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RBM4 Promotes Pancreas Cell Differentiation and Insulin Expression

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The RNA-binding protein RNA-binding motif protein 4 (RBM4) modulates alternative splicing of muscle-specific mRNA isoforms during muscle cell differentiation. To better understand the physiological function of RBM4, we exploited a gene knockout strategy in the present study. Mice with targeted disruption of one of the two *Rbm4* genes exhibited hyperglycemia coincident with reduced levels of serum insulin and reduced size of pancreatic islets. The embryonic pancreases of *Rbm4*-deficient mice showed reduced expression or aberrant splicing of many transcripts encoding factors required for pancreas cell differentiation and function. Using pancreatic acinar AR42J cells, we demonstrated that RBM4 promoted insulin gene expression by altering the isoform balance of the transcription factors Isl1 and Pax4 via alternative splicing control. RBM4 overexpression was sufficient to convert AR42J cells into insulin-producing cells. Moreover, RBM4 may mediate glucose-induced insulin expression and insulin receptor isoform switches. These results suggest that RBM4 may have role in promoting pancreas cell differentiation and endocrine function, essentially via alternative splicing regulation.

The proteomic complexity of mammalian genomes is greatly expanded by the selective use of exons via alternative splicing. The spatiotemporal expression of alternatively spliced mRNA isoforms contributes substantially to cell differentiation and fate specification and hence influences organogenesis. Reprogramming of alternative splicing during cell differentiation can be achieved by induction of a tissue-specific splicing regulator or by switched expression of one factor to another one that has different or even antagonistic splicing activities. Alternative splicing also provides a mechanism for responding to metabolic prompts (1, 2). Gaining comprehensive insight into alternative splicing remains an imperative goal in the postgenome era.

The RNA-binding motif 4 (RBM4) protein has multiple functions in mRNA metabolism; it primarily modulates alterative splicing of precursor mRNAs and regulates mRNA translation (3). We previously reported that RBM4 represses expression of the splicing factor polypyrimidine tract-binding protein (PTB) via alternative splicing-coupled nonsense-mediated mRNA decay during muscle cell differentiation (4). Moreover, RBM4 also modulates the use of alternative exons of many mRNAs encoding muscle differentiation factors or cytoskeletal proteins. Therefore, we deduced that RBM4 promotes myogenesis through its role in altering the repertoire of mRNA isoforms. To further our understanding of the physiological function of RBM4, we attempted to exploit the gene knockout strategy in mice. Previous splicing factor knockout and transgenic studies have revealed the functional consequences of alternative splicing in mammalian development and physiological processes (5). For example, cardiac tissue-specific disruption of the splicing factor gene *Srsf1* resulted in abnormal cardiac phenotypes in mice and, moreover, revealed a group of transcripts that are targets of SRSF1 and are involved in sarcomere function and Ca^{2+} signaling (6).

Here, by using *Rbm4* knockout mice, we found that RBM4 may also play a role in pancreas cell differentiation. The pancreas is a heterogeneous organ that comprises exocrine acinar cells and ducts that produce and deliver, respectively, digestive enzymes, and it contains islets of Langerhans that secrete various endocrine hormones (7). Differentiation of pancreatic endocrine cells is tightly controlled by the sequential expression and activation of transcription factors (8). The pancreatic and duodenal homeobox 1 gene (*Pdx1*) governs early pancreas development. Pdx1-expressing endodermal cells are the common progenitors of both endocrine and exocrine lineages that appear at the 15-somite stage and diverge in mice at embryonic days 9.5 to 13.5. The transcription factor neurogenin-3 (Ngn3) is expressed only in endocrine progenitor cells and is required for the specification of the endocrine lineage. Subsequently, two paired-box family transcription factors, Pax4 and Pax6, trigger the differentiation of the endocrine pancreatic α (glucagon-producing) and β (insulin-producing) cells, respectively. Moreover, Pax4 and Pax6, as well as several other transcription factors, including the LIM/homeodomain protein islet 1 (Isl1), differentially regulate the expression of pancreatic endocrine hormones. The isoforms of Pax4 and Pax6 and Isl1 are generated by alternative splicing and possess different transcriptional potencies (9–11). It is as yet unclear how the expression of these isoforms is regulated and how they coordinately modulate endocrine expression and even fine-tune pancreas development.

Herein, we provide evidence for defective pancreas cell differentiation and function in Rbm4-deficient mice and demonstrate that RBM4 promotes the expression of pancreatic differentiation factors and insulin essentially via its role in the regulation of alternative splicing.

MATERIALS AND METHODS

Generation and characterization of *Rbm4* knockout mice. The *Rbm4a* and *Rbm4b* targeting constructs were established by using a recombineer-

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ing procedure (12). In the mouse genome, the *Rbm4a* and *Rbm4b* genes are oriented in opposite directions, but their exons and introns are similarly organized, with four exons and three introns (see Fig. S1A in the supplemental material). A 13-kb DNA genomic fragment consisting of the *Rbm4a* and *Rbm4b* genes was retrieved from the bacterial artificial chromosome (BAC) clone BMQ-447O5 (Wellcome Trust Sanger Institute) and inserted into the vector ploxpNT (13) to replace the *loxP-neo* cassette (see Fig. S1A). To generate the *Rbm4a* targeting vector, an *Rbm4a* gene fragment containing most of exon 2 was deleted and replaced with a cassette containing a promoterless enhanced green fluorescent protein (EGFP) gene (14) and a neomycin resistance gene *(neo)* flanked by two *loxP* sites from plasmid pL452 (12). The *Rbm4b* targeting vector was analogously constructed but contained a promoterless DsRed-mammalian sterile 20 (MST) gene. Therefore, the EGFP and DsRed-MST reporter genes were under the transcriptional control of the *Rbm4a* and *Rbm4b* promoters, respectively. The targeting vectors were then linearized with NotI and electroporated into R1 mouse embryonic stem cells. We confirmed integration of the targeting sequences in G418- and ganciclovir-resistant embryonic stem clones using Southern blotting. Subsequently, the homologous recombinant clones were injected into C57BL/6 blastocysts and transferred to pseudopregnant imprinting control region (ICR) mice to produce chimeras. The resulting chimeric males were mated with C57BL/6 females to transmit the disrupted *Rbm4* allele to the agouti 129-C57BL/6 hybrid offspring. Following the standard procedure (15), heterozygous mice were generated and their genotypes confirmed by PCR analysis (see Fig. S1B in the supplemental material).

Glucose metabolic assays. The prestudy body weights (BW) of the mice were recorded. We followed standard procedures (16) for performing glucose and insulin tolerance tests on 8-week-old *Rbm4* knockout mice and wild-type littermates. Essentially, after an overnight fast, mice were orally fed glucose (1.5 mg/g BW) or intraperitoneally injected with recombinant human insulin (0.75 U/g BW) (Sigma) for the glucose tolerance and insulin tolerance tests, respectively. The blood glucose levels were measured using a glucometer (Roche) at various time points.

Histological examination and fluorescence microscopy. For immunohistochemical analyses, the pancreases of 16-week-old Rbm4 knockout or wild-type mice were fixed in 4% paraformaldehyde at 4°C overnight and embedded in paraffin. Deparaffinized sections (5 μm thick) were incubated with antibodies against insulin or glucagon (1:1,000) (Abcam). The secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (1:200) (Jackson ImmunoResearch Labs). Staining was performed using 3,3-diaminobenzidine tetrahydrochloride (Dako) as the substrate. Hematoxylin-eosin staining was performed using pancreas tissues from 8-week-old mice; samples were examined at low magnification with an Olympus BX51 microscope. On embryonic day 13.5 (E13.5), mouse embryos of wild-type or Rbm4a or Rbm4b knockout mice were placed in petri dishes filled with phosphate-buffered saline (PBS) and examined by fluorescence microscopy using a Zeiss Lumar V12 fluorescence microscope equipped with a charge-coupled-device (CCD) camera (AxioCam HRc) and filters for DsRed (excitation, 545 ± 15 nm; emission, 620 ± 30 nm) and GFP (excitation, 470 ± 20 nm; emission 525 ± 25 nm).

Preparation of MEFs. Mouse embryonic fibroblasts (MEFs) were prepared as reported (17) from individual E14.5 embryos bearing the wild-type, *Rbm4a*$^{-/-}$, or *Rbm4b*$^{-/-}$ genotype. In brief, the head and internal organs were removed, and the rest of the trunk was minced and dispersed in 0.25% trypsin for 20 min at 37°C. Cells were grown, maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, and used for experiments within four passages.

Cell cultures and transfection. HEK293 cells were cultured and transfected as described previously (18). Rat AR42J cells were grown in DMEM containing 2.5 mM glucose and 10% fetal bovine serum; the medium was refreshed every 2 days. For glucose challenges, cells were treated with 40 mM glucose for 1 h.

Adenovirus transduction. To overexpress the RBM4 protein, we used the adenovirus expression system. The full-length mouse *Rbm4a* cDNA was PCR amplified and inserted into the KpnI-XhoI sites of the vector pShuttle-CMV (cytomegalovirus) (Agilent Technologies). Recombinant Rbm4-expressing adenovirus Ad-Rbm4 was produced by homologous recombination of pShuttle-CMV-Rbm4 with the pAdEasy-1 adenoviral vector (Agilent Technologies) in *Escherichia coli* strain BJ5183 according to the manufacturer's instructions. The adenoviral vector expressing an *Rbm4*-targeting short hairpin RNA (Ad-shRbm4) has been described (18). Adenovirus propagation and titers were determined according to the manufacturer's instructions. The adenoviral vector expressing Ngn3 was a kind gift of Chia-Ning Shen (Academia Sinica).

Plasmid construction. The pGL4.10-hINS (pGL-hINS) reporter containing the human insulin promoter was a kind gift of Kevin Ferreri (Beckman Research Institute of City of Hope, CA). The expression vector encoding FLAG-tagged RBM4 has been described (18). The cDNAs encoding mouse Isl1 and Pax4 and their isoforms were obtained by reverse transcriptase PCR (RT-PCR) using total RNA prepared from MEFs as the template and then inserted into pcDNA3.1 (Invitrogen) to generate the FLAG-tagged expression vectors; the constructs were confirmed by DNA sequencing.

ELISAs. Measurements of the insulin levels in mouse serum and in AR42J cell culture supernatants were performed with the rat/mouse insulin enzyme-linked immunosorbent assay (ELISA) kit from Millipore according to the manufacturer's protocol.

RNA extraction and RT-PCR analyses. Total RNA was extracted from cultured cells or tissues using TRIzol reagent (Invitrogen). For RT-PCR, 2 μg of RNA was reverse transcribed with SuperScript III (Invitrogen) in a 10-μl reaction mixture followed by PCR using specific primers. After electrophoretic separation, the resulting products were blotted and probed with specific ^{32}P-labeled forward primers.

Immunoblotting. Immunoblotting of cell lysates was performed using the enhanced chemiluminescence system (Millipore). The images were analyzed by the LAS-3000 imaging system (Fujifilm). The primary antibodies used in this study included polyclonal antibodies against RBM4 (18), FLAG epitope (Sigma-Aldrich), tubulin (Thermo Fisher Scientific), total and phosphorylated p38 mitogen-activated protein kinase (MAPK) (Cell Signaling Technology), AKT (Cell Signaling Technology), and p44/p42 MAPK (Cell Signaling Technology).

Transcription assay. HEK293 cells were seeded in 12-well plates (2×10^5 cells/well) 24 h prior to transfection. The transfection reaction mixture contained 0.25 μg of the pGL-hINS firefly luciferase reporter, 1 μg of effector-expressing vector, and 0.1 μg of the pRL-SV40 *Renilla* luciferase vector (Promega) as a reference. After 24 h, the transfectants were collected and lysed with Promega passive lysis buffer, and cell debris was removed following centrifugation. The activities of firefly and *Renilla* luciferases were determined using the dual-luciferase assay kit (Promega).

RESULTS

Aberrant splicing in *Rbm4*-deficient mice. Mouse genomes have two adjacent copies of *Rbm4* (*Rbm4a* and *Rbm4b*) that are oriented in opposite directions on the same chromosome, but their exons and introns are organized similarly (Fig. 1A). We disrupted each *Rbm4* gene by a conventional strategy (see Materials and Methods; see also Fig. S1A in the supplemental material) and generated homozygous mutant progeny, namely, *Rbm4a*$^{-/-}$ and *Rbm4b*$^{-/-}$ mice, whose genotypes were also confirmed by PCR (Fig. 1B). These homozygous mice were viable and fertile without an obvious phenotype. *Rbm4a* disruption completely abolished *Rbm4a* mRNA expression in mouse embryonic fibroblasts (MEFs), as was expected, but somewhat increased the *Rbm4b* mRNA level (Fig. 1C). An analogous result was observed in *Rbm4b*$^{-/-}$ MEFs (Fig. 1C); the mechanism by which ablation of one *Rbm4* copy resulted in increased expression of the other is still

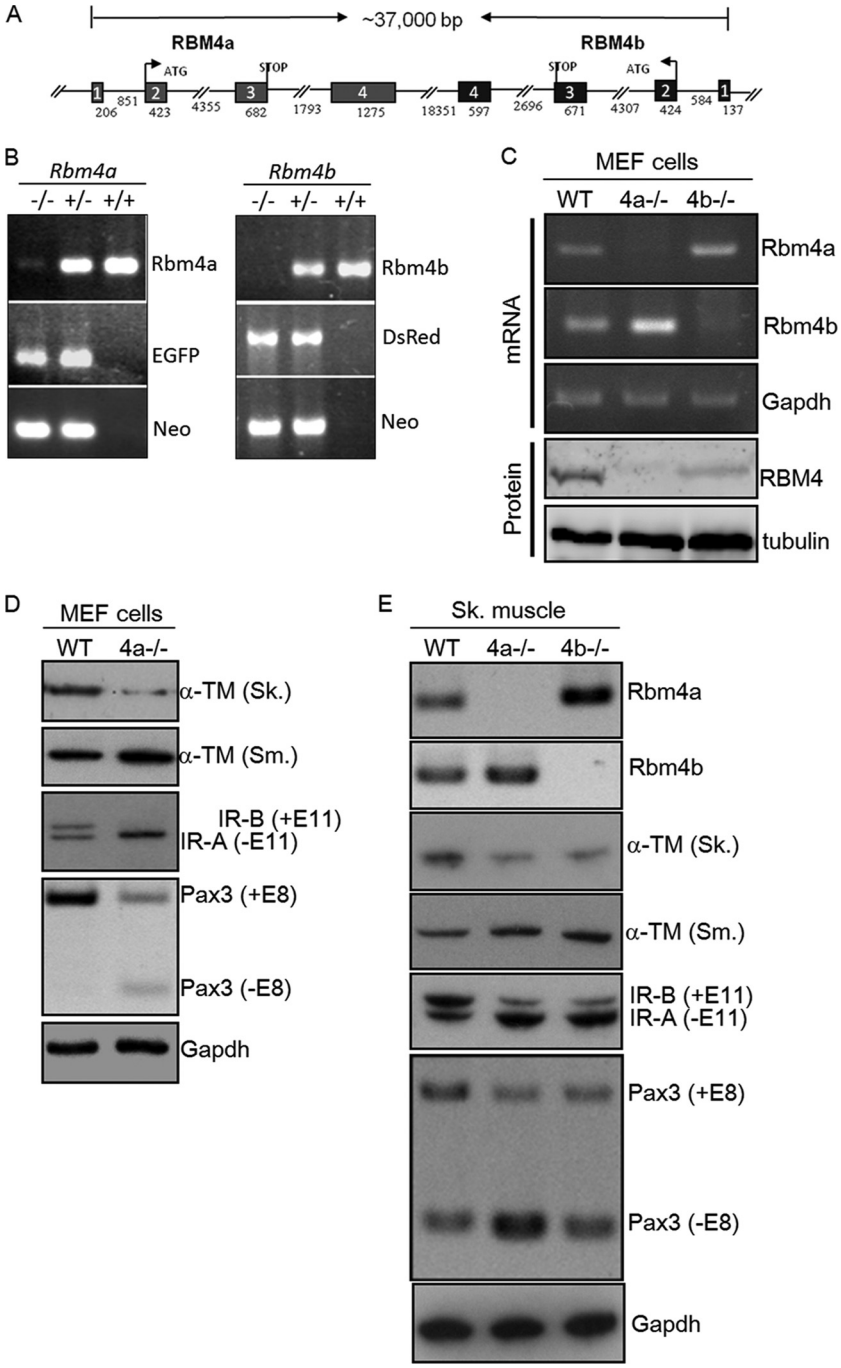

FIG 1 *Rbm4*-deficient mice exhibit aberrant splicing. (A) Schematic of mouse *Rbm4* gene organization. (B) Genotyping of *Rbm4* knockout mice. Genomic DNA of wild-type, *Rbm4a*, or *Rbm4b* knockout mice was analyzed by PCR using specific primers (see Table S1 in the supplemental material). (C) Top, total RNA of wild-type or *Rbm4* knockout MEFs was examined by RT-PCR using gene-specific primers. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as a control. Bottom, immunoblotting of whole-cell lysates was performed using an antibody against RBM4 or α-tubulin. WT, wild type. The anti-RBM4-reactive band with a slightly lower rate of gel mobility in *Rbm4b*$^{-/-}$ MEFs represents RBM4a. (D) Total RNA of WT or *Rbm4a*$^{-/-}$ MEFs was examined by RT-PCR using primer sets (see Table S1) that distinguish splice isoforms of α-tropomyosin (α-TM) (sk, skeletal; sm, smooth), IR, and Pax3. Gapdh was used as a loading control. (E) RT-PCR was performed using total RNA extracted from skeletal muscles of the WT and *Rbm4* knockout mice. For panels D and E, the PCR products were blotted and detected with ^{32}P-labeled forward primers.

unclear. Nevertheless, immunoblotting showed a significant reduction of RBM4 protein levels in *Rbm4a*$^{-/-}$ MEFs and a moderate reduction in *Rbm4b*$^{-/-}$ MEFs (Fig. 1C). Because the expressions of the EGFP and DsRed-MST transgenes were under the control of the *Rbm4a* and *Rbm4b* promoters, respectively, we examined their distributions in *Rbm4* knockout mouse embryos. As shown in Fig. S1C in the supplemental material, both fluorescent proteins were expressed in all tissues, but the signal of DsRed-

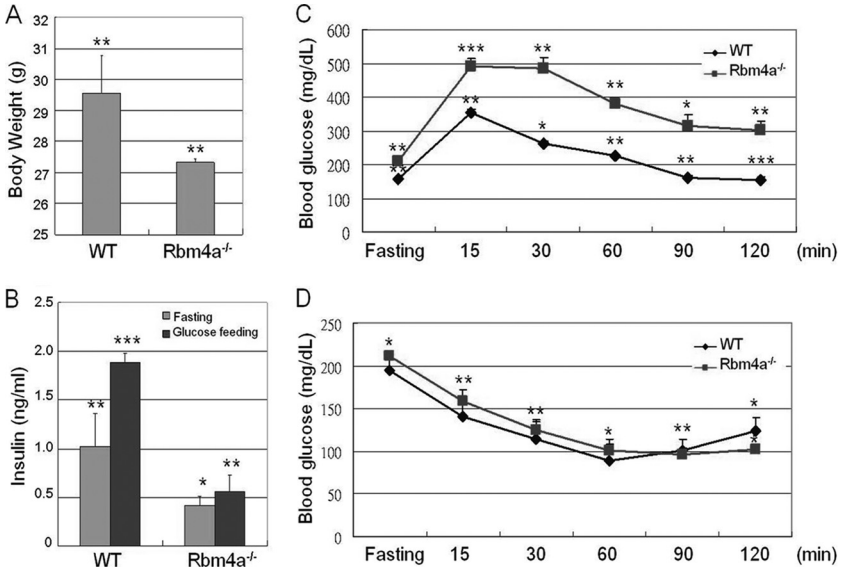

FIG 2 *Rbm4*-deficient mice exhibit aberrant glucose metabolism. Eight-week-old wild-type (WT) and *Rbm4a*$^{-/-}$ male mice were subjected to the following measurements or tests: body weight (WT, 30.90 ± 1.247 g [$n = 5$], *Rbm4a*$^{-/-}$, 27.10 ± 0.509 g [$n = 5$]; $P < 0.01$) (A), fasting and postprandial levels of serum insulin (B), glucose tolerance tests of fasting and refed mice (C), and insulin tolerance tests of fasting and refed mice (D). For panels B through D, the mean and standard deviation were obtained from three mice (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

MST was high in the neural tube, suggesting that RBM4b may have a role in the developing central nervous system.

Next, we checked the alternative splicing patterns of several RBM4 target transcripts in *Rbm4* knockout mice. RT-PCR showed that the expression level of the skeletal muscle isoform of α-tropomyosin was reduced, with a concomitant increase in the smooth muscle isoform level, in *Rbm4a*$^{-/-}$ and *Rbm4b*$^{-/-}$ MEFs and in the muscle and heart of 8-week-old knockout mice (Fig. 1D and E; see also Fig. S1D and E in the supplemental material), consistent with our previous observations in RBM4-depleted HEK293 cells (18). Moreover, we observed that *Rbm4* knockout MEFs and adult muscles had higher levels of exon 11-skipped insulin receptor (IR), i.e., the insulin-insensitive IR-A isoform encoded by *IR* (19), and exon 8-skipped *Pax3*, which encodes a dominant negative isoform of the muscle transcription factor Pax3 (20) (Fig. 1D and E). Therefore, this study has confirmed the involvement of RBM4 in the alternative splicing regulation of transcripts involved in muscle cell differentiation, as observed previously *in vitro* (4).

Defective glucose metabolism in Rbm4-deficient mice. Newborn *Rbm4* knockout mice did not exhibit any substantive growth defects, but at 8 weeks their body weights were decreased by ~10% compared to their wild-type littermates (Fig. 2A). We measured glucose and insulin metabolism indices for *Rbm4* knockout mice. The results show that fasting *Rbm4a*$^{-/-}$ male mice had significantly lower levels of serum insulin (~0.45 ng/ml) than did the wild-type mice (~1 ng/ml) and had an inefficient insulin response to glucose challenge (Fig. 2B). The results of the glucose tolerance test show that *Rbm4a*$^{-/-}$ mice were hyperglycemic (~218 mg/dl versus ~158 mg/dl in wild-type mice) after an 8-h fast, and their blood glucose levels were 1.5- to 1.7-fold higher than those of the wild-type mice after glucose feeding (Fig. 2C). Similar results were observed for the *Rbm4b*$^{-/-}$ mice (see Fig. S2 in the supplemental material). This hyperglycemic tendency of *Rbm4* knockout mice was consistent with their lower insulin levels (Fig. 2B). Nevertheless, within 60 min after intravenous injection of insulin, *Rbm4a*$^{-/-}$ mice showed rates of glucose decline similar to those of wild-type mice (Fig. 2D), indicating the apparently normal insulin sensitivity of *Rbm4a*$^{-/-}$ mice. However, blood glucose levels of the *Rbm4* knockout mice recovered slowly from hypoglycemia in the later stages of the insulin tolerance test, which indicates that they might have had additional defects in glucose metabolism (Fig. 2D). Together, these results suggest a role for RBM4 in glucose metabolism.

RBM4 promotes the expression of endocrine differentiation factors and insulin. Next, we examined pancreas morphology in Rbm4 knockout mice. Hematoxylin and eosin staining of mouse pancreas sections showed significantly and moderately reduced islet sizes in *Rbm4a* and *Rbm4b* knockout mice, respectively (Fig. 3A, top row). This observation was confirmed by immunohistochemical analyses using anti-insulin and antiglucagon (Fig. 3A, top row) and also coincided with the reduced levels of serum insulin (Fig. 2B). Next, using RT-PCR, we observed that the insulin mRNA levels in *Rbm4a*$^{-/-}$ embryonic pancreases were largely reduced (~16% of wild-type levels) and that the glucagon and somatostatin levels were also reduced by ~50% (Fig. 3B). Moreover, RT-PCR showed that the expressions of transcription factors for islet differentiation, including *Pdx1*, *Ngn3*, *Neurod1*, and *MafA*, in *Rbm4a*$^{-/-}$ embryonic pancreases decreased to different extents compared to those in wild-type mice (Fig. 3C). These results revealed that Rbm4 deficiency impaired the formation of pancreatic islets and diminished the expression of genes governing endocrine cell fate.

Acinar tumor-derived AR42J cells exhibit endocrine properties upon overexpression of Ngn3 and produce insulin upon glucose challenge (21). Using adenoviral vector-mediated gene transfer into AR42J cells, we observed that RBM4 overexpression sufficed to induce the expression of several pancreatic transcription factors that we examined as described above and that the effect of RBM4 was, in general, more efficient than that of Ngn3 (Fig. 3D). More-

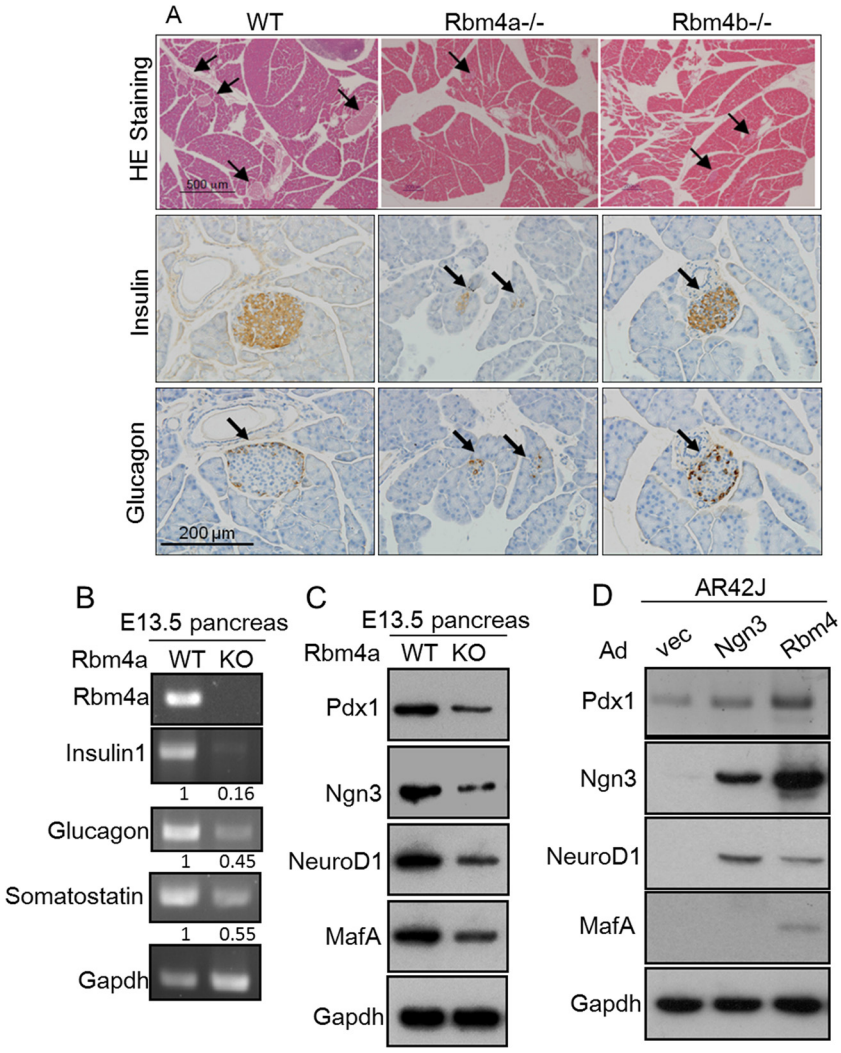

FIG 3 RBM4 promotes the expression of pancreatic differentiation factors. (A) Hematoxylin and eosin (HE) staining and immunohistochemical analyses using anti-insulin and antiglucagon were performed on pancreatic sections of wild-type (WT), *Rbm4a*$^{-/-}$, and *Rbm4b*$^{-/-}$ mice. (B) Total RNA extracted from WT and *Rbm4a*$^{-/-}$ E13.5 pancreases was analyzed by RT-PCR using gene-specific primers (see Table S1 in the supplemental material). The number below each panel indicates the relative level of each RT-PCR product of *Rbm4a*$^{-/-}$ compared to that of WT. Gapdh was used as a loading and normalization control. (C) RT-PCR was performed as described for panel B using primers (see Table S1 in the supplemental material) specific to the indicated factors, and the PCR products were then blotted and detected with ^{32}P-labeled forward primers. (D) Total RNA was extracted from mock-infected (vec) or Ad-Ngn3- or Ad-Rbm4-infected AR42J cells and subjected to RT-PCR and blotting analyses as described for panel C.

over, the overexpression of RBM4 significantly elevated the level of insulin mRNA in AR42J cells, whereas Ngn3 was insufficient to induce insulin expression, as reported previously (21) (see Fig. 5A). Therefore, RBM4 may act as a promoting factor in the conversion of AR42J cells into an endocrine lineage.

RBM4 promotes insulin expression by modulating alternative splicing of pancreas-specific transcription factors. Next, we attempted to explore whether RBM4 regulates insulin expression via its role in alternative splicing regulation. We evaluated spliced isoform expressions of several islet-specific transcription factors, such as Isl1, Pax4, and Pax6, and membrane receptor and transporter proteins, such as IR and glucose transporter 2 (Glut2) (9–11, 19, 22) in *Rbm4* knockout embryos. Total RNA from E13.5 pancreases was analyzed by RT-PCR using specific primer pairs that could distinguish splice isoforms (see Table S1 in the supplemental material). The results show that the splicing patterns of all examined transcripts were altered in the *Rbm4a*$^{-/-}$ embryonic pancreases (Fig. 4A), indicating that RBM4 may participate in the control of alternative splicing of at least some pancreatic transcripts that encode factors important for pancreas cell differentiation and function. We assessed this possibility by overexpressing RBM4 in AR42J cells. The results show that RBM4 overexpression reversed the altered pattern of isoform expression observed for all transcripts in *Rbm4*-deficient embryonic pancreases (Fig. 4B and 5A; see also Fig. S3 in the supplemental material). RBM4 could promote the use of an alternative 3′ splice site within exon 5 of *Isl1* and exon 7 skipping of *Pax4*, resulting in the expression of Isl1β and Pax4v, respectively. Using immunoprecipitation-RT-PCR, we observed that RBM4 associated with endogenous *Isl1* and *Pax4* transcripts (see Fig. S4 in the supplemental material), suggesting that RBM4 plays a direct role in alternative splicing regulation of these two transcripts. Moreover, RBM4 overexpression raised the

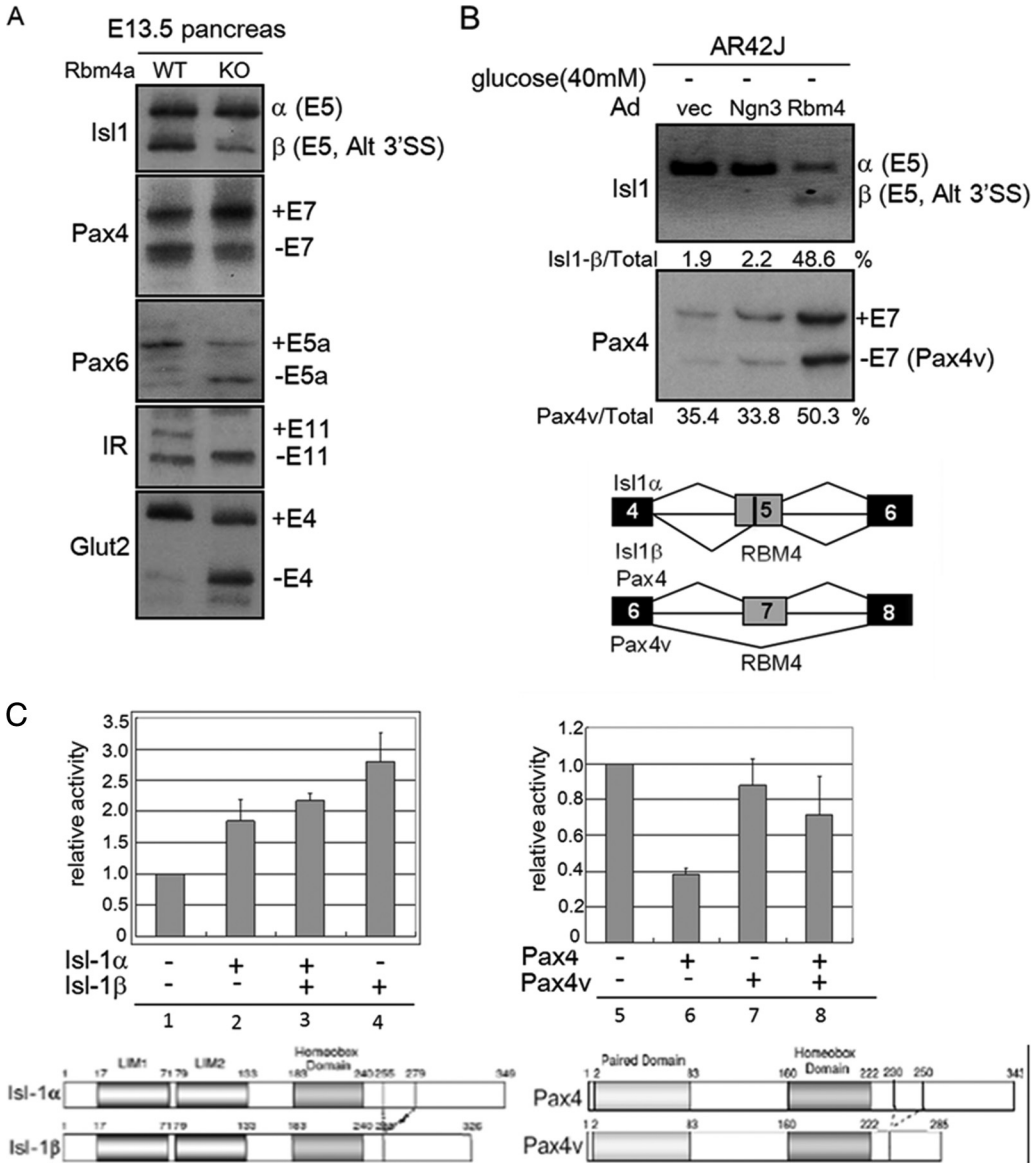

FIG 4 RBM4 promotes insulin expression by modulating alternative splicing of pancreas-specific transcription factors. (A) RT-PCR was performed on total RNA obtained from wild-type (WT) and *Rbm4a*$^{-/-}$ embryonic mouse pancreases using primers (see Table S1 in the supplemental material) that could specifically distinguish the splice isoforms of the indicated genes. The PCR products were detected with ^{32}P-labeled primers as described for Fig. 3C. E, exon; Alt 3′SS, alternative 3′ splice site. (B) Alternative splicing of Isl1 and Pax4 was examined as described for panel A using total RNA extracted from mock-infected (vec) or Ad-Ngn3- or Ad-Rbm4-infected AR42J cells. The percentage below each panel shows the Isl1β/total Isl1 and Pax4v/total Pax4 ratios. The schematic diagram shows alternative splicing of Isl1 and Pax4. (C) HEK293 cells were cotransfected with the pGL-hINS firefly luciferase reporter, the expression vector of effectors, and the pRL-SV40 *Renilla* luciferase vector as a control. The bar graph shows the relative firefly versus *Renilla* luciferase activities; the means and standard derivations were obtained from three experiments. The diagram shows Isl1 and Pax4 proteins and RBM4-induced isoforms.

overall *Pax4* transcript level, suggesting that RBM4 may induce *Pax4* expression, reminiscent of a similar effect of RBM4 on other pancreatic transcription factors (Fig. 3D). Overall, our data indicate a direct role for RBM4 in alternative splicing regulation during pancreas development.

The transcription factors Isl1 and Pax4 directly regulate insulin promoter activity (23). The Isl1β and Pax4v isoforms are expressed in rodent insulinomas and appear to exhibit altered transcriptional properties (9, 11). Thus, we suspected that the reduced expression of insulin in *Rbm4* knockout mice resulted from unbalanced isoform expression of some transcription factors. Therefore, we examined the activity of RBM4-induced transcription factor isoforms in insulin expression. Using the firefly luciferase reporter driven by the human insulin promoter (pGL4.10-hINS [pGL-hINS]) (24), we observed that overexpression of either Isl1 isoform could stimulate insulin promoter activity but that Isl1β was more potent than Isl1α in transactivation (Fig. 4C, Isl1), as has been reported (9). Moreover, FLAG-tagged Pax4 suppressed the insulin promoter by ~60%, whereas its exon 7-skipped isoform Pax4v had only a minor effect (Fig. 4C, Pax4, lanes 6 and 7). Coexpression of Pax4v with Pax4 compromised the suppressive effect of Pax4 on transcription (lane 8), suggesting that an in-

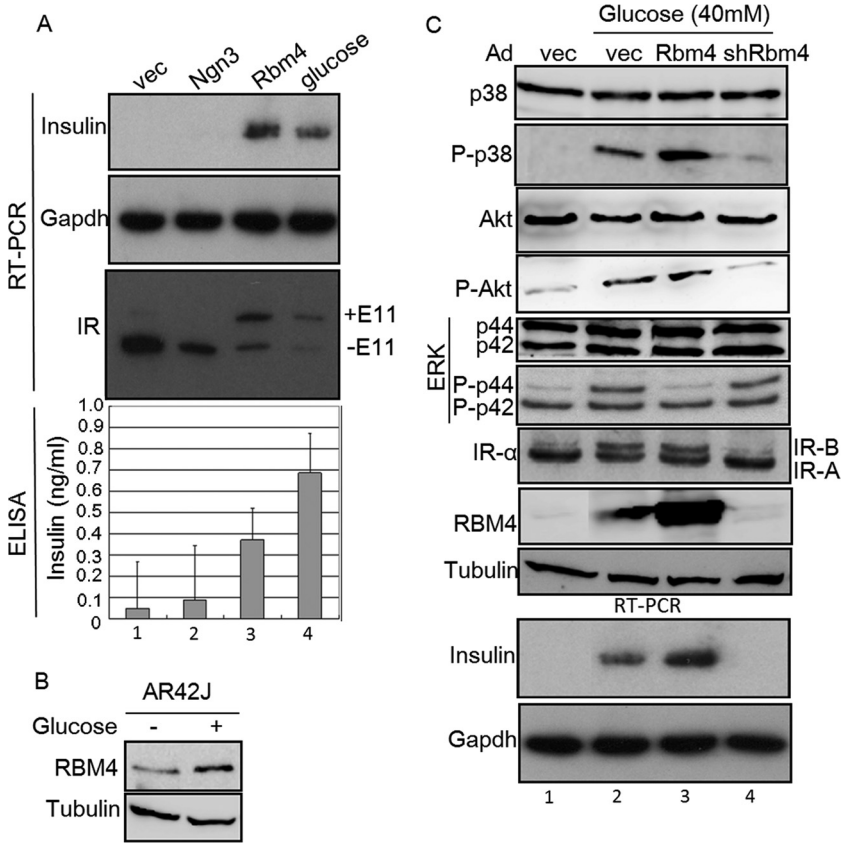

FIG 5 RBM4 plays a role in glucose-induced signaling pathways. (A) AR42J cells were infected with an empty vector (vec), Ad-Ngn3, or Ad-Rbm4 for 24 h or treated with 40 mM glucose for 1 h. The expression levels of insulin and Gapdh mRNAs were examined by RT-PCR and blotting analyses. Alternative splicing of IR was examined as described for Fig. 4A. The bar graph shows the levels of secreted insulin in virus-infected AR42J cells as determined by ELISA; the means and standard deviations were obtained from three independent experiments. (B) Immunoblotting was performed using specific antibodies against RBM4 or α-tubulin in lysates prepared from mock- or glucose-treated AR42J cells. (C) AR42J cells were infected with empty vector (vec), Ad-Rbm4, or Ad-shRbm4 and then treated with 40 mM glucose, except for lane 1. Immunoblotting of cell lysates was performed using a specific antibody against each indicated protein. The bottom two panels show RT-PCR analyses of insulin and Gapdh mRNA as described for panel A.

creased Pax4v/Pax4 ratio could relieve Pax4-suppressed insulin expression. Nevertheless, none of the tested transcription factors affected the transcription activity of the mock reporter (see Fig. S5 in the supplemental material). Therefore, RBM4-mediated alternative splicing of Isl1 and Pax4 may play a role in modulating insulin expression. Moreover, alternative splicing may act as a mechanism for controlling the balance between the isoforms of pancreas-specific transcription factors during development.

RBM4 plays a role in glucose-induced IR signaling. Glucose can stimulate insulin expression and activate alternative splicing of IR transcripts to produce the IR-B isoform (25). Indeed, we obtained the same result in AR42J cells treated with 40 mM glucose (Fig. 5A, lane 4). RBM4 overexpression also induced insulin mRNA expression and promoted exon 11 inclusion of the IR transcript (lane 3). The similar effects of glucose treatment and RBM4 overexpression prompted us to further examine whether RBM4 mediates glucose-induced cell signaling. Moreover, our ELISA result showed a modest increase in secreted insulin upon RBM4 overexpression compared to glucose treatment (Fig. 5A, ELISA), suggesting that RBM4 induces insulin expression primarily at the transcriptional level, whereas glucose can also stimulate insulin secretion (26).

We explored how RBM4 may mediate glucose signaling in AR42J cells. Using RT-PCR, we observed that glucose treatment differentially increased the mRNA levels of both *Rbm4* genes (see Fig. S6 in the supplemental material). Accordingly, the RBM4 protein level was also increased (Fig. 5B). Next, we examined whether RBM4 influences glucose-induced signaling effects. Glucose treatment activates several protein kinase signaling pathways. As has been reported (27–29), we observed enhanced phosphorylation of Akt (on Thr308), extracellular signal-regulated kinase 1/2 (ERK1/2) (Thr202/Tyr204), and p38 kinase (Thr180/Tyr182) in glucose-treated AR42J cells (Fig. 5C, lane 2). Glucose also induced the expression of the IR-B isoform detected by antibodies against the IR-α subunit as well as insulin mRNA expression (lane 2). RBM4 overexpression in AR42J cells treated with glucose caused a prominent enhancement in p38 phosphorylation (lane 3). More importantly, RBM4 knockdown mediated by a short hairpin RNA completely abolished glucose-induced p38 MAPK and Akt activation and the expression of IR-B and insulin (lane 4), suggesting an important role for RBM4 in glucose-induced signaling. Moreover, we noted that RBM4 overexpression prevented glucose-induced ERK phosphorylation, whereas its absence had no effect on ERK (lanes 3 and 4). We reason that this was because RBM4 induced the expression of IR-B, which may further the p38 MAPK and Akt pathways but disfavor ERK activation (see Discus-

sion for details) (30). Therefore, RBM4 may act as an effector in glucose-induced IR signaling pathways.

Overall, our data demonstrate that RBM4 promotes the expression of insulin and a splicing switch of IR toward the B isoform, which exhibits a high affinity for insulin. Therefore, we conclude that RBM4 likely plays a positive role in modulating gene expression toward the endocrine cell lineage.

DISCUSSION

We have shown that mice carrying a knockout of a single *Rbm4* gene developed essentially normally and survived long after birth, unlike mice with knockouts of several other splicing factors, each of which often causes early arrest of embryogenesis (5). This difference was probably due to the presence of two highly homologous copies of *Rbm4* and their presumed redundant functions. Nevertheless, ablation of a single copy of *Rbm4* still perturbed alternative splicing of many muscle and pancreatic transcripts in embryos and adults (Fig. 1 and 4), similar to the scenario of pathological dysregulation or tissue-specific depletion of other splicing factors (31). This study not only confirms the role of RBM4 as an important splicing regulator during development but also may assist future identification of tissue- or development-specific mRNA isoform expression owing to RBM4 deficiency.

A hierarchical role for RBM4 in pancreatic gene expression. Our study showed that *Rbm4*-deficient mice exhibited reduced pancreatic islet sizes, lower fasting serum insulin levels, and glucose intolerance (Fig. 2). Depletion of a single *Rbm4* gene was sufficient for reducing the expression or altering the splicing pattern of several pancreatic transcripts during embryogenesis (Fig. 3 and 4). Using AR42J cells, we demonstrated that RBM4 regulated the alternative splicing of several pancreatic transcription factors; hence, RBM4-induced isoforms elevated insulin gene transcription (Fig. 4). Moreover, we showed that RBM4 acts as an important mediator in glucose-induced cellular signaling and insulin expression (Fig. 5C). Overall, our results indicate that RBM4 modulates gene expression and signaling to promote pancreas function and differentiation.

Recent reports have begun to reveal how alternative splicing plays a critical role in stem cell programming, tissue differentiation, and development (2). For example, the neuronal splicing factor nSR100 promotes the inclusion of a neuron-specific exon of the transcription factor REST and thereby attenuates its repressive effects on the transcription of neuron-specific genes; this alternative splicing event consequently modifies transcriptional networks to promote neurogenesis (32). Analogous to nSR100, RBM4 modulated alternative splicing of at least two pancreatic transcription factors and generated the isoforms that could coordinately augment insulin gene transcription (Fig. 4). Moreover, RBM4 enhanced the expression of several pancreatic transcription factors (Fig. 3), perhaps via a role in modulating pancreas cell signaling (see "The potential role of RBM4 in the pancreas cell signaling circuit" below). Therefore, RBM4 may modulate the expression levels and spliced patterns of mRNAs during pancreas cell differentiation. This is somewhat analogous to the scenario of RBM4 in myoblasts, in which RBM4 downregulates the expression of the splicing regulatory factor PTB and also antagonizes its activity in splicing regulation and thereby promotes the expression of muscle-specific mRNA isoforms (4). Therefore, RBM4 plays a hierarchical role in transcriptome regulation in muscles and the pancreas.

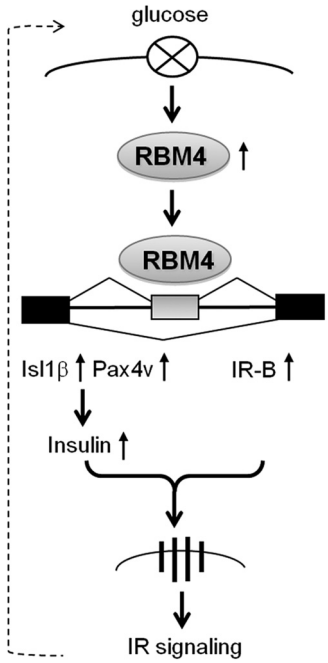

FIG 6 Model for an RBM4-regulated gene expression and signaling circuit in the pancreas. RBM4 modulates alternative splicing of many pancreatic factors, including Isl1, Pax4, and IR. RBM4-induced Isl1 and Pax4 isoforms can increase insulin gene transcription. Moreover, the RBM4-induced IR-B isoform likely exhibits higher signaling activity upon insulin binding, which in turn increases glucose uptake and signaling (dashed line).

The potential role of RBM4 in the pancreas cell signaling circuit. We repeatedly observed that RBM4 modulates exon 11 inclusion of IR and promotes IR-B isoform expression in various cell lines and tissues (Fig. 1, 4, and 5)(4). We also showed that RBM4 acted as a positive effector in glucose signaling pathways and that its overexpression was sufficient to mimic the effect of glucose that induces insulin and IR-B isoform expression (Fig. 5). IR-B is the predominant isoform in differentiated pancreatic β cells and shows higher sensitivity to insulin (30). IR-B-activated p38 MAPK signaling is important for both pancreatic development and insulin production from β cells (19). Insulin also activates the phosphatidylinositol-3-kinase–Akt pathway to promote β-islet survival and function (33). Therefore, increased insulin and IR-B levels may further enhance the p38 MAPK and Akt signaling pathways. Coincidently, we showed that RBM4 was responsible for signal-activated p38 MAPK and Akt signaling when cells were challenged with glucose (Fig. 5). Perhaps glucose-induced RBM4 expression permits a more efficient transmission of insulin and/or IR signaling and further increases glucose uptake (Fig. 6). Therefore, we hypothesize that RBM4 acts as a positive mediator in the pancreas cell signaling circuit.

In conclusion, our data show for the first time that RBM4 can function by integrating both the transcriptional and splicing regulatory networks into coherent gene expression programs and may establish a positive-control circuit that promotes pancreatic endocrine activity. Furthermore, the findings of RBM4-mediated gene expression and signaling that help to promote β-islet function may have clinical implications with respect to restoring impaired islet cell function and insulin sensitivity in patients with diabetes.

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We declare no conflicts of interest.

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