

Neonatal diabetes: What can genetics teach us about the endocrine pancreas?

C. Polychronakos

Introduction

Neonatal diabetes (NDM) is rare, estimated to occur at a frequency of 1:400,000 live births. One might question the importance of investing much effort in elucidating the molecular pathology of such a rare form of the disease. However, new discoveries in the past decade have pointed out a number of important reasons to do so. These discoveries have created a compelling paradigm of personalised medicine and pointed out mechanisms and pathways of the normal function of the pancreatic b-cell that were previously unknown. In this presentation I shall provide an overview of the subject, following which I will concentrate on the molecular and functional investigation of two forms of neonatal diabetes that have revealed previously unsuspected functional insights into pancreatic islet development as well as insulin synthesis and release by the mature b-cell, which may lead therapeutic targets for common form of diabetes.

Overview of neonatal diabetes mellitus

NDM is characterised by persistent hyperglycaemia manifesting in the first few days or weeks of life, with ketosis and evidence of insulin deficiency. It typically requires insulin treatment. Approximately half of the cases are transient (TNDM), with a gradual reduction in insulin requirements and establishment of normoglycaemia without treatment by 3-4 months of age (Slide #). In the remaining cases, diabetes is permanent (PNDM). The two forms are clinically indistinguishable in the first few weeks of life. However, molecular diagnostics will, in most cases, permit the distinction between PNDM and TNDM, knowledge of obvious importance to the family of the affected infant.

Transient NDM. Most cases of TNDM are due to an unusual abnormality at chromosomal locus 6q24 related to parental imprinting, the property of some autosomal genes to express RNA only from the paternal, or only from the paternal gene copy¹. Two genes at 6q24 are expressed from the paternal copy only. These are *PLAGL1* (Pleiomorphic Adenoma Gene-Like 1), a zinc-finger transcription factor and *HYMAI* (Hydatiform Mole-Associated Imprinted), an untranslated RNA of unknown function. The disease is due to a double dose of either or both of these genes that can

happen when the effect of imprinting is negated by molecular lesion, which can be:²

1. Paternal isodisomy, in which both copies of chromosome 6 are of paternal origin and both express the two genes. Isodisomy is not heritable and cases due to it are all sporadic.

2. Duplication of a chromosomal fragment containing 6q24. The disease shows a familial pattern of dominant inheritance but only when the transmitting parent is the father.

3. A methylation abnormality, in which the maternal copy of the locus, normally silenced by methylation, remains unmethylated. The methylation defect can be inherited as an autosomal recessive trait³.

Molecular diagnosis is straightforward in #1 and #3, by a simple assay that shows absence of methylation. A family history can usually be elicited and helps diagnose #2. The duplication can be confirmed by quantitative PCR, array comparative genomic hybridisation or by multiple ligation-mediated probe amplification (MLPA).

PLAGL1 encodes a zinc-finger protein with anti-proliferative activity whose loss of function contributes to tumorigenesis⁴. Its function in the pancreatic b-cell and the consequences of its overexpression have not been studied in any detail and the possibility that they may be relevant to the dysfunction seen in more common forms of diabetes

has not been explored.

Permanent NDM. The causes of PNDM are much more diverse and not known in all cases. Most cases are sporadic but autosomal inheritance, both dominant and recessive, can be seen.

The most common and best understood forms are due to mutation of *KCNJ11* (K⁺ Channel inwardly rectifying family J, member 11) encoding the potassium channel KIR6.2, or its regulatory subunit, SUR1 (sulfonylurea receptor), encoded by the *ABCC8* gene (ATP-Binding Cassette, subfamily C, member8). Closure of KIR6.2 is essential for the b-cell depolarisation that triggers insulin release. Loss-of-function mutations of either gene cause recessive infantile hyperinsulinism, while gain-of-function mutations keep the channel constitutively open and prevent depolarisation, causing insulin deficiency and dominantly transmitted PNDM. Depending on the severity of the mutation, the diabetes can be transient in some cases.

KCNJ11 or *ABCC8* mutations are important to diagnose because the resulting PNDM responds to high doses of sulfonylureas, with regulated endogenous insulin secretion. Figure 1 illustrates the gradual switching of a 17-year old female PNDM patient with *KCNJ11* mutation from insulin to glibenclamide. Shortly after the locus was discovered in 2005, insulin (which she had been treated with since birth) could be tapered off within days with

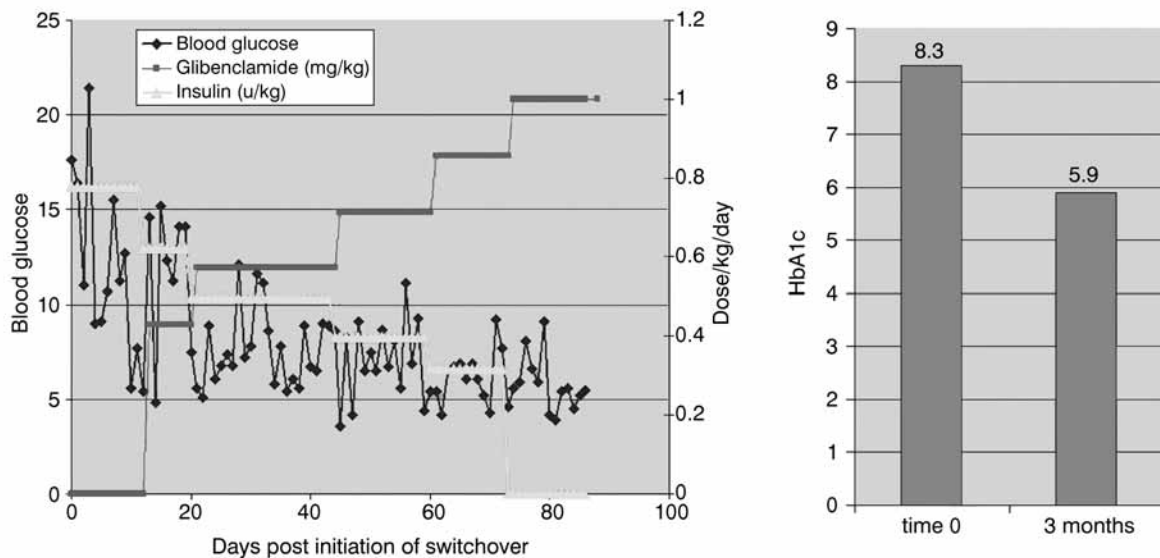


Fig. 1. Example of the successful use of high-dose glibenclamide to treat permanent neonatal diabetes in a 17-year old female who had been treated with insulin since birth. Insulin was tapered as doses of the oral agent was being decreased, on an outpatient basis, with frequent control of blood glucose and ketones. Three months later, her glycated haemoglobin had normalised.

increasing doses of the oral agent, on an outpatient basis. Notice the drastically reduced blood glucose fluctuations and the dramatic improvement in HbA1c. Newborns can be switched as soon as a molecular diagnosis is made. *KCNJ11* has a single exon, permitting rapid sequencing and diagnosis. *ABCC8* has multiple exons but should be the next gene to be sequenced in cases with normal *KCNJ11* and normal 6q24 methylation, because of the therapeutic implications.

PNDM can also be due to dominant mutations of the insulin gene that cause faulty post-translational processing that produces an abnormal peptide, toxic to the b-cell⁵ Diagnosis is useful for genetic counselling. At least two genes mutated in MODY (Maturity-Onset Diabetes in the Young), a dominantly inherited disease, can cause recessively inherited PNDM, when both parents transmit a mutated allele. These are *GCK* (Glucokinase, the b-cell glucose sensor, mutated in MODY 2) and *IPF* (insulin promoter factor, necessary for pancreatic development, mutated in MODY4)⁶. The latter form is due to complete pancreatic agenesis and also requires supplementation with pancreatic enzymes.

Finally, mutations of the *FOXP3* (Forkhead

Box P3) transcription factor, necessary for the development and function of regulatory T-lymphocytes, cause the IPEX syndrome (Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked), with fulminant multi-system autoimmunity, including type 1 diabetes, starting shortly after birth.

RFX6: an essential controller of the formation and function of the pancreatic islet

In recent years, our group has studied the Mitchell-Riley syndrome (OMIM #601346), a rare condition of neonatal diabetes that also includes multiple intestinal atresias and absent gallbladder⁷. The proband was a 6-month old female infant with neonatal-onset insulin-dependent diabetes, offspring of second cousins. She came to autopsy after succumbing to gastrointestinal complications following repair of multiple intestinal atresias. A male sibling had also died under similar circumstances. Autopsy revealed a well-formed pancreas with the exocrine component of normal gross and microscopic appearance (Fig. 2). Islets were completely absent and there was no staining for insulin, glucagon or somatostatin. There was some sca-

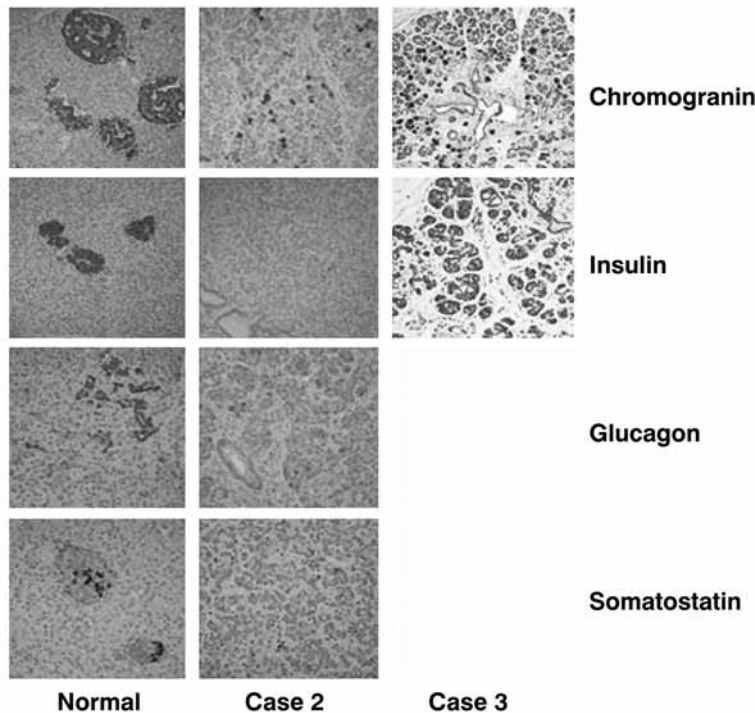


Fig. 2. Immunohistochemistry showing complete absence of islet structures or cells staining for the three main endocrine hormones of the pancreas, compared to an autopsy sample of a control infant that came to autopsy for an unrelated reason.

tered staining for chromogranin (a generic marker of endocrine cells) but no structure resembling islets. By an international collaboration, we assembled DNA from six cases with PNDM and intestinal atresia, all sporadic. Two of the additional cases was also consanguineous (offspring of first cousins), an observation strongly supporting autosomal recessive inheritance.

Assuming that the proband was homozygous for a mutation inherited from one of the two common great-grandparents, we used homozygosity mapping to narrow down the location of the mutated gene⁸. With the *Illumina* Hap550 array, we genotyped the proband for 550.000 single-nucleotide polymorphisms (SNPs) and searched the results for long (>1Mb) regions of uninterrupted homozygosity at all markers. This allowed us to narrow down the mutated gene to nine regions totalling ~130 Mb, or ~3% of the genome, as expected of the offspring of second cousins. No known gene involved in the formation of pancreatic islets mapped to these regions, suggesting that the syndrome involved a novel gene.

Further homozygosity mapping of one additional consanguineous case (offspring of first cousins), narrowed down potential position of the locus to three regions totalling 24 Mb and containing 194 known genes (RefSeq database). A search in the EST (expressed sequence tags) databases revealed that only one of these 194 genes, *RFX6*, had specific expression in pancreatic islets, making it the more likely candidate. However, definitive proof required the sequencing of all genes in the

region, a task that would have taken years by the traditional methods.

In one of the first uses of combining exon capture with next-generation sequencing (NGS), we sequenced all 194 genes (~1.400 exons). First, a custom *Nimblegen* oligonucleotide array was designed, complementary to the exons of interest. The patients' sample was hybridised to the array and, after the rest of the DNA was washed away, it was eluted and all captured fragments sequenced using the *Roche* 454-FLX Titanium technology. Thirty novel sequence variants, not present in the existing SNP databases were identified. Of these, only three were in coding regions and only one changed protein sequence. It was a 217 Ser>Pro substitution in a highly conserved region of exon 6 in *RFX6*, pinpointing this gene once more as the most likely candidate⁸.

To provide definitive proof, we then sequenced all 19 exons of the remaining known cases. Overall, a convincing mutation was found in five out of the six known cases (Fig. 3). Four of the five were homozygous, even in the one non-consanguineous case. He was the offspring of unrelated French parents sharing a 2 Mb chromosomal segment carrying the mutation, inherited from a very remote ancestor. The child was homozygous in every single marker over this 2 Mb region. A few such regions can be found in almost every individual in the European population, probably dating from the exit of *Homo sapiens* from Africa. They cause disease only if they happen to carry a mutation.

A very interesting genotype-phenotype corre-

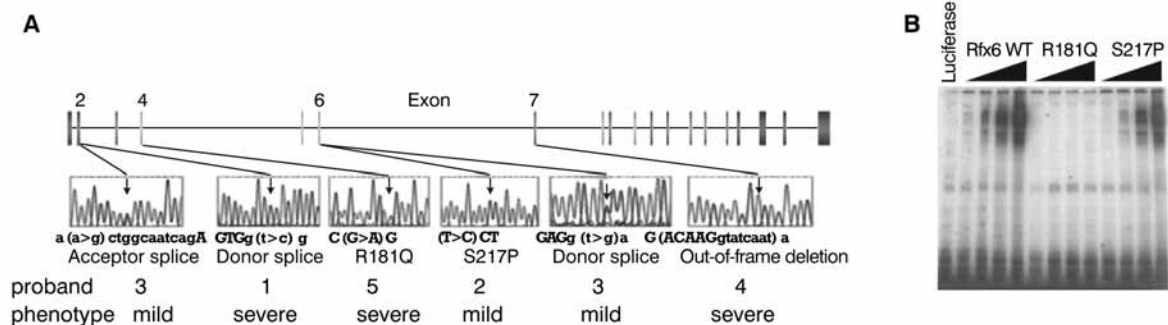


Fig. 3. (a) The mutations causing Mitchell-Riley syndrome are shown. (b) The functional significance of the two missense mutations was further explored with electrophoretic mobility shift assay (EMSA). In vitro translated wild-type or mutant *RFX6* protein was labeled with a DNA probe corresponding to the DNA binding element known to interact with all members of the *RFX6* family. In the presence of binding, the mobility of the complex is slowed down and it appears as a band high up in the gel. The 181 R>Q mutation, replacing a Arg, crucial for DNA interaction, with Gln completely abolishes binding and is associated with a severe phenotype. The 217 S>P mutation, on the other hand, has little effect and must be interfering with some other aspect of *RFX6* function. It is also associated with a much milder phenotype.

lation was evident. The most severe phenotype (multiple intestinal atresias, complete insulin deficiency and absence of islets at autopsy, when performed) was found in the cases with homozygosity for mutations that completely abolished gene expression or function: One donor splicing mutation in exon 2, causing the inclusion of an intron in the mRNA, one out-of-frame 13-bp deletion in exon 7, causing a nonsense protein and a 181 Arg>Gln mutation, affecting a highly conserved arginin in the DNA-binding domain that completely abolished DNA binding (Fig. 3). The case with the mildest mutations (compound heterozygosity for one donor and one acceptor splicing mutations) is now ten years old, well controlled with small doses of insulin and with relatively mild gastrointestinal complaints. Another long-term survivor with mild phenotype was the case in which the 217 Ser>Pro substitution was found with exon capture and NGS. It is in a highly conserved part of the protein, away from the DNA-binding module, of unknown function.

What is *RFX6*, and what does it do? The gene had never been studied before our discovery that it is mutated in the Mitchell-Riley syndrome. It belongs to a family of transcription factors widely expressed in different tissues, and it is the only one with exquisite specificity for the pancreatic islets. It is also expressed in the intestine, where it must also play an important role in early embryonic development, as indicated by the coexistence of multiple intestinal atresias accompanying its absence. In collaboration with Dr. Renian Wang, University of Western Ontario, we show that its expression in human foetal pancreas increases dramatically between 12 and 20 weeks of gestation, concurrent with the histological appearance of hormone-staining b-cells.

Targeted inactivation of the mouse *Rfx6*, done in parallel with the human genetic work by our collaborators of Dr. Michael German's group at UCSF, showed a striking similarity of the mouse knockout phenotype with the human Mitchell-Riley syndrome. Both the complete absence of endocrine cells in what was otherwise a normal exocrine pancreas, and the intestinal atresia were reproduced. This constitutes additional proof that *RFX6* is absolutely required for the existence of the endocrine pancreas.

It is known that the master switch for the development of the endocrine component of the pancreas in the second trimester of gestation is

Neurogenin3 (*NEUROG3*). However, *NEUROG3* is expressed within a very narrow time window to initiate the development of the islets, and then its expression drastically diminishes. By contrast, *RFX6* remains highly expressed in adult pancreas. What does it do there? Does it have a functional role in addition to its crucial role in the embryonic development of the islets?

To answer this question we knocked *Rfx6* expression down using siRNA (small inhibitor RNAs) in the min6 cells, mouse b-cell line that preserves the ability to finely control glucose synthesis and release as a function of ambient glucose, and often used as an experimental model to study this function. We found that decreasing expression to about 40% of control decreased glucose-regulated insulin release by 50% (Taleb and Polychronakos, unpublished data). Interestingly, intraperitoneal injection of a glucose load in mice also resulted in the doubling of *Rfx6* mRNA, indicating that this gene does not only coordinate response to glucose but that it dynamically interacts with glucose levels. The importance of these regulatory connections will need to be elucidated with further *in vitro* and *in vivo* work.

***PLAGL1* as a regulator of glucose-stimulated insulin release**

PLAGL1, the gene whose double expression causes TNDM has been extensively studied for its tumour suppressor properties but its role in the pancreatic islet remains largely unexplored. To answer this question, we engineered a clone of the INS-1 rat b-cell line to express the human *PLAGL1* in a tetracycline-regulated manner. We show that induction of slight *PLAGL1* overexpression with the appropriate dose of tetracycline, quantitatively close to the doubling of its expression level in human TNDM, results in a substantial decrease of GSIS (Du et al., submitted). Interestingly, *in vitro* exposure of mouse islets to high glucose downregulates the endogenous *Plagl1*, indicating the presence of another previously unsuspected regulatory loop.

What can these rare cases teach us about common forms of diabetes?

RFX6: The greatest hope for a permanent cure of diabetes (type 1 or type 2, or of any other aetiology) is that a method can be developed for the regeneration of the destroyed or malfunctioning b-

cells. Indeed, a large world-wide effort in high-profile laboratories are developing approaches to generate cells that produce insulin in a glucose-dependent manner through guided differentiation of stem cells, or the transdifferentiation of components of the non-endocrine pancreas. In the mouse, it has been shown that both acinar and ductal exocrine cells can differentiate into b-cells. Despite some progress, the problem is that such transformations are neither complete nor durable. This is perhaps not surprising as these efforts involved manipulation of gene expression without knowledge of RFX6, the crucial molecular player. This knowledge offers great promise in engineering new b-cells, using RFX6 as biomarker for successful transformation and/or finding ways of inducing its expression.

Our work on *PLAGL1* also indicates that it is a crucial player in a previous unsuspected and unstudied functional pathway in glucose regulation.

It is known that the main regulator of insulin release is glucose. However, the effect of glucose is also known to be modulated by other metabolic inputs, such as lipids and aminoacids. This requires additional control pathways, not all of which has been elucidated. *PLAGL1* may be an important lead to such a previously unstudied pathway.

The beta-cell failure in type 2 diabetes almost certainly has different aetiologies in different patients, that affect different pathways. Better understanding of these pathways is a requirement for the development of novel therapeutic approaches, tailored to the metabolic disturbance responsible for the diabetes in the particular case (personalized medicine).

In conclusion, we have seen that rare Mendelian forms of diabetes can give us insights that would not have been suspected from the ordinary approaches of conventional research. It is to be

hoped that these insights can be translated into better treatments for common forms of diabetes.

References

1. *Giannoukakis N, Deal C, Paquette J, Goodyer CG, Polychronakos C.* Parental genomic imprinting of the human IGF2 gene. *Nat Genet.* 1993; 4(1): 98-101.
2. *Mackay DJ, Temple IK.* Transient neonatal diabetes mellitus type 1. *Am J Med Genet C Semin Med Genet.* 2010; 154C(3): 335-42. Review.
3. *Mackay DJ, Callaway JL, Marks SM, White HE, Acerini CL, Boonen SE, Dayanikli P, Firth HV, Goodship JA, Haemers AP, Hahnemann JM, Kordonouri O, Masoud AF, Oestergaard E, Storr J, Ellard S, Hattersley AT, Robinson DO, Temple IK.* Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. *Nat Genet.* 2008; 40(8): 949-51
4. *Spengler D, Villalba, M, Hoffmann, A, Pantaloni, C, Housami, S, Bockaert, J, Journot, L.* Regulation of apoptosis and cell cycle arrest by Zac1, a novel zinc finger protein expressed in the pituitary gland and the brain. *EMBO J* 1997; 16: 2814-2825.
5. *Støy J, Edghill EL, Flanagan SE, Ye H, Paz VP, et al.* Neonatal Diabetes International Collaborative Group. Insulin gene mutations as a cause of permanent neonatal diabetes. *Proc Natl Acad Sci USA* 2007; 104(38): 15040-4.
6. *Stoffers DA, Zinkin NT, Stanojevic V, Clarke WL, Habener, JF.* Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. *Nature Genet* 1997; 15: 106-110.
7. *Mitchell J, Punthakee Z, Lo B, Bernard C, Chong K, Newman C, Cartier L, Desilets V, Cutz E, Hansen IL, Riley P, Polychronakos C.* Neonatal diabetes, with hypoplastic pancreas, intestinal atresia and gall bladder hypoplasia: search for the aetiology of a new autosomal recessive syndrome. *Diabetologia.* 2004; 47(12): 2160-7.
8. *Smith SB, Qu HQ, Taleb N, Kishimoto NY, Scheel DW, et al.* Rfx6 directs islet formation and insulin production in mice and humans. *Nature.* 2010; 463(7282): 775-80.