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COMMENTARY

Identification of islet cell characteristics in humans with type 2 diabetes by single-cell sequencing

In a recent *Journal of Clinical Investigation* article, Son *et al.*[1] described their efforts to identify an alteration in regulatory protein activity in human β-cells, which is elicited in the state of type 2 diabetes, and explored therapeutic options for preventing β-cell failure.

First, the authors carried out single-cell ribonucleic acid sequencing using human pancreatic islet cells from donors with or without type 2 diabetes, and endeavored to build islet-specific transcriptional regulatory networks, using the Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNe)[2]. Employing this strategy, the authors identified transcriptional networks composed of regulons, which are regulated by 1,813 transcription factors, 969 cofactors and 3,370 signal transduction proteins. Based on the results obtained, they next measured the activity of these transcription factors and cofactors in each single cell using virtual inference of protein activity by enriched regulon analysis (metaVIPER)[3]. Expression levels of transcription factors or cofactors are often very low or even undetectable in many cell types, and their expression levels do not necessarily reflect their activity because of the complex modifications that occur after transcription of such regulatory proteins. However, metaVIPER overcomes these limitations, as the analysis measures the expression of ≥50 of its transcriptional targets, enabling reliable assessment of protein activity. Activities of MAF bZIP transcription factor A, specific transcription factors of β-cells, and iroquois homeobox, specific transcription factors of α cells, in each of the islet cells assessed by ARACNe and metaVIPER showed strong correlations with the expressions of insulin (*INS*) and glucagon (*GCG*), respectively, confirming the validity of these analyses.

Next, combining the ARACNe and metaVIPER data with the unsupervised hierarchical clustering analysis using iterative clustering (iterClust)[4], the authors classified islet cells from donors with or without type 2 diabetes into 11 cluster groups. Protein-based cluster analyses showed that there are two main clusters, depending on the difference in metabolic inflexibility/stress-response marker activity (MI), between MI^{+} and MI^{-} (Figure 1). MI^{+} cells are characterized by increased metabolic stress responses, endocrine progenitor features and cell stemness. Further iterative clustering resulted in classification of the MI^{+} cluster into five subclusters, which were designated $M1^{+1}$ to MI^{+5}. The MI^{-} cluster was divided into MI^{-1} to MI^{-5} subclusters. Among these subclusters, four ($M1^{+2}$, $M1^{+4}$, MI^{-1} and MI^{-2}) showed β-cell features, such as high expression levels of *INS* and enhancement of β-cell-specific transcription factor activity. Especially in the MI^{-1} subcluster, *INS* expression was the highest, and features of metabolic inflexibility/stress response, progenitor/stem cell and α-cell identity were inactive, suggesting this subcluster contained healthy β-cells (Figure 1). In contrast, six subclusters ($M1^{+1}$, MI^{+3}, MI^{+5}, MI^{-3}, MI^{-4} and MI^{-5}) showed α-cell features, such as high expression levels of *GCG* and high activity of iroquois homeobox 2 (Figure 1). Among these subclusters, MI^{+5} showed the strongest α-cell identity.

The clustering described above was carried out using mixtures of islet cells from non-diabetic and type 2 diabetic donors. Accordingly, it was expected that the clustering might lead to unbiased identification of specific cells residing in both non-diabetic and type 2 diabetic islets. Therefore, the authors sought to determine which clusters were, independently, enriched in non-diabetic versus type 2 diabetic islets. These analyses showed that non-diabetic islets were enriched in the MI^{-1} subcluster, which showed β-cell characteristics along with an inactive metabolic inflexibility/stress response and progenitor/stem cell features. Therefore, this subcluster was regarded as harboring healthy β-cells. Meanwhile, type-2 diabetic islets were enriched in the MI^{+2} and MI^{-5} subclusters. Notably, the MI^{+2} cluster consisted of β-cells with an active inflexibility/stress response and progenitor/stem cell features. Therefore, this subcluster was regarded as harboring type 2 diabetic β-cells. In addition, MI^{-5} subclusters were α-like cells with an inactive metabolic inflexibility/stress response, and this subcluster was regarded as consisting of type 2 diabetic α-cells. Notably, MI^{-5} subclusters showed elevated β-cell-like features (Figure 1). MI^{+5} showed the strongest α-cell identities, and the authors defined this subcluster as being comprised of healthy α-cells. These results are consistent with a previously proposed model in which, under pathological

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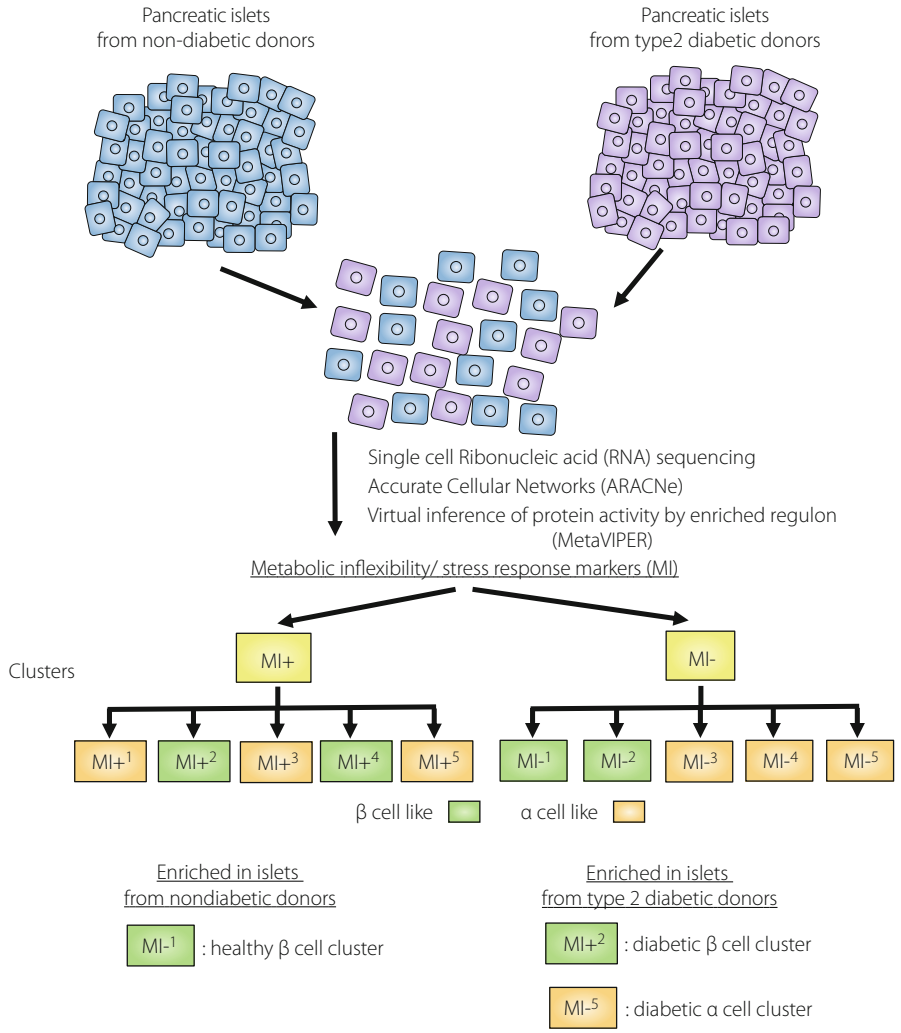

Figure 1 | The identification of type 2 diabetes-specific subpopulations characterized by metabolic inflexibility and stress response markers based on islet-specific regulatory network generation and protein activity analyses at the single-cell level.

conditions, stress responses are elicited in β-cells, leading to de-differentiation of these β-cells, thereby triggering their transdifferentiation into α-cells.

To identify master proteins regulating the transcriptional identity of islet cells in the setting of type 2 diabetes, the authors assessed activated and inactivated proteins in the MI^{+2} (type 2 diabetic β), MI^{-5} (type 2 diabetic α), MI^{-1} (healthy β) and MI^{+5} (healthy α) subclusters using metaVIPER analyses. Based on the results of these analyses, they selected 15 candidate master regulatory proteins; that is, AF4/FMR2 family member 3, BTB domain and CNC homolog 2 (BACH2), basonuclin 2, cut-like homeobox 2, EBF transcription factor 2, forkhead box O1, growth arrest specific 7, myelin transcription factor 1 like, nuclear factor of activated T cells 3, retinoic acid receptor beta, regulatory factor X7, transcription factor 4, teashirt zinc finger homeobox 2, zinc finger protein 385D and zinc finger RANBP2-type containing 3, and carried out gain-of-function experiments on these candidate proteins using islet cells from non-diabetic donors. The authors infected islet cells with adenoviruses encoding candidate genes, as well as a unique guide barcode sequence, followed by analyzing the infected islet cells, which were identified by the barcode, using single-cell ribonucleic acid sequencing. They measured the abilities of the candidate proteins to alter transcriptional identity from non-diabetic cells to type 2 diabetic cells. Consequently, they found that type 2 diabetic β-cell signatures were induced in cells transduced with BACH2/TSZH2, retinoic acid receptor beta/growth arrest specific 7/zinc finger protein 385D, zinc finger RANBP2-type containing 3/myelin transcription factor 1 like and cut-like homeobox 2/regulatory factor X7 combinations. Meanwhile, type 2 diabetic α-cell signatures were induced in cells transduced with AF4/FMR2 family member 3 alone, as well as with BACH2/TSZH2 and zinc finger RANBP2-type containing 3/myelin

transcription factor 1 like combinations. In addition, the authors also carried out experiments involving loss-of-function of candidate proteins, using a strategy similar to that used in gain-of-function experiments. These analyses showed conversion of type 2 diabetic β- and α-cell populations into healthy β- and α-cells, respectively, in response to BACH2 deficiency. Furthermore, functional analysis experiments showed that adenoviral transduction of BACH2 blunted glucose-responsive $Ca2^{+}$ flux into human non-diabetic β-cells.

Then, the authors focused on BACH2 and explored the clinical relevance of this regulatory protein in the development of type 2 diabetes. Histological analyses of pancreatic tissues from non-diabetic and type 2 diabetic patients showed that BACH2 protein is markedly increased in both β- and α-cells in type 2 diabetic samples, as compared with non-diabetic samples. In addition, the proportion of BACH2-positive cells in islet cells positive for both insulin and glucagon were significantly higher in type 2 diabetic than in non-diabetic samples. These data suggest that BACH2 activation mediates transition of islet cells from the healthy to the diabetic state, observations consistent with those obtained using metaVIPER analyses.

Finally, whether inhibition of BACH2 improves glycemia in the diabetic state was examined *in vivo* using *db*/*db* mice. Inhibition of BACH2 by compound 8, a dual BACH2/BACH1 inhibitor, decreased blood glucose levels of *db*/*db* mice in both the fasting and the non-fasting state. In addition, BACH2 inhibition partially blocked decrements in plasma insulin levels observed in vehicle-treated *db*/*db* mice, suggesting improvement of β-cell function in response to BACH2 inhibition. Furthermore, compound 8 treatment with isolated islets from *db*/*db* mice or donors with type 2 diabetes for 24 h increased glucose-stimulated insulin secretion, suggesting amelioration of β-cell failure with BACH2 inhibition. However, in these experiments, no evidence was presented showing whether reversal of islet cell characteristics from the diabetic to the healthy state had actually occurred in isolated islet cells exposed to 24-h BACH2 inhibition.

The present study unveiled a type 2 diabetes-specific subpopulation in human islet cells using a highly elegant experimental approach based on single-cell sequencing data. This novel and interesting study raises several issues that require further clarification. First, as type 2 diabetes is a highly heterogenous disease, it is important to investigate whether the diabetic signatures in islet cells identified in this study are actually present in numerous type 2 diabetes patients with a broad range of clinical features. In addition, from the therapeutic viewpoint, clarifying when these diabetic signatures are elicited in islet cells of type 2 diabetes patients is necessary, to determine the optimal time point for initiating preventive measures, such as BACH2 inhibition. Also, further studies are warranted to determine the so-called 'point of no return', when islet cell diabetic signatures become irreversible. In addition to BACH2, the authors identified several other master regulatory proteins, including AF4/FMR2 family member 3 and cut-like homeobox 2, that promote diabetic features in islet cells. Whether there is therapeutic potential in manipulating these regulatory proteins for type 2 diabetes remains an open question. Obviously, from the therapeutic viewpoint, developing strategies aimed at selectively manipulating the functions of master regulatory proteins in islet cells is another important issue.

The authors identified regulatory protein networks in human islets cells that show specifical alterations in type 2 diabetes. The results presented in this study offer novel insights applicable to future research on β cell failure.

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DISCLOSURE

The author declares no conflict of interest.

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