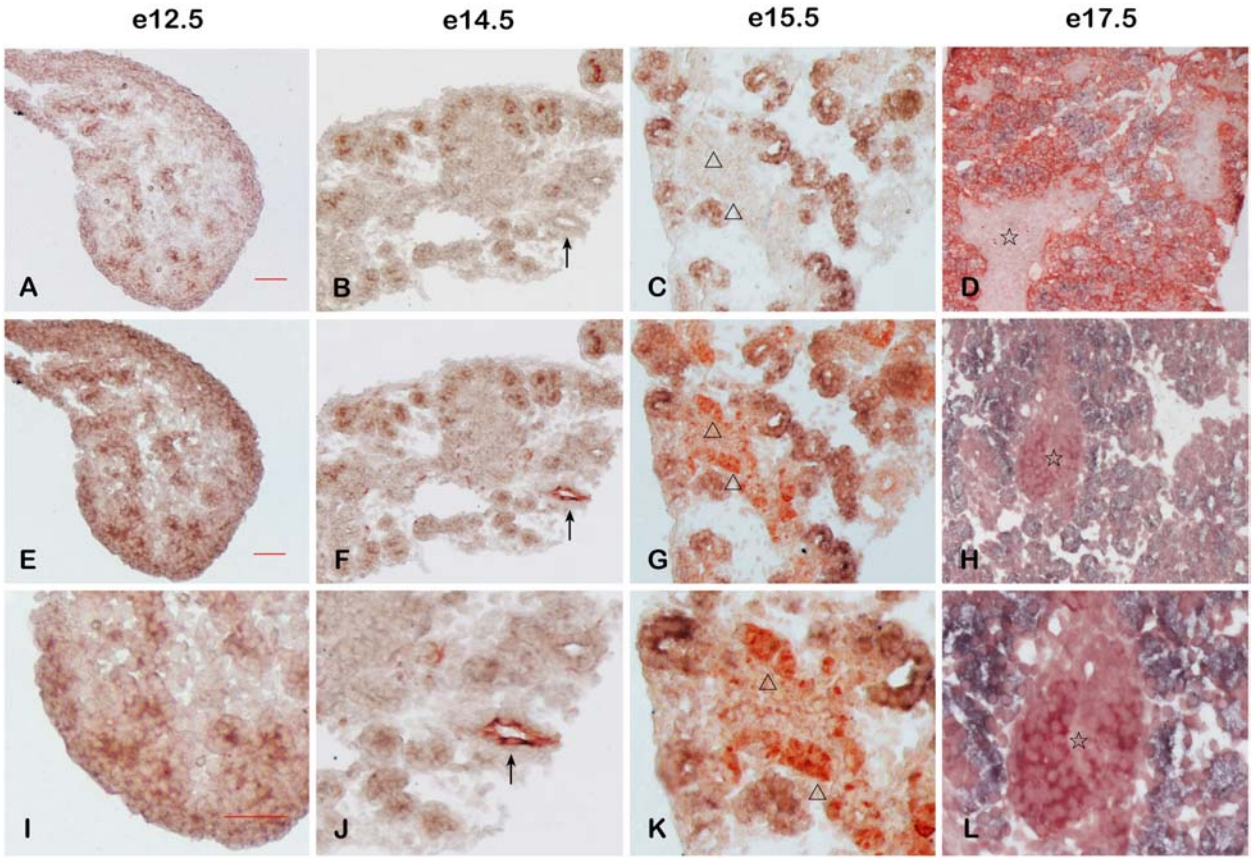

Figure 3. Co-localization of *D10Wsu52e* mRNA and Pax6, DBA, Amylase or insulin. To visualize the cell types expressing *D10Wsu52e* mRNA, post-hybridization sections (A-C) were histochemical stained (D-L, red) with anti-Pax6 antibody at e12.5 (E, I) and e15.5 (G, K), DBA-biotin at e14.5 (F, J), anti-Amylase antibody at e17.5 (D), anti-insulin antibody at e17.5 (H, L), respectively. I, J, K, L shows representative high-power image of E, F, G, H, respectively. The arrows in B and F indicate pancreatic duct of a same section. The triangles in C and G indicate the same regions of one section. The pentagons in D and H indicate pancreatic islets of two different sections. The signal of *in situ* hybridization is brown (A-C, E-G, I-K) or blue (D, H, L). Scale bar=50 μm.

which cell adhesion molecules play crucial roles in the cell differentiation, survival, and migration (4, 5). In this study, we found that the focal adhesion associated protein gene *D10Wsu52e* mRNA was mainly expressed in the brain, optic vesicles, otic vesicles, gut, limb buds and somites at e9.5 and e10.5, and also in the pancreatic buds at the primary transition. Thereafter, the expression of *D10Wsu52e* mRNA was present in all of the duct-like epithelial progenitor cells at e12.5, then decreased in the endocrine precursors and ductal cells, but still maintained at a high level in the exocrine precursors and mature cells after the second transition. This developmentally regulated expression pattern indicates that *D10Wsu52e* plays a role in the early pancreas development.

It has been found that the subcellular localization and function of FAAP are closely associated with integrins (6, 12), which are a large family of heterodimeric transmembrane receptors composed of disulfide bonded alpha- and beta-subunits. Integrins link the extracellular matrix proteins and the actin cytoskeleton through talin, vinculin, and paxillin to form focal adhesions and exert important roles in cell survival, mobility, proliferation and differentiation in tissue development (20, 21). Previous reports have found that integrin memebers alpha6beta4 and alpha3beta1 regulate the migration of pancreatic progenitors attracted by netrin-1(14), and beta1 integrin associated with alpha3, alpha5, and alpha6 mediate adhesion and migration of putative endocrine precursor cells within the developing pancreatic epithelium in rat and human pancreas (13, 22, 23). As members of the integrin-based focal adhesions, the expression of *paxillin* and *vinculin* mRNAs are found to kept at a high level in the pancreatic epithelial progenitor cells, while decreased in aggregated endocrine precursor cells. The similar expression pattern of *D10Wsu52e* mRNA is present at the same time. Combining the evidence that FAAP encoded by the *D10Wsu52e* gene can regulate vinculin-paxillin association in integrin-based focal adhesions (6), it is reasonable to expect that the differential expression of *D10Wsu52e* mRNA in the pancreatic progenitors and endocrine precursors is related to their adhesion and migration in the developing mouse pancreas.

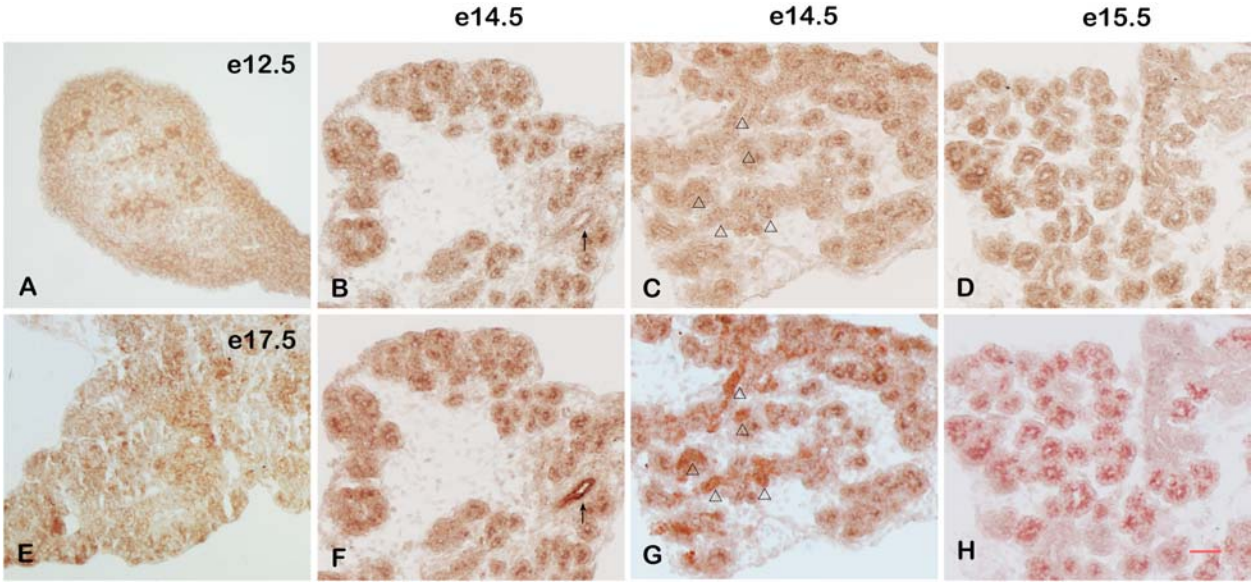

Figure 4. Expression of *paxillin* mRNA during mouse pancreas development. (A-E) *In situ* hybridization analysis (brown) of *paxillin* mRNA on e12.5 (A), e14.5 (B and C), e15.5 (D) and e17.5 (E) pancreas sections. (F-H) Histochemical analysis (red) of DBA (F), Pax6 (G) on e14.5 and Amylase (H) on e15.5 pancreas sections, respectively, after *in situ* hybridization. The arrows in B and F indicate pancreatic duct of a same section. The triangles in C and G indicate the same regions of one section. Scale bar=50 μm.

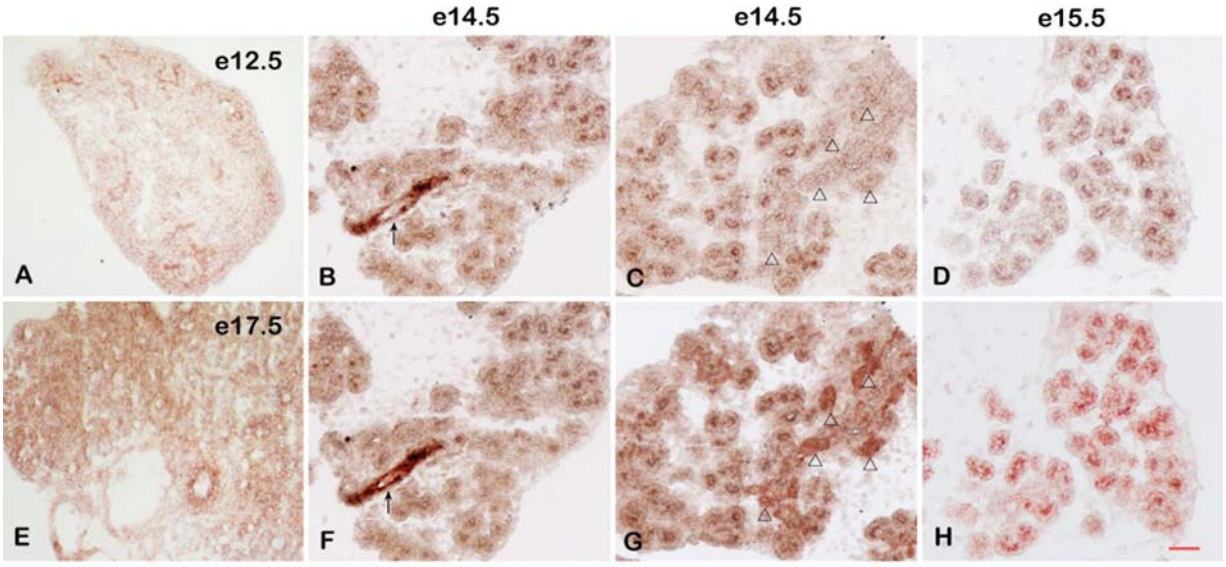

Figure 5. Expression of *vinculin* mRNA during mouse pancreas development. (A-E) *In situ* hybridization analysis (brown) of *vinculin* mRNA on e12.5 (A), e14.5 (B and C), e15.5 (D) and e17.5 (E) pancreas sections. (F-H) Histochemical analysis (red) of DBA (F), Pax6 (G) on e14.5 and Amylase (H) on e15.5 pancreas sections, respectively, after *in situ* hybridization. The arrows in B and F indicate pancreatic duct of a same section. The triangles in C and G indicate the same regions of one section. Scale bar=50 μm.

After the endocrine precursor cells differentiate and cluster into islets, the expression of *D10Wsu52e* mRNA in the islets decreases. Although some researches reported that integrins exert a role in insulin secretion of beta cells (15, 16), the other researches showed only weak existence of alpha3beta1 integrin in mouse islets (24). Moreover, major ECM proteins, including laminin, fibronectin, vitronectin, and elastin, are absent between islet cells *in vivo* (24, 25). These results indicate that *D10Wsu52e* is less related to functional maintenance of endocrine cells. However,

D10Wsu52e, *paxillin* and *vinculin* mRNAs were found to be successively expressed in the developing and mature acini. Their expression patterns coincide with some members of integrin family, such as alpha3, alpha6 and beta1 integrins, in human and rat pancreas (24, 26). Recently, it was found that conditional knockout of beta1 integrin in acinar cells resulted in focal acinar cell necrosis and disruption of acinar cell polarity (27). These results indicate that *D10Wsu52e* may participate in integrins-based regulation of acinar development and functional maintenence.

Besides the location in integrin-based focal adhesions, FAAP has been found to be deposited in cytoplasmic fractions (28). Human homologous protein HSPC117 of FAAP has also been identified in large RNase-sensitive granules as a binding partner of kinesin to transport RNA in dendrites (29) as well as several other protein complexes, such as TNF-alpha mRNA 3' AU-rich element binding complexes (30) and osmotic response element binding protein KIAA0827 (31). However, the function of FAAP in these protein complexes remains unknown. The expression analysis reported here is a prerequisite to the step of functional studies of FAAP in pancreas development.

6. ACKNOWLEDGMENTS

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