9-2019

(Epi)genomic heterogeneity of pancreatic islet function and failure in type 2 diabetes.

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Review

MOLECULAR METABOLISM

(Epi)genomic heterogeneity of pancreatic islet function and failure in type 2 diabetes

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ABSTRACT

Background: Pancreatic Islets of Langerhans are heterogeneous tissues consisting of multiple endocrine cell types that carry out distinct yet coordinated roles to regulate blood glucose homeostasis. Islet dysfunction and specifically failure of the beta cells to secrete adequate insulin are known precursors to type 2 diabetes (T2D) onset. However, the exact genetic, (epi)genomic, and environmental mechanisms that contribute to islet failure, and ultimately to T2D pathogenesis, require further elucidation.

Scope of review: This review summarizes efforts and advances in dissection of the complex genetic underpinnings of islet function and resilience in T2D pathogenesis. In this review, we will highlight results of the latest T2D genome-wide association study (GWAS) and discuss how these data are being combined with clinical measures in patients to uncover putative T2D subtypes and with functional (epi)genomic studies in islets to understand the genetic programming of islet cell identity, function, and adaptation. Finally, we discuss new and important opportunities to address major knowledge gaps in our understanding of islet (dys)function in T2D risk and progression.

Major conclusions: Genetic variation exerts clear effects on the islet epigenome, regulatory element usage, and gene expression. Future (epi) genomic comparative analyses between T2D and normal islets should incorporate genetics to distinguish patient-specific from disease-specific differences. Incorporating genotype information into future analyses and studies will also enable more precise insights into the molecular genetics of islet deficiency and failure in T2D risk, and should ultimately contribute to a stratified view of T2D and more precise treatment strategies. Islet cellular heterogeneity continues to remain a challenge for understanding the associations between islet failure and T2D development. Further efforts to obtain purified islet cell type populations and determine the specific genetic and environmental effects on each will help address this. Beyond observation of islets at steady state conditions, more research of islet stress and stimulation responses are needed to understand the transition of these tissues from a healthy to diseased state. Together, focusing on these objectives will provide more opportunities to prevent, treat, and manage T2D.

Keywords Type 2 diabetes; Pancreatic islets; (Epi)genomics; Genetics; Environment; Dysfunction

1. TYPE 2 DIABETES: A DISEASE OF MANY FACES

Like all other forms of diabetes, type 2 diabetes (T2D) is fundamentally a genetic disease. It is a complex, polygenic disorder with substantial environmental contributions and multiple gene—environment interactions. It manifests when insulin resistance unmasks intrinsic flaws in islet sensory and/or secretion machinery or impaired resilience and compensation mechanisms, ultimately leading to beta cell failure and death and insufficient insulin secretion. In contrast to monogenic forms of diabetes, which overwhelmingly alter protein-encoding DNA sequences, genome-wide association studies (GWAS) have revealed that the large majority of DNA sequence changes linked to T2D reside in non-coding regions of the genome. Recent studies have highlighted the genetic variability of T2D through quantitative (epi)genomic and clinical measures of human islet function. As (epi)genomic editing technologies such as CRISPR-Cas9 become more widespread in the field, further studies will be able to perturb specific (epi)genetic elements in various islet cell line and whole animal models to bridge the gap between diabetes genetic susceptibility and phenotypes.

Examination of patient clinical measurements of islet function alone also provide substantial insights into T2D heterogeneity. One study that performed a topological analysis of 2,551 T2D patients and their corresponding clinical data identified 3 subtypes of T2D with each group showing a distinct and strong association with either diabetic microvascular complications, cardiovascular disease, or neurological diseases and allergies, respectively [1]. Three years later, Ahlqvist and colleagues analyzed a cohort of 8,980 Eastern European individuals with diabetes and subdivided patients into 5 distinct groups with different disease progression and clinical complications [2]. The first subgroup of patients ($n = 577$) was labeled as severe autoimmune diabetes based on a high prevalence of auto-antibodies for zinc transporter 8A (ZnT8A). Group two (severe insulin deficient diabetes; $n = 1575$) was characterized by poor metabolic control (highest glycated hemoglobin (HbA1c) levels) and insulin deficiency, while

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https://doi.org/10.1016/j.molmet.2019.06.002

individuals in group three (severe insulin resistant diabetes; n = 1373) had higher BMI and insulin resistance. The fourth group (mild obesity related diabetes; n = 1942) possessed the highest average BMI and individuals in the fifth group (mild age-related diabetes; n = 3513) had the latest average age of disease onset. Notably, individuals from groups 1 and 2 had an increased prevalence of ketoacidosis at diagnosis and poor sustained insulin use. Patients in group 2 were also at a high risk for diabetic retinopathy while group 3 patients had a higher risk for kidney disease and diabetic complications (e.g., stroke, etc.). Further collection of patient clinical data and stratification into different subgroups of T2D can help guide medical professionals to design more successful and precise medical solutions. Distinct sets of T2D risk variants were also enriched in each of these clusters, suggesting these may represent genetically distinct T2D subtypes.

2. T2D GENETICS, SUBTYPES, AND MODULES

In late August 2018, the DIAMANTE consortium reported the identification of 243 T2D risk loci, 135 of which were newly discovered in the latest T2D GWAS meta-analysis of almost 900,000 individuals (74,124 cases and 824,006 controls) [3]. They identified 403 signals in 243 T2D risk loci, with approximately two-thirds (n = 151) containing one association signal and the remaining comprising 2–10 distinct ones. Importantly, the approximately three-fold increase in effective sample size enabled the identification of 'credible' sets of putative functional SNPs in each locus using genetic fine-mapping approaches. These efforts reduced the putative functional variants to less than 50 for over half of the association signals. For 101 loci, they refined the credible set of SNPs to 10 or fewer variants. Qualitatively, this study underscored conclusions from previous work by the T2D-GENES and GOT2D consortia on the potential regulatory nature of these T2D-associated sequence changes [4], as the large majority of these credible set SNPs overlap non-coding regions of the genome. Most of the T2D risk alleles are considered to act at least in part through islets, given their correlation with quantitative measures of islet function.

Clinically, analysis of T2D genome wide association study (GWAS) data (consisting of 94 independent T2D variants and 47 diabetes associated traits) identified five distinct groups of T2D loci [5]. Two of these groups had variant trait associations related to reduced beta cell function and insulin deficiency, of which the two differed by their proinsulin levels. The remaining three groups were characterized by variants associated with insulin resistance and were subdivided into obesity mediated (via high body mass index (BMI) and waist circumference (WC)), lipodystrophy-like fat distribution (low BMI, high cholesterols and triglycerides), and disrupted liver lipid metabolism (low triglycerides). Interestingly, from 4 independent T2D populations of European ancestry, they observed that 5,449/17,365 (~30%) of individuals were classified to a single group based on clinical measures of BMI, WC, C-peptide, high density lipoprotein (HDL), and triglycerides. These findings are important because they not only highlight the genetic heterogeneity of T2D, but also show that these genetic modules are supported by patient clinical data. Additional, more precise phenotypic measurements, such as intravenous glucose tolerance test (IVGTT)-based measures of first phase insulin secretion [6], will be critically important to help the community more precisely parse the relevant tissue(s), cell type(s), and physiologic process (es) affected by T2D-associated SNPs.

3. FUNCTIONALIZING GENETIC VARIATION OF ISLET FUNCTION AND FAILURE

Given their non-coding locations in the genome, it seemed plausible that at least a subset of T2D GWAS SNPs might alter the use or activity of *cis*-regulatory elements (REs) controlling islet gene expression. Multiple groups completed epigenome profiling of islets at the level of chromatin accessibility, histone post-translational modifications, specific islet transcription factor binding, and DNA methylation to identify putative regulatory regions and specific *cis*-REs [7–13]. Integration of these epigenomic maps and overlap with T2D GWAS SNP locations revealed a significant and specific enrichment in active regulatory regions, most notably in islet-specific stretch enhancer chromatin states [8] (http://theparkerlab.org/tools/isleteqtl/) or enhancer clusters [9] (http://www.isletregulome.org/isletregulome/), than would be expected by chance. Naturally, this led to the hypothesis that GWAS variants alter islet *cis*-RE use and expression of their gene targets to contribute to islet dysfunction and failure.

Key challenges to translating these statistical associations into molecular, cellular, and phenotypic insights include: 1) identifying the putative function-altering variant(s) among the tens to hundreds of genetically linked variants in a given locus; (2) determining the gene or genes that are the targets of these variants; and (3) elucidating the effect(s) of these variants on *cis*-RE use and activity and target gene expression. Over the past few years, studies have employed a variety of complementary approaches leveraging new technologies to uncover fundamental regulatory programs of islet cell identity and function using human islets and surrogate human and rodent beta cell lines and understand their genetic programming.

3.1. Identifying putative functional variants

Complementary *in vitro* and *in vivo* approaches have been developed and employed to nominate putative causal variant(s) among a set of genetically linked SNPs. Traditional reporter assays such as luciferase have been used as *in vitro* tools to test putative promoter or enhancer sequences for regulatory activity and to successfully identify expression-modulating effects of select candidate T2D GWAS SNPs, usually at the scale of one to tens of test sequences at the most. Unfortunately, they are too expensive and time consuming to systematically assess the hundreds to thousands of T2D credible set SNPs for their ability to modulate reporter activity. Massively parallel reporter assays (MPRA) allow one to create pooled libraries of thousands of DNA sequences and test for their effects on reporter gene expression in a single transfection. MPRA can be used both to test relative regulatory element activity of distinct sequences and to assess and compare the gene regulatory effects of GWAS SNPs (Figure 1). Barcoded reporter gene (e.g., *gfp*) transcripts are sequenced and quantified by RNA-sequencing. To identify sequences possessing *cis*-RE activity, barcode counts in the RNA-sequencing data are compared to those in the original plasmid library. Moreover, expression-modulating effects of GWAS SNP effects on *cis*-RE activity can be identified by comparing RNA-seq barcode counts of the DNA sequences containing the T2D risk and non-risk alleles. To date, most MPRA studies have tested the ability of selected DNA sequences to enhance the transcriptional activity of a minimal promoter, but there is potential to apply more sophisticated techniques to test combinatorial enhancer-promoter sequence effects.

Although these assays have not been conducted yet with T2D GWAS/credible set SNPs, their utility has been demonstrated for the study of other traits and diseases. For example, Tewhey et al. tested approximately 30,000 SNPs associated with expression differences in lymphoblastoid cell lines (LCLs) [14]. 12% of these sequences significantly altered reporter activity, with 95% of those exhibiting increased, enhancer, activity. These sequences overlapped epigenetic hallmarks of active regulatory elements in LCLs, including active histone tail modifications (such as H3K27ac), open chromatin sites, and ENCODE

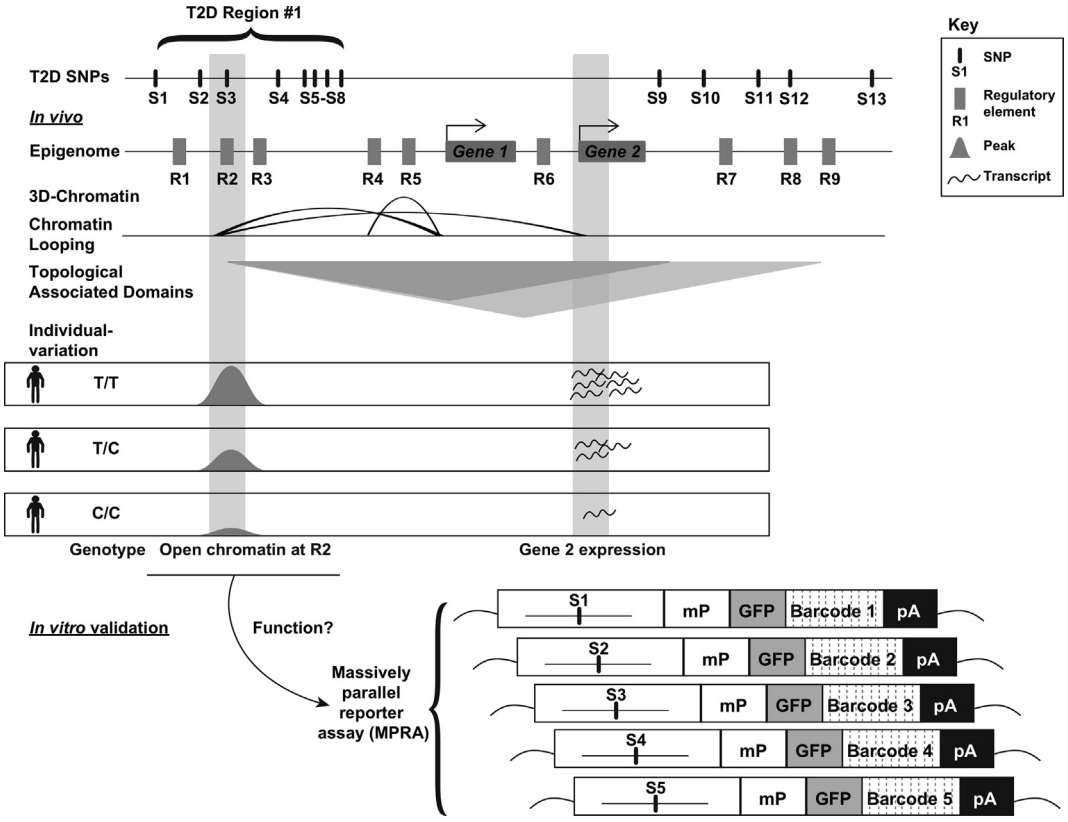

Figure 1: Assessing the relationship between T2D-associated genetic variation and islet (dys)function. Multiple SNPs (S1—S13) in close proximity that are associated with T2D occur in a non-coding region of the human genome (denoted as T2D Region #1). Epigenomic profiling identifies distinct regulatory elements (R1-R9) across the genome. In this example, *cis*-regulatory element R2 and T2D SNP S3 directly overlap. Chromatin looping analyses identify physical interactions/proximity between regulatory elements to identify gene targets of given *cis*-regulatory elements. Individual genetic variation influences chromatin accessibility at this region and also impacts expression levels of interacting genes (Gene 2). Massively parallel reporter assays (MPRA) provide an *in vitro* system to determine how each of thousands of T2D-associated SNPs affect transcriptional activity of a reporter gene. SNPs = single nucleotide polymorphisms; T2D = type 2 diabetes; mP = minimal promoter; GFP = green fluorescent protein; pA = polyadenylation signal.

LCL transcription factor binding sites. As anticipated, these active sequences were enriched for DNase hypersensitive sites that were unique to LCLs and not those for other cell types. Together, these data suggest that MPRA are capable of predicting DNA sequence regulatory activity relevant to *in vivo* contexts and warrant their use as a tool to identify putative functional variants among the hundreds to thousands of T2D credible set SNPs.

Alternatively, SNP effects on *in vivo* regulatory element use can be deciphered from (epi)genomic profiles if the individual from which they were generated was genotyped. This information can be leveraged in two ways. If these profiles are generated from multiple genotyped individuals, chromatin accessibility quantitative trait locus (caQTL) analysis can be performed to identify SNP alleles that alter chromatin accessibility (as depicted in Figure 1). Khetan et al. recently completed ATAC-seq analysis of human islets from 19 donors to identify approximately 150,000 open chromatin regions (OCRs) genome-wide [15]. caQTL analyses identified 2949 OCRs whose accessibility was modulated by the genotype of a SNP residing within it. caQTL were located in distal (putative enhancer) OCRs at a rate three-fold higher than proximal (promoter) OCRs (30% vs. 12%). Open chromatin regions containing caQTLs were enriched for sequence motifs and empiric binding (as determined by ChIP-seq) of FOXA2, PDX1, MAFB, and NKX6-1. It therefore appears that the chromatin-modulating effects of these variants may be mediated through differential binding of islet transcription factors. As might be anticipated based on their predominantly distal locations and overlap with islet transcription factor binding sites, these caQTL are fairly islet-specific. For the SNPs demonstrating significant effects on islet gene expression (eQTL SNPs; [11,16,17]), directions of the chromatin and gene expression effects were consistent, i.e., T2D risk alleles closing chromatin were also linked to decreased gene expression. Importantly, overlap with the locations of T2D-associated SNPs in the NHGRI/EBI GWAS catalog nominated putative functional SNPs for 13 T2D-associated loci. This included rs11708067 in the *ADCY5* locus, which also exhibited significant allelic imbalance in an independent study [13] and has been linked to altered *ADCY5* expression in human islet eQTL analyses [11,16,17]. Of note, the T2D risk allele increased chromatin accessibility for seven of these SNPs and decreased it for the other six. These data suggest that both decreased and increased activity of certain genes and pathways contribute to islet dysfunction and T2D.

3.2. Identifying target genes

Two complementary approaches can and have been employed to identify the putative gene targets of islet *cis*-REs, particularly those related to central islet functions such as insulin biogenesis or overlapping GWAS SNPs. Similar in principle and approach to caQTL analyses, expression quantitative trait locus (eQTL) analyses assess how genetic variation influences gene expression levels. To date, analyses of steady state gene expression in two independent cohorts identified 2341 and 616 islet eQTLs, respectively. Among the 216 eQTLs detected in both cohorts, 14 were linked to T2D-associated SNPs. This modest overlap between T2D associated SNPs and steady state islet

eQTL may be due to (1) limited power to detect eQTLs with these sample sizes; (2) the distinct locations of the majority of detected eQTL (promoters) compared to GWAS SNPs (enhancers); or the possibility that many T2D GWAS SNPs modulate physiologic or pathologic islet responses. eQTL meta-analyses of the gene expression from hundreds of islets across multiple groups should help resolve these questions.

Techniques that interrogate the 3-dimensional architecture of human islets and beta cells have revealed important regulatory features and uncovered extensive enhancer—enhancer, enhancer-promoter, and promoter-promoter links. Initial insights were derived from targeted approaches such as circular chromatin conformation capture (4C) to identify regions interacting with promoters of the *INS* gene [18,19] or those of other select genes, such as the transcription factors *PDX1* and *ISL1* [9]. These targeted approaches revealed extensive intra-chromosomal looping interactions with multiple putative enhancers, some at ranges up to hundreds of kilobases to megabases away. When Xu and colleagues blocked *INS* promoter activity, both contacts with and expression of the interacting genes *SYT8* and *ANO1* decreased [19]. Interestingly, both of these genes promote insulin secretion, which led the authors to suggest a model wherein physical association of genes involved in insulin metabolism with the highly transcribed *INS* locus stimulates their expression. Surprisingly, 4C-seq in human EndoC-βH1 beta cells detected contacts between the *INS* promoter and gene loci on multiple other chromosomes, including both type 1 and type 2 diabetes GWAS loci [20]. As the technical vs. biological nature of *trans*-chromatin interactions and their meanings is still debated, it will be exciting to see if the existence or dynamics of interactions between these GWAS loci and *INS* in these potential transcription factories contribute to the underlying molecular mechanisms of their association with either type 1 or type 2 diabetes in people. Nonetheless, these studies of a handful of specific sites reveal surprising intricacies and potential functional importance of transcriptional regulation in islet cell nuclei.

More broadly, the 'all-by-all' Hi-C approach was recently applied to define the genome-wide location of chromatin interactions and define chromatin territories/neighborhoods in pancreatic islets from one individual and EndoC-βH1 [21]. The majority (>90%) of Hi-C loops detected were shared with other cell types. Moreover, the anchor points of these shared loops were significantly enriched for CTCF and CTCFL motifs, which suggests that the large majority of chromatin neighborhoods are dictated by the relatively limited flexibility in conformations to pack the chromatin into each cell type's nucleus. In contrast, approximately 10% (n = 1,078) of interactions were unique to EndoC and islets, such as those in the ZnT8 transporter-encoding *SLC30A8* locus, and were enriched for beta cell-specific transcription factor binding site motifs [21].

These large-scale Hi-C chromatin neighborhoods in EndoC-βH1 were further refined into hundreds of actively transcribed promoter-promoter, promoter-enhancer, and enhancer—enhancer interactions that were revealed by RNA Polymerase 2 ChIA-PET [21]. RNA Pol2 looping sites were notably enriched for beta-cell transcription factors and brought into close proximity genes associated with glucose sensing and insulin secretion. Surprisingly, some loci exhibited an extensive, highly connected network of chromatin interactions, often consisting of >5 distinct connections. These included gene loci encoding transcription factors associated with beta cell identity and development (e.g., *PDX1*, *ISL1*, *NKX6-1*, *MAFB*, and *miR-375*) as well as glucose sensing and insulin metabolism and secretion (e.g., *PCSK1*, *RIMBP2, RGS7*, and *CDC42*) [21]. Overlap of T2D-associated GWAS SNPs with any ChIA-PET interactions was modest, however, suggesting that (1) the sensitivity of Pol2 ChIA-PET technology was insufficient; (2) Pol2-mediated looping with GWAS SNP-containing *cis*-REs differs significantly between primary adult human islets and EndoC-βH1, which appears to retain some epigenomic signatures of its fetal pancreas origin looping; and/or (3) the looping effect(s) of some GWAS SNP-containing *cis*-REs may be condition-specific, as has been shown in other cell types [22,23]. Additionally, EndoC-βH1 genotypes may not have permitted assessment of certain GWAS SNPs impacts on *cis*-RE usage as only 20—30% of associated loci were heterozygous in the cell line. The large majority of disease variants were homozygous in EndoC-βH1 for the non-risk allele. Thus, if the risk allele creates or activates a *cis*-RE, it will not be detected in this particular individual. Nonetheless, these analyses nominated T2D-associated SNPs mapping to target genes *CEP41* and *C11orf54* with consistent alterations on *cis*-RE activity and gene expression in islets and EndoC-βH1 [21]. Several studies have demonstrated that the physiology of EndoC-βH1 cells, including insulin secretion and response to glucose, closely resembled that of islets [24,25]. However, these cells are not responsive to cytokines [26] suggesting there are significant differences in some of their functional properties. Further studies of specific pathways and processes should therefore exercise caution when extending results found in EndoC-βH1 into primary human beta cells. Integration of islet and corresponding cell line multi-genomic datasets with genome-wide genetic variation information, and designing tools to make this information accessible in an interactive, searchable format such as in a web application (https://shinyapps.jax.org/endoc-islet-multi-omics/), will be crucial for nominating further T2D gene targets.

4. PRECISE CELLULAR GENOMICS

Elucidating the molecular mechanisms that lead to beta cell dysfunction and T2D pathogenesis has been a major focus of diabetes research for decades. However, advances in single cell genomic profiling techniques have led to greater understanding of non-beta cell type transcriptional regulation and suggest that they may play important roles in hallmark features of beta cell insufficiency and failure linked to T2D genetic risk and pathophysiology. Single cell transcriptome analysis of human islet cells indicate that multiple monogenic diabetes genes are highly expressed in beta cells (e.g., *PDX1, PAX4, INS, HNF1A,* and *GCK*) [27]. However, other non-beta cell types express genes mutated in monogenic diabetes (such as *PAX6* and *RFX6*), congenital hyperinsulinemia (*HADH, UCP2*) and those implicated as T2D GWAS target/effector genes [28].

Recent study of type 1 diabetic (T1D) human islets has provided surprising insights into alpha cell biology. In T1D islets, the alpha cell proportions remain relatively unchanged despite abnormal glucagon secretion [29]. This dysregulated glucagon secretion is instead accompanied by decreased expression of important islet transcription factors including *ARX, MAFB,* and *RFX6* and increased expression of stress response factors such as *ATF4, ERN1,* and *HSPA5* [29] suggesting that changes in alpha cell identity may ultimately lead to their dysfunction. Analysis of normal and T2D islet single cells with simultaneous RNA-seq and patch clamping (patch-seq) also revealed subpopulations of alpha cells with varying enrichment for ER stress response genes (e.g., *DDIT3, XBP1, PPP1R15A*) [30]. Interestingly, this transcriptomic heterogeneity was consistent in normal and T2D islets and associated with variability in alpha cell electrophysiological measures; ER stressed alpha cells had lower cellular size and Na^+ peak current. Prior single cell transcriptomic analyses have also noted subpopulations of ER-stressed beta cells [31,32] which implicates the dysfunction of both alpha and beta cells in diabetes pathogenesis. Similarly, the integrity of beta and alpha cell functions seem to be

dependent on each other, as under hypoglycemic conditions, T2D islets show reduced insulin, C-peptide, and glucagon secretion [33]. Additionally, during a glycemic clamp experiment, an increase in glucagon secretion was positively correlated with beta cell function suggesting that signaling between the two islet cell types is crucial for maintaining glucose homeostasis.

Studies of delta cells in *Sst-Cre* transgenic mouse models [34–36] reveal that timely regulation of insulin secretion is controlled by various delta-cell specific pathways. Induction of the ghrelin receptor (Ghsr) in delta cells was correlated with enhanced somatostatin release and ultimately reduced insulin and glucagon secretion [35,36]. Furthermore, the peptide hormone Ucn3 was shown to be co-released with insulin from beta cells to activate type 2 corticotropin-releasing hormone receptor (Crhr2) on delta cells in an alternate pathway that promotes somatostatin release and negatively regulates insulin levels [34]. Delta cells are also notably enriched for G protein-coupled receptors (e.g., *GLP1R, GIPR, GPR120*) which exert careful control over metabolism [37]. These receptors are also common therapeutic targets of T2D, suggesting that treatment and management of the disease should not neglect delta cell (dys)function and/or survival.

Efforts to characterize the epigenomes of each islet cell type are emerging and revealing new insights of cellular fate and differentiation. Two groups have performed open chromatin profiling of purified beta and alpha cell fractions [10,12] and identified between 1850 and 3999 beta and 5316-27,000 alpha-specific peaks. These cell-specific regions were enriched for transcription factor motifs implicated in cell development and were enriched for diabetes-associated SNPs. Arda and colleagues also suggest that the beta cell epigenome is plastic and capable of being derived from other endocrine and exocrine precursor cells. Discrepancies in the numbers of cell-specific peaks determined by both groups are likely due to the cell surface markers used to enrich for each. CD26/DPP4, used by Arda et al., is a strong positive selector for alpha cells, which then enables negative selection for beta and other minor cell populations. However, this method of enrichment for beta cells will not remove contaminating delta and PP/gamma cells. Continued development of new tools and markers for islet cell enrichment, such as NTPDase3 [38] should continue to help us to understand changes elicited by genetic and environmental factors in each distinct cell type.

Iterative proteomic screens in human islets are also proving useful for identifying putative cell-specific surface markers for isolation [39], wherein beta and delta cell populations were obtained by co-enrichment for CD9 and CD56. Challenges currently remain to exclusively enrich for the minor islet cell types (delta, gamma/PP), thus strategies that negatively select for these cells may be needed. Study of the rarer gamma/PP cells, which constitute roughly $<$1–5% of the total islet volume, remain limited due to the lack of known cell-surface markers for enrichment and purification (Figure 2). Whole islet analyses are unable to capture cell type-specific changes and therefore preclude analysis of their potential roles in T2D genetics and pathophysiology. Given the clear and extensive genotype effects on *cis*-RE usage [13,15] and gene expression [11,16,17] in islets, more extensive analysis of sorted cell types from multiple individuals is warranted to define a representative set of islet cell-specific REs and distinguish condition-specific from genotype-driven effects on their use and activity.

5. ISLET RESPONSES; MOVING BEYOND STEADY STATE MEASUREMENTS

To date, the overwhelming majority of studies including and assessing genetic variation have profiled the steady state patterns of epigenetic modifications and gene expression in islets or their constituent cell types. Others have compared how these steady state measures differ between T2D and non-diabetic (ND) individuals [13,16,40–44]. Surprisingly, these studies, especially transcriptome analyses, have identified only modest alterations despite clear phenotypic differences in HbA1c and other metabolic traits in T2D vs. ND donors. This suggests that alterations in transcriptional regulation may not contribute to T2D pathogenesis, or that these (epi)genomic comparative studies are not effectively capturing the alterations associated with islet (patho) physiologic decline or T2D onset. Genomic assays such as RNA-seq provide only a snapshot of tissues' or cell types' transcriptomes at a given point in time. Genes that are important for islet function and resilience (e.g., Gene A) and genes whose expression induces islet failure (e.g., Gene C) would be detected in a comparative analysis between islets at healthy and T2D states (Figure 3). In contrast, genes that are temporarily induced by the initiation of islet stress or in the compensation or pre-diabetic stages (e.g., Gene B) before decline towards disease state would be missed.

Furthermore, T2D is a complex disease with dynamic ranges of severity and secondary health complications across individuals. Thus, comparing single snapshots of gene expression in T2D individuals at different stages of islet health and disease progression may simply lead to obfuscation. Longitudinal studies of *in vivo* epigenetic and gene expression changes in islets of severe, early onset (*db/db*) or polygenic, late-onset (Tallyho, NZO) [45–47] diabetic mouse models may be the only practical solution to identify the temporal nature of these changes and identify the molecular features of islet dysfunction, compensation, and failure in T2D pathogenesis. Indeed, longitudinal analyses of aging islets in mice identified DNA methylation changes in key genomic regions associated with beta cell proliferation and metabolism [48]. These findings suggest that changes in the islet (epi)genome and transcriptome may also be dynamic during the course of T2D development and progression.

Alternatively, *in vitro*, it may be possible to subject human islets to diabetic-like conditions through the use of inflammatory cytokines and/or oxidative and ER stress. Already, studies from a few groups have demonstrated clear differences in islet gene expression, including the modulation of putative T2D target genes, during stimulatory or stress responses, and certain epigenetic and gene expression features in islets are only revealed upon these *in vitro* or *in vivo* exposures, such as glucose-stimulated insulin secretion, palmitate, inflammatory cytokines or other response defects [49–53]. Examining the transcriptomic and (epi)genomic changes of human islets under these various stressors over time may provide greater knowledge of the epigenetic and gene expression changes preceding islet stress, failure, and ultimately diabetes onset.

6. FROM CORRELATION TO CAUSATION: MODELING T2D VARIANT EFFECTS AND TARGET GENE FUNCTION

Together, the studies highlighted above combine to unveil important information necessary to translate T2D GWAS statistical associations into biological knowledge of the genes and mechanisms underlying T2D risk and progression: the putative functional variant(s), the gene or genes they regulate, whether the T2D risk alleles activate or inactivate their targets, and the cell types in which they elicit these effects. The final, and most critical, step is to take these data, generate testable hypotheses, and create accurate, or perhaps more importantly, relevant models to determine the molecular, cellular, and physiologic functions of the T2D-associated target genes and thereby close the association-biology gap.

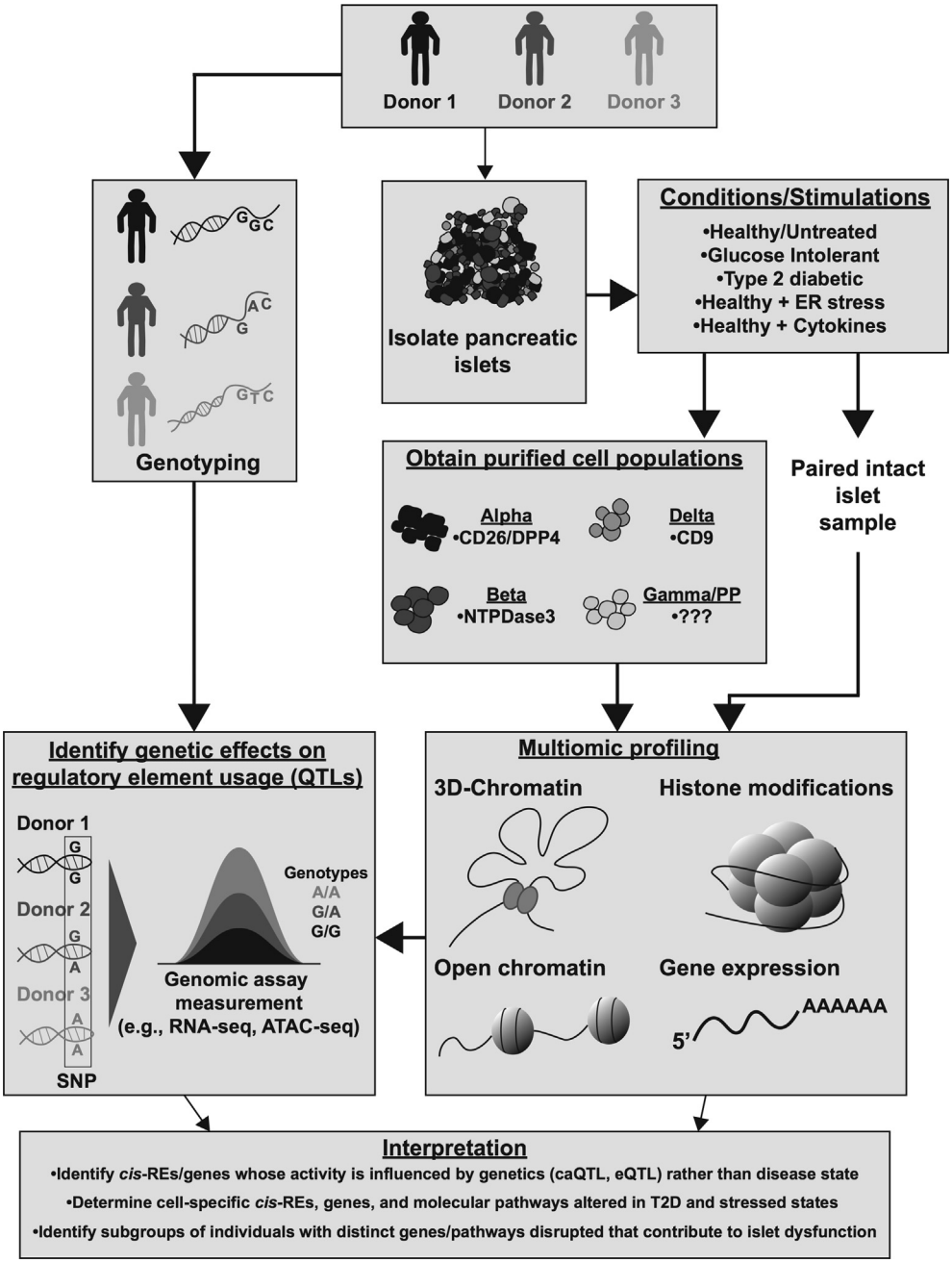

Figure 2: Moving towards a more precise understanding of islet cellular genomics and responses. Proper elucidation of islet (dys)function and its association with T2D pathogenesis is confounded by individual genetic variation as well as islet cellular heterogeneity. To obtain a better understanding of both, future studies must prioritize strategies to obtain purified islet cell type populations (e.g., beta, alpha, delta, gamma/PP) via sorting with specific cell surface markers. Characterization of each cell type-specific genomic profile at baseline, stimulated, and diseased conditions will provide clearer understanding of key cellular and molecular processes that are altered and important in T2D development. Additionally, by sampling islets from multiple individuals and leveraging genotypes, it will be possible to identify *cis*-regulatory elements and genes that are influenced by genetics rather than disease state. SNP = single nucleotide polymorphism; QTL = quantitative trait locus; ER = endoplasmic reticulum.

A variety of cellular and animal models have been developed and applied over the past few years to experimentally manipulate *cis*-regulatory elements and their target gene function as it related to beta cell/islet function, glucose homeostasis, and T2D pathogenesis. CRISPR/Cas9 has revolutionized our ability to modify genomes and epigenomes almost at will. Unsurprisingly, CRISPR (epi)genome editing tools can and have been used to target putative T2D target genes [54] or *cis*-REs [55] in beta cell lines and assess their functions. As some of the T2D risk variants have been linked to increased *cis*-RE activity and target gene expression, such as the *C2CD4A/B* locus [56], the CRISPR activation systems, comprising a catalytically dead Cas9 (dCas9) protein linked to epigenetic activator proteins such as the histone acetyltransferases p300/CBP [57] or synthetic activation module (SAM) [54], will be essential tools to model the molecular and cellular effects of the T2D risk variant.

Experimental modeling of select T2D-associated islet 'enhancero-pathies' have both yielded success and presented challenges. The

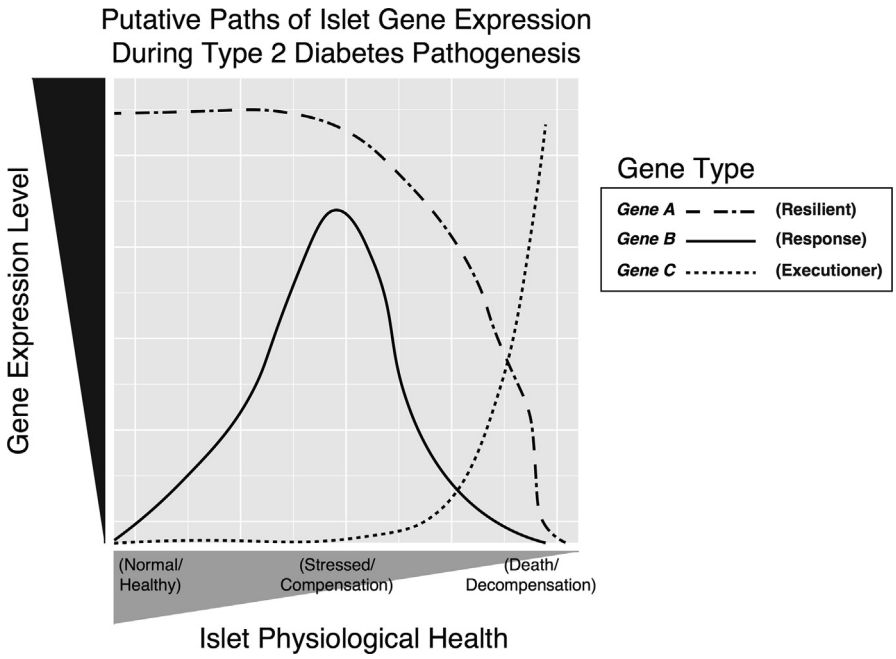

Figure 3: Challenges with identifying gene expression alterations in type 2 diabetes. Gene expression measurements from RNA-seq data typically represent only a snapshot of tissues' or cell types' transcriptome at a given point in time. In recent comparative analyses of islet intact and single cell transcriptomes from T2D and ND individuals, relatively few genes are significantly altered despite the clear phenotypic differences between them. This may suggest that the mechanisms that precede islet failure and T2D pathogenesis are post-transcriptional and cannot be detected in conventional RNA-seq analyses. However, it is also possible that the putative paths of these genes' alterations over the course of islet physiological decline and T2D development are simply being missed. Genes that are important for islet function and resilience (e.g., Gene A) and those whose expression directly induces or is the consequence of islet failure (e.g., Gene C) may be detected in a comparative analysis between islets at healthy and decompensated states. However, response genes that are temporarily induced by islet stress (e.g., Gene B) would not be detected in this comparison.

ADCY5 locus provides an example of how the complementary approaches described above can converge to translate the T2D risk variant statistical associations into consistent phenotypes and increased biological knowledge. Among several putative functional SNPs in this T2D-associated locus, one (rs11708067) overlaps an islet enhancer and alters islet chromatin accessibility [13,15]. The T2D risk allele reduces chromatin accessibility [13,15] and is linked to reduced islet *ADCY5* expression [11,17,55,58]. Cross-species mapping of functional regulatory elements identified an equivalent, sequence-conserved and functional *cis*-RE in the rat INS-1 (832/13) beta cell line, and both *Adcy5* expression and insulin content were substantially reduced in INS-1 (832/13) clones in which this *cis*-RE was deleted using CRISPR/Cas9 compared to wildtype clones [55]. Finally, *ADCY5* silencing in human islets impaired glucose signaling and glucose-stimulated insulin secretion.

Other loci, such as *TCF7L2,* illustrate a challenge of studying 'enhanceropathies'. The genetic and molecular pathophysiology seems more complicated, with potentially pleiotropic effects. The rs7903146 T2D-associated SNP overlaps an islet open chromatin region (OCR) and the risk 'T' allele is associated with increased *TCF7L2* expression and decreased insulin content and secretion in human islets [59]. However, *TCF7L2* is expressed in multiple metabolically active tissues, and tissue-specific deletions implicate distinct roles for *Tcf7l2* function in liver and in both pancreatic islet alpha and beta cells to maintain glucose homeostasis. Some of these complications likely reflect the differing functional impacts of mutations in the regulatory element vs. the protein-coding gene. *SHH* provides an extreme but informative example of this [60]. *SHH* is used iteratively during development for fundamental patterning decisions in different tissues, and *Shh* whole body knockouts are embryonic lethal. However, homozygous deletion of a distal limb enhancer sequence >1 million nucleotides away from the *Shh* promoter produces mice with no limbs, and single nucleotide changes in the equivalent *SHH* distal limb enhancer in humans cause polydactyly. We anticipate that direct modeling of the 'enhanceropathies' by (1) explicitly removing the *cis*-RE or switching SNP alleles within it using genome editing or (2) epigenetically modulating its activity will help to resolve the pleiotropic and contradictory effects of certain GWAS loci, such as *TCF7L2*.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

Stratification of genetic risk and progression in T2D and precise understanding of the molecular, cellular, and physiologic effects of T2D risk variants are key objectives moving forward. Studies to date have provided important insights into and surprises about fundamental features of islet gene regulatory programs and the genetics of T2D. The extensive effect of genetic variation on islet *cis*-RE use and gene expression that has been uncovered, even under steady state, emphasizes the importance of sampling multiple individuals for a given process that is to be studied and of evaluating and incorporating genetic variation into future human islet studies to identify representative, not 'reference', islet responses to stimuli and diabetogenic stressors. Genetic and environmental factors appear to be impinging on the same territory of the genome to elicit their (potentially deleterious) epigenetic effects on islet cell identity and function, namely distal enhancer elements. Incorporation of genotype information for each human islet sample will allow us to decode the genetic programming of islet environmental responses and identify T2D SNPs that enhance or suppress these responses. Together with improving techniques to purify and obtain distinct islet cell populations, we can better

understand each cell type's genomic architecture and better characterize their roles in islet resilience and failure. Experimental manipulation of the regulatory elements and/or the target genes identified by (epi)genomic approaches described above and modeling the putative pathways and processes they implicate in human islet cell lines (e.g., EndoC-βH1-H3) is essential to progress from correlation to causation. Similarly, transitioning from "the" mouse (C57BL/6) to multiple mouse models for insights into the effects of naturally occurring genetic variation on islet function and physiology [61] and for manipulation of key genomic elements should also help characterize the dynamic range of islet behavior and response.

T2D is a heterogeneous, complex, and progressive disorder, as multiple subtypes have been identified and associated with different genetic risk and clinical outcome profiles. Future islet genomics studies that focus on identifying the distinct subgroups of individuals with distinct genes/pathways that are disrupted and/or contributing to islet (dys)function at basal and/or responsive states are needed. Furthermore, priority should be given to profiling more islets from pre-diabetic and T2D individuals to characterize the transition between basal to stressed to T2D state and determine if there are intermediate signatures for islet failure and T2D onset. Together, this multi-pronged approach toward studying T2D genetics and islet pathophysiology will help identify additional targets and opportunities for intervention that can be exploited for more precise and effective preventative, treatment, and management options for T2D.

FUNDING

This article is part of a supplement entitled 'Biomarkers of Beta-Cell Health and Dysfunction: Towards Personalised Diabetes Care. Proceedings of the 20th Servier-IGIS Symposium'. The Symposium and the publication of the Supplement have been made possible by an unrestricted educational grant from Institut la Conférence Hippocrate — Servier Group.

ACKNOWLEDGEMENTS

N.L. and M.L.S. wrote and revised the manuscript.
This study was funded by DOD W81XWH-16-1-0130 and ADA grant 1-18-ACE-15 to M.L.S. Opinions, interpretations, conclusions, and recommendations are solely the responsibility of the authors and do not necessarily represent the official views of the American Diabetes Association or the Department of Defense.

CONFLICT OF INTEREST

None declared.

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