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Expression patterns of epiplakin1 in pancreas,pancreatic cancer and regenerating pancreas

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ABSTRACT

Epiplakin1 (Eppk1) is a plakin family gene with its function remains largely unknown, although the plakin genes are known to function in interconnecting cytoskeletal filaments and anchoring them at plasma membrane-associated adhesive junction. Here we analyzed the expression patterns of Eppk1 in the developing and adult pancreas in the mice. In the embryonic pancreas, Eppk1+/Pdx1+ and Eppk1+/Sox9+ pancreatic progenitor cells were observed in early pancreatic epithelium. Since Pdx1 expression overlapped with that of Sox9 at this stage, these multipotent progenitor cells are Eppk1+/Pdx1+/Sox9+ cells. Then Eppk1 expression become confined to Ngn3+ or Sox9+ endocrine progenitor cells, and p48+ exocrine progenitor cells, and then restricted to the duct cells and α cells at birth. In the adult pancreas, Eppk1 is expressed in centroacinar cells (CACs) and in duct cells. Eppk1 is observed in pancreatic intraepithelial neoplasia (PanIN), previously identified as pancreatic ductal adenocarcinoma (PDAC) precursor lesions. In addition, the expansion of Eppk1-positive cells occurs in a caerulein induced acute pancreatitis, an acinar cell regeneration model. Furthermore, in the partial pancreatectomy (Px) regeneration model using mice, Eppk1 is expressed in 'ducts in foci', a tubular structure transiently induced. These results suggest that Eppk1 serves as a useful marker for detecting pancreatic progenitor cells in developing and regenerating pancreas.

INTRODUCTION

Plakins are large multi-domain molecules, which link cytoskeletal elements together and to connect them to junctional complexes, such as desmosomes and hemidesmosomes (Sonnenberg & Liem 2007). Some of the genes belonging to this family, e.g. Desmoplakin, Plectin and BPAG1, are well characterized. Knockout mice of these genes showed phenotypes of skin blistering and embryonic or neonatal lethalities (Guo *et al.* 1995; Smith *et al.* 1996; Gallicano *et al.* 1998). In human, autoimmune diseases of these genes are reported, consistent with the skin blistering phenotype of the gene knockout mice. Epiplakin1 (Eppk1) is one of the plakin family genes and is involved in the formation of intermediate filament network by binding with keratin and vimentin (Jang *et al.* 2005). Eppk1 was originally cloned as an autoantigen of a human subepidermal blistering disease (Fujiwara *et al.* 2001; Spazierer *et al.* 2003). However, Eppk1 deficient mice develop normally without an evident epidermal phenotype (Goto *et al.* 2006; Spazierer *et al.* 2006), leaving its function *in vivo* elusive. Eppk1 was identified as one of the proteins bnding to EGF receptor by proteomics (Blagoev *et al.* 2003), thus suggesting its novel function as a component of EGF signaling.

Eppk1 was reported to be expressed in the pancreas by Northern blot analysis or immunohistochemistry (Fujiwara *et al.* 2001; Spazierer *et al.* 2003), and EGF signaling was reported to be involved in the development of pancreas (Miettinen *et al.* 2000) and carcinogenesis (Miyamoto *et al.* 2003). In an attempt to gain insights into the potential function of Eppk1, the expression of Eppk1 with respect to markers for pancreatic progenitors and differentiated cell types during embryonic development and in regenerating pancreas were carried out. Eppk1 was expressed in pancreatic progenitor cells in the early embryos as well as in centroacinar cells (CACs) in adult pancreas. The results suggested that Eppk1 is useful as a marker for progenitor cells in developing and regenerating pancreas.

RESULTS

Expression pattern analyses of Eppk1 in embryo and adult pancreas

Eppk1 expression was first examined with respect to Pdx1, a pancreatic progenitor marker. In this study, two kinds of anti-Eppk1 antibodies raised against distinct epitopes within Eppk1 (Fujiwara *et al.* 2001; Spazierer *et al.* 2003) were used to detect the expression of Eppk1. Both anti-Eppk1 antibodies gave almost the same results and did not recognize any proteins in *Eppk1* KO mice (data not shown). Since both anti-Eppk1 antibodies and anti-Pdx1 antibody were raised in rabbits, a *Pdx1*/GFP transgenic mouse line in which GFP was driven under the *Pdx1* promoter was used to examine the co-expression of Eppk1 and Pdx1. The expression of GFP completely overlapped with that of Pdx1 in the *Pdx1*/GFP transgenic mice throughout their lifetimes (supplementary Fig. 1). Eppk1 expression was found in *Pdx1*/GFP-positive (*Pdx1*/GFP+) pancreatic progenitor cells in the E10.5 pancreas epithelium (Fig. 1A-C). The expression of Eppk1 is also examined with respect to Sox9, a Sry/HMG box transcription factor shown to mark a population of Pdx1+ cells and be required for the maintenance of the progenitor cell pool (Seymour *et al.* 2007). Eppk1 was found to co-localize with Sox9 at this stage (Fig. 1D-F). Therefore, the multipotent progenitor cells are positive for Eppk1, Pdx1 and Sox9 at this stage.

In the E12.5 pancreas epithelium, most cells co-expressed Pdx1 and Eppk1. However, there are a population of Eppk1+ cells, which turned out to be Pdx1-negative (Pdx1-) (arrowheads in Fig. 1G-I) or Sox9- cells (solid lines in Fig. 1J-L). A co-localization of the Sox9 and Pdx1 persisted until this stage (arrows in supplementary Fig. 2), and Pdx1+/Sox9- cells (arrowheads in supplementary Fig. 2) begin to emerge. Taken together, the Eppk1+/Pdx1+/Sox9+ cells at E10.5 diverged into Eppk1+/Pdx1-/Sox9- and Eppk1+/Pdx1+/Sox9- cells within the Eppk1+/Pdx1+/Sox9+ pancreatic epithelium at E12.5 (Fig. 8).

In E15.5 pancreas, a divergence of expressions of Eppk1 and *Pdx1*/GFP was observed

(Fig.2A-C). Most of the *Pdx1*/GFP+ cells were Insulin expressing cells at this stage (supplementary Fig. 3), suggesting these cells are differentiating into β cell lineage. Taken together, the expression of *Pdx1*/GFP is excluded from Eppk1+/Pdx1+/Sox9+ pancreatic progenitor cells. At this stage, Eppk1+/Sox9+ cells retained confined to the 'central duct-like structures' (solid lines in Fig. 2) (Seymour *et al.* 2007). Ngn3 was also co-expressed with a population of Eppk1-expressing cells, which reside along the 'central duct-like structure' (Fig. 3G-I), indicating the possible endocrine progenitor characteristics of the Sox9+/Eppk1+ cells. On the other hand, in the forming acini (dotted lines in Fig. 2D-F), Sox9 expression decreased, and the expression of p48, which was reported to be expressed in exocrine progenitor cells and to be essential for exocrine differentiation (Krapp *et al.* 1996; Krapp *et al.* 1998), began to emerge at this stage, which expression found to be co-localized with that of Eppk1 (Fig. J-L). Furthermore, Eppk1 expression was observed to co-express with Hes1 in the centroacinar position of the forming acini (Fig. 2M-O) (Esni *et al.* 2004; Jensen 2004). These results thus indicate that Eppk1 is expressed in both endocrine and exocrine progenitor cells at E15.5.

In neonatal pancreas at P0, the expression of Eppk1 no longer overlapped with that of *Pdx1*/GFP (Fig. 3A-C). Eppk1 expression was mainly observed in ducts, with some positive cells in the islets (Fig. 3A-C), which turned out to be Glucagon-expressing α cells (Fig. 3D-F). The expressions of Eppk1 in islets overlapped with that of Cytokeratins (Fig. 3G-I). Cytokeratins were expressed in Glucagon+ cells (supplementary Fig. 4), which agreed with that reported previously (Bouwens *et al.* 1994). On the other hand, co-expression of Sox9 and Eppk1 was not retained (Fig. 3J-L).

In adult pancreas, Eppk1expressions are observed in relatively small cells surrounded by Amylase+ acinar cells (Fig. 4E, H), but no longer in Glucagon+ α cells (Fig. 4A-C). The Eppk1 expression almost completely overlapped with Cytokeratins (Fig. 3E-H), indicating that they were

duct cells. Detailed examination revealed that there were two kinds of Eppk1+ cells, one was surrounded by acinar cells (arrowheads in Fig. 4I-L) and the other was outside them (arrows in Fig. 4I-L). The Eppk1+ cells located in the acinar stem position correspond to a terminal ductal position that intercalates with the distal duct cells, and they are called CACs (Slack 1995). The Eppk1+ cells outside acinar cells were duct cells, which could be distinguished by the relatively strong reactivity against DBA, a kind of lectin that binds strongly to duct cells of pancreas, but relatively weakly to CACs (Fig. 4J). In CACs, Eppk1 expression overlapped with Sox9 and Hes1, an effector of Notch signaling (arrowheads in Fig. 4M-R) (Miyamoto *et al.* 2003). In duct cells, only Eppk1, but not Sox9 or Hes1, was detected (arrow in Fig. 4M-O).

Expression patterns of Eppk1 in pancreatic cancer

The next experiment was designed to determine whether Eppk1 was expressed in human pancreatic intraepithelial neoplasia (PanIN) cells, one of the pancreatic ductal adenocarcinoma (PDAC) precursor lesions, because CACs are regarded to be the origin of PanIN (Hezel *et al.* 2006). PanINs are graded from stages 1 to 3, increasing grades reflect increasing atypia, and eventually transform into frank PDAC. Normal duct is composed of a single-layer with low cuboidal cells. PanIN1 is considered early cancer precursor lesion, characterized by its columnar and mucinous epithelium (Fig. 5A), which is detected by DBA (Fig. 5C, F), with the nuclei regularly localized along the basement membrane (Fig. 5E, F). In this stage, Eppk1 was expressed in the PanIN cells, which were characterized by the expression of Cytokeratins (Fig. 5G-J). Late PanIN lesions, PanIN2 and PanIN3, represent a distinct step toward invasive carcinoma. The mucinous epithelium becomes thinner, and the epithelium appeared curved with evident invasion beyond the basement membrane as luminal budding (Fig. 5K, P). In late PanINs, the DBA-positive epithelium layers become thinner, whereas the DAPI-positive cell layers become

multi-layers (Fig. 3L-P). However, the expressions of Eppk1 and Cytokeratins were down regulated in this stage (Fig. 5Q-T), indicating that PanIN cells lost their ductal characters.

Expression patterns of Eppk1 in regenerating pancreas

CACs are known to be involved not only in the origin of PanIN, but also in the regeneration of acinar cells in acute pancreatitis (Gasslander *et al.* 1992). The next question was whether Eppk1+ cells proliferate in regenerating pancreas in acute pancreatitis. Caerulein mediated pancreatitis has been shown to trigger acinar cell regeneration. We induced weak pancreatitis by caerulein injection (Jensen *et al.* 2005). One day after caerulein injection, acinar cell degeneration began to occur, and the acinar cells thereafter regenerated and almost completely recovered in one week after the initial injection (Fig. 6A-L), and the number of Eppk1+ cells increased by a factor of 1.97 (Supplementary Fig. 5 show low magnification pictures; compare supplementary Fig. 5C, D with supplementary Fig. 5A, B). This resulted in a change in the cellular alignment from linear before treatment into a cluster of cells (compare Fig. 6F with Fig. 6B). At day 7 after the caerulein injection, they almost returned to their original linear alignments, though many cells remained clustered (compare Fig.6J with Fig. 6B). We next examined the Eppk1+ cells during this regenerating period (Fig. 6M-X). Cytokeratins were still expressed in Eppk1+ cells at day 4 after the caerulein injection (Fig. 6O, P), though most of the Eppk1+ cells lost the reactivity with DBA (Fig. 6Q, R). The co-expressions of Eppk1 with PCNA (Fig. 6S, T) and Sox9 (Fig. 6U, V) indicated that the Eppk1+ cells proliferated and had multipotency (Seymour *et al.* 2007). p48, a marker for acinar progenitor cells, was expressed in Eppk1+ cells with acinar morphology (arrowheads in Fig. 6W, X), thus suggesting a dedifferentiation and redifferentiation might have occurred. Since p48 was also expressed in acinar cells themselves (arrows in Fig. 6W, X), acinar cells might dedifferentiate and acquire an

acinar progenitor character, which then redifferentiate into acinar cells. These cells renewed by themselves, which agreed with previous reports that preexisting acinar cells contribute to acinar cell regeneration (Jensen *et al.* 2005; Desai *et al.* 2007). Another possible interpretation is that ductal-to-acinar transdifferentiation occurred from Eppk1+ cells.

A partial pancreatectomy (Px) was also performed. It has been reported that a partial Px induce not only the proliferation of β cells (Dor *et al.* 2004), but also the regeneration, which recapitulated development of pancreas in the focal region. It was previously reported that small ductules were observed in the focal region (Bonner-Weir *et al.* 1993). These cells are called 'ducts in foci', which are proposed to dedifferentiated/transdifferentiated from duct cells and differentiated into new lobes of pancreas, comprising both endocrine and exocrine tissues (Bonner-Weir *et al.* 1993). HE staining of the pancreas on the next day after a partial Px revealed that eosin-stained acinar cells degeneration occurred and that leukocytes infiltrated into the focal region (Fig. 7A, B). On the 4th days after a Px, acinar cells degenerated and 'ducts in foci' were observed (Fig. 7C, D, arrowheads in Fig. 7D). As previously reported (Bouwens *et al.* 1995), Cytokeratins were expressed in the 'ducts in foci' (Fig. 7G), and Eppk1 was also observed in these cell (Fig. 7H). DBA reactivities were lost in these Eppk1+ cells (Fig. 7I, J) similar with the case in the caerulein induced pancreatitis (Fig. 6Q, R). In some cells, Eppk1 expression overlapped with that of Amylase (arrowheads in Fig. 7E, F). There are also Eppk1+/Amylase- cells (arrows in Fig. 7E, F), thereby suggesting that some of the 'ducts in foci' were dedifferentiated/transdifferentiated by acinoductal metaplasia (Lardon & Bouwens 2005). The number of Eppk1+ cells also increased by a factor of 4.96 (Supplementary Fig. 5 show low magnification pictures; compare supplementary Fig. 5E, F with supplementary Fig. 5A, B), as in the caerulein treated pancreas (supplementary Fig. C, D). The ducts were Sox9+ (Fig. 7K, L) and PCNA+ (Fig. 7M, N), as seen in the ducts in the caerulein treated pancreas (Fig. 6S-V). In

Eppk1 KO mouse, Cytokeratins+ ‘ducts in foci’ were observed (Fig. 7P) and PCNA was also observed (Fig. 7O), indicating that Eppk1 is not essential to form ‘ducts in foci’ and that Eppk1 was not essential for the proliferating activity, either.

Eppk1 expression was observed in the islets in pancreata had a partial Px (Fig. 7Q, R). Further investigation revealed that the Eppk1-positive cells were Glucagon-expressingα cells (Fig. 7S, T), which was similar to that seen in P0 islets (Fig. 3D-F).

DISCUSSION

Eppk1 has been reported to be expressed in various organs by Northern blot analyses, including the pancreas (Fujiwara *et al.* 2001; Spazierer *et al.* 2003), but detailed expression patterns have not been examined. Studies of Eppk1 might reveal the molecular mechanism of signaling transduction underlying early pancreatic differentiation, and might shed lights on our future development of regenerative medicine for the cure of diabetes mellitus (Kume 2005a, 2005b; Shiraki *et al.* 2005; Shiraki *et al.* 2008). In the present study, we focused on the expression patterns of Eppk1 in pancreas. We summarized the results in Figure 8.

The Pdx1-positive pancreatic progenitor cells have been shown to give rise to the cells of the endocrine, exocrine and duct lineages, that is, all lineages of cells existing in the adult pancreas (Jonsson *et al.* 1994; Offield *et al.* 1996; Gu *et al.* 2002). At E10.5, the pancreatic progenitor cells composed of cells with an almost complete overlap of Eppk1, Sox9 and Pdx1 expression (Fig. 1A-F; Fig. 8, E10.5 Pdx1+/Eppk1+/Sox9+ cells). Sox9 has been recently reported to maintain the multipotency of the pancreatic progenitor cell activity (Seymour *et al.* 2007).

Then, in E12.5 pancreas Pdx1-/Sox9- cells appeared within the Eppk1+ cells (Fig. 1G-L). Gu *et al*. showed that pancreatic duct progenitors derived from multipotent pancreatic progenitor cells by E12.5, and differentiate into Pdx1- duct lineage cells (Gu *et al.* 2002). Thus, Eppk1+/Pdx1- cells (arrowheads in Fig. 1G-I) were possibly the progenitor cells of the ductal lineage (Fig. 8, E12.5 Pdx1-/Eppk1+/Sox9- cells). Since the expression of Pdx1 and Sox9 almost overlapped (supplementary Fig. 2), Eppk1+/Sox9- cells (solid lines in Fig. 1J-L) and Eppk1+/Pdx1- cells (arrowheads in Fig. 1G-I) were possibly the same population. Therefore, the Eppk1+/Sox9- cells were presumaly to be Eppk1+/Pdx1-/Sox9- and also the progenitor of the ductal lineage. At this stage, Pdx1+/Sox9- cells began to emerge, which located at the edge of

the pancreatic epithelium at this stage (arrowheads in supplementary Fig. 2). These Pdx1+/Sox9- cells might be the progenitors of the exocrine cells (Fig. 8, E12.5 Pdx1+/Eppk1+/Sox9- cells). On the other hand, Sox9 is required for the expression of Ngn3 (Lynn *et al.* 2007), and for the endocrine cell differentiation to be switched on (Gradwohl *et al.* 2000; Jensen *et al.* 2000; Schwitzgebel *et al.* 2000; Gu *et al.* 2002). Pdx1+/Eppk1+/Sox9+ population correspond to multipotent progenitor cells (Fig. 8, E12.5 Pdx1+/Eppk1+/Sox9+ cells).

In the pancreas of E15.5 embryo, the expression of Eppk1 was not detected in Pdx1+ cells (Fig. 2A-C), most of which expressed insulin (supplementary Fig. 3), suggesting that these cells are differentiating toward a β cell fate. Eppk1+/Sox9+ cells form a 'central duct-like structure', in which the expression of Ngn3 was observed (Fig. 2D-I), indicating that Eppk1 marks the endocrine progenitor at this stage (Fig. 8, E15.5 Pdx1-/Eppk1+/Sox9+ cells; red circle). On the other hand, the exocrine cell progenitors form acini at this stage (Jensen 2004), in which strong Eppk1 and weak Pdx1expression was observed (Fig. 2J-O; Fig. 8, E15.5 Pdx1±/Eppk1+/Sox9- cells), suggesting that Eppk1 serves also as an exocrine progenitor cell marker. The expressions of Hes1 in the center position and p48 in the outer side were observed in the forming acini (Fig. 2J-O). p48 is a subunit of Ptf1α, which is essential for differentiation of pancreatic exocrine cells (Krapp *et al.* 1996; Krapp *et al.* 1998). Hes1 is a Notch signaling related transcriptional factor, which sustains immaturity of the cells (Kageyama *et al.* 2005). Esni and colleagues reported that p48+/Hes1+ double positive common progenitor cells diverged into p48+/Hes- exocrine precursor cells, and p48-/Hes1+ Notch-regulated progenitor cells (Esni *et al.* 2004).

In the adult pancreas, Eppk1 was expressed in duct cells and in centroacinar cells (CACs), which were Sox9+/Hes1+ cells (Fig. 4). Thus, it was suggested that p48+/Hes1+ common progenitor cells differentiated into the pancreatic exocrine cells (Fig. 8, Pdx1±/Eppk1-/Sox9-) and CACs (Fig. 8, Pdx1-/Eppk1+/Sox9+, red circle), which was possibly

Notch-regulated progenitor cells. Among the hormone expressing cells in postnatal pancreas, Eppk1 was detected only in α cells in perinatal pancreas (Fig. 8, P0 Pdx1-/Eppk1+/Sox9-), which were also Cytokeratin+ (Fig. 3). The expression of Eppk1 and Cytokeratins disappeared in the adult (Fig. 4A-C; Fig. 8, α cells Pdx1-/Eppk1-/Sox9-).

It is hypothesized that some cancers represent an aberrant recapitulation of normal development (Hezel *et al.* 2006). It is further deducted that some cancers can be originated from adult tissue stem cells. The activations of PI3K (Stanger *et al.* 2005), Notch and TGFα signalings (Miyamoto *et al.* 2003) in CACs gave rise to PanIN, which has been shown to be a precursor lesion of PDAC (Hezel *et al.* 2006). Eppk1 was also expressed in the early stage of PanIN cells (Fig. 5A-J). Hes1, a transcription factor downstream of Notch signaling, was expressed in CACs (Fig. 4P-R) (Miyamoto *et al.* 2003), and Eppk1 was reported to bind to EGF receptor (Blagoev *et al.* 2003). However, the expression of Eppk1 in PanIN cells gradually decreased, similarly with that of Cytokeratins (Fig. 5Q-T). The relation between Eppk1 and EGF were not clear at the moment.

Next we observed the expression patterns of Eppk1 in regenerating pancreas. Based on the findings of genetic tracing experiments, it is reported that β cells (Dor *et al.* 2004) and acinar cells (Desai *et al.* 2007) normally renew themselves slowly. However, when injured, immediate repair is taken place in the pancreas of rodents. Acinar cell regeneration in an acute pancreatitis model (Gasslander *et al.* 1992; Jensen *et al.* 2005) by injection of caerulein and islet regeneration in a partial Px model (Bonner-Weir *et al.* 1993) are well known. Here, in both caerulein treatment and a partial Px, Eppk1+/Cytokeratins+ cells appeared among acinar cells, which were possibly formed by acinoductal metaplasia (a brown curved arrow in Fig. 8) (Lardon & Bouwens 2005) or dedifferentiated/transdifferentiated from duct cells, in which PCNA and Sox9 were expressed later (Fig. 6 and 7; a red curved arrow in Fig. 8). Recently, it was shown that new

endocrine cells were differentiated from duct cells in a partial duct ligated pancreas (Xu *et al.* 2008). In the report, Ngn3+ cells appeared from duct cells and differentiated into α and β cells. Since the gene expressions and the morphologies of the duct cells of injured pancreata and 'central duct-like structure' in E15.5 pancreas were similar (compare Fig. 2, 6 and 7; red circles in Fig. 8), the characteristics of the cells may also be similar. Ngn3 was reported to be expressed in Sox9+ cells (Lynn *et al.* 2007), which correspond to the Pdx1-/Eppk1+/Sox9+ cells in the 'central duct-like structure' (Fig. 8 E15.5 red circle). According to our above hypothesis, the Ngn3+ cells derived from the duct cells of the partial duct ligated pancreas (Xu *et al.* 2008) are similar with Pdx1-/Eppk1+/Sox9+ CACs, which might give rise to endocrine cells upon duct certain injury (a green arrow in Fig. 8) (Lynn *et al.* 2007). Furthermore, we found p48 to be expressed in the Eppk1+ cells (arrowheads in Fig. 6W, X), indicating that Eppk1 was a marker of the progenitor cells of acinar cell regeneration. p48 was also detected in the acinar cells themselves, indicating that during regeneration of acinar cells, either dedifferentiation from acinar cells (a brown arrow in Fig. 8), differentiation of progenitor cells (a purple arrow in Fig. 8) or simply proliferation of existing acinar cells (a lime green line in Fig. 8) is supposed to occur. Cytokeratins+ 'duct in foci' in *Eppk1* KO mouse were also PCNA+ (Fig. 7O, P), indicating that Eppk1 was not essential for maintaining stem cell activity. In islets, Eppk1 was expressed in α cells of perinatal pancreas (Fig. 3D-F), which gradually disappeared toward the adult (Fig. 4A-C). In the perinatal pancreas, islets were located near by the duct cells, which were also Eppk1+ (Fig. 4I-L; blue circles in Fig. 8). α cells and duct cells may have similar characteristics and some interactions at this stage. Islets at this stage often were 'dump-bell' shaped (supplementary Fig. 6A), though they were usually spherical in the adult (supplementary Fig. 6B), as a result of fission (Seymour et al., 2004). The expression of Eppk1 in α cells was observed again in the islets of pancreas that had a partial Px (Fig. 7S, T; a blue arrow in Fig. 8), which also showed 'dump-bell' shape

(supplementary Fig. 6C). Eppk1 may be involved in the motility of islets.

In summary, we examined the expression patterns of Eppk1 in pancreas and showed that Eppk1serves as an excellent marker to analyze the cell lineage of pancreas.

EXPERIMENTAL PROCEDURES

Animals and pancreas regeneration models

A transgenic mouse line *Pdx1*/GFP (Gu *et al.* 2004) was a gift from Dr. Melton of Harvard University. Wild type ICR mice were obtained from SLC (Kanagawa, Japan). The date of conception was established by the presence of a vaginal plug and recorded as E0.5. 8-week-old mice were used as adults. Caerulein treatment was carried out as previously described (Jensen *et al.* 2005). A pancreatectomy (Px) was carried out as previously described (Bonner-Weir *et al.* 1993).

Antibodies

Two kinds of rabbit anti-Eppk1antibodies were used: one is described previously (Fujiwara *et al.* 2001) and another is a gift from Dr. G. Wiche of University of Vienna (Spazierer *et al.* 2003). Guinea pig anti-Hes1 antibody was a gift from Dr. R. Kageyama (Kyoto University) (Hatakeyama *et al.* 2006). Rabbit anti-p48 antibody was kindly provided by Dr. H. Edlund (Li & Edlund 2001). Mouse anti-Ngn3 (Cat #F25A1B3) (Zahn *et al.* 2004) antibody was obtained from Developmental Studies Hybridoma Bank. The other primary antibodies used were mouse anti-Insulin (Cat# I-2018, Sigma, St. Louis, MO), mouse anti-Glucagon (Cat #G-2654, Sigma), mouse anti-GFP (Cat #11814460001, Roche, Indianapolis, IN), mouse ant-pan Cytokeratin (Cat #ab11213, Abcam, Cambridge, U. K.), mouse anti-PCNA (Cat #NA03, Oncogene Research Products, Boston, MA), rabbit anti-Pdx1 (Cat# KAL-KR059, Trans Genic Inc., Kumamoto, Japan), rabbit anti-human Glucagon (Cat #A0565, Dako Cytomation, Glostrup, Denmark), goat anti-Amylase (Cat #sc-12821, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and goat anti-Sox9 (Cat #sc-17340, Santa Cruz Biotechnology, Inc.) antibodies. Fluorescein isothiocyanate conjugated DBA

(*Dolichos biflorus* agglutinin, Vector Laboratories, Inc., Burlingame, CA) was also used.

Immunological analyses

Immunohistochemical analyses of the frozen-sections and paraffin sections were performed as described previously (Yoshida *et al.* 2003). First, sections were boiled in Target Retrieval Solution (Dako Cytomation,) for 10 minutes at 105˚C for antigen retrieval. Bound first antibodies were visualized with Alexa 488 or Alexa 568 labeled appropriate secondary antibodies (Invitrogen, Carlsbad, CA). Double immunostaining using rabbit anti-Eppk1 and rabbit anti-p48 antibodies was performed as follows: First anti-p48 antibody was bound to a frozen section and visualized by Alexa 488-conjugated anti-rabbit IgG antibody. Then, anti-Eppk1 antibody, which was biotinylated by Biotin Labeling Kit-NH2 (Dojindo Raboratories, Kumamoto, Japan) according to the procedure recommended by the manufacturer, was bound after blocking using non immunized rabbit serum. The expression pattern of Eppk1 was visualized by streptavidin-conjugated Alexa 588. The nuclei were counterstained using DAPI (Roche). Optical sections were viewed using a scanning laser confocal imaging system (TCSSP2 AOBS, Leica Microsystems, Wetzler, Germany). Non confocal images were acquired using an Olympus IX-71 microscope (Olympus Optical, Tokyo, Japan) equipped with a Nikon digital sight DS-5M (Tokyo, Japan). Images were processed using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). The numbers of stained cells in supplementary Figure 5 were quantified by analyzing the areas of Eppk1-positive staining images using Lumina Vision program (Mitani Corporation, Fukui, Japan).

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FIGURE LEGENDS

Figure 1. The expression patterns of Eppk1 at early stages of pancreatic development.

Immunohistochemical analyses of Eppk1 in E10.5 (A-F) and E12.5 pancreatic buds (G-L). (A, D, G, J) The expressions of Eppk1 (red), (B, H) the images of GFP in *Pdx1*/GFP mice pancreata, (E, K) the expression patterns of Sox9 are shown. The arrowheads in G-I represent Eppk1+ *Pdx1*/GFP- cells. The areas surrounded by solid and dotted lines in J-L are Eppk1+/ Sox9- cells and Eppk1+/ Sox9+ cells, respectively. Asterisks in J-L indicate non-specific signals. C, F, I and L are merged images. Scale bar, 100 μm (A-F), 170 μm (G-L).

Figure 2. The expression patterns of Eppk1 at secondary transition of pancreatic development.

Immunohistochemical analyses of Eppk1 in E15.5 pancreas (A, D, G, J and M). The expression patterns of GFP in *Pdx1*/GFP mouse (B), Sox9 (E), Ngn3 (H), p48 (K) and Hes1 (N) are shown. The areas surrounded by white solid lines in A-C are *Pdx1*/GFP+ Eppk1- cells. The areas surrounded by solid and dotted lines in D-O are ‘central duct-like structure’ and forming acini, respectively. The arrowheads in M-O represent centroacinar region. C, F, I, L, and O are merged images. Scale bar, 100 μm (A-L), 30 μm (M-O).

Figure 3. In P0 perinatal pancreas, Eppk1 is expressed in α cells in islets.

Immunohistochemical analyses of Eppk1 in P0 pancreas (A, D, G and J). The expression patterns of GFP in *Pdx1*/GFP mouse (B), Glucagon (E), Cytokeratins (H, CKs), Sox9 (K) are shown. C, F, I and L are merged images. Scale bar, 100 μm.

Figure 4. Eppk1 is expressed in duct cells and centroacinar cells of adult pancreas.

Immunohistochemical analyses of Eppk1 in adult pancreas (A, E, I, M and P) are shown. The expression patterns of GFP in *Pdx1*/GFP mouse (B), Cytokeratins (F, CKs), Sox9 (N), Hes1 (Q) and DBA+ cells (J) are shown. Arrowheads and arrows in I-L represent the cells lie inside or outside of acinar cells, respectively. Arrowheads and arrows in M-O represent Sox9+ or Sox9- cells, respectively. The arrowheads in P-R are Hes1+ /Eppk1+ cells. C, G, K, O and R are merged images. Expressions of Amylase are also shown (D, H and L). Scale bar, 100 μm (A-L), 50 mm (M-R).

Figure 5. Eppk1 is expressed in the early stage but not in the late stages of PanINs.

Histological analyses of the early (A-J) and late stages (K-T) of PanINs. (A, K) Bright-field images of HE-stained PanINs. Boxes in A, K depict regions shown in B-E, or L-T, respectively. The expressions of Eppk1 are examined (B, G, L and Q). DBA+ (C, M) and Cytokeratins+ (H, R; CKs) cells are also shown. D, I, N and S are merged images and blue dots show nuclei (E, J, O and T). Schematic drawings of the intracellular expressions of DBA (green) and Eppk1 (red) are shown in F and P. Scale bar, 400 μm (A, K), 125 μm (B-E, G-J, L-O, Q-T).

Figure 6. Eppk1 is expressed in the proliferating cells during acinar cell regeneration in caerulein induced acute pancreatitis.

(A-L) Histochemical analyses of the caerulein treated pancreata. The images of HE stainings of sham treated (A) and day one (E) or day 7 (I) after caerulein treatment are shown. Immunohistochemical analyses using anti-Eppk1 (B, F, J) and anti-Amylase (C, G, K) antibodies were also done. D, H and L are merged images. (M-X) Double immunostainings of Eppk1 with other markers in the pancreas on the 4th day after caerulein treatment are shown. Expressions of

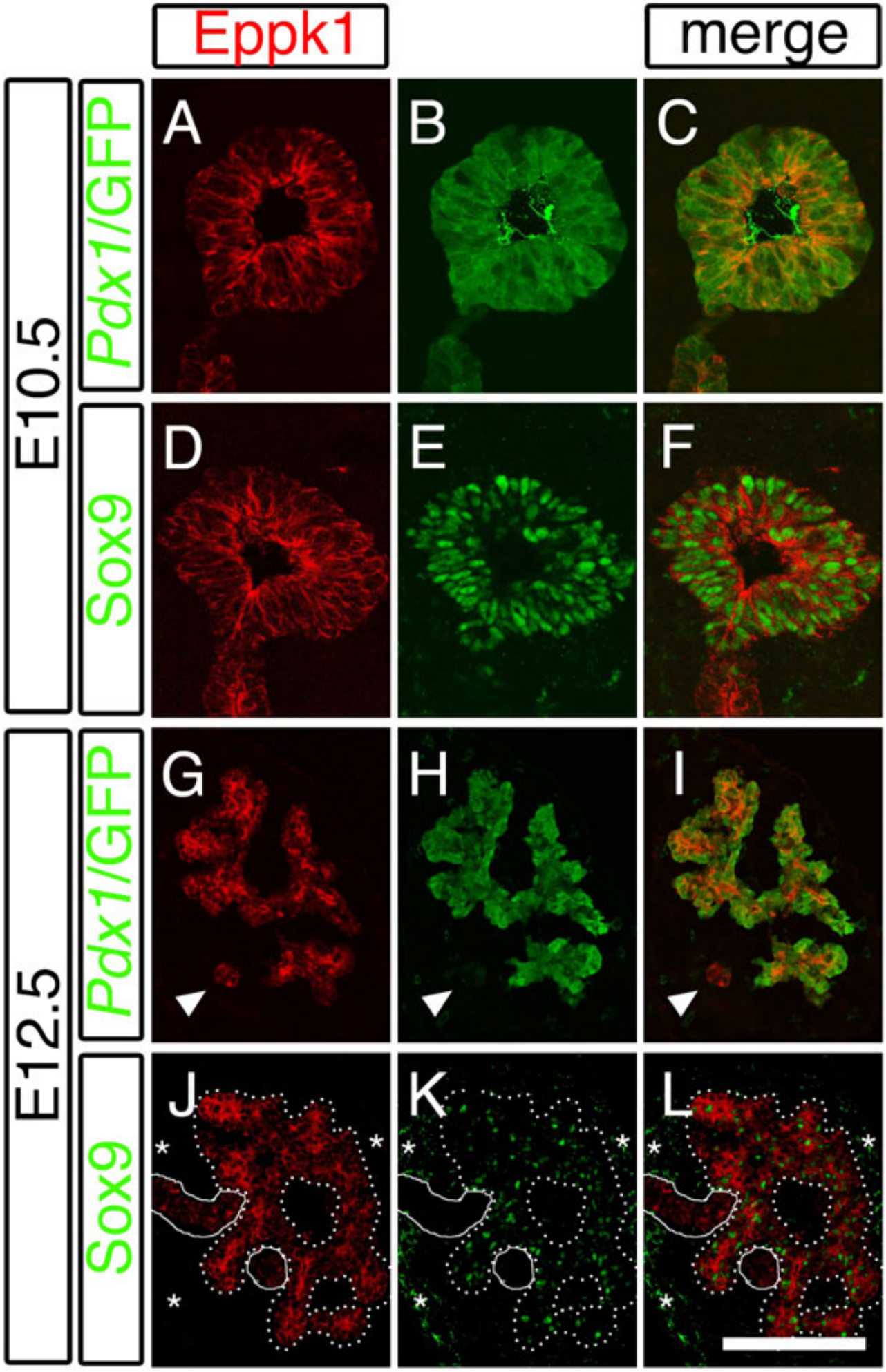
Amylase (M), Cytokeratins (O; CKs), DBA (Q), PCNA (S), Sox9 (U) and p48 (W) in Eppk1+ cells (N, P., R, T, V and X) were investigated. Arrowheads and arrows depict acinar progenitor cells or acinar cells. Scale bar, 100 μm (A-L), 50 μm (M-X).

Figure 7. Eppk1 is expressed in the ducts in foci and α cells in the pancreas which has received a partial pancreatectomy.

(A-D) The images of HE stained sections of the pancreas one day (A, B) and 4 days (C, D) after a partial Px. B and D are magnified images in the focal region depicted by the squares in A and C, respectively. Arrowheads in D depict 'ducts in foci'. (E-T) Double immunostainings of Eppk1 and various markers in the focal region (E-P) and islet (Q-T) of the pancreas at day 4 after a partial Px was taken place. Arrows or arrowheads show Eppk1+/Amylase- or Eppk1+/Amylase + cells, respectively.. Expressions of Amylase (E), Cytokeratins (G; CKs), DBA (I), Sox9 (K), PCNA (M, O), GFP of *Pdx1*/GFP mouse (Q) and Glucagon (S) in Eppk1-positive cells (F, H, J, L, N, R and T) were investigated. Eppk1 KO mouse was used in O and P with the counter staining done by using anti-panCK antibody (CKs). Scale bar, 1.2 mm (A, C), 400 μm (B, D), 100 μm (E-T).

Figure 8. A schematic representation of Eppk1 expression.

Expressions of Pdx1, Eppk and Sox9 are indicated in each cell lineage. Cells indicated by red and blue are those showing the same expression patterns of the three genes, respectively. Colored curved arrows show changes in gene expressions. See text in Discussion.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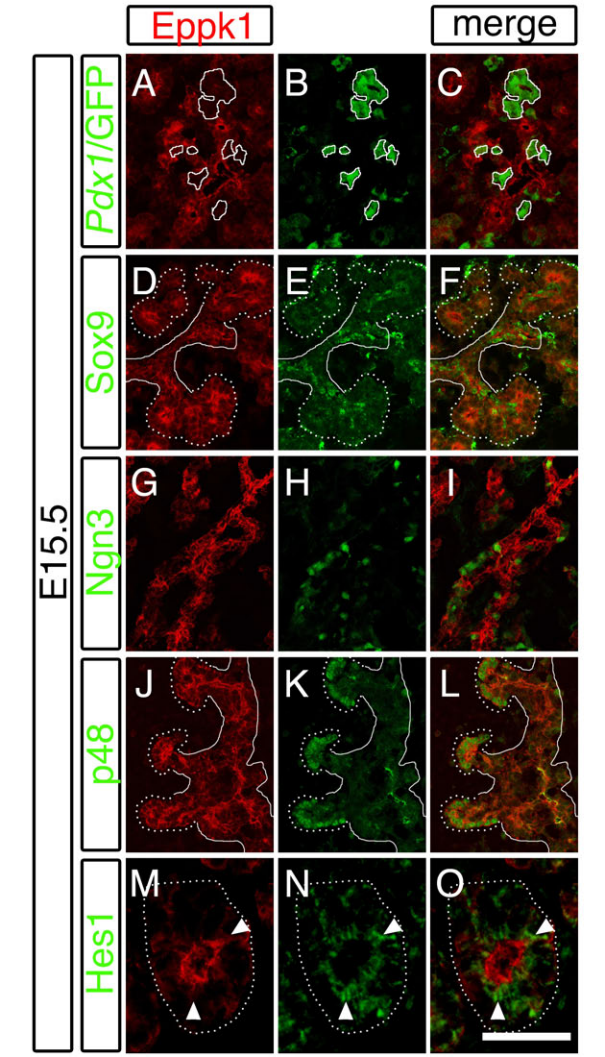
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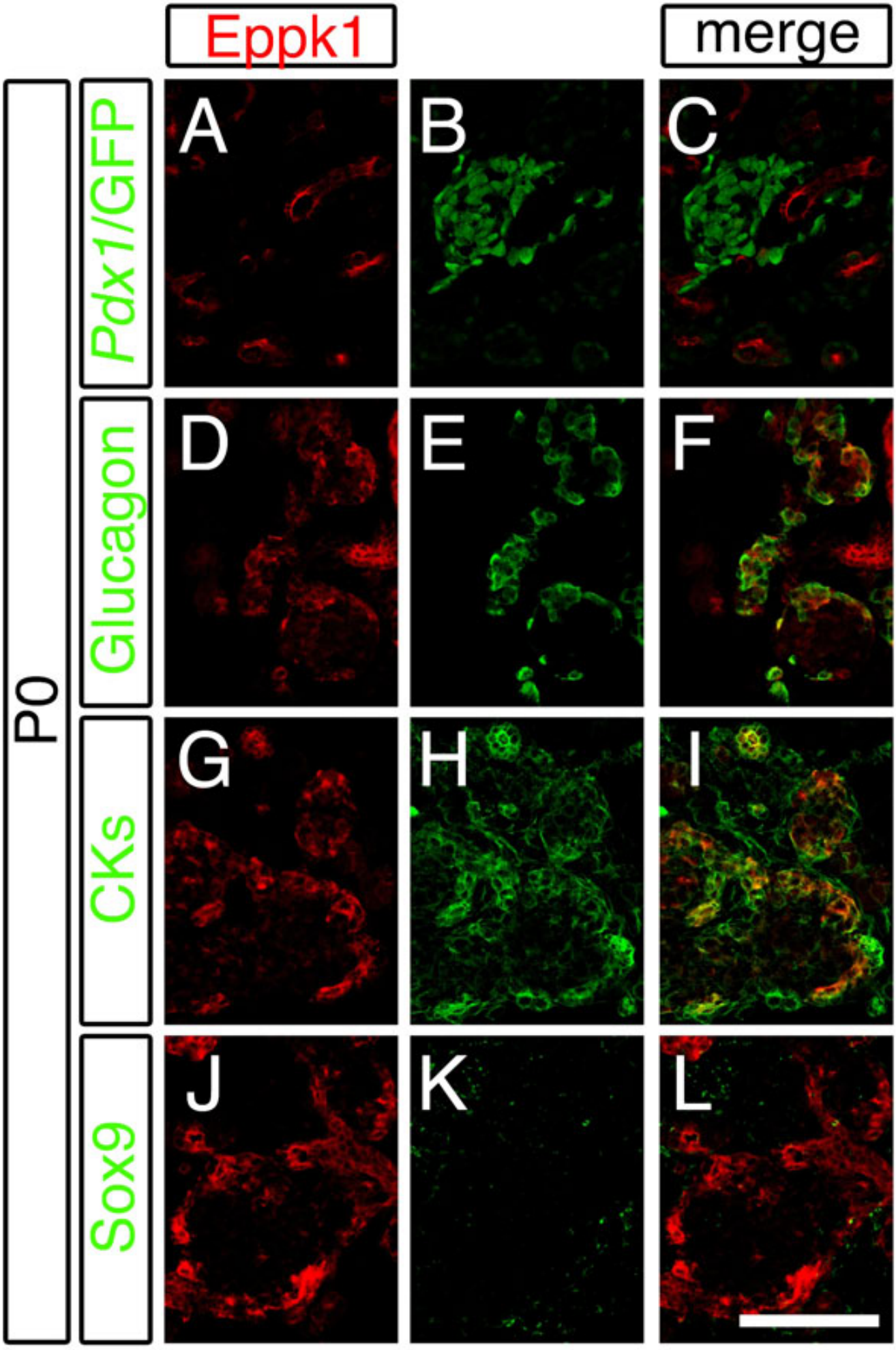
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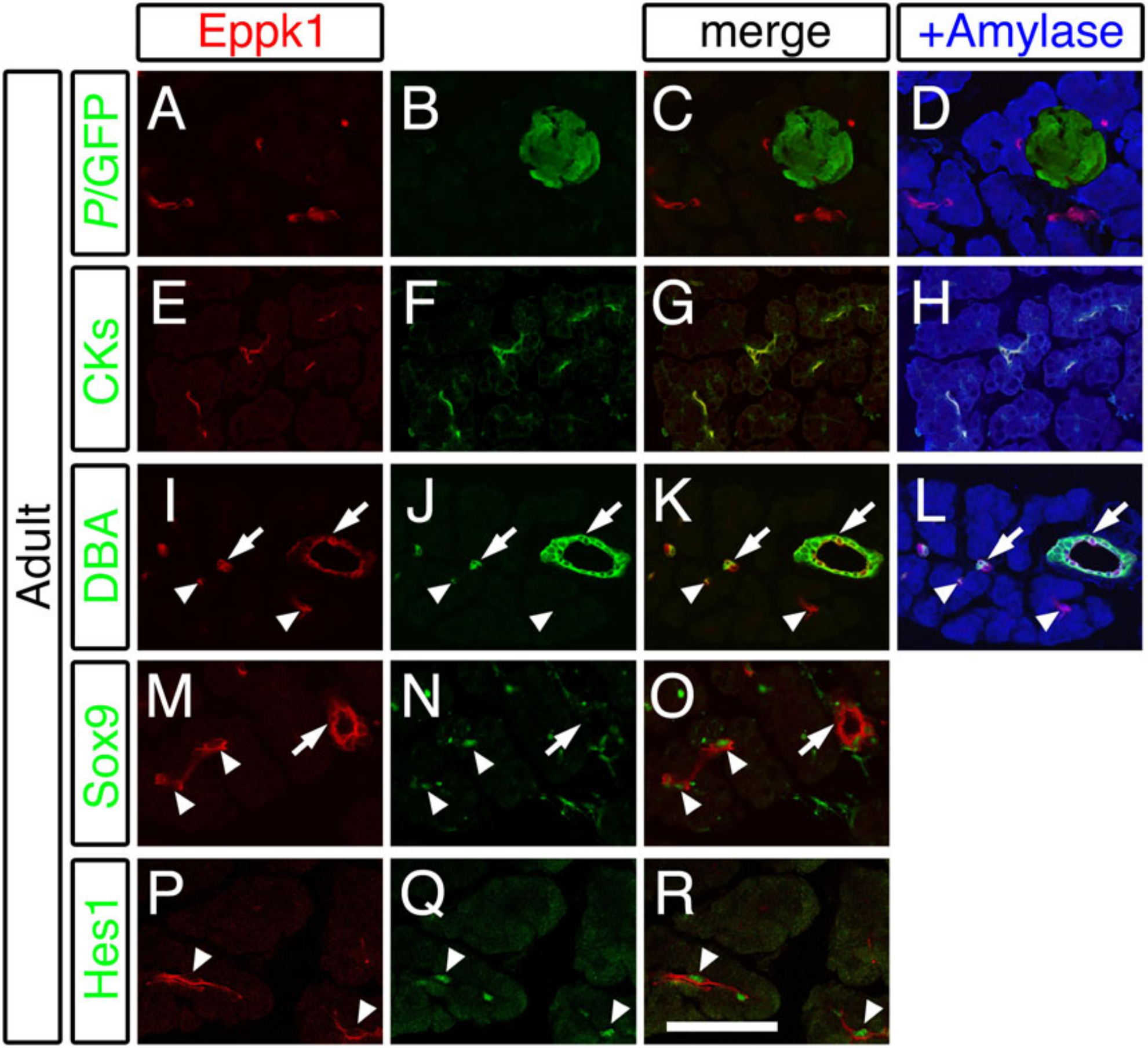
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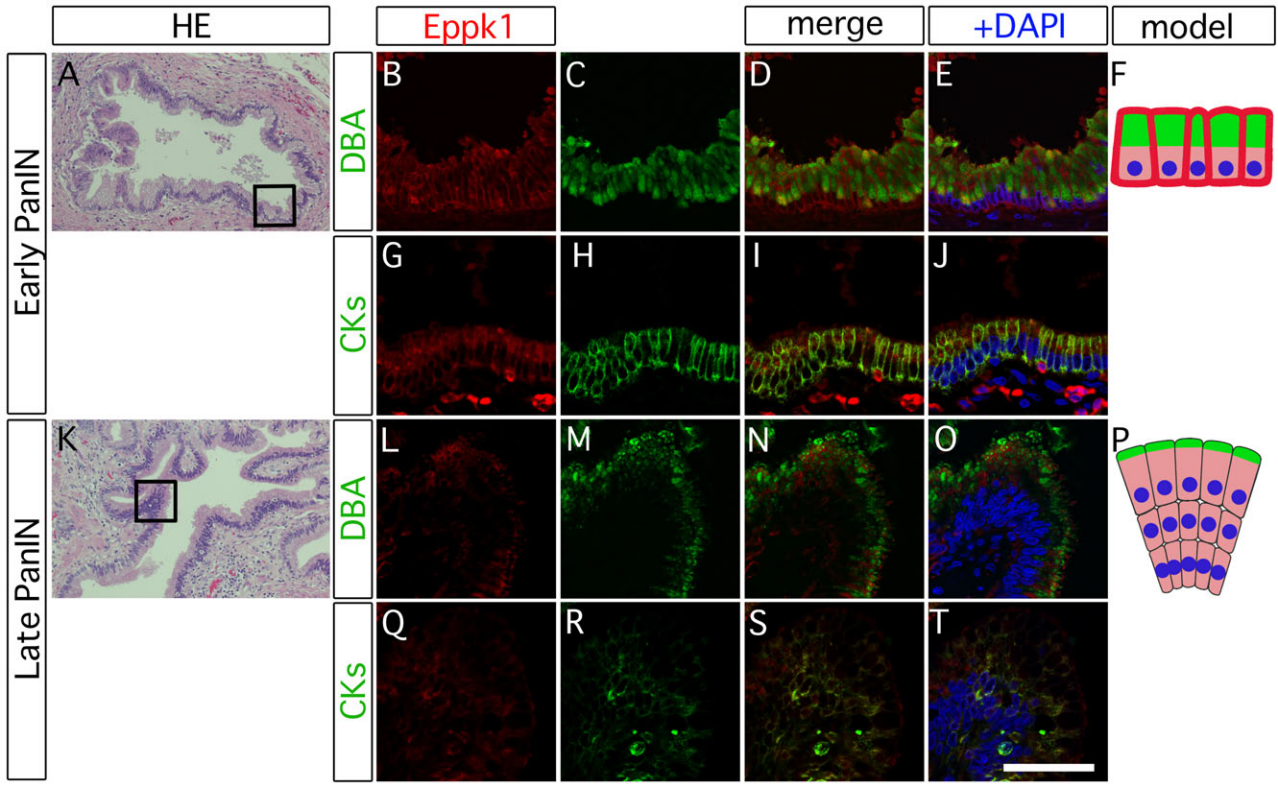
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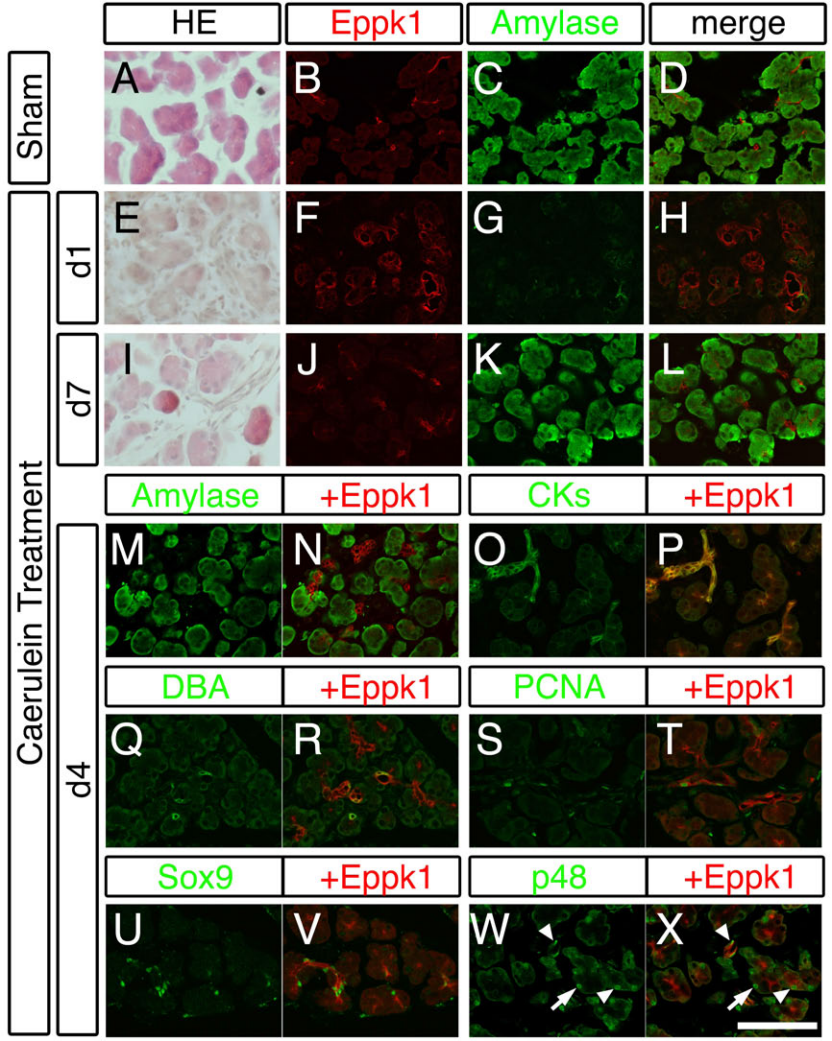
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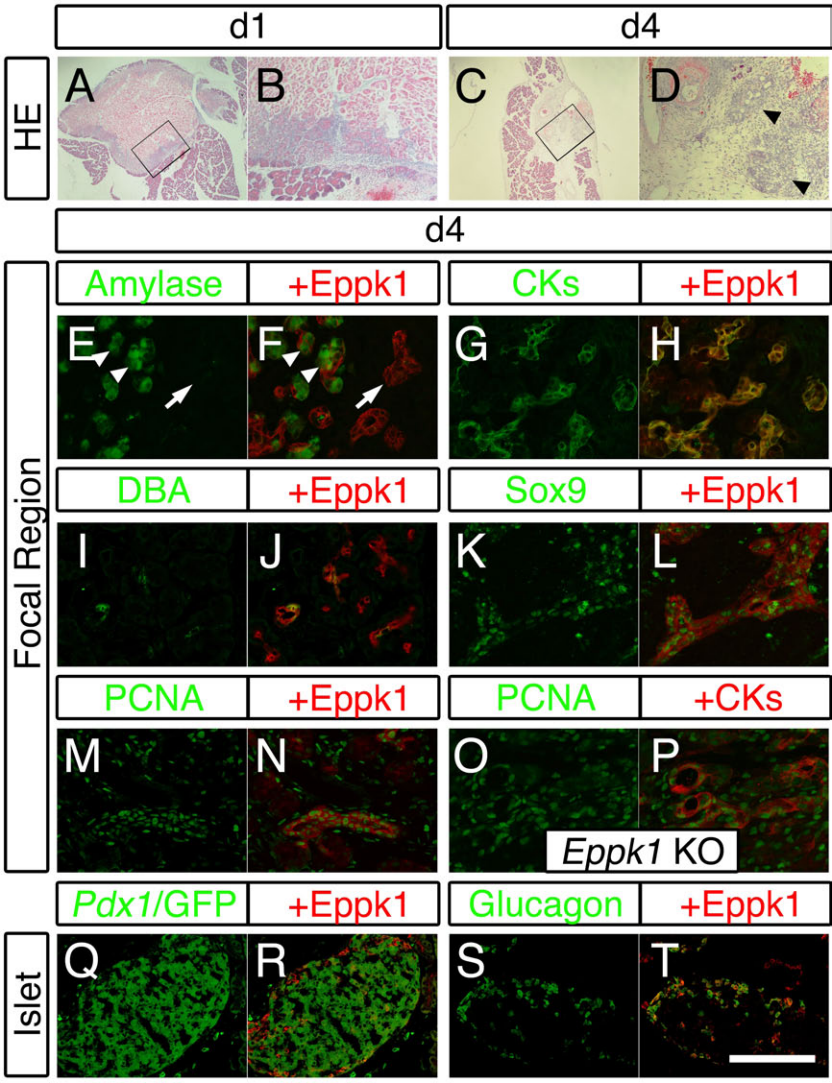
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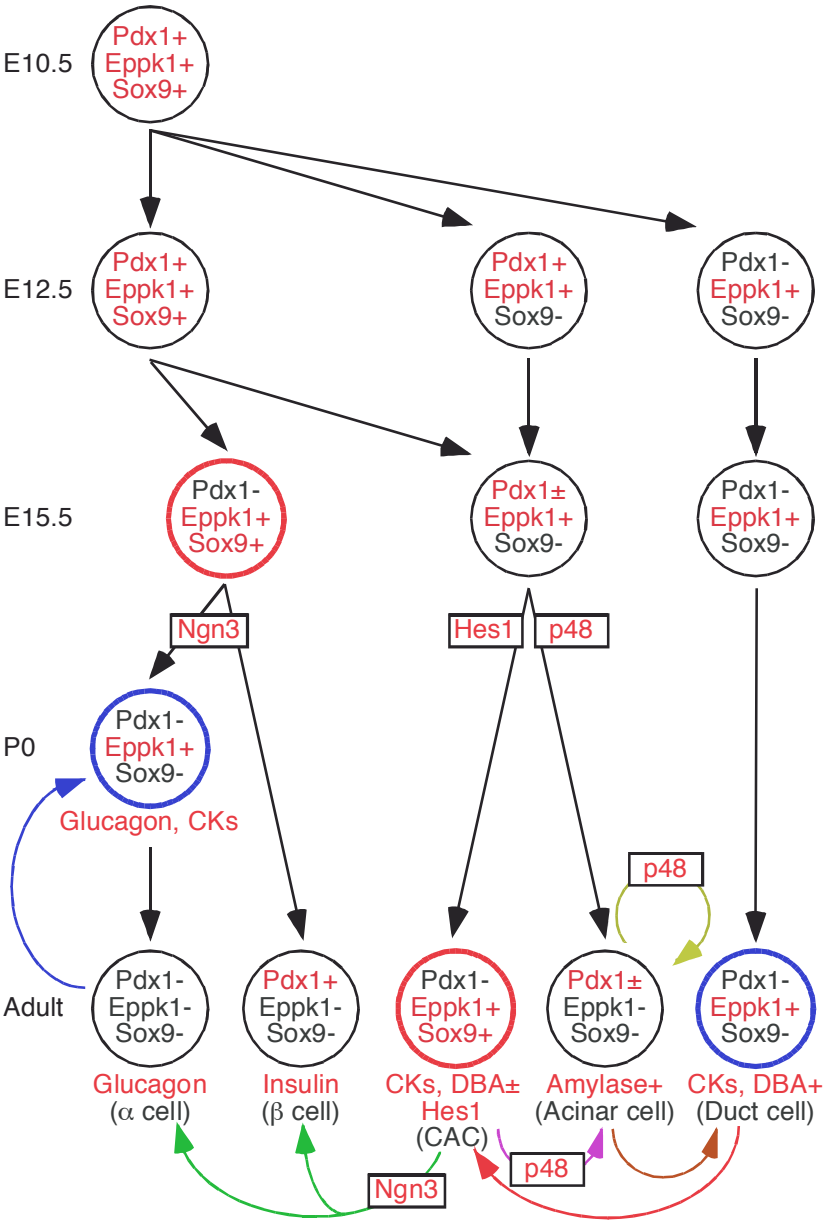
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(β cell)
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Hes1
(CAC)
Pdx1±
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Sox9-
Amylase+
(Acinar cell)
Pdx1-
Eppk1+
Sox9-
CKs, DBA+
(Duct cell)
Ngn3
p48

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1.

The expressions of GFP are well concerned with those of Pdx1 in all of the developmental stages of *Pdx1*/GFP mouse.

The expression patterns of GFP in E10.5 (A-D), E12.5 (E-H), E15.5 (I-L), P0 (M-P) and adult (Q-T) of *Pdx1*/GFP mouse are shown. (A, E, I, M, Q) The expressions of Pdx1 are shown. (B, F, J, N, R) The images of GFP in *Pdx1*/GFP mice are shown. C, G, K, O and S are the merged images. Blue dots in D, H, L, P and T represent nuclei. Scale bar, 100 μm.

Supplementary Figure 2.

Sox9 is not expressed in all of the Pdx1-positive cells of E12.5 pancreas.

The expression patterns of Pdx1 (A) and Sox9 (B) in E12.5 pancreas are shown. The areas surrounded by white solid lines are Pdx1+ pancreatic epithelial cells. The arrowheads and the arrows represent Pdx1+/Sox9- cells and Pdx1+/Sox9+ cells, respectively. Most of the Pdx1+ cells were Sox9+ and no Pdx1-/Sox9+ cells were observed. C is the merged image. Blue dots mean nuclei in D. Asterisks are non-specific signals. Scale bar, 170 μm.

Supplementary Figure 3.

Almost all Pdx1-positive cells are β cells in E15.5 pancreas.

The expression patterns of Pdx1 (A) and Insulin (B) in E15.5 pancreas are shown. C is the merged image. Blue dots represent DAPI staining in nuclei in D. Scale bar, 50 μm.

Supplementary Figure 4.

Cytokeratins are expressed in α cells of P0 pancreas.

The expression patterns of Glucagon (A) and Cytokeratins (B, CKs) are examined in P0 pancreas. The arrowheads are Glucaon+/CKs+ cells. C is the merged image. Blue dots represent DAPI staining in nuclei in D. Scale bar, 100 μm.

Supplementary Figure 5.

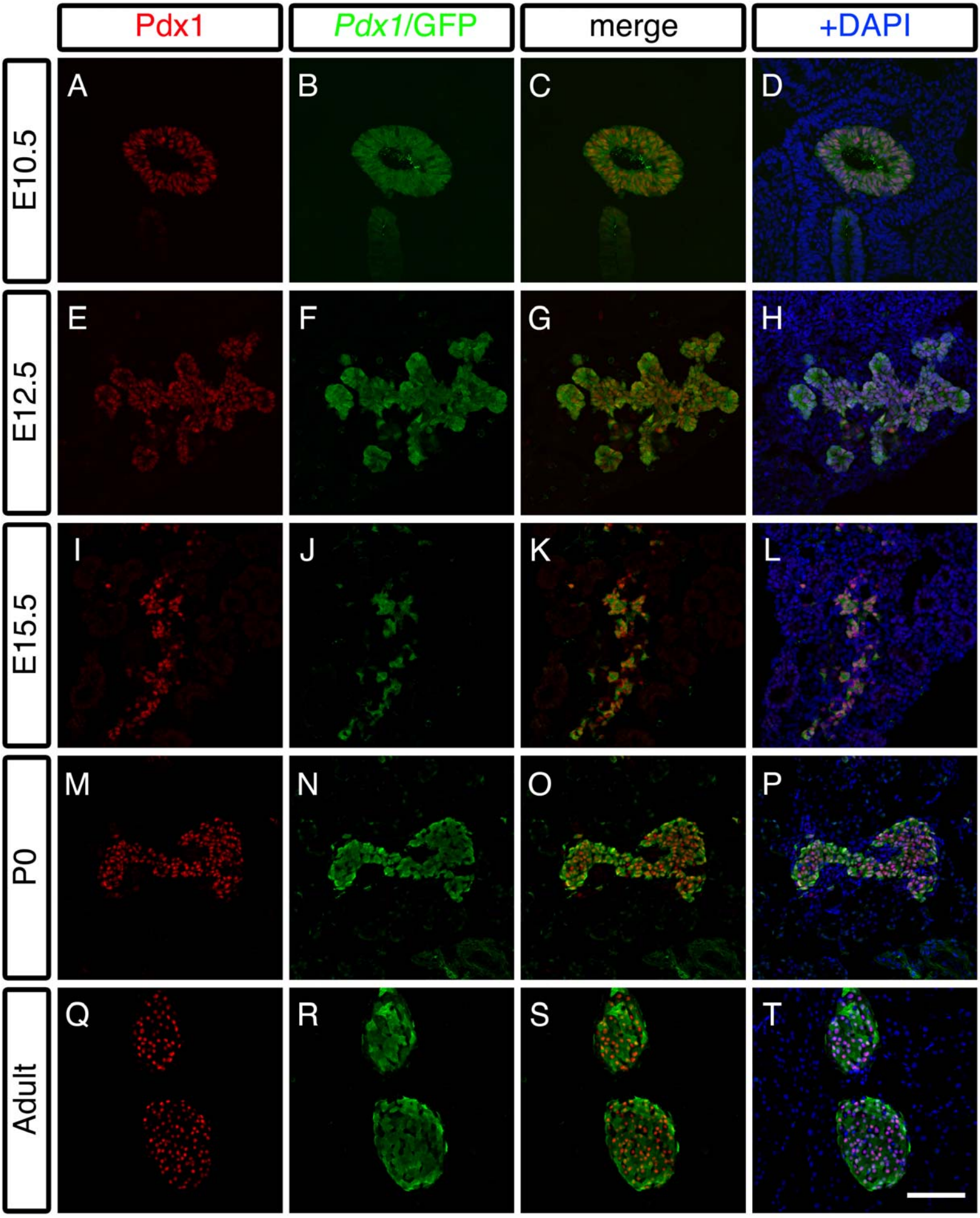
Low magnification pictures of Eppk1+ cells after injury.

The expressions of Eppk1 in sham (A), in caerulein (C) treated pancreas and in the pancreas which has received a partial pancreatectomy (E) were investigated. The sections were observed by low magnification (x20). Blue dots represent nuclei in B, D and F. Scale bar, 200 μm.

Supplementary Figure 6.

Morphologies of the islets of P0, adult and adult Px pancreas.

The images of islets (arrowheads) of a P0 pancreas (A), adult pancreas (B) and an adult pancreas which underwent a partial Px (C) are shown. Note the islets of P0 and Adult Px are smaller than the adult islets and some islets are ‘dumb-bell shaped’. Scale bar, 100 μm.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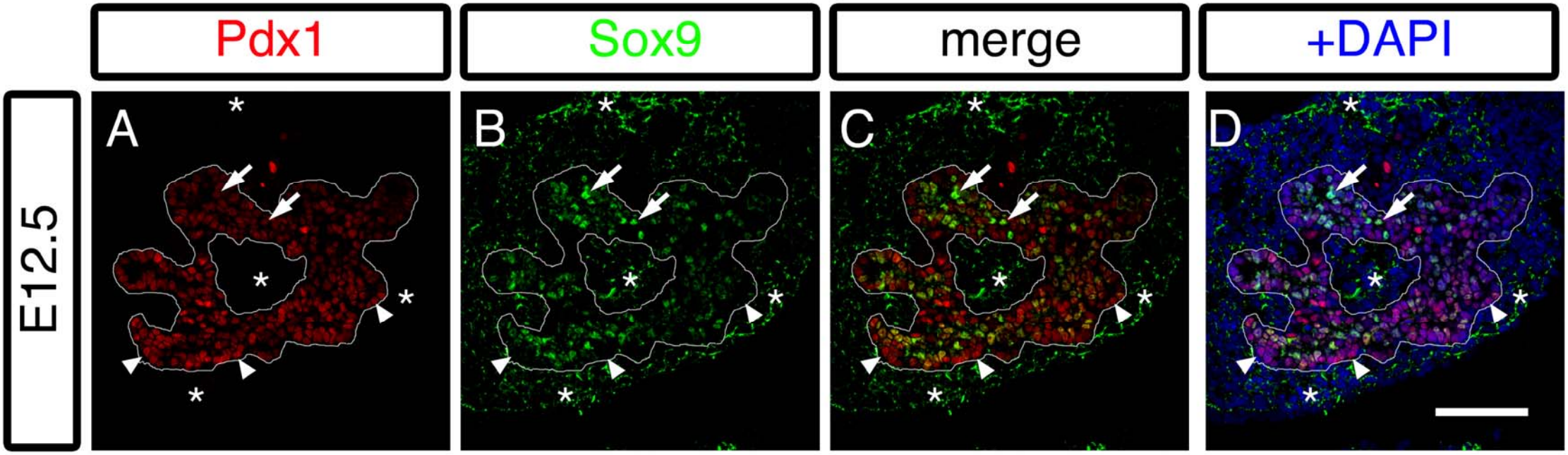
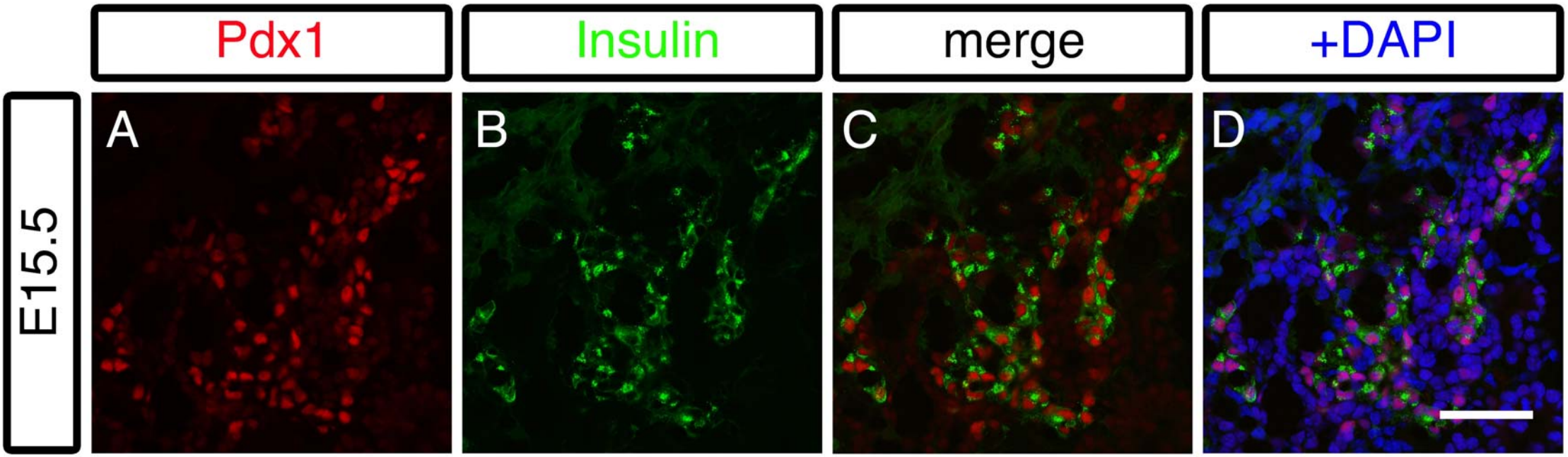
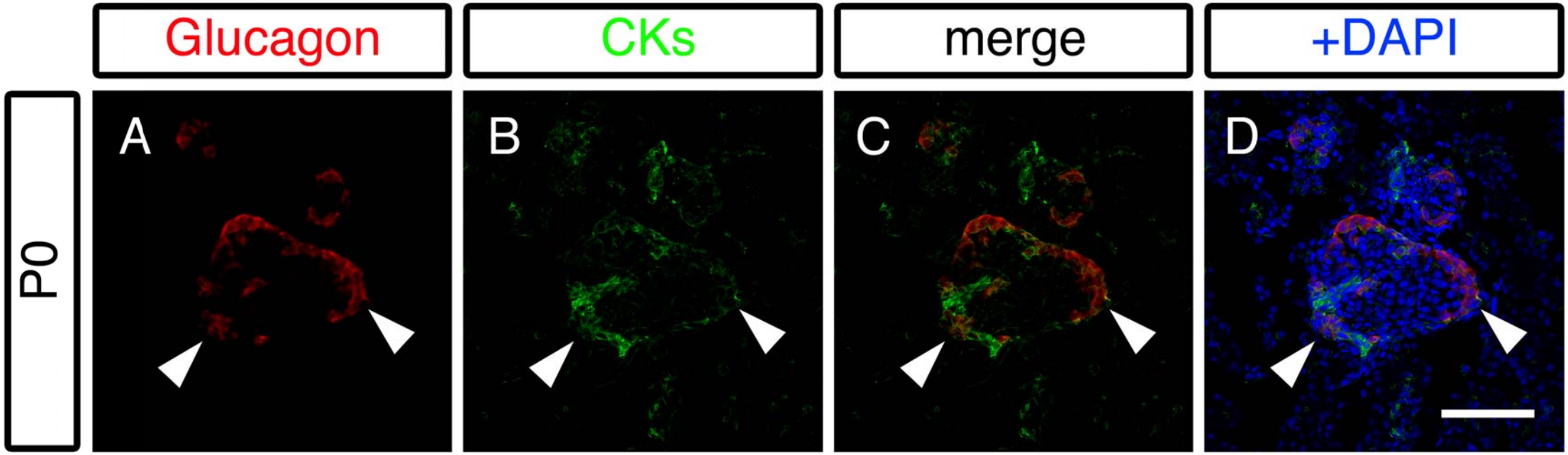
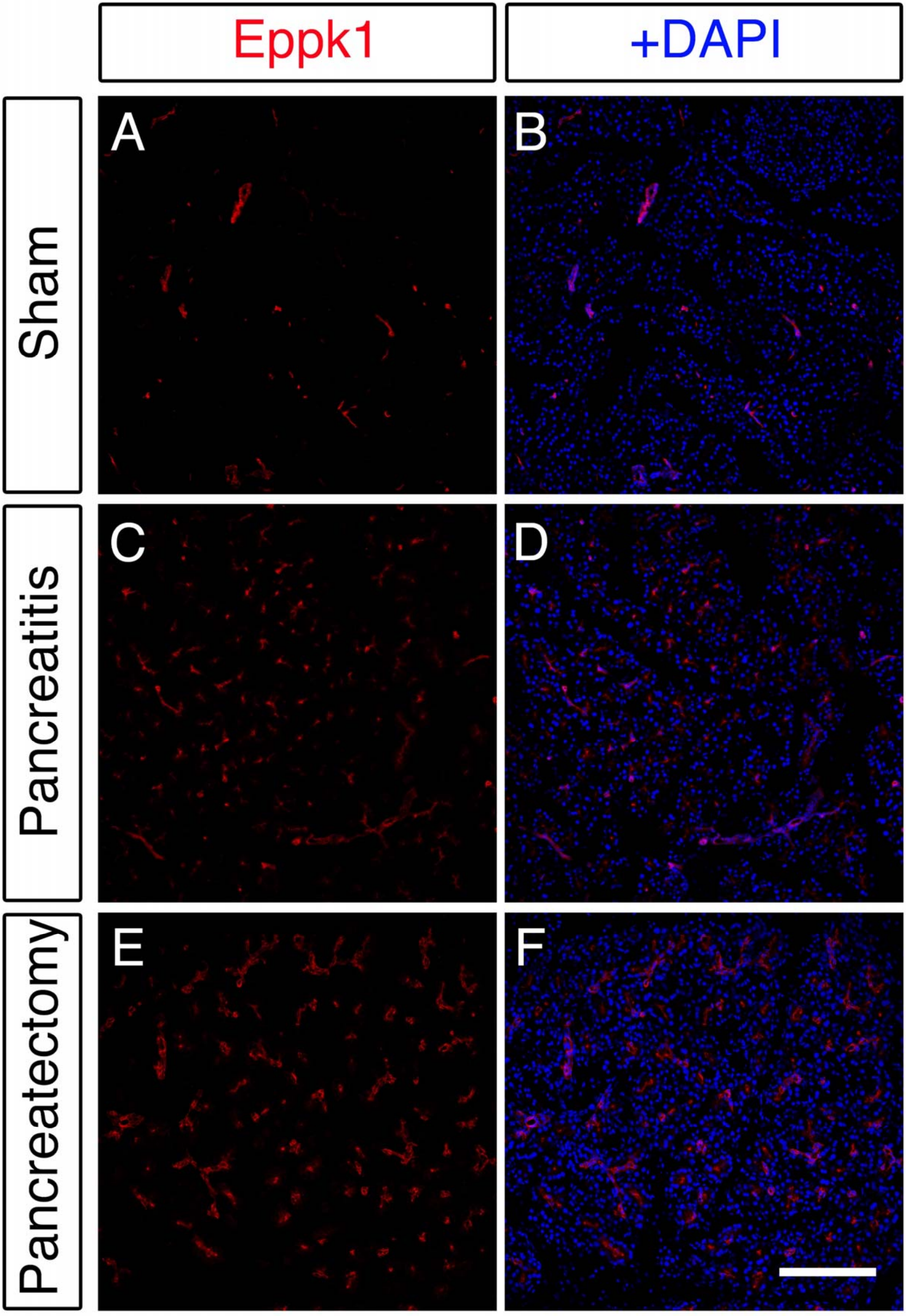

Pdx1
Sox9
merge
+DAPI
E12.5
A
B
C
D


Pdx1
Insulin
merge
+DAPI
E15.5
A
B
C
D


Glucagon
CKs
merge
+DAPI
P0
A
B
C
D


Eppk1
+DAPI
Sham
Pancreatitis
Pancreatectomy
A
B
C
D
E
F

P0
Adult-Sham
Adult-Px
A
B
C