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Dynamic expression of *Tbx2* and *Tbx3* in developing mouse pancreas

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Abstract

Tbx2 and *Tbx3* are closely related members of the T-box family of transcription factors that are important regulators during normal development as well as major contributors to human developmental syndromes when mutated. Although there is evidence for the involvement of *Tbx2* and *Tbx3* in pancreatic cancer, so far there are no reports characterizing the normal expression pattern of these genes in the pancreas. In this study, we examined spatial and temporal expression of *Tbx2* and *Tbx3* in mouse pancreas during development and in the adult using *in situ* hybridization and immunohistochemistry. Our results show that *Tbx2* and *Tbx3* are both expressed in the pancreatic mesenchyme throughout development beginning at embryonic day (E) 9.5. In addition, *Tbx2* is expressed in pancreatic vasculature during development and in epithelial-derived endocrine and ductal cells during late fetal stages, postnatal development and in adult pancreas. In contrast, *Tbx3* is expressed in exocrine tissue in the postnatal and adult pancreas. Further our results demonstrate that *Tbx2* and *Tbx3* are expressed in tumor-derived endocrine and exocrine cell lines, respectively. These dynamic changes in the expression pattern of these transcription factors lay the foundation for investigation of potential roles in pancreas development.

Keywords

Tbx2; *Tbx3*; T-box; Pancreas; Islets

Tbx2 and *Tbx3* are closely related members of the *Tbx2* subfamily of the T-box transcription factor family that play essential roles in cell proliferation, cell fate and tissue identity during development (Papaioannou, 2001; Papaioannou and Silver, 1998). *Tbx2* and *Tbx3* share over 90% sequence identity in their DNA binding domain (Agulnik et al., 1996) and during embryonic development, *Tbx2* and *Tbx3* have overlapping expression patterns in many tissues (Chapman et al., 1996; Gibson-Brown et al., 1998). Targeted mutagenesis studies have revealed crucial roles for both genes during embryonic development. Mice homozygous for a *Tbx2* null mutation show lethal cardiovascular defects and abnormal limb development (Harrelson et al., 2004; Suzuki et al., 2004). Mice homozygous for a *Tbx3* null mutation die during midgestation with abnormalities in the yolk sac, limbs, mammary glands and heart (Davenport et al., 2003; Mesbah et al., 2008).

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In addition to important roles in development, increasing evidence suggests a role for these two genes in a variety of cancers, including pancreatic cancers (Fan et al., 2004; Jacobs et al., 2000; Mahlamäki et al., 2002; Prince et al., 2004; Sinclair et al., 2002; Vance et al., 2005). *Tbx2* was amplified in 50% of 31 pancreatic cell lines tested (Mahlamäki et al., 2002) and *Tbx2* protein and RNA expression was found in human pancreatic tumors (Chen et al., 2008; Duo et al., 2009). There is also a report that *Tbx3* is over expressed in metastatic endocrine neoplasm's (Hansel et al., 2004). As pancreatic cancer is the fourth leading cause of cancer death in the United States (Postier, 2003) a new biomarker could be valuable for early detection of the disease.

In recent years, several reports identified *Tbx3* as one of the self renewal regulators of embryonic stem cells (ESC), as well as a factor increasing the germ-line competency of induced pluripotent stem cells (iPSC) (Galan-Caridad et al., 2007; Han et al., 2010; Ivanova et al., 2006). Thus, characterization of *Tbx3* and the closely related *Tbx2* in the developing pancreas could provide valuable information on the control of self renewal of pancreatic progenitor cells and the differentiation of cells from ES cells.

Although there is one report of *Tbx3* expression in pancreatic mesenchyme at E 9.5 (Zhou et al., 2007), no detailed expression has been previously reported. In this study, we examine the spatial and temporal expression of *Tbx2* and *Tbx3* in mouse pancreas throughout development and in the adult using a combination of *in situ* hybridization (ISH), immunohistochemistry (IHC) and comparison of expression with known markers of differentiation. Our results show that *Tbx2* and *Tbx3* are both expressed in the pancreatic mesenchyme throughout development beginning at E9.5. In addition, *Tbx2* is expressed in pancreatic vasculature during development and in epithelial-derived endocrine and ductal cells during late fetal stages, postnatal development and in the adult pancreas. In contrast, *Tbx3* is expressed in exocrine tissue in the postnatal and adult pancreas. Additional studies demonstrate that *Tbx2* and *Tbx3* are expressed in tumor-derived endocrine and exocrine cell lines, respectively.

1. Results and discussion

1.1. Mesenchymal expression of *Tbx2* and *Tbx3* during budding and early morphogenesis of the pancreas

Tbx2 and *Tbx3* expression was first detected in the developing pancreas at E9.5 and E10.5 in the mesenchyme surrounding the pancreatic buds, which are identified by the epithelial marker, *Pdx1* (Fig. 1). During early pancreas morphogenesis at E12.5 and E13.5 a diffuse pattern of mesenchymal expression of *Tbx2* and *Tbx3* was observed (Fig. 2A-F) with the most intense expression at the dorso-lateral angle of the embryonic pancreas, which is composed mostly of mesenchymal cells. Expression is only in the mesenchyme and never in the epithelium, as shown by comparison with a known epithelial marker, *Pdx1*, in adjacent section at E10.5 (Fig. 3A-D). At E13.5, analysis of adjacent sections using probes for either *Tbx2* or *Tbx3* and anti-E-cadherin antibody, a marker of epithelium, confirmed that neither *Tbx2* nor *Tbx3* are expressed in the pancreatic epithelium (Fig. 3E-H). The pattern of mesenchymal expression, however, differs between the two genes: *Tbx2* is expressed uniformly in the mesenchyme and in close contact with epithelial branches (Fig. 3E, F); *Tbx3* is expressed around the epithelial branches but with intense localized expression at the bifurcation of branching tubes (Fig. 3G, H), indicating a potential role for *Tbx3* in pancreatic epithelial branching morphogenesis.

1.2. *Tbx2* and *Tbx3* expression during mid and late stages of pancreas development

E14.5 marks the transition point of pancreas development when differentiation of the progenitor cells begins (Zhou et al., 2007). At this stage, *Tbx2* is expressed throughout the

pancreas (Fig. 2G) and, by E15.5, is expressed uniformly in the pancreatic mesenchyme in close contact with the epithelial branching network, marked by E-cadherin (Fig. 3I, J). In addition, *Tbx2* expression was seen in a few clusters of epithelial cells, which were E-cadherin positive in adjacent sections (arrows, Fig. 3I, J). *Tbx3*, on the other hand, is expressed in a punctate pattern (Fig. 2H) in the mesenchyme at these stages (Fig. 3K, L).

At E17.5, *Tbx2* is expressed both in mesenchyme and epithelial-derived tissues. It is present in mesenchyme surrounding clusters of E-cadherin positive endocrine cells (Fig. 4A, B), and where well-formed islets are evident morphologically, *Tbx2* is expressed in the endocrine cells (Fig. 4C, D). Double immunofluorescence (IF) using anti-*Tbx2* with anti-insulin or anti-glucagon showed that *Tbx2* is co-expressed in the insulin-positive cells and glucagon-positive cells of endocrine clusters at E17.5 (Fig. 5A-F), suggesting that *Tbx2* might play a role in the maturation of major hormone producing cells. Some (Fig. 4E, F) but not all (Fig. 4A, B) E-cadherin-positive epithelial ducts show *Tbx2* expression. In contrast, *Tbx3* is expressed only in the mesenchyme with epithelial tissues completely devoid of signal (Fig. 4G, H).

Tbx2 expression was also observed in pancreatic vasculature from E12.5 to E17.5 (Fig. 2A, D, G, arrows; Fig. 4E, F, arrowheads). Vessels were identified in sections at E17.5 as duct-like structures lined by endothelial cells or as structures lacking E-cadherin expression in adjacent sections (Fig. 4E, F, white arrowheads). The importance of vascular signals in pancreas development and differentiation has been noted (Lammert et al., 2001).

1.3. *Tbx2* and *Tbx3* expression during postnatal and in adult pancreas

At postnatal day (P) 7, *Tbx2* is expressed in the cytoplasm of islet cells, including the insulin-positive cells (Fig. 5G-I) and in pancreatic ducts (data not shown), whereas *Tbx3* is not in the islets but is expressed in exocrine tissue (Fig. 5J-L). In the adult, *Tbx2* is present in the islets in cells (Fig. 6A-C), glucagon-positive cells (Fig. 6D-F), Dolichos Biflorus Agglutinin (DBA)-positive ducts (Fig. 6G-I), but not in carboxypeptidaseA1 (CPA1)-positive exocrine tissue (Fig. 6J-L). *Tbx3* is not expressed in the islet (Fig. 6M-O) but is expressed in exocrine tissue (Fig. 6P-R).

1.4. *Tbx2* and *Tbx3* expression in pancreatic cell lines

We investigated *Tbx2* and *Tbx3* protein expression in tumor-derived endocrine pancreatic cell lines TC and TC, which were derived from mouse insulinoma and glucagonoma and maintain adult differentiated and cell phenotypes, respectively (Efrat et al., 1988; Hamaguchi et al., 2003) and an exocrine cell line 266-6, derived from a pancreatic acinar cell tumor (Ornitz et al., 1987). *Tbx2* is expressed in the cytoplasm of both endocrine cell lines (Fig. 7A-D) and in the exocrine cell line (Fig. 7E, F). *Tbx3* is not expressed in β TC cells (data not shown) but was present in line 266-6 (Fig. 7G, H). These findings are consistent with our *in vivo* results in adult pancreas with the exception that *Tbx2* is expressed in the 266-6 exocrine cell line, but not in exocrine tissue in normal pancreas. This *Tbx2* expression may be due to the tumor origin of cells. In all three cell lines, both *Tbx2* and *Tbx3* are expressed in the cytoplasm rather than the nucleus, similar to postnatal and adult pancreases (Fig. 7A-H).

1.5. Conclusion

The dynamic changes in expression of *Tbx2* and *Tbx3* expression throughout pancreas development and in pancreatic tumor cell lines (summarized in Table 1) lay the foundation for investigation of potential roles of these transcription factor genes in pancreas development and their potential roles in pancreatic cancer.

2. Experimental procedures

2.1. Animals, in situ hybridization and immunohistochemistry

Random bred ICR mice (Taconic, Germantown, NY) were used to generate embryos from E9.5 to E17.5, where noon on the day of the vaginal plug detection was designated E0.5. Embryos were dissected in cold phosphate buffer saline (PBS), fixed overnight in 4% paraformaldehyde, and washed two times in PBT (PBS containing Tween-20), dehydrated in 100% methanol and stored at -20°C until use. For whole-mount ISH and IHC at E9.5 and E10.5, embryos were partially dissected to expose the foregut for visualization of the pancreatic area. At E12.5-E14.5, pancreases were dissected out, along with the stomach, spleen and duodenum. Pancreases from P7 pups and two months old adults were collected for analysis. Samples for section ISH and IF were treated with 30% sucrose overnight following fixation, embedded in Tissue Tek OCT compound (Sakura Fine Technical Co, Ltd, Tokyo, Japan), and snap-frozen on dry ice in ethanol and stored at -80°C until use.

Sense and antisense digoxigenin labeled riboprobes for *Tbx2* and *Tbx3* (Chapman 1996) were generated by *in vitro* transcription in the presence of digoxigenin-labeled dUTP (Roche, Nutely, NJ). Standard procedures were used for whole-mount ISH (Wilkinson, 1992). For section ISH analysis, 10µm frozen sections were made of E10.5-E17.5 embryos. Section ISH was performed as described previously (Prado et al., 2004). Sections were counter stained with Nuclear Fast Red.

Whole-mount IHC with anti-Pdx1 antibody was performed using standard protocol (Davis, 1993). IF was performed on 10 µm frozen sections from whole embryos (E10.5-13.5) and pancreases (E17.5, P7, and adult) using protocols as previously described (Begum et al., 2009). The antibodies used were as follows: guinea pig anti-human insulin, guinea pig anti-glucagon (both from Linco Research, St. Charles, MO), rabbit and goat anti-Pdx1 (a gift from Dr. Christopher Wright), rabbit anti-Tbx2 (Bioworld Technology Inc, MN, USA), rabbit anti-Tbx3 (Invitrogen, CA, USA), goat anti-mouse carboxypeptidase A1 (R&D system, MN, USA), rat anti-E-cadherin (Sigma, St. Louis, MO), Fluorescein Dolichos Biflorus Agglutinin (Vector Laboratories, CA, USA), Alexa fluor donkey anti-rabbit (Molecular Probes Inc. Eugene, OR, USA), Texas redconjugated anti-guinea pig, Cy3 conjugated anti-rabbit, Cy3 conjugated anti-rat, and Cy3 conjugated anti-goat (all from Jackson Immuno Research, West Grove, PA). Western blot analysis of the Tbx2 and Tbx3 antibodies indicated that they each recognize as single band of the expected size (74 kDa and 79 kDa, respectively; Bioworld Technology, Inc. and Invitrogen product data sheets) (Smith et al., 2011). All sections were stained with 4', 6'-diamidine-2-phenylindole dihydrochloride (DAPI) for nuclear visualization. Images of whole mount samples were taken under bright field on a Nikon SMZ1500 microscope (Nikon, Japan). Sections for ISH and IF were examined with a Nikon MICROPHOT-FXA microscope (Nikon, Japan), and images were captured using NIS-Elements D3.10 software. Each ISH and IHC result shown represents a minimum of 4-6 samples.

2.2. Cell culture

Mouse pancreatic cell lines, β TC, α TC, and 266-6, were obtained from ATCC (Manassas, VA, USA). β TC was grown in DMEM (Invitrogen) and supplemented with 15% fetal bovine serum (FBS) (Hyclone, Utah, USA), α TC in RPM1 (Invitrogen) and supplemented with 10% FBS, and 266-6 in DMEM supplemented with 10% FBS. Cultures were grown at 37°C under humidified conditions in 5% CO₂/95% air. For IF, cells were grown in chamber slides (NunC, NY, USA), fixed in 4% paraformaldehyde for 10 minutes, and following a wash with PBS, fixed cells were treated with antibodies. DAPI was used to visualize the

nucleus in all IF analyses. IF staining was examined with a Nikon MICROPHOT-FXA microscope (Nikon, Japan), and images were captured using NIS-Elements D3.10 software.

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Abbreviations

CPA1	carboxypeptidase A1
DAPI	4', 6' -diamidine-2-phenylindole dihydrochloride
DBA	Dolichos Biflorus Agglutinin
E	embryonic
ESC	embryonic stem cells
IF	immunofluorescence
IHC	immunohistochemistry
iPSC	induced pluripotent stem cells
ISH	in situ hybridization
P	postnatal

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Highlights

- We examined spatial and temporal expression of *Tbx2* and *Tbx3* in mouse pancreas.
- *Tbx2* and *Tbx3* are expressed in the pancreatic mesenchyme throughout development.
- *Tbx2* and *Tbx3* are expressed in adult pancreatic islet and acini respectively.
- *Tbx2* and *Tbx3* are expressed in tumor-derived pancreatic cell lines.

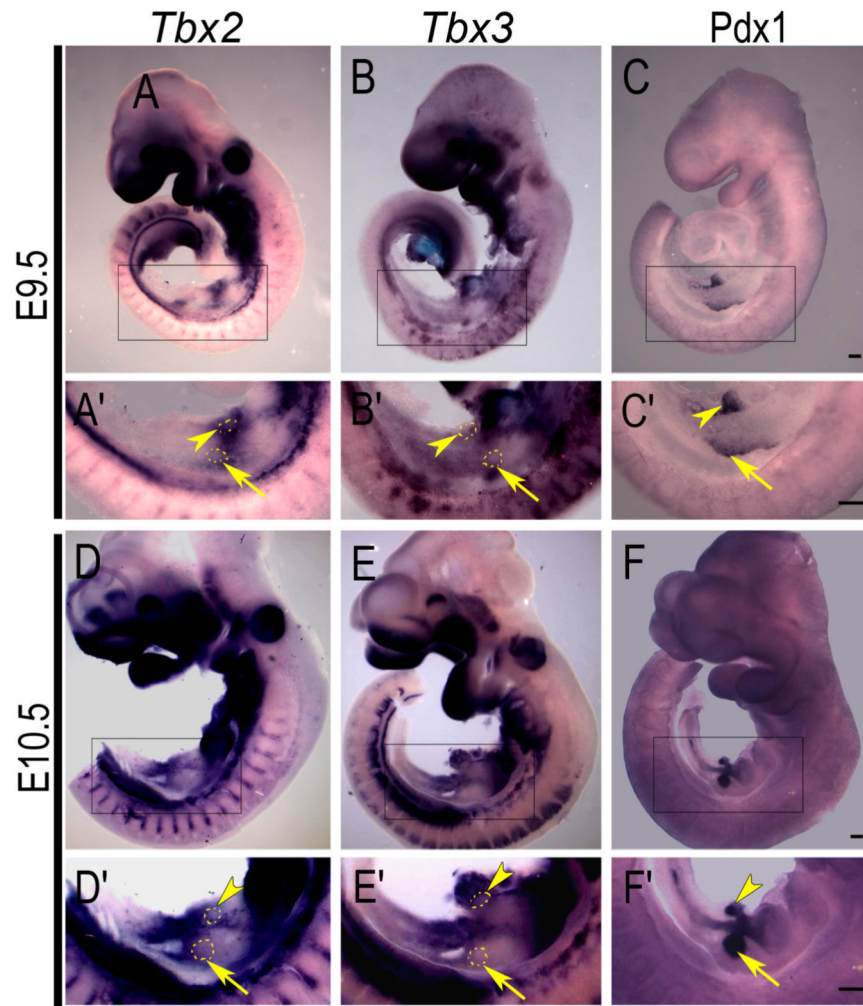


Fig. 1. *Tbx2* and *Tbx3* RNA expression at bud stages of pancreas development. Whole-mount ISH was performed for *Tbx2* (A, D) and *Tbx3* (B, E) on dissected whole embryos at E9.5 and E10.5. *Pdx1* expression (C, F) was detected by whole-mount IHC to highlight pancreatic epithelium. Boxed areas in A-F show the pancreatic region at higher magnification in A'-F'. The approximate position of the epithelial buds are marked by dotted lines (A', B', D' and E'). Arrows indicate the dorsal pancreas and arrowheads indicate ventral pancreas. Scale bars in C, C', F and F' = 200 μ m (A- F).

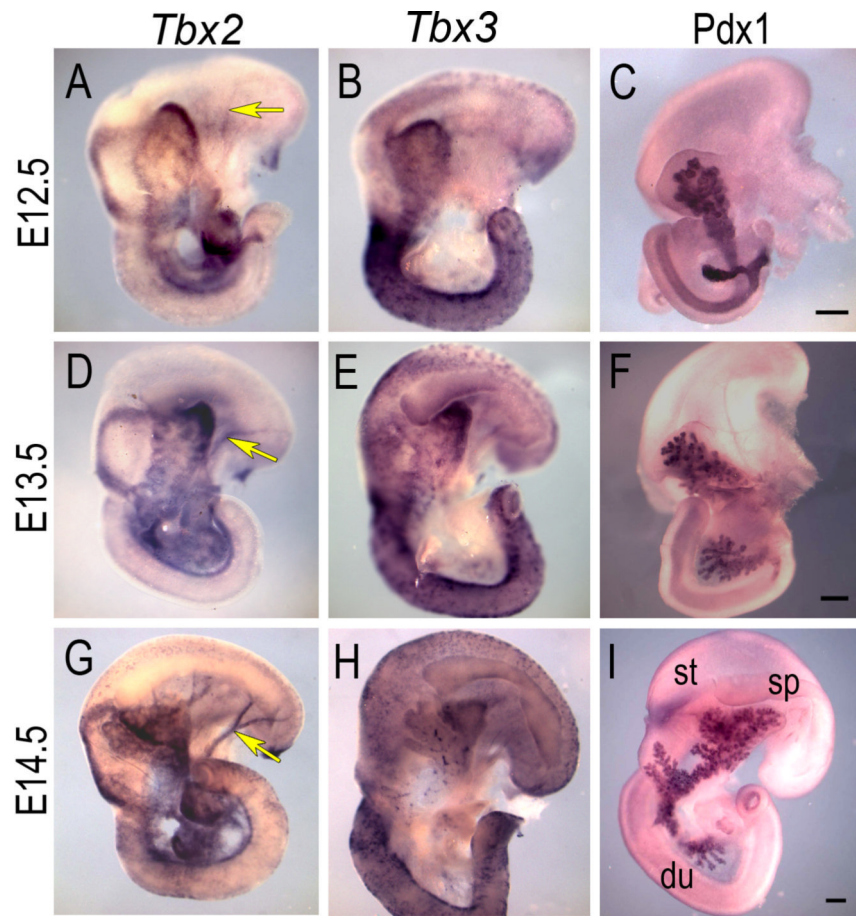


Fig. 2. *Tbx2* and *Tbx3* RNA expression in the developing pancreas, E12.5-E14.5. Whole-mount ISH of the pancreas (dorsal view) isolated with stomach (st), duodenum (du), and spleen (sp). Both *Tbx2* (**A, D, G**) and *Tbx3* (**B, E, H**) are expressed in the pancreatic mesenchyme. *Pdx1* epithelial expression by whole-mount IHC is shown for comparison (**C, F, I**). Arrows show *Tbx2* expression in the pancreatic vasculature (**A, D, G**). Scale bars in **C, F and I** = 200 μ m (**A-I**).

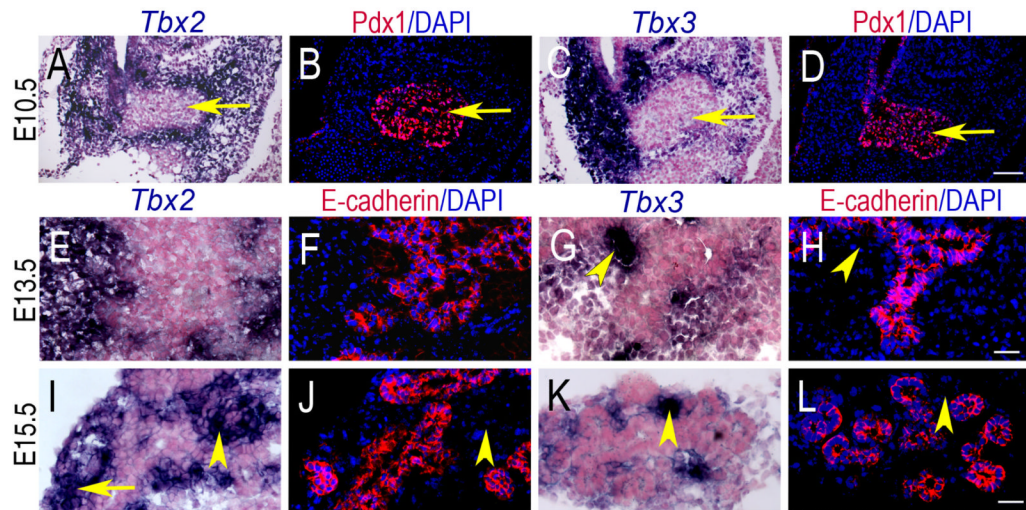


Fig. 3. *Tbx2* and *Tbx3* RNA expression in sections of the developing pancreas, E10.5- E15.5. *Tbx2* (A) and *Tbx3* (C) are expressed in the pancreatic mesenchyme at E10.5 but not in the epithelium (arrows), as indicated by Pdx1 expression (B, D) in adjacent sections. At E13.5, *Tbx2* (E) is expressed in the mesenchyme around the epithelial branching network, as indicated by E-cadherin (F) in adjacent sections. *Tbx3* (G) shows localized expression (arrowhead) in the mesenchyme at epithelial bifurcations, shown by E-cadherin in adjacent sections (H). At E15.5, *Tbx2* (I) is expressed mostly in the mesenchyme (arrowhead), but is also present in epithelial cell clusters (arrows) which are positive for E-cadherin (J). *Tbx3* (K) maintains localized expression in the mesenchyme (arrowhead) and is not present in the epithelium as indicated by E-cadherin in adjacent sections (L). A, E, I, C, G, K are section ISH on frozen sections counterstained with Nuclear Fast Red. B, F, J, D, H, L are section IHC counterstained with DAPI. Scale bar in D = 50µm (A-D); scale bar in H, L = 20µm (E-L).

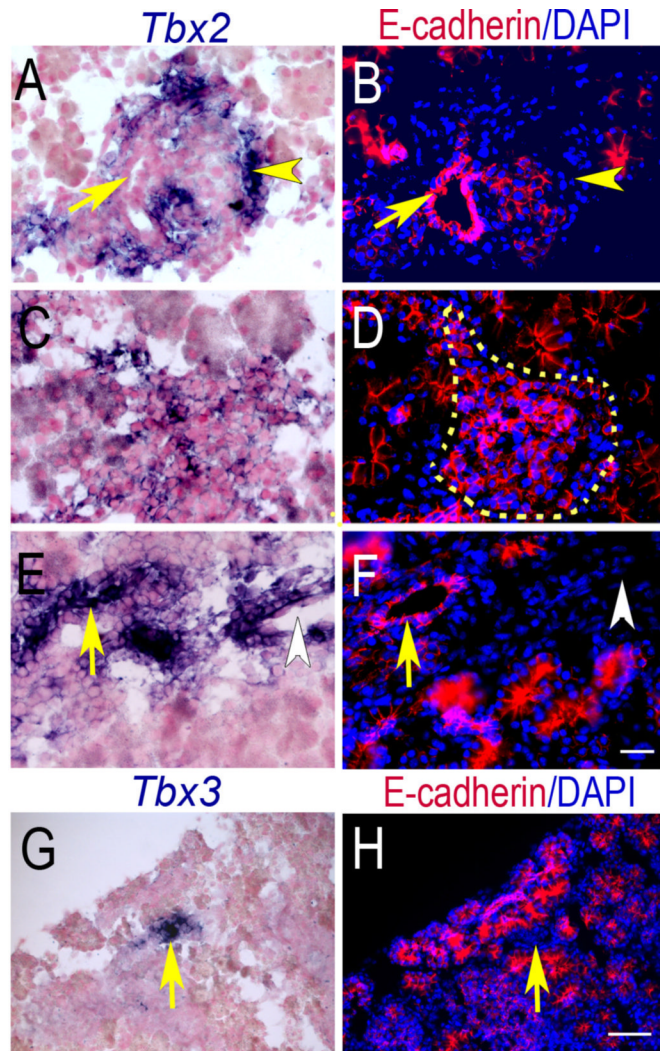


Fig. 4. *Tbx2* and *Tbx3* RNA expression at E17.5. *Tbx2* is expressed in mesenchyme (yellow arrowheads) surrounding E-cadherin-positive developing islets (**A**, **B**) and morphologically distinct islets (**C**, **D**, dotted line). *Tbx2* is expressed in some epithelial ducts (arrows in **E**, **F**) but not all (arrows in **A**, **B**) and is expressed E-cadherin-negative blood vessels (white arrowheads in **E**, **F**). *Tbx3* is expressed only in E-cadherin-negative mesenchyme (**G**, **H**). Section ISH was performed on frozen sections counterstained with Nuclear Fast Red (**A**, **C**, **E**, **G**). Adjacent sections stained with anti-E-cadherin (**B**, **D**, **F**, **H**). Scale bar in **F** = 20 μ m (**A**-**F**); scale bar in **H** = 50 μ m (**G**-**H**).

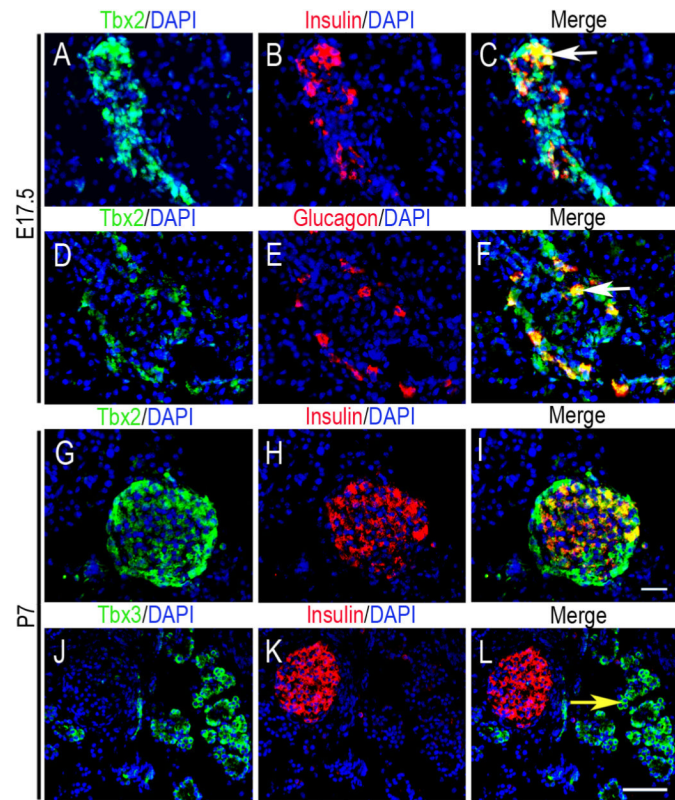


Fig. 5. Tbx2 and Tbx3 protein expression in epithelial derived tissues at late fetal and early postnatal stages. (A-F) Double IF staining on frozen sections at E17.5 shows Tbx2 is expressed in the islets in both insulin-positive cells and glucagon-positive cells (white arrows in C and F show double labeled cells in the merged images). Similar expression is seen at P7 (G-I). (J-L) Tbx3 expression is limited to exocrine tissue at P7 (yellow arrow in L). Scale bar in I = 20 μ m (A-I); scale bar in L = 50 μ m (J-L).

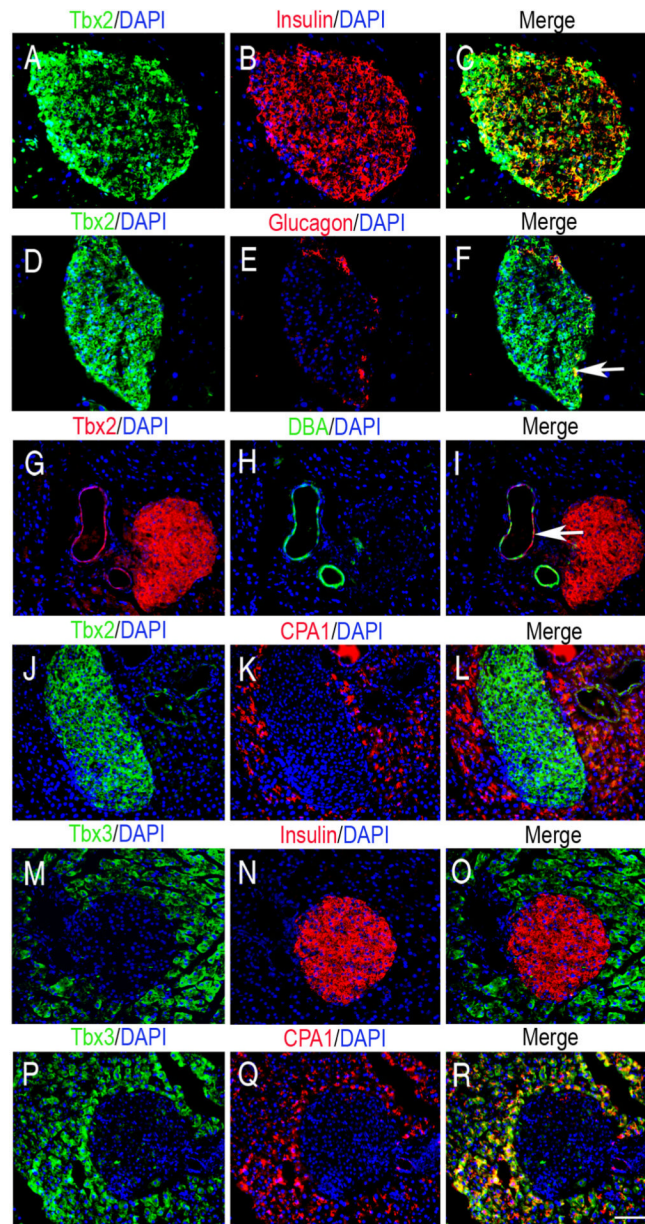


Fig. 6. Tbx2 and Tbx3 protein expression in the adult pancreas. (A-L) Double IF staining on frozen sections shows Tbx2 expression in insulin-positive cells and glucagon-positive cells of islets (arrow **F** shows colocalization in merged images), in some DBA-positive pancreatic ducts (arrow in **I**), but not in CPA1-positive exocrine tissue. (M-R) Double IF staining shows Tbx3 expression is not in the insulin-positive cells but is present in the CPA1-positive exocrine tissue. Scale bar = 50µm.

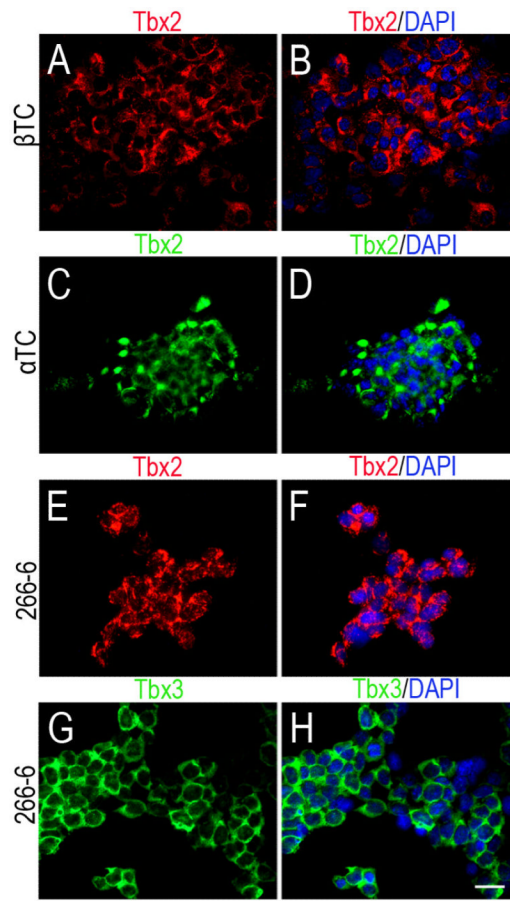


Fig. 7. Tbx2 and Tbx3 protein expression in pancreatic cell lines. IF on cell lines shows Tbx2 is expressed in the insulinoma-derived β cell line, β TC (**A, B**), in the glucagonoma-derived cell line, α TC (**C, D**) and in the acinar tumor-derived line, 266-6 (**E, F**). Tbx3 is expressed in 266-6 (**G, H**). Scale bar = 20 μ m.

Table 1
Tbx2 and Tbx3 expression in mouse pancreas throughout development and in tumor cell lines.

Gene	E9.5-E10.5	E12.5-E14.5	E15.5	E17.5	P7	Adult	β TC	α TC	266-6
Tbx2	Mesenchyme around epithelial buds	Mesenchyme, uniformly around epithelial branches, vessels	Mesenchyme, some epithelial cells	Mesenchyme, islet α and β cells, ducts, vessels	Islet α and β cells, ducts	Islet α and β cells, ducts	Yes	Yes	Yes
Tbx3	Mesenchyme around epithelial buds	Mesenchyme, punctate	Mesenchyme, punctate	Mesenchyme	Exocrine tissue	Exocrine tissue	No	ND ¹	Yes

¹ ND=not done