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Single-cell transcriptome conservation in a multispecies comparative analysis of fresh and cryopreserved insulinoma cell lines

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Abstract

Background Insulinoma is the most common pancreatic neuroendocrine tumour in dogs and humans. The understanding of driving factors and critical survival genes in insulinomas is limited and overall survival is poor for canine and human malignant insulinoma. This study aimed to use single-cell RNA-sequencing to conduct a multispecies analysis of insulinoma cell lines to understand their single-cell transcriptomic landscape. Secondly, the impact of freeze-thawing on the pancreatic beta single-cell transcriptome was investigated, to determine whether cryoarchiving of primary insulinoma samples may be feasible in future studies.

Methods Single-cell transcriptomic analysis was performed using fresh and cryopreserved multispecies insulinoma cell lines (canINS, CM, INS-1 and MIN6). R and Seurat were used to perform cell clustering and specific cluster marker genes were identified by the FindMarkers function. Metascape was used to identify statistically enriched pathways for specific cell clusters. Differentially expressed genes between fresh and cryopreserved single-cell transcriptome profiles, were defined as genes with a log2 fold change > 0.25 and a Bonferroni-adjusted $P < 0.05$, based on the Wilcoxon rank sum test.

Results Based on the specific cell line single-cell transcriptome profiles, five or six cell clusters were constructed per cell line. All cell lines expressed neuroendocrine markers and additionally INS-1 and MIN6 displayed a gene signature indicative of mature/functional pancreatic islet/beta-cells. *DEPTOR*, *BICC1*, *GHR*, *CCNB2, CENPA, LMO4, VANGL1,* and *L1CAM* were identified as cross-species conserved insulinoma cluster marker genes. Little effect was found of cryopreservation and thawing on overall gene expression at the single-cell level in insulinoma cell lines: only 6 and 29 genes had a log2 fold change > 1 in cryopreserved versus fresh canINS and CM, respectively.

Conclusions canINS, CM, INS-1 and MIN6 are all principally relevant as insulinoma models and the demonstrated differences in their single-cell transcriptomic profiles could aid researchers in selecting the appropriate cell lines for specific study objectives. Cross-species conserved insulinoma cluster marker genes were identified that harbour oncogenes and their involvement in insulinoma tumourigenesis should be investigated in future studies. The good comparability between cryopreserved and fresh insulinoma cells allows for inclusion of cryopreserved insulinoma patient samples in future studies, which allows for reduced assay-based variability.

Keywords Canine, Cryopreservation, Pancreas, Pancreatic β-cells, Pancreatic neuroendocrine neoplasm, Neuroendocrine tumour model, Single-cell RNA-sequencing

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Background

Insulinoma is the most common pancreatic neuroendocrine tumour in dogs and humans [1, 2]. Still, canine and human insulinoma are rare tumours, with estimated annual incidences of 30 cases per million dogs and 1–3 cases per million humans [3, 4]. Due to these low incidences, our understanding of driving factors and critical survival genes in insulinomas is limited. Canine insulinomas are regarded as malignant in > 95% of cases because they almost always metastasise to abdominal lymph nodes and liver [5]. Only 5–16% of human insulinomas develop lymph node and liver metastases [6]. Treatment protocols in both canine and human malignant insulinoma include aggressive multimodal therapy with a combination of surgery, and medical management with glucocorticosteroids, diazoxide, somatostatin receptor ligands and/or cytotoxic chemotherapy [7, 8]. Despite these multimodal treatment protocols, overall survival is poor for canine and human malignant insulinoma with a reported median survival time of 14 months (range 0–51 months) for surgically treated dogs and a 5-year survival of human malignant insulinoma patients of 24–67% [7, 8]. New, more precise adjuvant treatments are needed to increase the success rate of treatment modalities for malignant insulinoma.

To gain a deeper understanding of insulinoma biology and to identify novel therapeutic targets, researchers utilise in vitro and in vivo insulinoma models [9, 10]. Historically, CM (human), MIN6 (murine), and INS-1 (rat) insulinoma cell lines have been widely used [10–13]. Although these cell lines are valuable resources to study insulinomas, each of them has unique limitations [10, 14]. The CM cell line has large chromosomal re-arrangements involving the insulin gene, which has resulted in loss of insulin secretion in its early passages [15]. MIN6 and INS-1 cells secrete insulin, however, MIN6 is derived from a transgenic mouse and its genetic background does not resemble spontaneous insulinoma [16]. Similarly, INS-1 cells have mutations that are not seen in insulinoma [17]. Considering shared clinical and molecular features of canine and human malignant insulinomas, the canine insulinoma has been suggested as translational model for human malignant insulinoma [9, 10]. More recently, canINS a canine insulinoma cell line and NT-3, a human insulin-secreting pancreatic neuroendocrine cell line have been established, although NT-3 is not widely available [18, 19]. Using a veterinary comparative oncology approach, canINS and CM cells have been used to identify shared therapeutic targets in signalling pathways in canine and human insulinomas. It was demonstrated that Notch pathway inhibition successfully targeted chemoresistant cancer stem cells in both canINS and CM [18].

Aside from cell lines, tissue samples of canine insulinomas have also been used to better understand the underlying molecular mechanisms of insulinomas. Using bulk RNA-sequencing of canine insulinomas, similar transcriptomic profiles in normal pancreas and early clinical stage primary insulinomas were found, whereas late clinical stage primary insulinomas resembled the transcriptomic profile of metastatic lymph nodes [20]. However, bulk RNA-sequencing does not account for intra-tumoural heterogeneity, because it only determines average gene expression levels from the bulk of tumour cells present in a sample. Single-cell RNA-sequencing (scRNA-seq) on the other hand allows transcriptome analysis at the single cell level and can identify rare cell populations [21]. Therefore, scRNA-seq has become an established technique to identify novel cancer cell subpopulations and therapeutic targets [22].

To obtain accurate single-cell transcriptomic profiles of patient samples, ideally, upon retrieval of fresh patient samples, these samples are immediately dissociated into single cell suspensions to prevent ischaemia-related gene expression changes and RNA degradation, and the fresh single cell suspensions are subsequently partitioned into gel beads-in-emulsion for scRNA-seq [23, 24]. However, this workflow can pose logistical challenges [25]. The use of cryopreservation protocols of patient samples or single cell suspensions overcomes these challenges [26]. Cryopreservation, however, can influence the cellular transcriptome and the effect of cryopreservation on gene expression profiles and composition of cell clusters should be determined to validate the use of cryopreservation protocols in scRNA-seq [26, 27].

This study aimed to use scRNA-seq to conduct a multispecies analysis of insulinoma cell lines canINS, CM, INS-1, and MIN6 to understand their single-cell transcriptomic pancreatic endocrine differentiation landscape. Secondly, scRNA-seq of fresh and cryopreserved canINS and CM cells was conducted to understand the impact of freeze-thawing on the β-cell transcriptome, in order to determine whether cryoarchiving of primary insulinoma samples may be feasible in future studies.

Methods

Cell culture

The canine insulinoma cell line canINS was cultured in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin–streptomycin (Invitrogen) and 200 ng/mL growth hormone (GH) (ProSpec). The human insulinoma cell line CM was cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. The mouse insulinoma cell line MIN6 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) containing

25 mM glucose, supplemented with 15% FBS, 0.05 mM β-mercaptoethanol (Life Technologies) and 1% penicillin–streptomycin. The rat insulinoma cell line INS-1 was cultured in RPMI-1640 + Glutamax (Gibco) supplemented with 10% FBS, 1 mM sodium pyruvate (Life Tech), 10 mM HEPES (Life Tech), and 0.05 mM β-mercaptoethanol. All cell lines had a doubling time of 1.5–2 days, were cultured at 37 °C with 5% CO_2, and cells were passaged on reaching 70–80% confluence. Short tandem repeat (STR) analyses and *mycoplasma* testing were not performed immediately prior to scRNA-seq, as the cell lines exhibited no observable phenotypic abnormalities while maintained under standard culture conditions.

Cell cryopreservation

Freezing medium, consisting out of 90% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to aliquots of 1×10^6 cells/mL of the canINS and CM cell lines and cells were transferred to cryogenic vials. The vials were put into a CoolCell cell freezing vial container (Corning) and stored at -80 °C overnight. Vials of the frozen cell lines were then taken out of the CoolCell containers and stored at -80 °C for four weeks until use.

Sample preparation

Cryopreserved cells were thawed in a 37 °C water bath, after which they were washed using pre-warmed 1 × phosphate-buffered saline (PBS) (Gibco) supplemented with 0.04% bovine serum albumin (BSA) (Sigma-Aldrich). Single cell suspensions of both fresh and cryopreserved cells were created at a concentration of 1×10^6 cells/mL in 1 × PBS + 0.04% BSA according to the Cell Preparation for Single Cell Protocols Handbook CG00053 (10x Genomics). Using trypan blue exclusion, all cell suspensions were confirmed to have a viability greater than 90%. Samples were partitioned on a Chromium Controller (10x Genomics) within 30 min of preparation.

Single-cell RNA-sequencing

Frozen barcoded cDNA was sent to GENEWIZ from Azenta Life Sciences, Leipzig, Germany, where scRNA-seq was performed. Single-cell 3' gene expression libraries were prepared for each sample using a Chromium NEXT Gem Single Cell 3 v3.1 Kit and dual index library construction kit (10x Genomics) targeting 10,000 cells per sample. Sequencing was performed on a NovaSeq 6000 sequencer (Illumina) using a 28 × 10 × 10 × 90 bp configuration. CellRanger (version 7.1.0, 10x Genomics) was used to process raw FASTQ files and to align reads to either the canine CanFam3.1, human GRCh38, murine GRMch38/mm10, or rat mRatBN7.2 genome.

Data filtering and integration

The count matrix of each sample was imported into R (version 4.4.0) and converted to a Seurat (version 5) object. To estimate the number of dead cells, the percentage of reads mapping to mitochondrial chromosomes per cell (percent.mt) was calculated. Optimal percent.mt thresholds were determined for cell lines from different species by manually investigating the quality control plots demonstrating percent.mt for each cell line and the association between nCount_RNA versus percent.mt. Read count was well correlated with feature count for all samples based on the nCount_RNA versus nFeature_RNA plots (Additional file 1). Low quality reads and potential doublets were filtered, and only cells were retained which met the following requirements: $2000 < \text{nFeature\_RNA} < 8000$, and $\text{percent.mt} < 5$ (MIN6 and canINS), or $\text{percent.mt} < 10$ (INS-1 and CM). To mitigate the effects of cell cycle heterogeneity, each cell was assigned a cell score based on its expression of G2/M and S phase markers using the CellcycleScoring function and cell cycle scores were regressed out during data scaling using the ScaleData function [28]. The SCTransform function was used for normalising the count data for each dataset. Batch correction was performed for the fresh and cryopreserved canINS samples and the fresh and cryopreserved CM samples using the Data Integration function after selecting the most variable features which were used to extract integrating anchors using the FindIntegrationAnchors function. Principal component analysis was performed and the first 17 dimensions were used to compute a Uniform Manifold Approximation and Projection (UMAP).

Identification of cluster marker genes, enriched pathways, and differentially expressed genes

Cells were clustered using the FindClusters function and specific cluster marker genes were identified by the FindMarkers function. Because the different cell lines were likely at different stages of β-cell differentiation, an important part of the analysis was to characterise expression of a panel of neuroendocrine and specific β-cell differentiation markers. FeaturePlot and VlnPlot functions were used to visualise the expression of markers genes of neuroendocrine differentiation, epithelial/mesenchymal (E/M) differentiation, islet cell hormones and mature β-cell differentiation, and immature β-cells and lineage-specific pancreatic progenitors. If no cells were identified to express a specific marker gene on the FeaturePlot, the expression of that specific marker gene was classified as absent in that cell line sample. The cluster marker specificity of the top 10 markers with the highest log2 fold changes per cluster was investigated using the VlnPlot

Table 1 Single-cell RNA-sequencing metrics for fresh and cryopreserved insulinoma cell lines

Sample	Species	# Cells recovered	Mean Reads per Cell	Median genes per cell	Number of reads	Reads mapped to genome
CM-fresh	human	9,423	43,042	4,908	406 M	89.40%
CM-cryo	human	14,311	23,931	3,392	342 M	90.80%
canINS-fresh	dog	6,035	68,959	5,964	416 M	81.50%
canINS-cryo	dog	10,606	29,993	3,847	318 M	84.90%
INS-1	rat	9,111	44,680	4,153	407 M	85.70%
MIN6	mouse	5,427	65,279	3,606	354 M	76%

function. Marker genes that were found to be unique for specific clusters were novel characteristics of insulinoma cell lines and included putative candidate genes responsible for insulinoma cell immortality. To identify species-conserved candidate genes, for all cell lines it was determined if the unique cluster marker genes of one cell line were also expressed in the other three cell lines. Only cluster marker genes expressed in all four insulinoma cell lines with plausible cancer-related functions based on a PubMed literature search, were added to the list of candidate genes. To identify enriched pathways for specific cell clusters, differentially expressed genes (DEGs) per cluster with a log2 fold change > 1 were analysed for statistically enriched pathways, including Gene Ontology (GO), KEGG, Reactome and MSigDB, using Metascape (https://www.metascape.org) [29–33].The *Mus musculus* genome and the *Rattus norvegicus* genomes were used as background genomes for MIN6 and INS-1, respectively. The *Homo sapiens* genome was used as background genome for both canINS and CM. Associated gene networks were constructed using unsupervised clustering based on *k*-means. Differentially expressed genes between fresh and cryopreserved single-cell transcriptome profiles, were defined as genes with a log2 fold change > 0.25 and a Bonferroni-adjusted $P < 0.05$, based on the Wilcoxon rank sum test as implemented in Seurat's FindMarkers function.

Results

Clustering and single-cell phenotypic characterisation

Depending on the specific cell line, a total of 5,427 – 9,423 cells were analysed with a median of 3,606 – 5,964 genes per cell, and 43,042 – 68,959 mean reads per cell (Table 1). Based on the specific cell line single-cell transcriptome profiles, 5 or 6 cell clusters were constructed per cell line (Fig. 1A). INS-1 and MIN6 both expressed *Ins1* and *Ins2* whereas canINS and CM did not express insulin. The *ACVR1C* gene which encodes for activin receptor-like kinase 7 (ALK7), a receptor regulating insulin secretion and β-cell function in response to Nodal or Activin B binding, was the only marker of mature β-cells expressed in all four insulinoma cell lines. *ACVR1C* was the only mature β-cell marker, expressed in canINS, whereas CM expressed both *ACVR1C* and *MAFA*. INS-1 and MIN6 expressed all islet hormones and mature β-cell markers from the tested marker panel (*Acvr1c*, *Gcg*, *Ins1*, *Ins2*, *Mafa*, and *Pdx1*) (Fig. 1B). The expression of neuroendocrine differentiation markers and immature β-cells and lineage-specific pancreatic progenitors in the insulinoma cell lines is displayed in Additional files 2 and 3, respectively. Neuroendocrine differentiation markers expressed in all four insulinoma cell lines were *ENO2*, *MAP2,* and *NCAM1.* Additionally, CM, INS-1 and MIN6 also expressed *CHGA*, *SSTR2*, and *SYP*. *GATA4* and *ISL1*, which were the only lineage-specific pancreatic progenitors expressed in canINS, were expressed in all tested cell lines. Additionally, the other three cell lines expressed *GATA6*, *NEUROD1*, *NKX2-2* and *PAX6*, whereas INS-1 and MIN6 also expressed *FOXA2*, *NKX6-1* and *PAX4*.

Insulinomas have been hypothesised to derive from pancreatic ducts, and therefore should have an epithelial phenotype [34]. However, when cultured in vitro in monolayer conditions, pancreatic β-cells undergo epithelial mesenchymal transition (EMT) [35]. To assess the EMT status of the cell lines, the gene expression of ductal/epithelial markers (*ANXA4*, *CDH1* and *SLC4A4*) and mesenchymal marker vimentin (*VIM)* was investigated. All four insulinoma cell lines expressed *ANXA4*, *CDH1* and *SLC4A4.* The expression of vimentin was detected in all insulinoma cell lines, except for MIN6. *VIM* expression was significantly higher in canINS and CM, compared to INS-1 (Additional file 4). An overview of the average expression levels as well as the fraction of cells expressing each marker gene from the tested panel per cell line is provided in Additional file 5.

Cross-species conserved insulinoma cluster marker genes harbour oncogenes

Single-cell transcriptomics analysis enables the detection of cluster marker genes which are robustly expressed

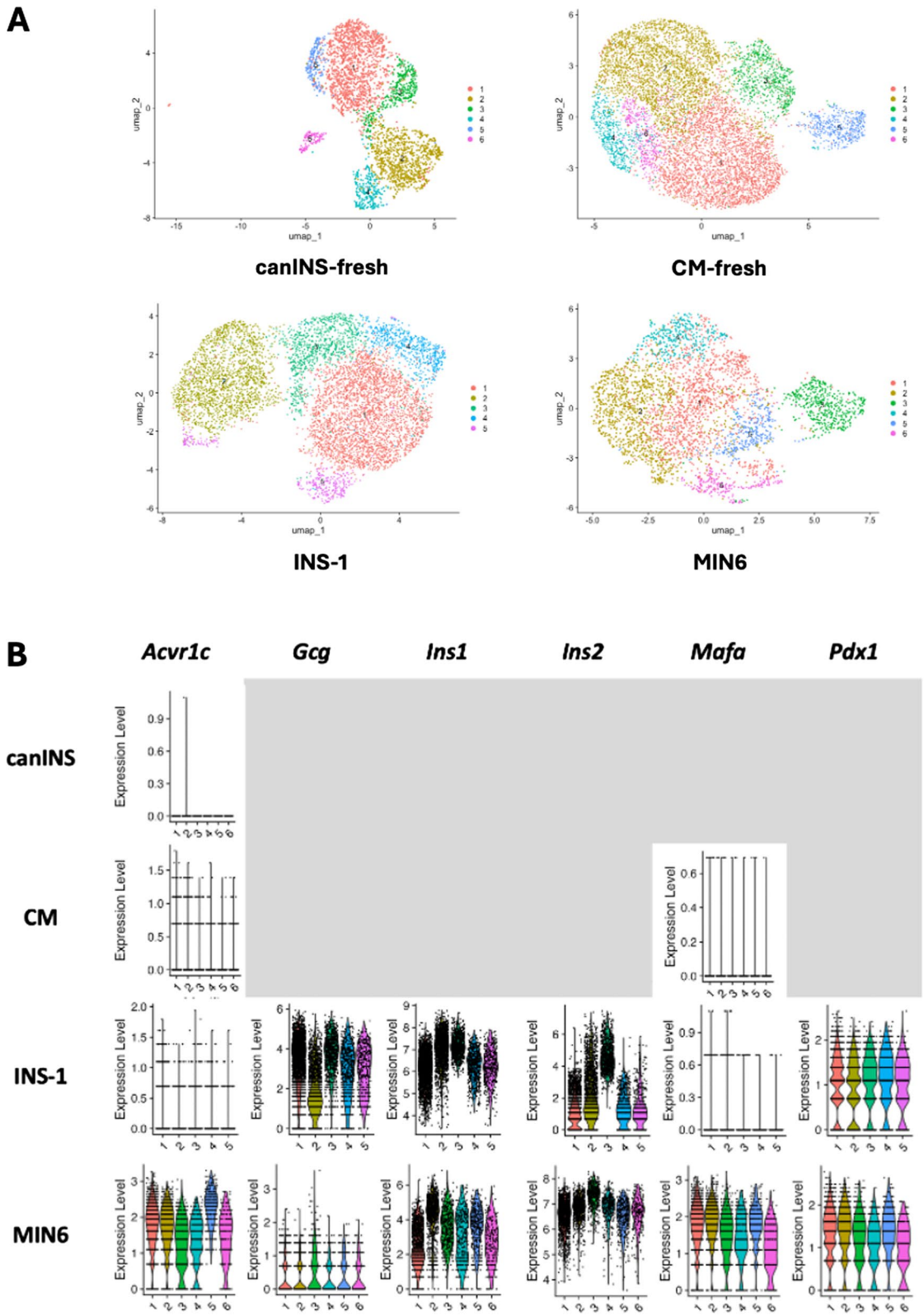

Fig. 1 Uniform Manifold Approximation and Projection (UMAP) plots of single-cell transcriptomes of insulinoma cell lines. **A** Six cell clusters were identified in canINS and CM, whereas five cell clusters were identified in INS-1 and MIN6. **B** Violin plots showing expression of islet hormones and mature β-cell markers. Grey blocks represent absent marker expression. *Acvr1c* was the only marker of mature β-cells expressed in all four insulinoma cell lines

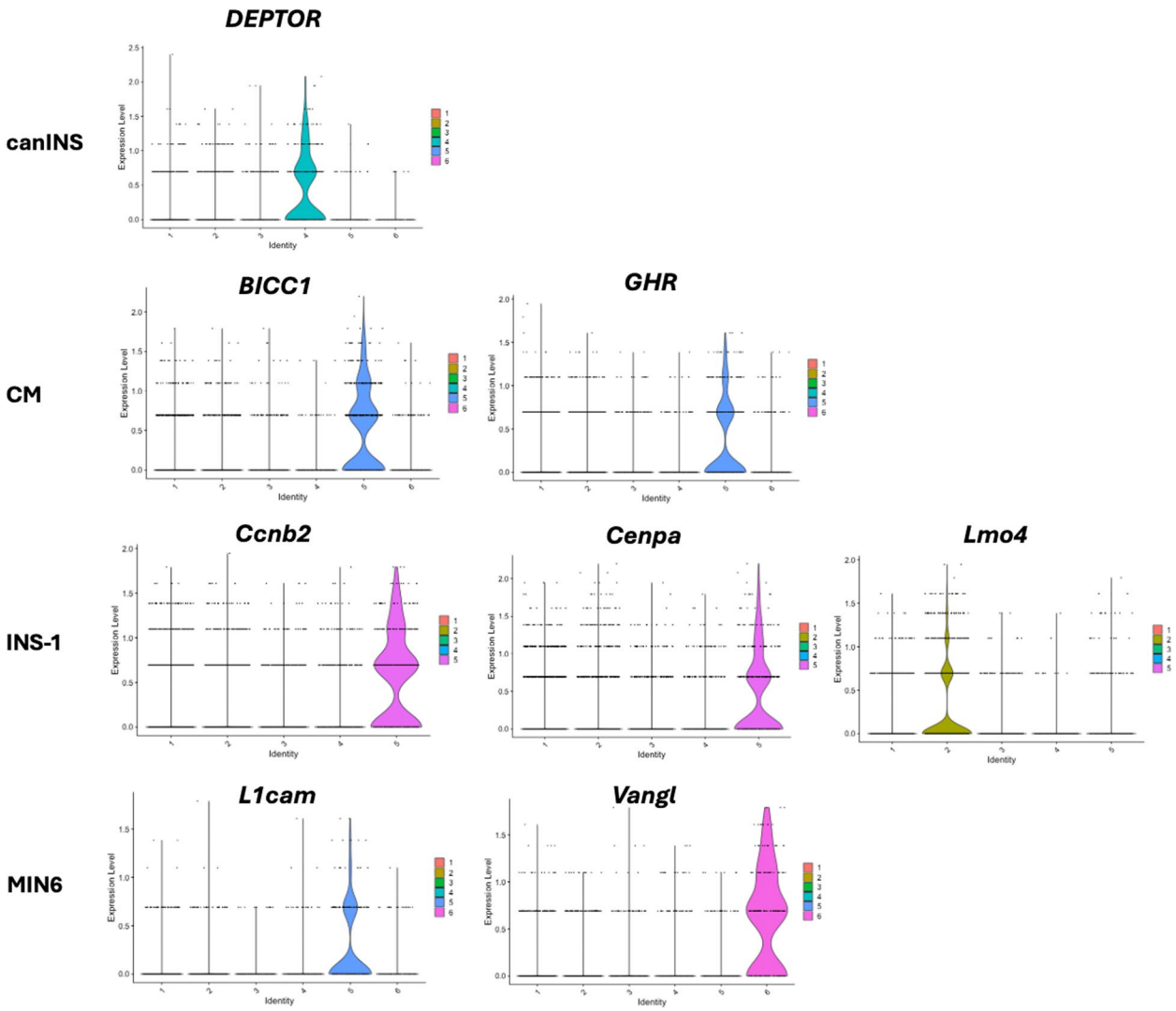

Fig. 2 Violin plots demonstrating cross-species conserved insulinoma cluster marker genes. These unique cell cluster markers that are conserved between species harbour oncogenes

genes in subpopulations of cells, that might otherwise be identified as low-expressing genes in bulk RNA-sequencing. Therefore, we identified the top 10 markers for each cluster of all insulinoma cell lines (Additional file 6). Out of the 23 clusters detected in total in the four cell lines, only four clusters (canINS cluster 1, CM clusters 4 and 6, and MIN6 cluster 1) had a top 10 marker gene signature that only contained genes uniquely expressed in that specific cell line (Additional file 6). The remaining 19 clusters had a top 10 marker gene signature characterised by genes that were consistently expressed in all cell lines. Because marker genes that were uniquely expressed in specific cell clusters could include oncogenes, it was investigated which of the unique cell cluster markers with plausible cancer-related functions were conserved between species and expressed in all four insulinoma cell lines. *DEPTOR*, *BICC1*, *GHR*, *CCNB2, CENPA, LMO4, VANGL1,* and *L1CAM* were identified as novel candidate genes with putative involvement in insulinoma tumourigenesis (Fig. 2).

Enriched signalling pathways

GO and KEGG analyses of DEGs per cluster were performed to investigate statistically enriched gene signalling pathways in the subclusters (Additional files 7–10). All four cell lines had at least one cluster that revealed a higher proportion of genes involved in neuroendocrine cell function: 'Export from cell' was enriched in canINS cluster 5, 'Secretion by cell' was enriched in CM cluster 2, 'Peptide hormone metabolism' and 'Response

to hormone' were enriched in INS-1 clusters 1 and 3, respectively, and 'Secretion by tissue' was enriched in MIN6 cluster 3. The neuroendocrine origin of canINS, INS-1 and MIN6 was further emphasised by neuronal system pathway genes being significantly enriched in canINS cluster 2, INS-1 cluster 2, and MIN6 cluster 5. Finally, canINS cluster 3, CM cluster 3, INS-1 cluster 5 and MIN6 cluster 4 were identified as clusters that were significantly enriched in genes involved in cell cycle regulation (Additional files 7–10).

Effect of DMSO cryopreservation on insulinoma single-cell transcriptomics

A total of 10,605 cryopreserved canINS cells and 14,311 cryopreserved CM cells were analysed with a median of 3,847 and 3,392 genes per cell, and 29,993 and 23,931 reads per cell, respectively (Table 1). Based on the single-cell transcriptome profiles, 5 canINS cell clusters and 7 CM cell clusters were constructed from the cryopreserved samples (Fig. 3A). Clusters 1, 2 and 3 from cryopreserved canINS cells were similar in gene expression to clusters 1, 2 and 4 from fresh canINS cells, respectively, sharing 5 – 6 marker genes from the top 10 cluster marker genes per cluster. Clusters 1, 2, 4 and 5 from fresh and cryopreserved CM cells were similar in gene expression, sharing 5 – 9 marker genes from the top 10 cluster marker genes per cluster (Fig. 3B, Additional file 6). After merging the canINS-fresh and canINS-cryo datasets and the CM-fresh and CM-cryo datasets the UMAPs demonstrated close to equal distribution of the cells of the fresh and cryopreserved samples in all clusters with strong correlations between the levels of expression of the top 10 marker genes per cluster between fresh and cryopreserved cells (Additional file 11). Expression of marker genes of neuroendocrine differentiation, E/M differentiation, islet cell hormones and mature β-cell differentiation, and immature β-cells and lineage-specific pancreatic progenitors was maintained in cryopreserved canINS and CM cells. To further evaluate gene expression changes associated with cryopreservation of insulinoma cells, differential gene expression between fresh and cryopreserved samples was investigated. 1,395 genes were differentially expressed between fresh and cryopreserved canINS cells, but only 6 genes had a log2 fold change > 1 and were classified as top DEGs (Additional file 12). *BTF3*, *MEI4*, *NUPR1*, *FOS* and *SLC25A6* were the top DEGs upregulated in cryopreserved canINS cells. 1,484 genes were differentially expressed between fresh and cryopreserved CM cells, of which 29 genes had a log2 fold change > 1 (Additional file 12). *TNFRSF12A*, *CKS1B* and *PTTG1* were the top DEGs upregulated in cryopreserved CM cells with the remainder of the top DEGs being downregulated in cryopreserved CM cells.

Discussion

Single-cell RNA-seq has become a powerful technique to investigate intra-tumoural heterogeneity, enabling the identification of rare subpopulations of tumour cells that may underlie treatment resistance or drive metastasis [21, 22]. Genetic diversity and interactions within the tumour microenvironment are key factors that drive intra-tumoural heterogeneity [36]. Although these factors are absent in vitro and cancer cell lines are considered clonal in origin, a recent scRNA-seq study demonstrated that a considerable fraction of intra-tumoural heterogeneity reflects intrinsic cellular plasticity which is maintained in the in vitro environment [37]. The fact that discrete subpopulations of cells can be identified in cancer cell lines, underpins the relevance of this study that for the first time has investigated multispecies single-cell transcriptomic profiles of insulinoma cell lines.

The first goal of the present study was to conduct a single-cell transcriptomic analysis of four insulinoma cell lines and to better characterise their state of pancreatic endocrine differentiation. Dedifferentiation of pancreatic β-cells resulting in loss of insulin-secretory capacity in adherent monolayer cell culture conditions is one of the main challenges in creating representative in vitro preclinical insulinoma models [9, 35, 38]. Insights into the differentiation status of different insulinoma cell lines will allow researchers to make well-informed decisions regarding what insulinoma cell lines to select to address specific research questions.

Single-cell RNA-seq identified individual clusters within each insulinoma cell line with unique expression profiles and different gene signalling pathways differentially up-regulated based on cluster. The INS-1 and MIN6 cell lines expressed all markers from the tested panel of islet cell hormones and mature β-cell differentiation, including insulin, whereas *ACVR1C* and *MAFA* were the only mature β-cell markers expressed by canINS and CM. To a different degree, all insulinoma cell lines did express markers genes of neuroendocrine differentiation, and markers of immature β-cells and lineage-specific pancreatic progenitors. These results confirm the neuroendocrine phenotype of canINS, CM, INS-1 and MIN6 cells albeit the marker expression in canINS and CM cells was generally lower than in INS-1 and MIN6 cells. The lack of insulin expression and secretion of canINS and CM has been previously reported [15, 18]. Interestingly, canINS however regained insulin expression and secretion when cultured in non-adherent conditions, which together with the expression of neuroendocrine markers, suggests

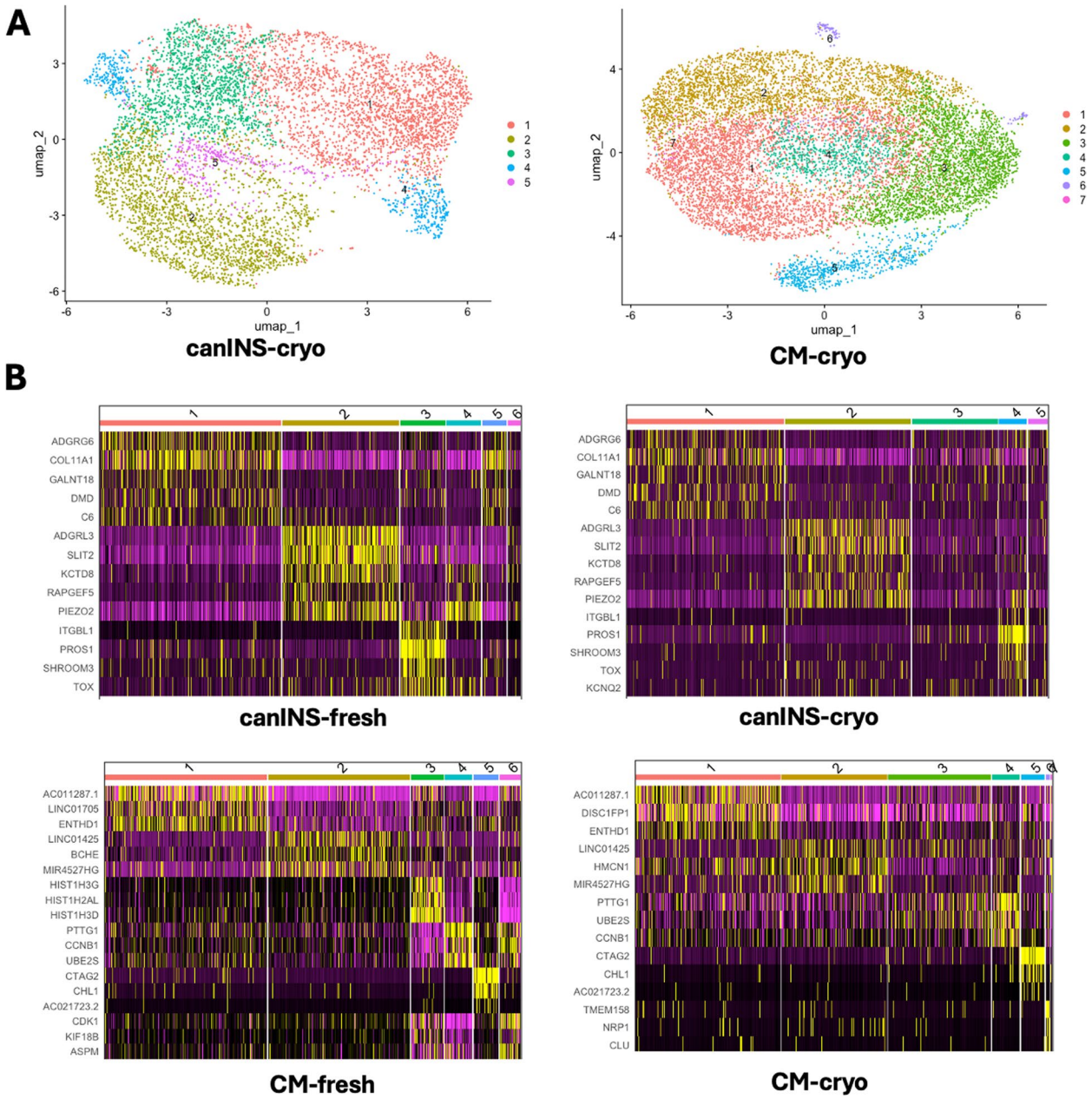

Fig. 3 Cell populations captured from scRNA-seq of cryopreserved canINS and CM cells. **A** Uniform Manifold Approximation and Projection (UMAP) plots of single cell transcriptomes of cryopreserved canINS and CM cells. **B** Heatmaps of the top 3 cluster marker genes per cluster demonstrate that cluster marker gene expression has largely been conserved between fresh and cryopreserved canINS and CM cells

that the epigenetic memory of these cells from their origin is intact [18]. Although, it has not been investigated whether CM cells are able to regain their insulin expression and secretion, the fact that insulin expression and secretion was regained in canINS implies that the absence of insulin expression per se does not exclude cell lines of being potential models for insulinomas. Moreover, the benefit canINS and CM cells have over INS-1 and MIN6 cells is that they have been established from spontaneously occurring insulinomas in contrast to INS-1 and MIN6. INS-1 has been established from a radiation induced rat insulinoma, whereas MIN6 originates from a transgenic mouse expressing the large T antigen under the control of the rat insulin promotor [12, 13]. Therefore, the genetic background of INS-1 and MIN6 do not resemble typical insulinoma,

which is a disadvantage when using these cell lines as preclinical insulinoma models.

When expanded in monolayer culture, pancreatic β-cells undergo EMT, resulting in highly proliferative mesenchymal cells that retain the ability to re-differentiate into insulin-producing cells [35]. In line with their insulin-producing capacity, INS-1 and MIN6 expressed epithelial/ductal markers, whereas INS-1 only demonstrated low expression of mesenchymal marker *VIM* and MIN6 did not express any *VIM*. Reflecting their neuroendocrine pancreatic progenitor phenotype, canINS and CM expressed higher levels of *VIM*, but these cell lines also maintained expression of epithelial/ductal markers, suggesting that these cell lines contain a large proportion of hybrid E/M cells. The occurrence of hybrid E/M cells co-expressing *CDH1* and *VIM* has been previously reported in prostate cancer, lung cancer and colorectal cancer cell lines [39]. EMT is not an all-or-none process, and E/M hybrid cells are considered to display collective cell migration by giving rise to clusters of circulating tumour cells, which enhances their metastatic potential compared to that of individually migrating ones [40]. The canINS and CM cell lines would be preferable over the INS-1 and MIN6 cell lines to study the role of E/M hybrid cells in metastatic potential of insulinomas.

Cross-species comparative transcriptomic analysis of cancer cell lines allows for the identification of conserved transcriptomic changes that despite species differences reflect significant changes that contribute to tumourigenesis [41, 42]. To harness the power of single-cell transcriptomic analysis that enables the detection of rare cell types that could drive tumourigenesis, this study focused on the detection of unique cluster marker genes that were conserved between species. *DEPTOR*, *BICC1*, *GHR*, *CCNB2, CENPA, LMO4, VANGL1,* and *L1CAM* were identified as cross-species conserved unique cluster marker genes in the insulinoma cells lines. Previously these genes have been demonstrated to display protumour effects in a variety of cancers [43–50]. Especially, *BICC1*, *GHR*, *CCNB2*, *CENPA*, *LMO4*, and *L1CAM* are interesting candidate genes for future functional validation studies investigating their precise role as potential driver genes of insulinoma tumourigenesis, as they have previously been identified as core genes, contributing to pathogenesis of either pancreatic neuroendocrine tumours, insulinomas, or pancreatic adenocarcinomas [44–48, 50]. Growth hormone receptor (GHR) expression has previously been demonstrated using immunohistochemistry in canine primary insulinomas and insulinoma metastases [44]. Moreover, GH and insulin-like growth factor 1 (IGF-1) expression were increased in metastases compared to primary canine insulinomas, and it has been hypothesised that therapeutic targeting of the GH/IGF-1 axis might inhibit canine insulinoma proliferation and prevent outgrowth of micrometastases after insulinoma surgery [44]. Currently, pegvisomant is the only clinically available GHR antagonist, which is FDA approved for the treatment of acromegaly, but more GHR antagonists have recently been developed [51]. Although there is growing evidence that GH/IGF-1 axis signalling can promote cancer progression through the GHR, so far only limited studies have investigated the anti-cancer effects of GHR antagonists. Promising results have been obtained with compound G, a pegvisomant variant. Both compound G and pegvisomant demonstrated single-agent efficacy against pancreatic cancer xenografts and, when combined with gemcitabine, reduced tumour growth more effectively than monotherapy [52]. The results of the current study provide a rationale to further investigate the effects of GHR inhibition on insulinomas, and all four tested insulinoma cell lines could serve as model for these experiments as *GHR* was identified as a cross-species conserved unique cluster marker gene.

The main limitation of our scRNA-seq cross-species comparative analysis is its descriptive nature. As this was a hypothesis-generating study, it was beyond the scope of this study to perform in depth functional characterisation of the different insulinoma cell lines. Previously, we demonstrated that canINS and CM cells demonstrate similar invasive ability and colony formation in vitro [18]. Future studies should however focus on further cross-species comparative analysis of the insulinoma cell lines using additional orthogonal functional assays. Another limitation of our study is the lack of STR analyses and *mycoplasma* testing immediately prior to performing scRNA-seq. Although we did not appreciate any phenotypic changes to the cells, without STR analyses we cannot guarantee the authenticity of the cell line samples used in our study, nor the absence of *mycoplasma* which could have potentially altered gene expression [53].

Single-cell RNA-seq has demonstrated to work well for fresh cells, whereas single-nucleus RNA-seq is suitable for frozen tissue. However, scRNA-seq captures both cytoplasmic and nuclear RNA, leading to higher transcript detection compared to snRNA-seq [54]. Therefore, the second goal of this study was to compare single-cell transcriptomic profiles from fresh and frozen insulinoma cell line samples to understand whether cryoarchiving of insulinoma samples is feasible in future studies without compromising data quality. Cryopreservation and thawing of canINS and CM cells did not impact the expression of marker genes of neuroendocrine differentiation, E/M differentiation, islet cell hormones and mature β-cell differentiation, and immature β-cells and lineage-specific pancreatic progenitors. Moreover, 3 clusters of each cell line were almost identical in fresh and frozen cells based

on their top cluster marker genes from the single cell transcriptomic profiles.

Finally, the similarity between fresh and cryopreserved canINS and CM cells was underpinned by the absence of many DEGs between fresh and frozen cells, which is in line with previous studies that demonstrated that cryopreservation only had minimal impact on single cell transcriptomics [25, 27, 55–57]. Out of 1,395 genes differentially expressed between fresh and cryopreserved canINS cells, only 6 genes had a log2 fold change > 1 and were classified as top DEGs. *BTF3*, *MEI4*, *NUPR1*, *FOS* and *SLC25A6* were the top DEGs upregulated in cryopreserved canINS cells. 1,484 genes were differentially expressed between fresh and cryopreserved CM cells, of which 29 genes had a log2 fold change > 1. *TNFRSF12A*, *CKS1B* and *PTTG1* were the top DEGs upregulated in cryopreserved CM cells with the remainder of the top DEGs being downregulated in cryopreserved CM cells. To the authors' knowledge, out of these top DEGs only upregulation of *FOS* has previously been reported to be associated with DMSO cryopreservation of human HEK293, murine NIH 3T3 cells and murine monocyte-derived macrophages [27]. FOS is a nuclear phosphoprotein which forms a complex with the JUN/AP-1 transcription factor and has an important role in signal transduction, cell proliferation and differentiation [58]. Although validation of the reported DEGs using quantitative real-time polymerase chain reactions was beyond the scope of the current study, it is well known that any cryopreservation method will cause some slight perturbations to the cells. This minor method-related bias will need to be considered when applying DMSO cryopreservation prior to processing cells for scRNA-seq.

Conclusions

In this study, it was demonstrated that canINS and CM cell lines display a neuroendocrine phenotype, but unlike the INS-1 and MIN6 cell lines, do not display a gene signature indicative of mature/functional pancreatic islet/β-cells. Rather, canINS and CM express genes normally activated during pancreatic development or in early endocrine progenitors. Nevertheless, canINS, CM, INS-1 and MIN6 are all principally relevant as insulinoma models and the results of this study could aid researchers in selecting the appropriate cell lines for specific study objectives. Whereas this study focused on the interrogation of the scRNA-seq data in terms of expression of neuroendocrine differentiation markers, immature β-cells and lineage-specific pancreatic progenitors, the raw data which have been made publicly available can be used in future studies to further investigate the relatedness between the multispecies insulinoma cell lines. In comparing relative differences between fresh and cryopreserved insulinoma cell line samples, little effect was found of cryopreservation and thawing on overall gene expression at the single-cell level. The good comparability between cryopreserved and fresh insulinoma cells allows for inclusion of cryopreserved insulinoma patient samples in future studies, which allows for reduced assay-based variability.

Abbreviations

BSA	Bovine serum albumin
DEGs	Differentially expressed genes
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
E/M	Epithelial/mesenchymal
EMT	Epithelial mesenchymal transition
FBS	Fetal bovine serum
GH	Growth hormone
GHR	Growth hormone receptor
IGF-1	Insulin-like growth factor 1
PBS	Phosphate-buffered saline
scRNA-seq	Single-cell RNA-sequencing
snRNA-seq	Single-nucleus RNA-sequencing
STR	Short tandem repeat
UMAP	Uniform Manifold Approximation and Projection

Supplementary Information

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Additional file 1. Dot plot of data quality control in scRNA-seq data. The colour of the dots represents the percentage of mitochondrial gene expression. The threshold to retain cells was set at 2000 < nFeature_RNA < 8000, and percent.mt<5 (MIN6 and canINS), or percent.mt<10 (INS-1 and CM).

Additional file 2. Violin plots of insulinoma cell lines demonstrating the expression of neuroendocrine differentiation markers. Grey blocks represent absent marker expression.

Additional file 3. Violin plots of insulinoma cell lines demonstrating the expression of markers of immature β-cells and lineage-specific pancreatic progenitors. Grey blocks represent absent marker expression.

Additional file 4. Feature plots of insulinoma cell lines demonstrating ductal/epithelial and mesenchymal marker expression. Grey block represents absent marker expression.

Additional file 5. Bubble plot demonstrating the average expression levels as well as the fraction of cells expressing each marker gene from the tested panel in canINS, CM, INS-1 and MIN6.

Additional file 6. Top 10 cluster marker genes of canINS, CM, INS-1 and MIN6 clusters. Marker genes in bold type were conserved in all four insulinoma cell lines. Highlighted in yellow are marker genes that were conserved in cryopreserved canINS and CM.

Additional file 7. Functional enrichment analysis of canINS clusters by Metascape. Bar charts of clustered enrichment ontology categories (GO and KEGG terms) are coloured based on *P*-values.

Additional file 8. Functional enrichment analysis of CM clusters by Metascape. Bar charts of clustered enrichment ontology categories (GO and KEGG terms) are coloured based on *P*-values.

Additional file 9. Functional enrichment analysis of INS-1 clusters by Metascape. Bar charts of clustered enrichment ontology categories (GO and KEGG terms) are coloured based on *P*-values.

Additional file 10. Functional enrichment analysis of MIN6 clusters by Metascape. Bar charts of clustered enrichment ontology categories (GO and KEGG terms) are coloured based on *P*-values.

Additional file 11. Merged UMAPs of canINS-fresh and canINS-cryo and CM-fresh and CM-cryo, respectively, demonstrating close to equal distribution of the cells of the fresh and cryopreserved samples in all clusters with strong correlations between the levels of expression of the top 10 marker genes per cluster between fresh and cryopreserved cells.

Additional file 12. Differentially expressed genes (DEGs) between fresh versus cryopreserved canINS and CM, respectively. 1,395 DEGs were identified between fresh and cryopreserved canINS cells with 6 top DEGs. 1,484 genes DEGs were identified between fresh and cryopreserved CM cells with 29 top DEGs.

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Authors' contributions
FOB and LJD conceived the study. FOB generated data, provided data analysis, visualisation and interpretation, and prepared the original manuscript. PYKC provided data analysis and visualisation. DX provided bioinformatics support. All authors reviewed, edited and approved the final manuscript prior to submission.

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Data availability
The datasets generated and analysed during the current study are available in NCIB's Gene Expression Omnibus (GEO) repository: GSE284573.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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