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Deletion distal to the PAX6 coding region reveals a novel basis for familial cosegregation of aniridia and diabetes mellitus

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ABSTRACT

Aims: Analyze cosegregation of aniridia and diabetes to identify genetic criteria for detection and early treatment of diabetes-susceptible aniridia patients.
Methods: We assessed a two-generation family: three individuals with aniridia, two previously diagnosed as type 2 diabetes. One individual with aniridia, with unknown diabetes status, was evaluated by oral glucose tolerance test. Genetic analysis of aniridia-associated genes was performed on all available family members. Candidate genes were functionally tested by gene silencing in MIN6 pancreatic β-cells.
Results: A 25 year old male with aniridia had a diabetic oral glucose tolerance test despite a normal fasting blood glucose. A 484–630 kb deletion ~120 kb distal to *PAIRED BOX 6* (*PAX6*) showed dominant cosegregation with aniridia and diabetes in all affected family members. The deleted region contains regulatory elements for *PAX6* expression and four additional coding regions. Knockdown of two of the deleted genes (*Dnajc24* or *Immp1l*) with *Pax6* impaired glucose-stimulated insulin secretion.
Conclusions: We demonstrate dominant cosegregation of diabetes and aniridia with a deletion distal to *PAX6*, which is clinically distinct from the mild glucose intolerance previously reported with *PAX6* coding mutations. Asymptomatic aniridia individuals appear at risk of diabetes (and its complications) and could benefit from earlier diagnosis and treatment.

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1. Introduction

1.1. Aniridia background

Aniridia is a rare condition: the diagnostic feature is absence or hypoplasia of the iris, commonly with associated conditions, such as glaucoma and cataracts [1]. The prevalence of congenital aniridia ranges from 1:64,000 to 1:96,000 [2]. Approximately two thirds of aniridia cases show highly penetrant autosomal dominant inheritance [2].

Aniridia is most commonly associated with haploinsufficient paired box 6 (*PAX6*) mutations. Mutations in the *PAX6* coding region as well as downstream regulatory regions have been reported in aniridia [3–7]. Roles for non-coding *PAX6* regulatory mutations in aniridia, are supported by functional analyses of the human *PAX6* locus in mice [8]. *PAX6* is a member of the *PAX* multi-gene family of transcription factors and is expressed in the developing central nervous system, eye, nose and pancreas [9], consistent with important regulatory roles during development. *PAX* genes, initially identified in Drosophila, are evolutionarily conserved across metazoans. In addition to its function as a transcriptional activator, mouse Pax6 also recruits histone methyltransferases to its target genes to remodel the local chromatin [10]. The PAX6 coding sequence is particularly well-conserved between mouse and human [11], exhibiting 99% amino acid identity. In addition to causing aniridia in humans, disruption of *PAX6* orthologs underlies the mouse *Small eye* and *Drosophila eyeless* phenotypes [12].

1.2. PAX6 and diabetes risk

Despite pancreatic expression of *PAX6* and its central role in β-cell development [13], diabetes prevalence was reported unchanged in an aniridia cohort compared to the general population [14], but half the aniridia subjects were under 20 years, hence lifetime risk of diabetes was unknown. Impaired glucose tolerance and low insulin secretion have been reported in people with aniridia with *PAX6* coding mutations [15–19]. However, *PAX6* haploinsufficiency alone does not appear to cause frank diabetes since additional *PAX6*-independent risk factors were reported in the cases where diabetes and aniridia co-exist. For example, in one diabetic subject with a *de novo PAX6* mutation both parents had insulin secretory defects and the father additionally had impaired fasting glucose [20].

There is a single report by Yasuda et al. documenting cosegregation of diabetes and aniridia in a father and daughter [15]. A single nucleotide polymorphism (SNP) in *PAX6* intron 9 was identified in this family that was present in family members without aniridia, indicating it was not likely pathogenic. This same cosegregation in our subjects was explored with an aim to define a subgroup of asymptomatic aniridia individuals at high-risk of insulin-deficient diabetes and diabetic complications who would benefit clinically from early diabetes diagnosis and appropriate treatment.

2. Subjects, materials and Methods

2.1. Setting and participants

A 54-year-old Caucasian woman with congenital aniridia, amblyopia and aphakia (Subject 1) was referred to the Diabetes Centre, St Vincent's Hospital, Sydney for management of diabetes mellitus. There was a family history of aniridia (Fig. 1): her deceased father had aniridia with unknown diabetes mellitus status; her sister (Subject 2) had aniridia and insulin-treated diabetes with nephropathy and retinopathy; her sister's son (Subject 4) was only known to have aniridia. Written consent was obtained for all subjects.

2.1.1. Subject 1

The referred woman was diagnosed as having type 2 diabetes aged 28 years following diagnosis in her older sister (Subject 2). Initially treated with oral diabetes agents, she required low dose insulin therapy within 12 months. Her diabetes was stable (HbA1c 7.5%) but peripheral neuropathy, her sole diabetic complication, was already present at diagnosis. She was treated with a basal-bolus insulin regimen with total daily insulin dose under 20 units. She had bilateral aniridia at birth and had required bilateral cataract surgery. With regular ophthalmologist review she had no subsequent complications from aniridia. She had right aphakia with vision corrected with a right contact lens (6/18) and a left intraocular lens implant (6/9).

Additional clinical history included mitral valve prolapse and mild hypercholesterolaemia. Medications were aspirin, glargine and aspart insulin. On examination, height was 169 cm, weight 59 kg and body mass index (BMI) 20.7 kg/m^2. She was normotensive: blood pressure 128/84 mmHg without significant postural drop. There was distal bilateral sensory loss to mid-shins with absent ankle jerks, with vibration sense present. She had bilateral fine nystagmus consistent with aniridia, without diabetic retinopathy or glaucoma.

2.1.2. Subject 2

Subject 2 (Subject 1's older sister, Fig. 1) had congenital aniridia and diabetes mellitus. Her diabetes was initially poorly-controlled despite insulin therapy, developing diabetic retinopathy and nephropathy. She recently improved control with major lifestyle changes. She described having an eye operation in childhood, an operation for Central Auditory Processing Disorder, breast and neck cancer and a renal transplant. Self-reported height was 170 cm, weight 63 kg and BMI 21.8. Her waist was 71 cm and hips were 91.4 cm.

2.1.3. Subject 3

Subject 3 was Subject 1's brother (Fig. 1). He did not have aniridia or diabetes mellitus. He reported no medical conditions. Self-reported height was 178 cm, weight 80 kg and BMI 25.2. His waist was 89 cm and hips were 96 cm.

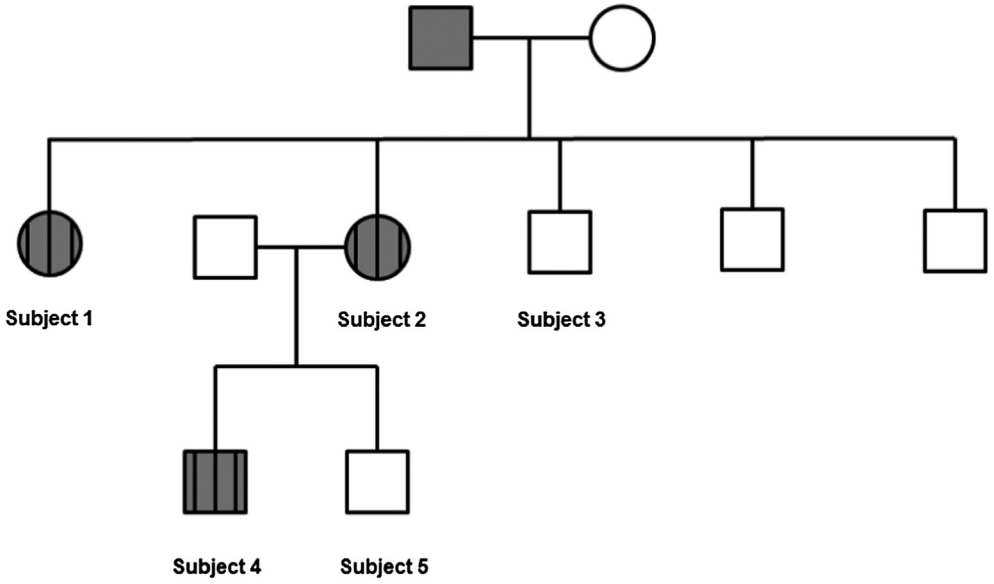

Fig. 1 – Pedigree. Squares represent males and circles represent females. Grey shading identifies family members with aniridia. Vertical lines identify family members with a diabetes diagnosis*. *Diabetes status of Subject 4 was determined in this study.

2.1.4. *Subject 4*

Subject 4 was Subject 1's nephew (Fig. 1). He visited our clinic aged 25 years and had congenital aniridia but no clinical signs of diabetes mellitus. On examination his height was 177 cm, weight 64.95 kg and BMI 20.7 kg/m^2. His waist was 80 cm and hips 95 cm. Blood pressure was 137/89 mmHg. His general health was good. He reported having asthma.

2.1.5. *Subject 5*

Subject 1's nephew (Fig. 1) did not have aniridia or diabetes mellitus but reported having asthma. Self-reported height was 182 cm, weight was 75 kg and BMI was 22.6. His waist was 83 cm and hips were 87 cm.

2.2. *Procedures and measures*

2.2.1. *Glucose tolerance tests*

Subject 1 had a 120 min mixed meal test after an overnight fast (liquid meal containing 50.1 g carbohydrate, 13 g protein, 11.4 g fat) as she had insulin-treated diabetes and an oral glucose tolerance test (OGTT) could elicit substantial hyperglycaemia. Her regular insulin glargine was withheld on the previous night. Blood samples for glucose, insulin, proinsulin and c-peptide were collected at 0, 15, 30, 45, 60, 90 and 120 min.

Subject 4, with no clinical signs of diabetes, was given a standard OGTT (75 g) after an overnight fast. Blood samples for glucose, insulin, proinsulin and c-peptide were collected at 0, 30, 60, 90 and 120 min. Insulin and c-peptide were assayed by automated two-site immunoassays (Roche Elecsys, Roche Diagnostics, Castle Hill, NSW, Australia). Proinsulin was measured using a radioimmunoassay (Merck Human Proinsulin HP1-15 K, EMD Millipore Billerica MA). Plasma glucose was measured using a hexokinase method (Roche Diagnostics, Castle Hill, NSW, Australia). Subject 4 was then tested for Glutamic acid decarboxylase (GAD) antibody and islet cell antibody to test type 1 diabetes status.

Areas under response curves (AUC) for glucose, insulin and proinsulin in both the meal test and OGTT were calculated using the trapezoidal method. The ratio $AUC_{insulin}/AUC_{glucose}$ is interpreted as an index of glucose-stimulated insulin secretion and is compared to values obtained from published data using identical OGTT and similar mixed meal protocols in non-diabetic subjects (n = 100) [21]. The ratio $AUC_{proinsulin}/AUC_{insulin}$ is interpreted as an index of proconvertase activity and is compared to published data obtained using a similar OGTT protocol (75 g in adults, less in children) in non-diabetic controls (n = 16), subjects with type 2 diabetes (n = 8) and subjects with a *PAX6* R240Stop mutation (n = 19) [16].

2.2.2. **PAX6 coding and regulatory region analysis**

The eleven coding exons and promoter regions (P1 and P0) of *PAX6* were sequenced in blood samples from Subjects 1 and 4 at the Garvan Institute of Medical Research with coding primers designed by Dr Giles S.H. Yeo, University of Cambridge (Supplementary Table 1; PCR-optimized conditions available upon request). PCR amplifications were inspected for single bands of expected sizes on agarose gels before purification with DNA Clean & Concentrator™-5 (Zymo Research). Samples were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (Applied Biosystems) and cleaned up with the BigDye-XTerminator kit (Applied Biosystems) and run on the ABI 3100 Genetic Analyzer. Sequences were assembled and analyzed with Sequencher software (GeneCodes). DNA samples were extracted and sequenced in a NATA ISO 15,189 certified facility at The Garvan Institute of Medical Research.

To identify potentially pathogenic copy-number variants, a comparative genomic hybridization (CGH) was performed by

South Eastern Area Laboratory Services (NSW Health Pathology) on Subject 1 using a custom Agilent 60 K microarray. This custom microarray is semi-targeted and has a backbone probe density of 25 Kb across the entire genome, with increased density in known pathogenic genes, including *PAX6*.

2.2.3. *DNA extraction from saliva*

Saliva collection and DNA extraction with Oragene saliva collection kits (OG-500) were undertaken in subjects with aniridia (Subjects 1, 2 and 4) and without aniridia (Subjects 3 and 5) according to the manufacturer's instructions.

2.2.4. *Deletion genotyping*

Subjects 1–5 were tested for the presence of the *PAX6* region deletion by quantitative PCR-based copy number analysis in a NATA ISO 15,189 certified facility at The Garvan Institute of Medical Research. Subject 1, whose blood sample was previously analysed by CGH, was used as a positive control, and an unrelated individual without aniridia or diabetes was used as a negative control (*PAX6* and control *GAPDH* primer sequences are listed in Supplementary Table 1).

2.2.5. *siRNA analysis*

MIN6 cells were maintained in DMEM (Invitrogen) containing 25 mM glucose, 10 mM HEPES, 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were transfected with *Pax6* and/or *Dnajc24*, *Elp4* or *Immp1l* ON-TARGET*plus* SMARTpool siRNA or Non-Targeting siRNA using DharmaFECT 3 (Dharmacon) according to the manufacturer's instruction. For insulin secretion assay, cells were incubated for 1 h at 37 °C in Krebs-Ringer HEPES buffer containing 2.8 or 25 mM glucose. Insulin was measured in an aliquot of the buffer by HTRF assay (Cisbio). Cell death was determined with the use of a Cell Death Detection ELISA (Roche Diagnostics) as previously described [22]. RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Real-time PCR was performed using *Power* SYBR Green PCR Master Mix (Applied Biosystems) on a 7900HT Real-Time PCR System (Applied Biosystems) as previously described [23]. Primer sequences are provided in Supplementary Table 2. The value obtained for each specific product was normalized to a control gene (*cyclophilin A*) and expressed as a fold-change of the value in control extracts.

2.3. *Statistical analysis*

2.3.1. *siRNA analysis*

All results are presented as mean ± SEM. Statistical analyses were performed using ANOVA with Bonferroni *post hoc* tests.

3. Results

3.1. *Clinical investigations*

The mixed meal test in Subject 1 showed a fasting glucose of 6.7 mmol/L with post meal test glucose of 17.2 mmol/L, consistent with the previously diagnosed diabetes mellitus. The OGTT in Subject 4 showed a fasting glucose of 4.7 mmol/L, peaking at 12.8 mmol/L, over the diagnostic threshold for diabetes mellitus. Subject 4 was negative for both Glutamic acid decarboxylase (GAD and islet cell antibodies.

Analysis of the mixed meal test and OGTT found insulin and glucose responses to the dynamic tests in both subjects consistent with impairment in glucose–stimulated insulin secretion. The ratio of Insulin AUC/glucose AUC was reduced in both subjects compared to the test-specific comparators calculated from summary data from 100 subjects using a similar test protocol (Fig. 2A) [21]. Wen et al reported elevated proinsulin/total insulin ratios in aniridia subjects with a R240X premature stop codon in *PAX6* [16], consistent with a defect in proconvertase activity. Our subjects had proinsulin/insulin ratios within the range expected for diabetic subjects (Fig. 2B), indicating that proconvertase impairment is not the primary defect in our subjects.

3.2. *Genetic analysis*

Sequencing of the *PAX6* coding and proximal promoter regions (P1 and P0) from Subjects 1 and 4 did not reveal any mutations. Therefore, an unbiased genome-wide CGH microarray was performed to identify potential pathological copy number variants in Subject 1. We identified a 484–630 kb deletion on 11p13 ~ 120 kb distal of *PAX6* (Chr11: 31,118,027–31,172,410 to 31,656,510–31,747,572; build hg19). This deleted region includes elongation factor protein 4

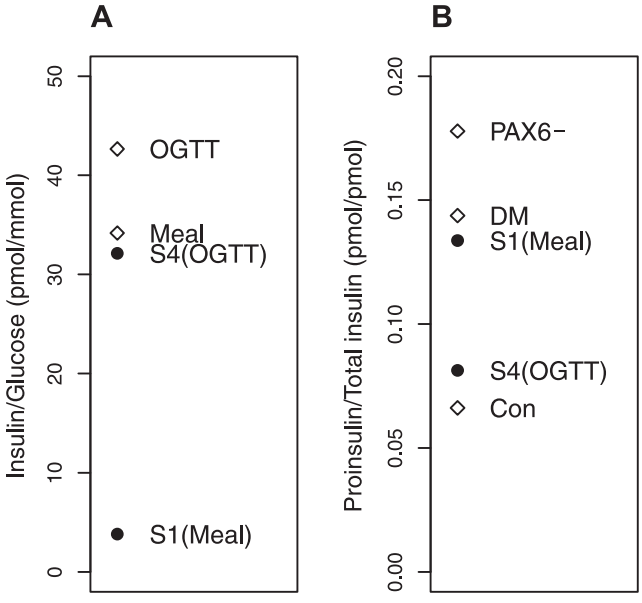

Fig. 2 – Ratios of AUCs in plasma during dynamic tests (2 hr OGTT, 2 hr mixed Meal test) in subjects (S1, S4, solid circles) and published comparators (open diamonds). A: Insulin/Glucose; comparator values are ratios of response AUCs in non-diabetic subjects (n = 100) from Dalla Man et al. [21]; B: Proinsulin/Total insulin; comparator values from 75 g OGTT in Wen et al. [16]; Con = non-diabetic control, DM = type 2 diabetes, PAX- = *PAX6* R240Stop mutation.

(*ELP4*), IMP1 inner mitochondrial membrane (*IMMP1L*), doublecortin domain containing 1 (*DCDC1*), DnaJ homolog subfamily C member 24 (*DNAJC24*) and contains known enhancers for *PAX6* expression.

3.3. *Segregation of the deletion distal to PAX6*

Targeted analysis of the deleted region identified in Subject 1 was performed on all available affected and unaffected family members using saliva samples. The deletion analysis confirmed the presence of a deletion distal to *PAX6* in Subjects 1, 2 and 4 who have aniridia and diabetes and its absence in the unaffected family members and in an unrelated control sample.

3.4. *Functional analysis of candidate genes in the deleted region*

To investigate the potential functional impact of the deletion distal to *PAX6* on β-cell function, we used siRNA gene silencing in the highly differentiated and glucose-responsive cell line MIN6 to knockdown each gene individually and in combination with *Pax6* (Fig. 3). Knockdown of *Pax6* in MIN6 cells reduced glucose-stimulated insulin secretion, without affecting basal insulin secretion (Fig. 4A). In contrast, knockdown of *Dnajc24*, *Elp4* or *Immp1l* alone had no effect on insulin secretion (Fig. 4A). Importantly, the combined knockdown of *Pax6* with *Dnajc24* or *Immp1l* potently inhibited glucose-stimulated insulin secretion (Fig. 4A). The knockdown of *Pax6* and/or *Dnajc24*, *Elp4* or *Immp1l* had no effect on cell death (Fig. 4B) or *insulin* mRNA expression (Fig. 4C). These data suggest that a combined reduction of *Pax6* with genes in the downstream deleted region impairs glucose-stimulated insulin secretion in β-cells.

4. Discussion

We proactively diagnosed overt diabetes in a 25 yr old individual with aniridia (Subject 4). We suspected this individual was at risk of diabetes because both aniridia and non-autoimmune insulin-treated diabetes were present in two of his family members, one being under our care. Although Subject 4's fasting glucose level was still normal, his OGTT glucose response met diagnostic criteria for diabetes.

Aniridia-associated *PAX6* mutations have been linked to glucose intolerance [15–19], although notably there have been

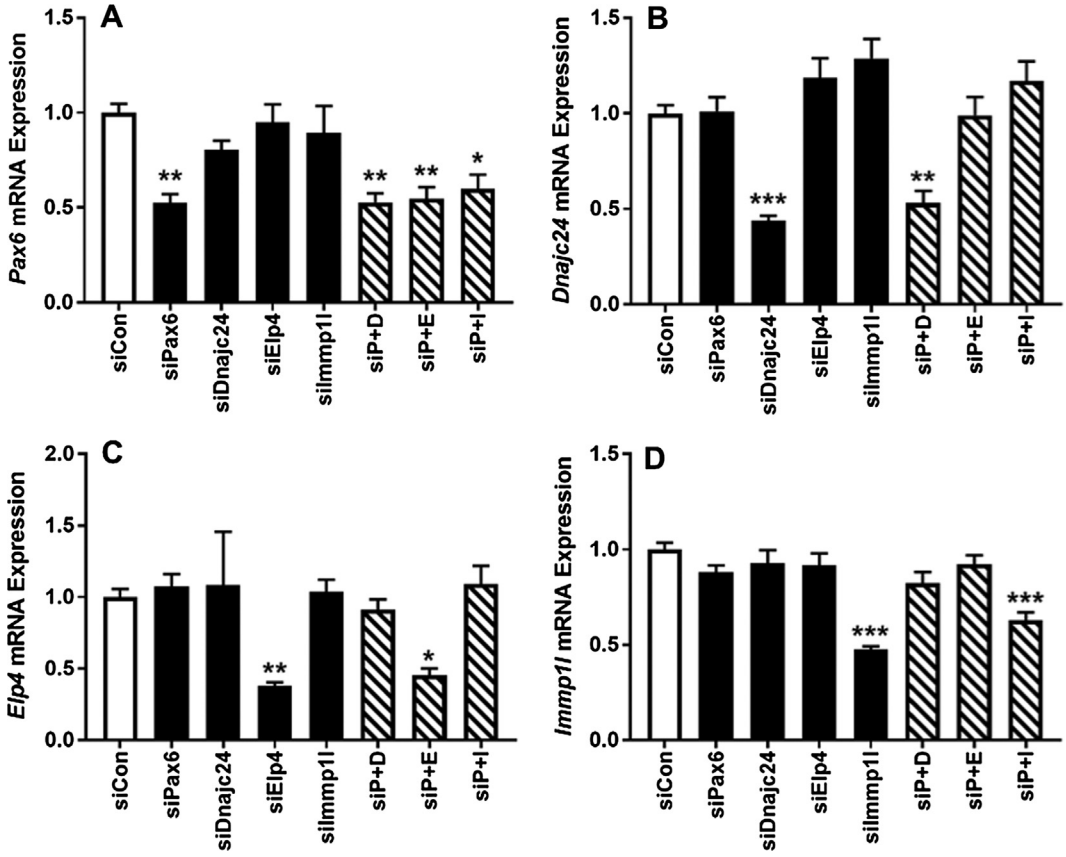

Fig. 3 – Effects of siRNA-mediated silencing of *Pax6* and/or *Dnajc24*, *Elp4* and *Immp1l* on mRNA expression. MIN6 cells were transfected with ON-TARGETplus SMARTpool siRNA or control Non-Targeting siRNA. Changes in (A) *Pax6*, (B) *Dnajc24* (C) *Elp4* and (D) *Immp1l* mRNA expression. All results are means ± SEM determined from 3 separate experiments in each group. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control siRNA-transfected cells. Transfection with combined siRNAs: siP + D = siPax6 + siDnajc24, siP + E = siPax6 + siElp4, siP + I = siPax6 + siImmp1l.

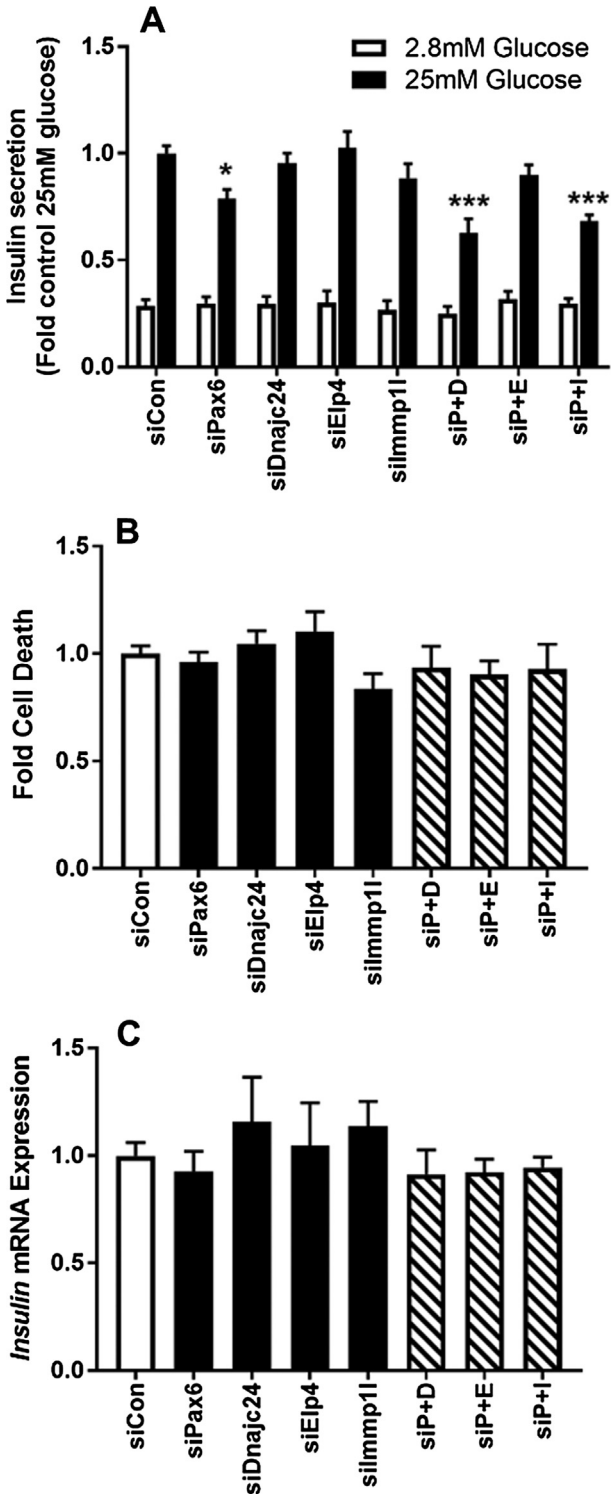

Fig. 4 – Effects of siRNA-mediated silencing of *Pax6* and/or *Dnajc24*, *Elp4* and *Immp1l* on β-cell function. MIN6 cells were transfected with ON-TARGETplus SMARTpool siRNA or control Non-Targeting siRNA. Changes in (A) insulin secretion at 2.8 or 25 mM glucose, (B) cell death and (C) insulin mRNA expression. All results are means ± SEM determined from 3 separate experiments in each group. *p < 0.05, ***p < 0.001 compared with control siRNA-transfected cells. Transfection with combined siRNAs: siP + D = siPax6 + siDnajc24, siP + E = siPax6 + siElp4, siP + I = siPax6 + siImmp1l.

very few reports of uncomplicated insulin-treated diabetes in these subjects. For example, a *de novo PAX6* mutation was identified in a subject whose parents both exhibited striking metabolic dysfunction, suggesting that this subject inherited additional substantial genetic risk for diabetes in besides the *PAX6* mutation [20]. In another case, Yasuda et al reported a pedigree showing co-segregation of aniridia and early-onset (22 years) insulin-treated diabetes [15] resembling the pedigree we report. Similarly to our subjects, no co-segregating pathogenic *PAX6* coding mutations were identified in this cohort [15]. However, by using genome-wide CGH technology unavailable when Yasuda et al reported their pedigree [15], we were able to identify a 484–630 kb deletion distal to the *PAX6* coding region. The deleted region overlaps a large regulatory region extending distally from the 3′ end of *PAX6* to intron 9 of the downstream *ELP4* gene, which is essential for PAX6 expression in specific tissues [8]. Deletions in this region have been identified in numerous independent aniridia cohorts [5–7,24–28]. Therefore, the deletion we identified distal to *PAX6* is most likely responsible for aniridia in our subjects.

The proinsulin/insulin profiles observed in our subjects differ from those observed in aniridia subjects with *PAX6* coding mutations [16]. Wen et al reported that elevated proinsulin/total insulin ratios in aniridia subjects with a R240X premature stop codon in *PAX6* correlated with defective proconvertase activity [16]. Consistent with this hypothesis, a heterozygous mouse model with a R266X premature stop codon in *Pax6* exhibited decreased prohormone convertase (PC) 1/3 expression [16]. In contrast, our subjects had proinsulin/insulin ratios within the range expected for newly-diagnosed (Subject 4) and long-term diabetic subjects (Subject 1) respectively. The human (R240X) and mouse (R266X) mutations that were previously investigated eliminate the PAX6 trans-activating domain but leave the paired domain, linker region and most of the homeodomain intact [16], suggesting that truncated PAX6 proteins may interfere with PC 1/3 expression. Additional molecular analysis of disease-associated PAX6 mutations will resolve this issue but our data do not support a role for defective insulin processing in aniridia patients with deletions distal to *PAX6*.

PAX6 is expressed during embryonic pancreas development and also plays a role in the normal function of adult endocrine cells, including the expression of glucagon and insulin in α-cells and β-cells respectively [29,13]. Although pancreatic enhancer elements have been mapped to regions upstream of the proximal *PAX6* promoter [30], it is unknown whether additional elements required for maintenance in the adult pancreas are located in the downstream deleted region. A paradox appears in which our subjects who lack downstream regulatory elements have a more extreme phenotype with respect to diabetes than individuals with loss-of-function mutations in the *PAX6* coding region [16,19]. This raised the possibility that additional genes within the deleted region contributed to β-cell failure in our subjects. To explore this concept, we performed knockdown for each conserved gene in the deleted region alone or in combination with *Pax6* in a murine β-cell model. Although the affected family members in this study did not have a *PAX6* coding mutation, the deletion distal to *PAX6* has been shown to abolish PAX6 expression in specific tissues [8]. As expected [31], *Pax6*

knockdown reduced glucose-stimulated insulin secretion (GSIS), while despite efficient knockdown individually other genes from the deleted region had no effect. However, *Dnajc24* or *Immp1l* in combination with *Pax6* knockdown produced a greater impairment of GSIS than *Pax6* knockdown alone without impacting basal insulin secretion, demonstrating an effect of these genes in β-cells. It will be interesting to further explore the functional consequences of combinatorial knockdown of all three genes in future studies. This *in vitro* data is consistent with our clinical findings from Subject 4, where basal insulin secretion and fasting glucose were normal but GSIS in response to an OGTT was impaired.

In summary, we proactively identified new-onset diabetes in a Caucasian family that showed co-segregation of aniridia and diabetes mellitus. The deletion distal to *PAX6* identified in our subjects overlaps with many deletions reported in individuals with aniridia [5–7,24–28]. However, these studies primarily focused on the ophthalmic features and did not report metabolic findings. In addition to the highly likely effect in our patients of the deletion on pancreatic islet developmental processes mediated by PAX6 [8] we provide evidence that downregulation of additional genes within the deleted region may contribute acutely to impaired GSIS with an early onset of an atypical insulin-treated diabetes in lean subjects who develop classical diabetic complications, providing possible diagnostic criteria. We suggest aniridia individuals who are asymptomatic for diabetes, do not have a *PAX6* coding mutation, and show a deletion distal to *PAX6* which includes the additional genes reported here, should be given an OGTT to detect possible diabetes. This can provide early diagnosis and treatment, preventing early diabetic complications as seen in affected family members in this study. If the cosegregation observed in the family pedigree reported here is confirmed in additional unrelated pedigrees it would be possible to provide new genetic diagnostic criterion for diabetes prediction in young individuals with aniridia. Accumulating evidence for non-coding mutations in aniridia have generated calls for routine deletion analysis distal to *PAX6* in this population [7,24]. Importantly, we identified an asymptomatic young family member with aniridia still with normal fasting glucose but a diabetic response to oral glucose challenge, whose two relatives with diabetes both had diabetic complications at diagnosis. Our data highlight that molecular identification of deletions distal to *PAX6* which produce an insulin-treated form of diabetes uncover a novel, clinically important aniridia subpopulation who should be specifically identified early for lifelong treatment, which could prevent diabetic complications.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2018.12.002.

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