Spatial Environment Affects HNF4A Mutation-Specific Proteome Signatures and Cellular Morphology in hiPSC-Derived β-Like Cells

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Studies of monogenic diabetes are particularly useful because we can gain insight into the molecular events of pancreatic β-cell failure. Maturity-onset diabetes of the young 1 (MODY1) is a form of monogenic diabetes caused by a mutation in the HNF4A gene. Human-induced pluripotent stem cells (hiPSCs) provide an excellent tool for disease modeling by subsequently directing differentiation toward desired pancreatic islet cells, but cellular phenotypes in terminally differentiated cells are notoriously difficult to detect. Re-creating a spatial (three-dimensional [3D]) environment may facilitate phenotype detection. We studied MODY1 by using hiPSC-derived pancreatic β-like patient and isogenic control cell lines in two different 3D contexts. Using size-adjusted cell aggregates and alginate capsules, we show that the 3D context is critical to facilitating the detection of mutation-specific phenotypes. In 3D cell aggregates, we identified irregular cell clusters and lower levels of structural proteins by proteome analysis, whereas in 3D alginate capsules, we identified altered levels of glycolytic proteins in the glucose sensing apparatus by proteome analysis. Our study provides novel knowledge on normal and abnormal function of HNF4A, paving the way for translational studies of new drug targets that can be used in precision diabetes medicine in MODY.

RESEARCH DESIGN AND METHODS

Cell Source and Reprogramming
We reprogrammed fibroblast cells as previously described (4,10) from a donor from the Norwegian MODY Registry.
after written informed consent and with experiments approved by the Regional Committee on Medical Ethics of Western Norway (REK 2010/2295). We used isolated human pancreatic islets from three deceased donors without diabetes (REK 2011/782) and the human embryonic stem cell line H1 as control.

**Genome Editing**
Alt-R CRISPR/Cas9 reagents were provided by Integrated DNA Technologies (Coralville, IA). Briefly, 10^5 iPSCs were mixed with 12.5 μL ribonucleoprotein, 5 μL electroporation enhancer, and 2.5 μL homology-directed repair (HDR) template (stock 3 nmol/L) in 40 μL Nucleofector solution 2 (Lonza); electroporated; and subsequently incubated for 48 h. Dissociated single cells were grown until colonies emerged. Ninety-six colonies were picked and screened. Positive clones from restriction fragment–length polymorphism assay, using BccI enzyme on exon 7–amplified segments, were validated by Sanger sequencing. Seven colonies, including D13, D94, E75, and E87, were found to be corrected by the HDR template.

**Evaluation of iPSC Integrity**
We characterized iPSCs as previously described (10), including assessment by flow cytometry and immunofluorescence as described below. The chromosome analysis was done at the Cell Guidance Systems Genetics Service, Cytogenetic Laboratory, Babraham Research Campus, Cambridge, U.K. We further evaluated pluripotency potential in mutated iPSC lines using an hPSC functional identification kit (SC027B; Bio-Techne Ltd.). Specifically, three uniquely formulated media were used to differentiate hPSCs into the three germ layers endoderm, ectoderm, and mesoderm. The three germ layers were detected by immunocytochemistry using the antibodies for the transcription factors SOX17 (endoderm), Otx2 (ectoderm), and brachyury (mesoderm) (SC027B; Bio-Techne).

**Directed Differentiation**
The directed differentiation of hiPSCs to β-like cells was performed as previously described (4,11). To reduce variability, we performed the different directed differentiations in parallel in the same multiwell plate for all conditions with the same batch of differentiation cocktail. At the end of stage 5 (S5), 5.0 million cells were embedded in alginate beads, as previously described (12), 1.2 million cells were used to form cell aggregates (8), and ~5 million cells were kept in adherent culture. The beads, aggregates, and planar cultures were differentiated until stage 7 (S7), as previously described (4,9).

**Western Blotting, Flow Cytometry, Immunofluorescence, and Confocal Staining**
As described elsewhere, we performed Western blotting (13), flow cytometry (14), immunofluorescence (4), and confocal imaging (9). For Western blotting, we used the primary antibodies HNF4A (1:1,000; Cell Signaling Technology), PDX1 (1:1,000, R&D Systems), or GAPDH (1:1,000, Santa Cruz Biotechnology); otherwise, we used the following; mouse anti-Oct-3/4 (sc-5279; Santa Cruz Biotechnology), rabbit anti-NANOG (ab21624; Abcam), goat anti-SOX17 (AF1924; R&D Systems), goat anti-PDX1 (AF2419; R&D Systems), mouse anti-HNF4A (PP-H1415-00; R&D Systems), mouse anti-Nkx-6.1 (F55A12-S; Developmental Studies Hybridoma Bank, University of Iowa), and guinea pig anti-insulin (A0564; Dako). The samples were mounted in Prolong Diamond Antifade Mounting Media (P36970; Life Technologies).

**Aggregate and Alginute Formation and Processing**
We used AggreWell 400 (34411; STEMCELL Technologies) as recommended by the manufacturer. Briefly, 1.2 × 10^6 cells were seeded per AggreWell in stage 6 (S6) medium, which generated aggregates of ~1,000 cells. After 48 h incubation, the aggregates were transferred to low-adherence plates and kept on an orbital shaker (100 rpm). We encapsulated cells with alginate and processed the capsules as described elsewhere (9).

**RNA Extraction, Processing and Next-Generation RNA Sequencing**
RNA was extracted from 2D Matrigel-differentiating cells, aggregates, and encapsulated cells using an RNeasy Micro Kit (QIAGEN) and processed and sequenced (mRNA sequencing for AggreWell samples and 3’ RNA transcriptomics [QIAseq UPX 3’ Transcriptome Kit; QIAGEN] for alginate samples; 2.5 and 10 ng purified RNA for the alginate and 2D samples, respectively) as previously described (9).

**Proteomics and Pathway Analysis**
The lysed cells and human islets (positive control) were processed and analyzed with mass spectrometry as previously described (4,15), using 25 μg of each sample and three TMT11plexes, with a reference mix of all samples in 2 of the 11 samples in each plex to allow crossplex analysis. Pathway analysis was performed as previously described (4).

**Glucose-Stimulated Insulin Secretion and Oxygen Consumption Rate Assays**
As previously described, we assessed S7 cells by assays of static glucose-stimulated insulin secretion (GSIS) (4), dynamic GSIS (16), and oxygen consumption rate (OCR) (17) using 60 handpicked S7 cell clusters in dynamic GSIS (perfused with high glucose [20 mmol/L] from 42 to 84 min, whereas other fractions were exposed to low glucose [1.67 mmol/L]) and 40–60 S7 cell clusters per 24 wells during OCR. The final concentrations of 20 mmol/L glucose, 5 μmol/L oligomycin (Cell Signaling Technology), 5 μmol/L carbonyl cyanide 3-chlorophenylhydrazone (Sigma-Aldrich), and 5 μmol/L rotenone (Sigma-Aldrich) were added in order. The OCR values were normalized to
the average baseline values measured in assay media containing 1.67 mmol/L glucose.

**Statistics**
Statistical analysis was performed in Excel version 14.7.7 and GraphPad Prism 7.0.0 software. A two-sided t test was used, and \( P < 0.05 \) was considered significant. Supplementary Fig. 11 was built in R 3.6.1 using the packages tidyr version 1.1.3, dplyr version 1.0.0, scico version 1.2.0, and ggplot2 version 3.3.5.

**Data and Resource Availability**
The RNA-sequenced data sets have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (accession no. GSE188827). The proteome data sets have been deposited in ProteomeXchange through the Proteomics Identification Database (accession no. PXD025054).

**RESULTS**
Generation of Mutated and Isogenic Control Cell Lines Using MODY1 iPSCs With an HNF4A Mutation
We generated several mutated (HNF4A\(^{+/c811dupA}\)) hiPSC lines (the hiPSC lines 6A, 6D, and 6F in Supplementary Fig. 1) by reprogramming fibroblasts from a patient with MODY1 (N904-1 in Fig. 1A and Supplementary Table 1) with a c.811dupA/p.Ile271fs mutation (Fig. 1B). Using these lines, we created corrected (HNF4A\(^{+/corrected}\))
mutation-free isogenic hiPSC lines (the E75, D13, D94, E87 hiPSC lines in Supplementary Fig. 1) by CRISPR/Cas9 HDR gene editing (Fig. 1C–E and Supplementary Figs. 2–3). We subsequently assessed the integrity of the mutated and corrected hiPSC lines (10) (Fig. 1F and Supplementary Figs. 1 and 4–7).

The 3D Environment Influences the Composition of the Cellular Proteome

The mutated and corrected hiPSCs were subjected to directed differentiation (Supplementary Fig. 8) toward pancreatic β-like cells, as done previously (4), but by adding two different 3D environments (aggregate formation in AggreWell and alginate encapsulation) to the standard 2D format (Fig. 2A). Using global proteomics analysis of end-stage pancreatic β-like cells (S7), we found that the cell lines cultured in AggreWell or in alginate capsules yielded distinctively different proteomics signatures (disregarding mutational status) that were also distinctively different from the lines cultured in 2D and from islet controls (Fig. 2B). The 2D and 3D environments did not differentially impact the single-protein levels (measured by proteomics) of key β-cell markers (Fig. 2C and Supplementary Fig. 9A), which were also expressed at a similar level by immunocytochemistry (Fig. 2D). Although proteomics analysis of S7 cells did not detect the HNF4A protein, we confirmed
Figure 3—Mutation-specific metabolic switch in the 3D alginate environment. A: Schematic overview of the study design where the proteome of cells in 3D alginate beads was compared with the proteome of cells in the 2D format for both the mutated cells (HNF4A<sup>1/c811dupA</sup>; 6D) and the corrected (HNF4A<sup>1/corrected</sup>; E75) S7 cells, resulting in 1,168 and 1,130 differentially abundant proteins (DAPs) (fold change > 1.5; *P* < 0.05), respectively. The DAPs were compared to distinguish the common DAPs caused by the alginate (bead) effect (class c, 525 proteins), DAPs caused by the mutation effect (class a, 637 proteins), and DAPs caused by the corrected effect (class b, 600 proteins).

B: Top canonical pathways (*z*-score > 1) identified by analysis of class a DAPs and class b DAPs (*z*-score filter = 1). Full canonical pathway names can be found in Supplementary Material.

C: Sequential events in glycolysis and the observed abundance level of quantified proteins involved in glycolysis in 3D alginate beads compared with 2D (yellow, no difference; blue, significantly lower abundance) demonstrated lower levels of fructose-biphosphate aldolase A (ALDO), α-enolase (ENO1), triphosphate isomerase (TPI1), and phosphoglycerate kinase 1 (PGK1). Although these four proteins are not known to be directly activated by HNF4A, they are all potential targets, as they contain HNF4A binding motifs (25). When we investigated the other parts of the glucose sensing apparatus of pancreatic β-cells in the context of the alginate-encapsulated 3D environment, we did not observe mutation-specific effects either in the levels of proteins involved in glucose influx (GLUT1) or in oxidative phosphorylation (OxPhos) (data not shown).

D: Schematic illustration summarizing the findings related to a metabolic switch. Observed data are represented by circles, and Ingenuity Pathway Analysis–predicted data are represented by squares. ROS, reactive oxygen species; VS, versus.
**HNF4A** expression at intermediary stages by other methods (Supplementary Figs. 8 and 10).

**The 3D Alginate Encapsulation Environment Potentially Triggers a Mutation-Specific Metabolic Switch**

Given that the alginate 3D environment had indeed produced a unique proteomics signature of S7 cells, we next assessed whether the mutational status affected the alginate-encapsulated S7 proteome (Fig. 3A). Focusing first on the mutated alginate-encapsulated S7 cells (class a in Fig. 3A), we identified glycolysis and gluconeogenesis (predicted as inhibited) as the top canonical pathway, consistent with the known role of HNF4A to regulate metabolic glucose sensing in pancreatic β-cells (18), followed by fatty acid β-oxidation and stearate biosynthesis (predicted as activated) (Fig. 3B and Supplementary Fig. 9B). In contrast, when extending the pathway analyses to the proteome of corrected cells (class b in Fig. 3A), we were not able to predict significant differences in glycolytic and gluconeogenetic pathways or in stearate biosynthesis and fatty acid β-oxidation pathways (Fig. 3B), supporting the notion that gene correction rescued the phenotype. Analogously, from the analysis of the proteome data set at the single-protein level (Fig. 3C and D and Supplementary Fig. 9C and D), four proteins among 10 enzymes in the glycolytic pathway displayed lower levels in the alginate-encapsulated 3D environment compared with 2D for mutated lines but not in corrected lines, although these were not reproduced in the transcriptome (Supplementary Fig. 11).

**Figure 4—Mutation-specific impaired 3D cell aggregation.** A: Phase-contrast representative images of mutated (HNF4A<sup>−/−; c811dupA</sup>; 6D) and corrected (HNF4A<sup>+/+</sup>; E75) S7 cell aggregates. B: Schematic overview of the study design where the proteomes of 3D cell aggregates were compared with the 2D proteomes for both the mutated cells and the corrected cells, resulting in 1,034 and 982 differentially abundant proteins (DAPs), respectively (fold change >1.5; *P* < 0.05). The DAPs were compared to distinguish the common DAPs caused by the 3D aggregation effect (class c, 504 proteins), DAPs caused by the mutation effect (class a, 530 proteins), and the DAPs caused by the corrected effect (class b, 478 proteins). C: Top canonical pathways (z score >1) identified by analysis of class a DAPs. D: Selected proteins involved in the GP6 signaling pathway, including collagen 2A (COL2A), collagen 5A (COL5A), and other ECM proteins, including laminin B2 (LAMB2) and LAMB3, were lower in the 3D cell aggregates of mutated cells (HNF4A<sup>−/−; c811dupA</sup>; 6D) compared with mutated cells cultivated in 2D. *P* < 0.05, ****P < 0.0001 by Student *t* test. Agg, aggregate; corr, corrected; mut, mutant; tRNA, transfer RNA; VS, versus.
The 3D Aggregation Cell Environment Precipitates a Mutation-Specific Cellular, Proteome-Specific, and Functional Phenotype

Since we found that also the aggregated 3D environment had produced a unique proteomics signature of S7 cells, we next assessed the mutated AggreWell-cultivated S7 lines. Strikingly, we observed that the mutated lines formed irregular spheroids that clustered together (Fig. 4A and Supplementary Fig. 12). In contrast, the corrected lines formed regular round shapes, which did not cluster (Fig. 4A and Supplementary Fig. 12). Next, we assessed whether the mutational status affected the alginate-encapsulated S7 proteome and transcriptome (Fig. 4B and Supplementary Fig. 11), first focusing on mutated AggreWell-encapsulated S7 cells (class a in Fig. 4B). We identified GP6 signaling and apoptosis (predicted inhibition) and EIF2 signaling and transfer RNA (predicted activation) as top canonical pathways (Fig. 4C). Since the GP6 signaling pathway plays a role in collagen-induced activation and aggregation (19), we assessed the proteome at the single-protein level and found that indeed, reduced levels of collagen and extracellular matrix (ECM)-related proteins (Fig. 4D), validated at the mRNA level (Supplementary Fig. 11). Finally, we assessed and identified mutation-specific functional readouts uniquely present in the aggregated 3D context (Supplementary Fig. 13).

DISCUSSION

In this study, we were able to identify for the first time a mutation-specific phenotype in MODY1 β-like cells facilitated by growing the cells in two different 3D environments and comparing the cellular and proteomic readouts to the 2D background. We were also able to suggest two specific molecular mechanisms whereby the two different 3D culture effects could be mediated. Each of these specific molecular mechanisms showed a dependency on the type of 3D context chosen. The cellular confinement in the alginate context helped to identify a metabolic phenotype with lower levels of glycolytic proteins, potentially affecting glucose sensing. The structural scaffolding in the AggreWell 3D context helped to identify a structural collagen-associated phenotype with irregular clusters, a unique proteome with lower levels of structural ECM proteins (laminins, collagens) as well as a functional readout.

The two chosen 3D environments challenge the differentiating pancreatic β-like cells in unique ways, including the levels of oxygen delivery, nutrient supply, and cell-to-cell contact. The 3D cell aggregation/AggreWell context is preferred by several investigators in generating pancreatic β-like cells (7,8,20) because it mimics thin in vivo pancreatic islets in size, level of cell-to-cell contact, and the self-organized compact spheroid arrangement (21). In the 3D alginate encapsulation context, the cells are immobilized and lack cell-to-cell contact; however, the alginate gel-pore network still allows transport of oxygen and diffusion of nutrients and waste products (22). Furthermore, alginate encapsulation is suitable for future therapeutic transplantation (23) and can also protect the cells in bioreactor culture systems (24).

In conclusion, our findings warrant a careful consideration and selection of an appropriate 3D context when studying stem cell models. Our study also provides a patient-specific diabetes model that can be further explored for potential new drug targets that can be used in precision diabetes medicine.

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**Author Contributions.** M.C. performed immunofluorescence imaging and analyses. M.C., C.W., A.M.S., Y.B., S.C., L.M.G., H.S., and H.R. interpreted the observations. M.C., C.W., Y.B., and J.G. performed the differentiation, sample preparation for proteomic and RNA sequencing analyses, and immunofluorescence staining, and analyzed data. M.C., A.M.S., Y.B., V.L., H.S., and H.R. conceived the experiments. C.W. performed the FACS analyses. A.M.S. performed the CRISPR/Cas9 editing and IPSC integrity studies. Y.B. and S.C. analyzed the proteomics data. Y.B., H.S., and H.R. wrote the manuscript. S.A. performed the immunoblotting. S.A. and H.S. generated human islet preparations. J.A.P. performed the tandem mass tag-labeling experiment and mass spectrometry analysis. E.T. provided the skin biopsy samples. E.T., P.N., I.N., and H.R. provided clinical data. M.V. performed the skin dem mass tagletting. A.H. Knudsen for technical help, Dr. Steven P. Gygi and the Taplin Mass Spectrometry facility at Harvard Medical School for use of mass spectrometers, A.H. Knudsen for technical help, and Lars A. Akslen at the Center for Cancer Biomarkers, Department of Clinical Medicine, University of Bergen, for strategic support. The confocal imaging was performed at the Molecular Imaging Center, Department of Biomedicine, University of Bergen.

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