A switch from MafB to MafA expression accompanies differentiation to pancreatic β-cells

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Major insulin gene transcription factors, such as PDX-1 or NeuroD1, have equally important roles in pancreatic development and the differentiation of pancreatic endocrine cells. Previously, we identified and cloned another critical insulin gene transcription factor MafA (RIPE3b1) and reported that other Maf factors were expressed in pancreatic endocrine cells. Maf factors are important regulators of cellular differentiation; to understand their role in differentiation of pancreatic endocrine cells, we analyzed the expression pattern of large-Maf factors in the pancreas of embryonic and adult mice. Ectopically expressed large-Maf factors, MafA, MafB, or cMaf, induced expression from insulin and glucagon reporter constructs, demonstrating a redundancy in their function. Yet in adult pancreas, cMaf was expressed in both α- and β-cells, and MafA and MafB showed selective expression in the β- and α-cells, respectively. Interestingly, during embryonic development, a significant proportion of MafB-expressing cells also expressed insulin. In embryos, MafB is expressed before MafA, and our results suggest that the differentiation of β-cells proceeds through a MafB+ MafA− Ins+ intermediate cell to MafB− MafA+ Ins+ cells. Furthermore, the MafB to MafA transition follows induction of PDX-1 expression (Pdx-1high) in MafB+ Ins+ cells. We suggest that MafB may have a dual role in regulating embryonic differentiation of both β- and α-cells while MafA may regulate replication/survival and function of β-cells after birth. Thus, this redundancy in the function and expression of the large-Maf factors may explain the normal islet morphology observed in the MafA knockout mice at birth.

Keywords: MafA; MafB; Maf factors; Insulin gene transcription factor; Pancreatic development; Endocrine differentiation; Pancreatic islets

Introduction

Transcription factors binding to three conserved insulin enhancer elements, A3 (Ohlsson et al., 1991; Boam and Docherty, 1989), RIPE3b/C1-A2 (Sharma and Stein, 1994; Shieh and Tsai, 1991), and E1 (Crowe and Tsai, 1989; Karlsson et al., 1987) are major regulators of insulin gene expression. The loss of PDX-1, the A3 binding factor, results in mice that are apancreatic (Jonsson et al., 1994; Offield et al., 1996), whereas mice homozygous null for NeuroD1, which binds to the E1 element, have a striking reduction in the number of insulin-producing β-cells (Naya et al., 1997). These observations underscore the importance of insulin gene transcription factors in the development and differentiation of pancreatic endocrine cells. We have identified and cloned MafA, a member of the large-Maf family of basic leucine zipper (bZIP) transcription factors, as the β-cell specific factor binding to the RIPE3b/C1-A2 element (Maf Response element or MARE) (Olbrot et al., 2002). RIPE3b1/MafA is a glucose-responsive factor expressed in insulin-producing cells (Sharma and Stein, 1994; Olbrot et al., 2002; Matsuoka et al., 2003; Kataoka et al., 2002).

The Maf family of transcription factors are important regulators of differentiation and regulate processes such as hematopoiesis, lens differentiation, and segmentation of hindbrain (Blank and Andrews 1997; Reza and Yasuda 2004; Ogino
and Yasuda, 1998; Ogata et al., 2004; Reza et al., 2002; Kim et al., 1999; Kawauchi et al., 1999; Ring et al., 2000). In the lens, Maf factors regulate expression of the major lens-specific protein crystallins (Ogino and Yasuda, 1998). Expression of avian MafA/L-Maf in neuroectodermal cells triggered lens differentiation program (Ogino and Yasuda, 1998), while the absence of cMaf prevented lens formation and the expression of crystallin genes (Kawauchi et al., 1999; Kim et al., 1999). Hence, it was surprising that the loss of MafA did not affect embryonic pancreatic development (Zhang et al., 2005a). However, after birth, the loss of MafA resulted in reduced proportion of β-cells in islets and impaired glucose tolerance, demonstrating the role of MafA in regulating β-cell replication/survival and function. In addition to MafA, pancreatic endocrine cells express other large-Maf family members (Olbrott et al., 2002; Matsuoka et al., 2003; Kataoka et al., 2004). Since all Maf family members recognize the consensus MARE element, cells expressing multiple Maf family members will have competition to regulate MARE-dependent gene expression. Thus, to understand the role of Maf factors in the differentiation of pancreatic endocrine cells, it is essential to determine the temporal and spatial expression patterns of these factors.

In this study, we demonstrate that in adult mice MafA and MafB are preferentially expressed in pancreatic β- and α-cells, respectively. Similarly, at E15.5, MafA and MafB expression was observed in the insulin- and glucagon-expressing cells. However, MafB was also expressed in nearly 90% of insulin-producing cells. The ability of MafB+ cells to express insulin was associated with Nkx6.1 expression, while MafB− Nkx6.1− cells expressed glucagon. Our results demonstrate a gradual shift in expression from MafB+ insulin+ MafA+ insulin+ cells during fetal development, which followed the enhanced PDX-1 expression in insulin+ cells. We suggest that this switch from MafB+ to MafA− cells is not essential for generation of insulin+ cells but may play a role in β-cell replication/survival and function. These results provide an explanation for the lack of effect on the embryonic development of pancreatic islets in MafA knockout mice.

Material and methods

Construction of expression vectors

Expression vectors were constructed by conventional molecular biology techniques. The insulin luciferase reporter constructs −238 WT LUC and its derivatives mutant 110−99m LUC have been described previously (Harrington and Sharma, 2001). The glucagon luciferase reporter construct GLUC LUC (Lee et al., 1992) was obtained from Dr. Dan Drucker (Toronto, Canada). The glucagon promoter from GLUC LUC was cloned into multiple cloning sites (MCS) of pEGFP (Clontech, Palo Alto, CA) to generate GLUC GFP. The expression vectors pcDNA-MafA and ΔN-MafA have been described previously (Olbrott et al., 2002). Mouse full-length MafB cDNA clone in pCMV-Sport6 was purchased from ATCC (Manassas, VA). cMaf cDNA from pCleMaf (Dr. I-C Ho, BWH, Boston) was cloned into MCS of pcDNA3.1 (Clontech, Palo Alto, CA) to generate pcDNA cMaf.

Mice and immunostaining

The day of vaginal-plug discovery was designated as E0.5 of C57BL/6 mice. Animals were anesthetized with intraperitoneal injection of Pentobarbital (50 mg/kg) and dissected. All animal protocols were approved by the Animal Care Committee of the Joslin Diabetes Center. Pancreata were excised, fixed in 4% paraformaldehyde, embedded in paraffin, and immunostained as described previously (Lotter et al., 2002). Antigen retrieval consisted of heating de-paraffinized tissue sections three times for 5 min each with 0.01 M citrate buffer in a microwave oven, followed by 20 min cool-down period before proceeding to the next step. The primary antibodies were guinea pig anti-insulin (Linco); guinea pig anti-glucagon (Linco); monoclonal anti-synaptophysin (Chemicon); mouse monoclonal anti-Ki-67 (BD Pharmingen); rabbit anti-Nkx6.1 (provided by P. Serup); goat anti-PDX-1 (provided by C. Wright); rabbit anti-MafB (Bethyl); rabbit anti-c-Maf (Bethyl); and rabbit anti-Pax6 (Covance). Our rabbit anti-MafA antibody was raised against the 329 to 347 carboxy-terminal amino acids of mouse MafA (ProSci, Poway, CA). The specificity of this antibody was confirmed using its blocking peptide in immunohistochemistry and Western blotting; the preimmune sera gave no staining at the same concentrations used in these experiments. Secondary antibodies for immunofluorescence were FITC- or Texas red-conjugated anti-rabbit, anti-mouse, or anti-guinea pig IgG (Jackson ImmunoResearch). For amplification, biotinylated anti-rabbit, anti-mouse, or anti-goat IgG antibodies (1:400, Jackson ImmunoResearch) were used followed by streptavidin-conjugated Texas red (1:400, Jackson ImmunoResearch) or streptavidin-conjugated Alexa fluor 488 (1:400, Molecular Probes). Nuclear counterstaining was achieved by DAPI mounting medium (Vector). The immunostaining shown in each figure was derived from several replicates of at least 5 sections from 3 different mice/embryos. At least 3 different pancreases were counted for quantitative results. Confocal images were taken on Zeiss LSM410 (Zeiss, Thornwood, NY); quantitations were performed using NIH Image J.

Cell culture and immunocytochemistry

HeLa cells were cultured in the Dulbecco’s modified Eagle’s medium supplemented with 10% (w/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C under 5% CO2. Cells were transfected with the various expression plasmids and 1 µg of GLU GFP reporter constructs using Lipofectamine (Invitrogen, CA). Forty-eight hours after transfection, cells were fixed with 4% (w/v) paraformaldehyde for 15 min, permeabilized with 0.25% (w/v) Triton X-100 in PBS for 15 min, and immunostained as described previously (Nishimura et al., 2002). After blocking with PBS containing 10% (w/v) BSA for 30 min, cells were incubated overnight with the primary antibody in PBS containing 3% (w/v) BSA, washed with PBS, and incubated with secondary antibody in PBS containing 3% (w/v) BSA for 1 h, followed by washing with PBS and mounting.

In situ hybridization

Fresh frozen slices were fixed in 10% buffered formalin (Fisher) for 20 min at −20°C. cMaf sense and antisense probes were diluted to 1 ng/µl and incubated overnight in a humidity chamber at 65°C. Next, slices were washed for 4×30 min in 1× Sodium Chloride-Sodium Citrate (SSC) (Sigma) and then for 1×30 min in Maleic Acid Buffer w/tween (MABT), Primary Alkaline-Phosphatase-DIG-antibody (Roche, 1:1000) was added, incubated overnight at 4°C. Staining was developed using BM Purple (Roche) for up to 48 h (4°C during over-night incubation and RT during daytime). Image of stained section was taken on Olympus BH2 light microscope. Immediately following in situ hybridization, slides were washed twice with PBS and incubated with guinea pig-anti-insulin (1:200) and rabbit-anti-glucagon (1:1000) antibodies for 2 h at RT. Secondary antibodies donkey-anti-guinea pig-Texas Red and donkey-anti-rabbit-FITC (Vector Labs, 1:200) were used for 1 h at RT, Confocal images were taken on Zeiss LSM410 (Zeiss, Thornwood, NY).

Luciferase assays

HeLa cells were transfected with the indicated amount of reporter constructs of −238 WT LUC, 110−99m LUC, or GLUC LUC and with 1 µg of pSV40-gal plasmid (Promega, Madison, WI). Whole cell extracts were prepared and luciferase activity was measured as previously described (Nishimura et al., 2005).
RT-PCR

Total RNA was extracted from MIN-6 or α-TC 1.6 cells and reverse-transcribed to cDNA, which was amplified by PCR with appropriate oligonucleotide primers as previously described (Olbrot et al., 2002). The results were confirmed from at least three independent samples. The following oligonucleotides were used for primers: cMaf3′ppT, 5′CTTGATGTTGCGCTATG-GATG3′, cMaf3′ppB, 5′GCTTGGTTGATTGACTTTTTCTG3′, MafA3′ppT, 5′TTTCTCGGACGCTCACCTGTA3′; MafA3′ppB, 5′GGGGTTCTCCGGGTTTCTTAAT3′; MafB3′ppT, 5′CTGGCC-CCCTAGCCCTGGACTC3′; MafB3′ppB, 5′GCGGCCCCTGGGACTCACA-A3′.

Western blot

Forty micrograms of nuclear extracts from indicated cell lines was run on 10% SDS-PAGE and transferred to PVDF membranes, which were subjected to Western blotting with indicated antibodies and visualized by enhanced chemiluminescence kit (Amersham Biosciences).

Results and discussion

Differential expression of large-Maf factors in pancreatic endocrine cells

Previously, we reported that, in addition to the MafA, other large-Maf factors, MafB and cMaf, were expressed in pancreatic endocrine cells (Olbrot et al., 2002). In a detailed study, Matsuoka and colleagues (2003) reported that in islets MafA was expressed in β-cells, MafB in more α- than β-cells, and cMaf at extremely low levels. Surprisingly, Kataoka et al. (2004) were unable to demonstrate MafB expression in α-cells but showed the expression of cMaf in these cells. Hence, we reexamined the expression profile of large-Maf factors in pancreatic endocrine cells. RT-PCR was performed on the RNA isolated from mouse islets, the β-cell line MIN-6, and the α-cell line α-TC1.6. Since the large-Maf factors share sequence homology in the coding region, PCR primers were designed in the unique 3′ untranslated regions. MafA and MafB expression was mostly restricted to β- and α-cell lines, respectively, while cMaf was expressed in both cell lines (Fig. 1A). To determine the relative expression of these factors, real-time PCR reactions were performed with the same cDNAs used in Fig. 1A. MafA was expressed at 250-fold higher in β- than in α-cells, while MafB expression was 450-fold higher in α-cells. Consistent with the results in Fig. 1A, cMaf expression was similar in both cell lines. This differential expression of MafA and MafB in pancreatic α- and β-cells is consistent with the results of Matsuoka et al. (2003). We determined the expression of large-Maf proteins in hormone-producing (αTC1.6 and MIN6) and non-hormone producing (HeLa) cell lines. Protein bands corresponding to MafA were detected only in the extract from the β-cell line, while cMaf protein was expressed in both α- and β-cell lines. Anti-MafA antibody recognized multiple protein bands (most likely phospho-MafA isoforms) and preincubation of the antibody with specific peptide prevented this recognition (Kondo et al., unpublished observation). Anti-MafB antibody does not work optimally in the Western blot (or the levels of MafB protein in αTC1.6 are very low), yet results from two independent samples convincingly showed expression of MafB in the α-cells (Fig. 1B). Band intensities corresponding to these large-Maf factors were significantly different in each cell line. Since the affinity of antibodies for these factors may be different, these intensities may not reflect true expression levels of these proteins. Even so, the Western blot analyses are consistent with the RNA expression pattern (Figs. 1A, B).

In adult pancreas, immunostaining for MafA was restricted to the pancreatic β-cells (Figs. 1C, D). Similarly, MafB expression was highly restricted to α-cell (Figs. 1E, F), and only a few rare β-cells in the adult pancreas immunostained for MafB. cMaf was expressed in both α- and β-cells (Figs. 1G, H). These data differ from the reported MafB expression in nearly a third (37%) of adult β-cells (Matsuoka et al., 2003). Differences in the mouse strains and/or antibodies may account for this discrepancy. Immunostaining results are consistent with those from RT-PCR and Western blot analyses, and together they confirm differential expression of MafA and MafB in pancreatic β- and α-cells.

Large-Maf factors activate expression from insulin and glucagon reporter constructs

Since large-Maf family members are expressed in pancreatic β- and α-cells, we examined their effect on regulating insulin and glucagon gene expression. Rat insulin II promoter: luciferase reporter constructs (−238 WT LUC) with or without mutations in the MARE element (Harrington and Sharma, 2001; Olbrot et al., 2002) were transfected into HeLa cells with MafA, MafB, or cMaf expression plasmids, or pcDNA3.1 as a control. All three large-Maf factors activated insulin gene expression from the wild type construct and not from the construct with the mutated MARE element (Fig. 2A). To determine if these factors regulate expression by forming heterodimers, cells were transfected with −238 WT LUC plasmid and large-Maf expression plasmids with or without the ΔNMafA plasmid that lacks the N-terminal activation domain of MafA (Olbrot et al., 2002). Activation of insulin gene expression mediated by these factors was inhibited by ΔNMafA (Fig. 2B). As observed for insulin gene expression, MafA, MafB, and cMaf activated glucagon gene expression, and this activation was inhibited by ΔNMafA (Fig. 2C). To assess the ability of Maf factors to activate gene expression at a single cell level, a glucagon:GFP reporter plasmid was transfected into HeLa cells with MafA, MafB, cMaf, ΔNMafA expression plasmids, or pcDNA3.1. Cells singly transfected with MafA, MafB, or cMaf induced expression from glucagon reporter construct (as observed by co-expression of Maf factors and GFP) (Figs. 2D–F) but not in those cells transfected with ΔNMafA or pcDNA3.1 (Figs. 2G, H). These results are consistent with the published reports (Planque et al., 2001; Kataoka et al., 2004; Zhao et al., 2005), and they demonstrate that MafA, MafB, or cMaf can activate expression from not only insulin but also glucagon promoter: reporter constructs.
Expression of MafB precedes MafA expression during pancreatic development

Large-Maf factors have highly conserved C-terminal DNA binding and protein dimerization domains (Blank and Andrews, 1997). However, homology in the N-terminal activation domain is less conserved, suggesting possible differences in their activation potentials and/or downstream targets. Thus, the expression of different large-Maf factors in the endocrine cells may differentially affect their function. Additionally, embryonic expression of these large-Maf factors may have significant impact on the differentiation of endocrine cell types. The anti-cMaf antibody that in adult pancreatic sections convincingly demonstrated cMaf expression in both α- and β-cells (Figs. 1G, H) was found unsuitable for detecting embryonic expression of this factor. Hence, to detect expression of cMaf during embryonic development, in situ hybridization was performed on the frozen pancreatic sections from E17.5 embryos; these sections were immunostained to detect insulin and glucagon expressing cells (Fig. 3). Our results demonstrate that as in pancreatic islets from the adult mice, cMaf was expressed in both insulin and glucagon producing cells. Unlike cMaf, MafA is exclusively expressed in β-cells and the expression of MafB is specific to α-cells in the adult pancreas. Thus, we hypothesized that MafA is important for the differentiation of β-cells while MafB has a similar role in the α-cells. Therefore, we focused our study on examination of spatial and temporal expression patterns of MafA and MafB during embryonic development to understand their roles in the pancreas.

Immunostained sections of pancreas from E10.5–P14 were examined for co-expression of MafA with insulin, and MafB with glucagon. Glucagon+ cells can be seen soon after pancreatic specification, and by E10.5 glucagon+ cells are seen in the pancreatic epithelium (Fig. 4C). Interestingly, cells in these early glucagon+ clusters express MafB, but occasional MafB+ cells near these clusters did not express glucagon (Figs. 4C, F). These data suggest that embryonically MafB is expressed in pancreatic cell types other than glucagon or that its expression precedes that of glucagon. In contrast to glucagon+ cells that are easily detected by E10.5, insulin+ cells are rare, and their number increases only after the secondary transition (around E13.5). At E10.5 and 11.5, neither insulin+ nor MafA+ cells were found, but the rare insulin+ cells at E12.5 and E13.5 expressed MafA (Figs. 4G, I). These results are similar to the report that MafA+ cells can be detected only from E13.5 (Matsuoka et al., 2004). The apparent discrepancy of our finding a few insulin+ MafA+ cells at E12.5 and Matsuoka et al. (2004) could be due simply to the limited number of insulin+ cells observed in the E12.5 pancreas. By E15.5, there is a significant increase in the number of insulin+ and glucagon+ cells (Figs. 4J, K). All of the MafA+ cells at this
stage co-expressed insulin, but a significant proportion of insulin+ cell did not express MafA. Interestingly, at E15.5 nearly all (98%) glucagon+ cells expressed MafB, but a significant number of MafB+ cells did not express glucagon. MafB is selectively expressed in the α-cells of the adult pancreatic islets; we determined that MafB expression gradually

Fig. 2. MafA, MafB, and cMaf can activate insulin and glucagon expression. (A) Luciferase reporter plasmids (−238 WT LUC or −122.121m LUC) were transfected into HeLa cells with the indicated expression plasmids (pcDNA3.1, MafA, MafB, or cMaf). Luciferase activity was determined 48 h after transfection. Results are presented relative to the activity of −238 WT luciferase construct transfected with pcDNA3.1 ± SE (n = 4). (B) Luciferase reporter plasmid (−238 WT LUC) and indicated expression plasmid were transfected into HeLa cells with or without ΔNMafA plasmid that lacks N-terminal activation domain. Results are presented as in panel A (n = 3). (C) Glucagon Luciferase reporter plasmid (−2.2 Glucagon LUC; Lee et al., 1992) and indicated expression plasmid were transfected into HeLa cells with or without ΔNMafA plasmid. Results are presented as in panel A (n = 3). Rat glucagon: GFP reporter plasmid was transfected into HeLa cells with (D) MafA, (E) MafB, (F) cMaf, (G) ΔNMafA expression plasmids, or (H) pcDNA3.1. Forty-eight hours after transfection, HeLa cells were immunostained to detect GFP (green), nuclei (DAPI blue), and Maf factors (Red), using anti-MafA (D and G), MafB (E), cMaf (F), and large-Maf (H) antibodies. Each figure shows combined three-color image, while the insets show signals from individual color channels. Scale bar = 20 μm.
becomes restricted to the α-cells after birth, and by 2 weeks few MafB+ glucagon− cells are seen (Figs. 4L–N).

**MafB is expressed in the insulin-producing cells during development**

The increased number of MafB+ glucagon− cells around secondary transition suggests an important role of this factor in regulating differentiation of other pancreatic cell-type(s). To determine if the E15.5 MafB+ glucagon− cells were endocrine cells, pancreatic sections were immunostained to detect MafB and synaptophysin expression. All MafB+ cells were synaptophysin−, but not all synaptophysin+ cells expressed MafB (Fig. 5A), suggesting that MafB is expressed in only some endocrine cells at this stage of development. Since the number of insulin+ cells increases at the secondary transition, we examined if the MafB+ Glucagon− cells expressed insulin and the MafA+ cells expressed glucagon. We observed that MafA+ cells did not express glucagon, while many MafB− cells were insulin+ (Figs. 5B, C). This was in direct contrast with the highly selective α-cell expression of MafB in adult islets. Quantitation showed that all the 137 MafA+ cells co-expressed insulin, while nearly 60% of MafB+ cells were insulin+ (Fig. 5F). Interestingly, only half (54%) of the insulin+ cells were MafA+ whereas nearly 90% of insulin− cells expressed MafB. These observations demonstrate that at E15.5 some insulin+ cells express MafB, a significant proportion express both MafA and MafB, and a minor population only express MafA.

![Fig. 3. During embryonic development, cMaf is expressed in both insulin− and glucagon− cells. (A) A light microscope image of E17.5 pancreas following in situ hybridization shows cMaf expression (dark staining). (B) Confocal image of the same section stained for insulin (red) and glucagon (green). Panels C–E are higher magnification images of inset shown in panel A and the corresponding region from panel B. One insulin- and one glucagon-expressing cell are numbered in the higher magnification image to clearly show that cMaf is expressed in both cell types. Scale bar = 20 μm.](image)

![Fig. 4. MafA and MafB expression pattern during embryonic development. Confocal images of immunostained pancreatic sections from indicated embryonic (E10.5–E15.5) and postnatal days 5, 8, and 14. Sections were stained with indicated antibodies that recognize transcription factors PDX-1, MafA or MafB (green), or hormones insulin or glucagon (red) and nuclei (DAPI blue). Results show that MafB is expressed earlier (E10.5) than MafA (E12.5) in developing pancreas. MafA expression is restricted to insulin− cells. At the earlier time points (E10.5–11.5), the majority of MafB− cells express glucagon, and only occasional glucagon− MafB− cells are seen (arrows in panels C and F), but around E15.5, there is a significant increase in the number of glucagon− MafB− cells. After birth, expression of MafB again becomes restricted to glucagon− cells. Scale bar = 20 μm for all images except panels Ja and Ka, where scale bar = 50 μm.](image)

To determine MafA and MafB co-expression with insulin, we stained thin (3 μm) consecutive sections of E15.5 pancreas with appropriate antibodies (Figs. 6A, B). Insulin staining in consecutive sections identified cells that were present in both
sections, thus permitting determination of MafB and MafA expression in the same cell. This analysis provides novel information regarding the differentiation of insulin+ cells during embryonic development: (1) all insulin+ cells expressed at least MafA or MafB; (2) in insulin+ cells, we can identify all possible combinations (only MafB, only MafA or both) of large-Maf factor expression (Figs. 6A, B, and insets). The observed proportion of these three cell-types was consistent with the results in Fig. 5. MafB is expressed earlier than the MafA (Figs. 4 and 5) and becomes restricted to α-cells during the postnatal period (Fig. 4), and adult insulin+ cells express MafA (Fig. 1). Furthermore, we found that during different stages of embryonic development less than 1% of insulin+ cells (E13.5 none, E15.5, and E18.5 0.6%) are apoptotic as judged by condensed nuclei visualized with propidium iodide (data not shown); these data suggest that the majority of insulin+ MafB+ cells at E15.5 survive and give rise to the insulin+ cells found after birth. We propose a model in which in any given insulin+ cell MafB expression precedes that of MafA, with a gradual inhibition in MafB expression in the MafA+ MafB+ insulin+ cells, resulting in the generation of insulin+ MafA+ cells. A future lineage tracing study should provide definitive proof for such switch. However, this model raises several important questions, including whether MafB turns on the expression of insulin and glucagon, that is, is MafB upstream of these hormones? What regulates the choice of MafB+ cells to express either insulin or glucagon? What turns on MafA expression in MafB+ insulin+ cells? Finally, why do insulin+ cells transition from MafB+ to a MafA+ cell-type when both factors can bind to the insulin MARE and activate insulin gene expression?

**Differential expression of Nkx6.1 in MafB+ cells defines the cells that express insulin or glucagon**

The generation and analysis of MafB knockout mice will eventually address whether MafB is upstream or downstream of insulin and glucagon gene expression. However, our results suggest that MafB is an important differentiation factor for both α- and β-cells. A significant proportion of insulin+ cells expressed both MafA and MafB, but glucagon+ cells expressed only MafB, so we decided to test whether the expression of Nkx6.1 correlated with the ability of MafB+ cells to express insulin. The transcription factor Nkx6.1 is critical for the differentiation of β-cells (Sander et al., 2000), and is required for the expression of MafA (Matsuoka et al., 2004). In consecutive sections of E15.5 pancreas, significantly more cells expressed Nkx6.1 than MafB+ (Figs. 7A, B). As reported previously (Sander et al., 2000), at this stage, a reasonable proportion of Nkx6.1+ cells are insulin+ (Fig. 7B). We observed that, at E15.5, the majority of insulin+ cells also expressed Nkx6.1 and MafB (arrow Figs. 7A, B, and insets) but that MafB+ glucagon+ cells did not express Nkx6.1 (closed triangle, Figs. 7C, D). Importantly, cells that were Nkx6.1+ MafB+ were glucagon− (arrows Figs. 7C, D). Thus, the ability of MafB-expressing cells to express glucagon or insulin depends on the co-expression of Nkx6.1.

**Fig. 5. MafB is expressed in insulin-producing cells during embryonic development.** (A) Confocal images of E15.5 pancreas stained for MafB (green), Synaptophysin (red), and nuclei (DAPI, Blue). All MafB+ cells are Synaptophysin+, but not all Synaptophysin+ cells are MafB+ (arrow). Images B and C show E15.5 pancreas stained for MafA (green), glucagon (red), and nuclei (DAPI, blue), or MafB (green) insulin (red) and nuclei (DAPI, blue), respectively. Images Bb and Cb show areas marked in images Ba and Ca at higher magnification. Quantitation is shown as percentage of MafA+(D) or MafB+(F) cells expressing insulin or the percentage of insulin+ cells expressing MafA (E) or MafB (G). Results are from at least five independent sections from three different pancreases. A significant proportion of MafB+ cells express insulin. MafB is expressed only in the insulin+ cells, but several insulin+ cells do not express MafA. Scale bar = 50 μm (Ba, Ca), 20 μm (A, Bb, Cb).

Nkx6.1 is upstream of MafA during pancreatic development and is important for the differentiation of insulin+ cells. However, Nkx6.1 is not likely to be upstream of MafB since glucagon+ MafB+ cells do not express Nkx6.1 (glucagon+ Nkx6.1− cells are extremely rare; Henseleit et al., 2005). Even so, we cannot rule out the possibility that Nkx6.1 is upstream of MafB in cells that will turn on insulin expression. Since a few insulin+ cells can be detected in Nkx6.1 knockout mice (Henseleit et al., 2005; Matsuoka et al., 2004), Nkx6.1 may not be essential for the
formation of insulin+ cells. This finding suggests two other possibilities for differentiation of insulin+ cells: (1) MafB can initiate differentiation of insulin-expressing cells and Nkx6.1 is required for the maintenance of insulin expression and induction of MafA; (2) Nkx6.1 and MafB together trigger the differentiation of insulin+ cells (we did observe a few rare MafB+ Nkx6.1+ cells that were insulin−). Demonstration of MafB expression in the remaining insulin+ cells in Nkx6.1 knockout mice would support the first possibility. The absence of insulin+ cells, even in the presence of Nkx6.1+ cells, in MafB knockout mice would also be consistent with the first possibility. However, reduced numbers of insulin+ cells in MafB knockout mice would support the second. The definitive answer for the importance of MafB and Nkx6.1 in the differentiation of insulin+ cells will only come from the analyses of these knockout mice.

Role of MafA in β-cell replication and maturation

Our results suggest a switch in the expression from MafB to MafA in insulin+ cells, raising questions such as, what regulates the switch in the transcription factor expression, and is it necessary? The MafA knockout mouse study (Zhang et al., 2005a) would suggest that the switch to MafA is required for the survival/proliferation and function of β-cells. The loss of MafA resulted in normal pancreatic morphology at birth, but after birth a reduction in the proportion of β-cells with resulting impaired glucose tolerance and diabetes (Zhang et al., 2005a).

Terminally differentiated β-cells have glucose-induced insulin secretion whereas fetal and neonatal islets are not yet glucose-responsive. The MafA knockout study showed Glut2 expression reduced in the knockout animals. Since more fetal and neonatal insulin+ cells express MafB than in adult mice, the transition from MafB to MafA expression may initiate the glucose-responsiveness of insulin+ cells by the selective induction of glucose transporter Glut2. In E15.5 pancreas (Fig. 8), Glut2 is expressed in some MafA+, MafB+, or Nkx6.1+ cells. Some of these MafB+ Glut2+ or MafA+ Glut2+ cells also express insulin. This suggests that the switch from MafB to MafA may not be needed for the maturation of β-cells, as judged by the induction of Glut2 expression. The observed expression of Glut2 in MafB− or MafA− cells may reflect differentiation of these cells from Glut2+ precursors (Pang et al., 1994). We find that, at E15.5, more MafB+ than MafA+ cells express Glut2, which is consistent with our hypothesis that during differentiation of insulin+ cells, MafB is induced first, followed by the expression of MafA. This would suggest that the Glut2 expression at E15.5 is a marker of precursor cells rather than the maturation of β-cells. The lack of a correlation between MafA and Glut2 expression in insulin+ cells (Fig. 8D) is not consistent with the observed reduction in the Glut2 expression in MafA null mice (Zhang et al., 2005a). However, as reported in other animal models (Thorens et al., 1990), reduction in the Glut2 expression could result from the hyperglycemia in these mice. Thus, our results demonstrate that the switch from MafB to MafA at E15.5 is not required for the induction of Glut2 expression but that MafA expression may turn-on additional genes critical for the function of adult β-cells.

The loss of MafA results in a reduced proportion of β-cells after birth, suggesting a possible role of the transition from MafB to MafA in the replication/survival of β-cells. To test for a role of these factors in β-cell replication, the co-expression of cell cycle marker Ki67 with MafA and MafB factors was examined in E15.5 and P14 pancreas. Consistent with previous observations that newly differentiated endocrine cells do not proliferate, we found in E15.5 mice that nearly 25% of the Nkx6.1+ cells are in the cell cycle (Ki67+) but none of the MafA+ cells and only a rare MafB+ cell

Fig. 6. Expression of MafA and MafB in the insulin+ cells. Two consecutive sections from E15.5 mouse pancreas stained with the indicated antibodies to detect insulin (red), nuclei (blue), and either MafA (A) or MafB (B). One can identify all possible combinations of MafA and MafB expression in the insulin+ cells. Cells indicated with arrows are MafB+ insulin+ but MafA−, cells indicated with the open arrowheads express both MafA and MafB, while cells indicated by filled arrowheads demonstrate the presence of MafA+ insulin+ MafB− cells. Lower images are higher magnification of the boxed areas in panels A and B; cells are numbered to identify the same cells in both sections. Total number of MafA and MafB expressing nuclei at E15.5 from five adjacent pairs of sections from three different pancreases (C) shows over 3-fold higher proportion of MafB+ cells at this stage (P = 0.01). Scale bar = 20 μm.
Fig. 7. MafB+ and insulin+ cells express NKX6.1 but MafB+ glucagon+ cells are NKX6.1−. Consecutive sections A and B show cells expressing insulin (red), nuclei (blue), and either MafB or Nkx6.1 (green). Arrows denote cells that are present in both sections and express both Nkx6.1 and MafB. All of the insulin+ cells are Nkx6.1+, but not all Nkx6.1+ cells were insulin+. Higher magnification image of areas marked in panels D and E is shown at the bottom. Total number of nuclei expressing the indicated factor at E15.5 from five adjacent pairs of sections from three different pancreases (C). Significantly more (50% \( P = 0.001 \)) Nkx6.1+ cells are seen at E15.5 than MafB+ cells. Panels D and E represent consecutive sections stained to detect glucagon (red), nuclei (blue), and MafB or Nkx6.1 (green). The arrowheads denote glucagon+ MafB+ cells that are Nk6.1−, while arrows indicate MafB+ Nkx6.1+ cells that are glucagon+. Scale bar = 20 \( \mu \)m.

Fig. 8. MafB to MafA transition is not essential for Glut2 expression. Triple IHC detection of (A) Nkx6.1, (B) MafA, or (C) MafB expression (green) with Glut2 (red) and nuclei (DAPI blue) shows that at E15.5 only some of the transcription factor expressing cells are Glut2+ (arrow). Staining in panel E shows that some MafB+ insulin+ cells are Glut2− MafA− (arrowhead), thus demonstrating that MafA expression in the insulin+ cells is not essential for the expression of Glut2 at this stage of development. Scale bar = 20 \( \mu \)m.
expressed Ki67 (1.3%, 3 out of 230 MafB+ nuclei) (Figs. 9A–D). However, from shortly before birth up to 2 weeks after birth, the replication rate of endocrine cells is significant (Figs. 9E–H). At P14, the proportion of Ki67+ Nkx6.1+ cells was reduced significantly (5.3%) and was comparable to the proportion of Ki67+ MafA+ cells (7.7%), possibly reflecting the restriction of Nkx6.1 to the β-cells. Nearly 17% of MafB+ cells expressed Ki67, showing that MafB expression does not impede replication. It is important to note that the increased replication rate of the MafA+ and MafB+ cells after birth accompanies the change from co-expression to single expression of these factors. Even though the majority of

Fig. 9. MafB expression does not inhibit replication of insulin+ cells. Mouse pancreatic sections at E15.5 were stained to detect (A) NKX6.1, (B) MafA, or (C) MafB (green) with Ki67 (red) and DAPI (blue). Insets show higher magnification of demarcated areas. Arrows indicate cells expressing both Ki67 and the indicated transcription factor. (D) Quantitation of Ki67+ cells showed that nearly 25% of Nkx6.1 cells are in cell cycle, while MafA and MafB expressing cells do not express Ki67 at this stage. Expression of NKX6.1 (E), MafA (F), or MafB (G) (green) in Ki67 (red) positive cells in pancreas at P14. Panels H and I show Ki67 (red), insulin (blue), and either MafA (H) or MafB (I) in green. (J) Quantitation of at least five independent sections from three different mice for each transcription factor shows similar levels of Ki67+, Nkx6.1+, and MafA+ cells, while a higher proportion of MafB+ cells express Ki67. Panel I shows insulin+ MafB+ cell expressing Ki67 at P14 in the pancreatic islet. Scale bar = 20 μm.
Fig. 10. Pax6 expression precedes MafA expression. Consecutive sections from E15.5 pancreas stained to detect MafA (A) and Pax6 (B) show that all insulin+ cells express Pax6 but not MafA. Arrows in panel A indicate MafA+ cells. Several insulin+ Pax6+ cells that are MafA- are shown at the higher magnification. Total number of nuclei expressing the indicated factor at E15.5 from five adjacent pairs of sections from three different pancreases (C). Nearly 6-fold more nuclei were Pax6+ than MafA- at this stage of development ($P = 0.004$).

Fig. 11. Induction of PDX-1high in the insulin+ cells precedes the switch in expression from MafB+ to MafA+. Co-expression of MafA (A) and PDX-1high (B) in the insulin+ cells was determined by staining consecutive sections. Total number of nuclei expressing the indicated factor at E15.5 from five adjacent pairs of images from three different pancreases (C, F). At E15.5, nearly 66% more nuclei were expressing high level of PDX-1high than MafA ($P = 0.006$), yet at this stage not all insulin+ cells were PDX-1high. Arrows indicate PDX-1high cells that were MafA-, and arrowheads indicate insulin+ cells that are MafA+ PDX-1low. Panels D and E show that some insulin+ MafB+ cells were PDX-1low (arrows), while others were PDX-1high. There are nearly 2-fold more MafB+ than the Pdx-1high cells at this stage (F, $P = 0.01$). These results show that PDX-1high expression is induced after MafB but before MafA expression.
MafB+ cells at P14 are α-cell (Fig. 4L), we can still detect a few Ki67+ MafB+ insulin+ cells. Thus, our results suggest that the presence of MafB in the insulin+ cell does not prevent their replication. However, the paucity of MafB+ glucagon− cells at this stage and the fact that some MafB+ insulin+ cells may already express MafA preclude our being able to conclude whether the expression of MafB in the insulin+ cells impairs but does not prevent β-cell replication.

**PDX-1 expression precedes induction of MafA in MafB+ insulin+ cells**

Transcription factor Nkx6.1 is upstream of MafA (Matsuoka et al., 2004), but at E15.5 not all Nkx6.1+ insulin+ cells express MafA with a significant proportion being MafB+ (Fig. 7 and data not shown), suggesting that signals in addition to Nkx6.1 are needed to trigger MafA expression. Transcription factors PDX-1, HB9, and Nkx2.2 are widely expressed in the early pancreatic epithelium. With time, their expression gradually becomes restricted while others, such as Pax4, Pax6, and NeuroD1, are expressed in the endocrine progenitors, following Ngn3 expression. Recent studies demonstrate that the transcription factors Nkx2.2 and Pax4 work in parallel to enable β-cell differentiation (Prado et al., 2004; Wang et al., 2004). Nkx2.2, its downstream target Pax6, and Pax4 enhance expression of PDX-1, HB9, and Nkx6.1, which are required for the maturation of β-cells. Interestingly, in a recent review, Sosa-Pineda (2004) reports that loss of Pax4 inhibits MafA expression, suggesting a possible link between MafA expression and β-cell maturation. Unfortunately, there are no good antibodies to monitor Pax4 expression during pancreatic development and hence we cannot determine the role of this factor in MafB to MafA switch. A recent lineage-tracing study demonstrates a role for Pax6 in the expression of terminal differentiation markers such as insulin and Glut2 (Ashery-Padan et al., 2004). Since the MafB to MafA switch may regulate terminal differentiation, we analyzed the role of transcription factors PDX-1 and Pax6 in inducing MafA expression.

During lens development in the chicken, Pax6 is required for the expression of MafA/L-Maf, thus placing Pax6 upstream of MafA (Reza and Yasuda, 2004; Reza et al., 2002). To determine if Pax6 is upstream of MafA expression in the insulin+ cells, consecutive sections were stained for MafA-insulin and Pax6-insulin. Pax6 is expressed in all endocrine cells, so at E15.5, all insulin+ and several insulin− cells expressed it (Fig. 10B). MafA is expressed only in some insulin− cells (Fig. 10A), so several Pax6− insulin− cells were MafA− (indicated by arrows) while some were MafA− (shown in inset). Pax6 is expressed in all insulin+ cells, so we can conclude that Pax6 is upstream of MafA during β-cell differentiation but cannot determine if Pax6 is required for switching the expression from MafB to MafA.

To assess the role of PDX-1 in triggering MafA expression in MafB+ cells, consecutive sections from E15.5 pancreases were stained for insulin and MafA, MafB, or PDX-1 (Figs. 11A–D). As previously reported (Oster et al., 1998; Jensen et al., 2000), PDX-1 expression can be varied at this stage with most PDX-1high cells being insulin+ while few PDX-1low cells expressed insulin+. All MafA− cells were PDX-1high and only a few insulin+ PDX-1high cells did not express MafA (insets Figs. 11A, B). Since insulin− cells express at least one of the two large-Maf factors, Pdx-1low insulin+ MafA− cells and PDX-1high insulin+ MafA− cells must express MafB. Figs. 11C and D

![Fig. 12. Model of Maf factor transition and the differentiation of insulin+ cells. Endocrine precursors expressing both MafB and Nkx6.1 differentiate towards an insulin+ phenotype, while precursors expressing MafB alone follow a pathway to glucagon-expressing cells. In addition to MafB and Nkx6.1, initial insulin+ cells are Pax6+, PDX-1low, and MafA+. Subsequently, there is induction of PDX-1high expression, which is followed by expression of MafA in the insulin+ cells. Towards the later stages of embryonic development and the early neonatal period, insulin+ MafA+ MafB− cells can be seen and these then differentiate into insulin+ MafA+ MafB− cells.](image-url)
confirm these predictions and demonstrate that, at E15.5, insulin \(^{-}\) MafB\(^{+}\) cells could express either PDX-1\(^{\text{high}}\) or PDX-1\(^{\text{low}}\) but that MafA is expressed only in cells with high levels of PDX-1. These data suggest that PDX-1 is enhanced prior to the expression of MafA but after MafB expression and so may play a role in turning on MafA in MafB\(^{+}\) cells. MafA can regulate PDX-1. These data suggest that the MafB to MafA switch proceeds through a PDX-1\(^{\text{high}}\) intermediate stage. Analysis of endocrine-cell specific PDX-1 knockout mice (PDX-1\(^{\text{CreER}}\); Zhang et al., 2005b) could determine the role of PDX-1 in this switch.

In summary, we have shown that the differentiation of \(\beta\)-cells (insulin \(^{-}\) MafA\(^{+}\)) from pancreatic endocrine precursors goes through an intermediate stage of insulin \(^{-}\) MafB\(^{+}\) that after the induction of PDX-1 gives rise to insulin \(^{-}\) MafB MafA\(^{+}\) cells, which eventually become insulin \(^{-}\) MafA\(^{+}\) cells (Fig. 12). Since insulin \(^{-}\) MafB\(^{+}\) cells are detected first and can be seen even after birth, our results suggest that MafA expression is not necessary for the formation of insulin\(^{-}\) cells during embryonic development and that MafB is sufficient for the formation of insulin\(^{-}\) cells. This observation provides an explanation for the normal looking pancreatic islets in MafB knockout mice at postnatal day 1 (Zhang et al., 2005a). Our data provide strong evidence for a role of MafB in regulating differentiation of both \(\alpha\)- and \(\beta\)-cells, with the possibility that MafB, either independently or together with Nkx6.1, triggers insulin expression. Analyses of MafB and Nkx6.1 knockout mice for the expression of MafB, MafA, and insulin should further define the role of these factors in triggering insulin expression.

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