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RESEARCH ARTICLE

The long noncoding RNA *MALAT1* predicts human islet isolation quality

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Human islet isolation is a cost- and resource-intensive program for generating islets for cell therapy in type 1 diabetes. However, only one-third of cadaveric pancreases get to clinical transplantation because of low quality/number of islets. There is a need to identify biomarkers that predict the quality of islets, before initiating their isolation. Here, we sequenced transcriptomes from 18 human islet preparations stratified into 3 groups (group 1: best quality/transplantable islets; group 2: intermediary quality; and group 3: inferior quality/nontransplantable islets) based on routine measurements, including islet purity/viability. Machine-learning algorithms involving penalized regression analyses identified 10 long noncoding RNAs (lncRNAs) that were significantly different across all group-wise comparisons (group 1 vs. group 2, group 2 vs. group 3, and group 1 vs. group 3). Two variants of metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) lncRNA were common across all comparisons. We then confirmed RNA-Seq findings in a validation set of 75 human islet preparations. Finally, in 19 pancreas samples, we demonstrated that assessing the levels of *MALAT1* variants alone (receiver operator characteristic curve AUC: 0.83) offers higher specificity in predicting postisolation islet quality, further improving the predictive potential for clinical islet transplantation when combined with Edmonton Donor Points/BMI/North American Islet Donor Score. We present this resource of islet quality–stratified lncRNA transcriptome data and identify *MALAT1* as a biomarker that significantly enhances current selection methods for clinical-grade (good manufacturing practice–grade) islet isolation.

Introduction

Type 1 diabetes (T1D) is currently managed via multiple daily insulin injections or continuous glucose monitors. Transplantation of human cadaveric islets is the only approved/health care–supported cell therapy for T1D in countries, such as Australia, Canada, France, Italy, Switzerland, and the United Kingdom. In the United States, although islet autotransplants (in chronic pancreatitis) are reimbursable, islet allotransplantation in individuals with T1D is performed only through clinical trials. Usually, costs of islet isolation from a human cadaveric pancreas are very high (~US$40,000/cadaveric pancreas) because of good manufacturing practice–grade (GMP-grade) reagents and workflows (1) necessary for clinical islet transplantation. Donor characteristics are important in selecting cadaveric pancreas for GMP-grade islet isolation procedures (2). Although some studies (3, 4) demonstrated donor BMI as a positive predictor of islet yield, islet viability, and insulin secretion, the Collaborative Islet Transplant Registry (CITR) data failed to validate these observations (5). Although older donors tend to yield higher numbers of islets than younger donors (6, 7), islet function appears to deteriorate with age (8). Therefore, islet isolation centers often face the difficult question of whether cost- and resource-intensive GMP-grade ($40,000) or the less pricey (~$700) research-grade reagents should be used for an available donor cadaveric pancreas. There is a need to identify biomarkers that can predict the quality and not just higher numbers of islets within donor pancreases, before undertaking this resource-, time-, and cost-intensive islet isolation procedure. Such a biomarker should not only

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independently predict the quality of islets before their isolation from the donor pancreas, but also add value when combined with existing parameters for donor pancreas selection.

Long noncoding RNAs (lncRNAs) constitute a group of RNA molecules more than 200 nucleotides long that are not translated into proteins. LncRNAs are associated with different diseases (9, 10), including diabetes (11–14), and are known to orchestrate biologically relevant (15) signaling networks that regulate cell- and tissue-specific gene expression (16–18). Recent studies have examined lncRNAs in human pancreatic islets (19–23); one of these landmark studies (22) uncovered human islet cell lncRNAs that are associated with key pancreatic transcription factors. A recent follow-up study from the same group (19) identified 2373 β cell lncRNAs in a pool of 41 human islet preparations. The lncRNA *PDX1-AS1/PLUTO* (19), although expressed at low abundance in human islets, demonstrated a positive correlation with *PDX1* repression in islets from donors with type 2 diabetes (T2D) or impaired glucose tolerance (IGT). These studies describe the potential role of lncRNAs in the development of IGT or T2D.

Here, we sought to examine and compare the lncRNA profiles of isolated human islet sample preparations that were stratified into 3 groups based on their postisolation islet quality. Whole-transcriptome RNA-Seq followed by implementation of machine-learning statistical analytical algorithms identified lncRNA candidate biomarkers of islet quality. Next, we validated the expression of these lncRNAs using TaqMan-based real-time quantitative PCR (qPCR) in 75 additional islet sample preparations. Finally, the potential of these lncRNAs to predict postisolation islet quality was assessed in 19 donor pancreatic tissue samples. The lncRNA metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) predicted the quality of islets within the cadaveric pancreas, before their isolation. Pancreatic *MALAT1* lncRNA expression alone could predict the suitability of donor pancreases for clinical islet transplantation (receiver operator characteristic [ROC] curve AUC: 0.83), better than the current BMI-alone criterion (ROC curve AUC: 0.79). A combined mathematical model generated to predict higher islet number (BMI) and better islet quality (*MALAT1* lncRNA expression) delivered higher predictive power (ROC curve AUC: 0.94) that substantially improves the predictive power over either of the currently used donor criteria (BMI, Edmonton donor points, or North American Islet Donor Score/NAIDS) alone. When combined with existing pancreas donor scores, such as the North American Islet Donor Score (NAIDS) (24) and the Edmonton Donor Points (2), *MALAT1* offered the highest predictive power.

We present here our discovery (n = 18), validation (n = 75), and prediction (n = 19) data sets, which to the best of our knowledge, represent the first resource of lncRNA expression in human islets stratified on the basis of their postisolation islet quality. Because tissue quality prediction is of utmost importance in clinical islet transplantation, which currently is the only approved cell therapy for T1D, our study presents molecular tools to advance selection of cadaveric tissues for stratification into GMP-grade workflows.

Results

LncRNA expression profiles are different across groups with varying islet quality. We had access to 93 pancreatic islets samples and 19 human pancreatic tissue samples from the Westmead Islet Isolation Centre (ref. 25 and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.129299DS1). Based on the results of standard quality control protocols (26), isolated islet samples from each of the pancreases were stratified into 3 groups (group 1: high quality/transplantable; group 2: intermediary quality, meaning good quality but low yield; and group 3: inferior-quality islet preparations). This stratification was performed using the pancreas islet quality score (Supplemental Tables 1–3) derived after assessment of 4 key postisolation measurements: (a) islet purity, (b) islet viability, (c) β cell viability, and (d) transplantability (Table 1). Human islet samples within group 2 are also of high quality. These islet samples could not be transplanted in individuals with T1D because the number of islet equivalents (IEQ) from these isolations was fewer than the desired number (4000 IEQ/kg of recipient body weight) for clinical transplantation. We randomly selected 18 islet samples from 3 groups for high-depth RNA-Seq (rRNA depletion library construction) using the HiSeq4000 platform. All the samples passed postsequencing quality control (QC), and on average 77,127,891 clean reads were obtained from these samples of the discovery set. A total of 6983 annotated lncRNAs and 450 potentially novel lncRNAs were identified (Supplemental Figure 2). An unsupervised hierarchical cluster analysis performed on the annotated lncRNAs revealed that all (n = 5) of the group 1 (transplantable) islet samples clustered closely with the intermediary-quality group 2 islet samples (n = 6) but not with the inferior-quality (n = 7) group 3

Table 1. Criteria assessed for islet categorization

Postisolation islet purity (%)	Postisolation islet viability (%)	Postisolation islet β cell viability index	Transplantability: transplantable (yes = 1), not transplantable (no = 3)
Score of 1 = 81–100	Score of 1 = 95–100%	Score of 1 = 0.5–1.0	Score of 1 = YES
Score of 2 = 61–80	Score of 2 = 85–94%	Score of 2 = 0.2–0.49	No score
Score of 3 = ≤60	Score of 3 = ≤84	Score of 3 = <0.2	Score of 3 = NO

Categorization of islet sample preparations into 3 groups was carried out by the surgical/islet isolation team based on the donor characteristics, procurement characteristics, and isolation characteristics. The isolation characteristics represent islet quality, which is derived from measurement of postisolation islet viability, islet purity, β cell viability, and transplantability. The transplantability is based on donor characteristics (the Edmonton Donor Points) and the availability of a sufficient number (4000 IEQ/kg recipient body weight) of isolated islet yield. The categorization of islets into 3 groups is a potentially novel aspect of this paper, and the isolation team adapted the following procedure for categorization. A measurement range of the 4 isolation characteristics was used to assign a criterion score (1, 2, 3) to each islet preparation, and this is presented in the respective columns. For islet isolation outcome, islets were scored as either 1 (transplantable) or 3 (not transplantable). Criteria scores were used to calculate a "weighted islet quality score" using the following formula: weighted islet quality score = A/C, wherein A = average of the criteria scores, and C = number of available criteria. For example, an islet sample preparation may have a score of 1 (if postisolation islet purity is 85%), a score of 2 (if postisolation islet viability is 89%), a score of 1 (if postisolation islet β cell viability index is 0.75), and a score of 1 (if that islet preparation was suitable for clinical transplantation). For this sample the value of A in the above formula would be = ([1 + 2 + 1 + 1]/4) = 1.25, and C = 4. The islet quality score therefore would be 1.25/4 = 0.3125. A weighted score system was used because some islet preparations did not have measurements for all 4 isolation characteristics. In this case, the islet isolation team compared procurement and donor characteristics (including well-known contributing factors, such as cold/warm ischemia: see Supplemental Tables 1–3) to assign a final category score to the islet preparation. For example, an islet sample preparation may have a score of 1 (if postisolation islet purity is 85%), a score of 2 (if postisolation islet viability is 89%), no data for postisolation islet β cell viability index, and a score of 3 (if that islet preparation was unsuitable for clinical transplantation). For this sample the value of A in the above formula would be = ([1 + 2 + 3]/3) = 2 and C = 3. The islet quality score therefore would be 2/3 = 0.667. These islet scores were then rounded off to the hundredth place and classified into respective groups as per predefined cutoff values: group 1 islet samples had an islet quality score ≤0.55, group 2 islet samples had an islet quality score >0.55 but ≤0.85, while group 3 islet samples had an islet quality score >0.85. All the scoring and categorization of islets into group 1, group 2 or group 3 were carried out independent of the measurements of lncRNA in these samples. The molecular biomarkers team was blinded to this information.

islet sample preparations (Figure 1A). We then compared the levels and abundance (fragments per kilobase of transcript sequence per million base pairs [FPKM]) of all lncRNAs expressed between the 3 groups of these discovery set islet samples. A significantly large number (246–322 lncRNA candidates) was identified to be differentially expressed (>2-fold, $P < 0.05$) in group-wise comparisons of lncRNAs (Figure 1, B–D).

Penalized regression analyses identify key lncRNAs associated with islet quality. To identify key lncRNAs that possess discriminatory capacity to identify the high-quality islet sample preparations, we performed penalized logistic/linear regression (PLR) analyses (27). To confirm that the outcome of this regression analysis was not a result of the sampling bias, we implemented resampling validation using bootstrapping workflows. These analyses identified 5–10 lncRNAs in each group-wise comparison (Supplemental Table 4). Of these, *MALAT1* was found to be common and significantly different across all comparisons (Figure 2, A–C). Intriguingly, 2 (of 8) splice variants of *MALAT1* (ENST00000616691.1, denoted henceforth as "*MALAT1*-1.1," and ENST00000619449.1, denoted henceforth as "*MALAT1*-9.1") were identified to be common in each bootstrap comparing group 1 islet samples, while another *MALAT1* variant (ENST00000620902.1, "*MALAT1*-2.1") was identified in comparisons involving group 3 islet samples (Figure 2, A–C, and Supplemental Figure 3). ROC curves for the significantly dysregulated lncRNAs demonstrated the capacity of *MALAT1* variants and variant ENST00000450589.5 (*GAS5*) lncRNAs to stratify islet samples into the relevant groups (Figure 2, D–F).

We also performed penalized linear (islet purity, islet viability, β cell viability) or logistic (transplant outcome: yes/no) regression analyses and bootstrapping on lncRNAs for each of these 4 parameters. Four *MALAT1* splice variants (*MALAT1*-9.1; *MALAT1*-2.1; ENST00000508832.2, "*MALAT1*-2.2"; and *MALAT1*-1.1) were identified to be common across individual penalized regression comparisons (Supplemental Table 5) between lncRNA levels and each of the 4 islet quality score criteria (outlined in Table 1). In univariate correlation analysis, the same 4 *MALAT1* variants showed significant positive correlation with postisolation islet viability, islet purity, or β cell viability index (Supplemental Table 6). We also identified other potentially novel and annotated lncRNAs that showed significance ($P < 0.05$) in univariate analyses for each of the 4 islet quality score criteria (data not shown). However, these were significantly lower in abundance (at least 10- to 48-fold less) than the highly abundant *MALAT1* splice variants (*MALAT1*-1.1 and *MALAT1*-9.1). The biological relevance of these low-abundance lncRNAs can be the subject of future investigations.

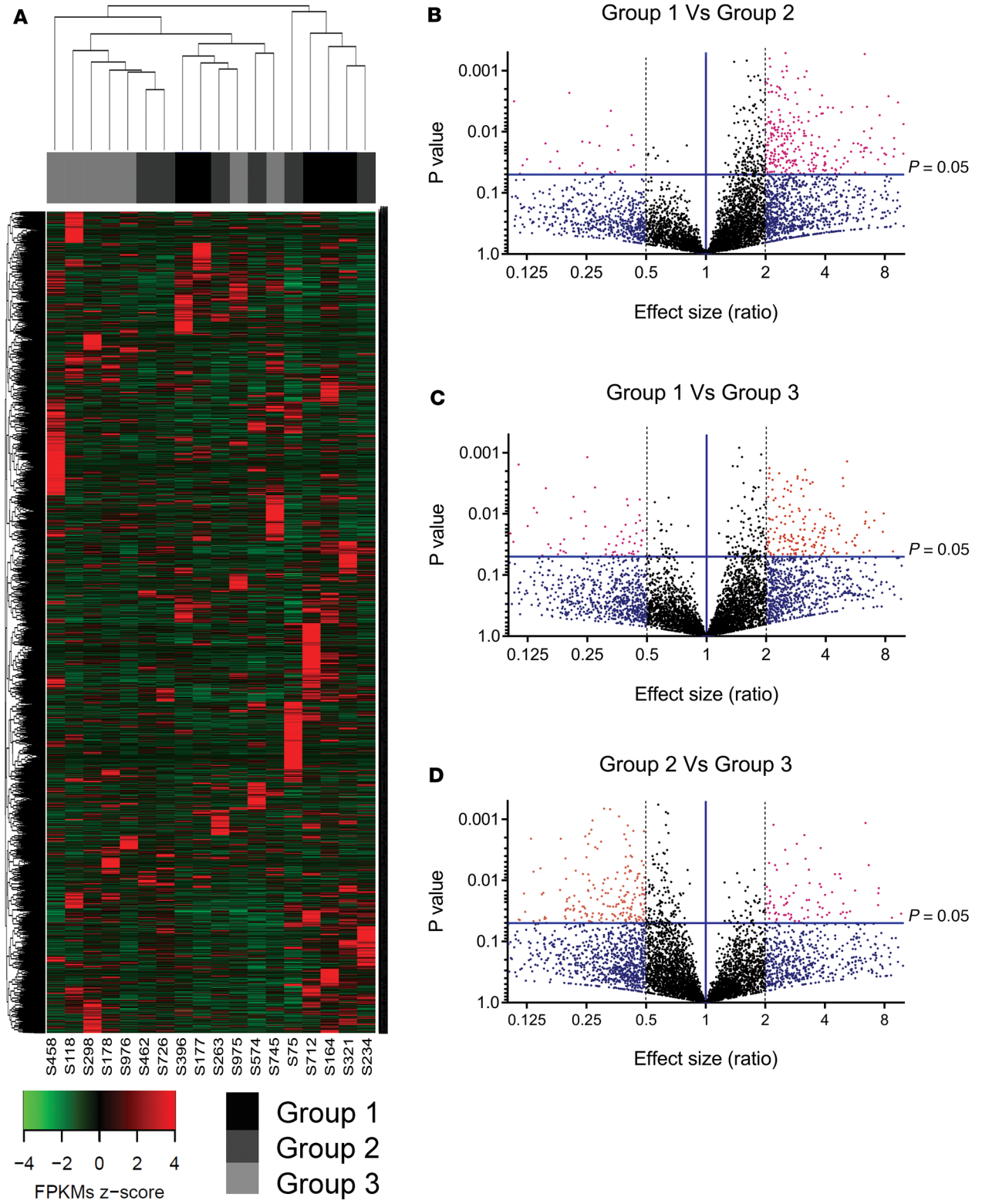

Figure 1. Discovery analyses using next-generation sequencing of human islets categorized on the basis of postisolation quality. (**A**) Unsupervised hierarchical euclidean (complete) cluster heatmap of the annotated (6721) lncRNAs in n = 18 human islets, categorized into 3 groups. (**B**–**D**) Volcano plots of annotated lncRNAs in the human islets categorized group 1 versus group 2 (**B**), group 1 versus group 3 (**C**), and group 2 versus group 3 (**D**). The effect size differences are depicted on the x axis while the $-\log_{10}$ P value is depicted on the y axis. The horizontal blue line represents the significant P value of 0.05, while the vertical blue line represents no difference.

The lncRNA MALAT1 is a potential biomarker for islet isolation outcome. Because high expression of *MALAT1*-1.1 and *MALAT1*-9.1 was identified in group 1 islet samples, we decided to confirm our findings in a validation set of 75 human islet samples for which all the relevant information to categorize them into 3 groups (group 1, $n = 40$; group 2, $n = 18$; group 3, $n = 17$), as well as RNA for qPCR-based *MALAT1* measurement, were available. Although group 1 islet samples had significantly higher insulin content than group 3 islet samples (Figure 3A), the levels of insulin gene transcript (Figure 3B) were similar. The inferior-quality (group 3) islet samples had significantly lower levels of *MALAT1* gene transcript (assay-907) (Figure 3C), but not the other (assay-177) *MALAT1* gene transcript assay (Figure 3D). To test whether these *MALAT1* variants could be biomarkers of postisolation islet quality, it was essential to measure *MALAT1* before islet isolation. We assessed *MALAT1* levels in a set of 19 (preisolation) pancreas samples, where a small piece of tissue was stored from the middle of the pancreas at the time of transection for cannulation, usually close to the duct itself, before islet isolation. The surgical team isolated islets from each of these 19 pancreatic samples (Supplemental Figure 1 and Figure 3E) and categorized them into group 1 or group 3 islet samples (group 1: transplantable, $n = 14$; group 3: nontransplantable, $n = 5$) while the lab team assessed the levels of *MALAT1* transcripts (assay-907 and assay-177) in each of the 19 deidentified pancreas samples. ROC curves, based on the pancreatic levels of the 2 *MALAT1* variants and donor BMI, returned an AUC of 0.79 for BMI alone, 0.83 for both *MALAT1* assays (907 and 177), and 0.94 for both *MALAT1* variants plus BMI (Figure 3F and Table 2). Because islet isolation centers across the world follow several criteria, we tested whether *MALAT1* measurements had any added benefit to the current donor pancreas selection score. A combination of *MALAT1* measurements with any of the standard scores, such as Edmonton Donor Points or the NAIDS (24), offers the highest predictive power (Table 2).

The stimulation index for group 1 islet samples appeared to be higher, although there was no statistically significant difference across the groups (Supplemental Figure 4A). Islet samples from the 3 groups were transplanted under the kidney capsule of diabetic animals, and the time for diabetes reversal was monitored to further classify the transplanted animals as early or late responders (Supplemental Figure 4B). A higher proportion of mice showed diabetes reversal within the first 3 weeks after transplantation (early responders) for group 1 and 2 islets as compared with group 3 islet recipients (Supplemental Figure 4C).

Discussion

The present study was designed to generate lncRNA profiles of human islet sample preparations that were stratified based on their postisolation islet quality. Currently, there are no predictive biomarkers of postisolation islet quality, and data from the CITR (5) have failed to validate the potential of clinical characteristics, such as BMI, in predicting the quality of islet sample preparations isolated from each cadaveric human pancreas. There is a need to identify molecular biomarkers that could allow real-time analysis and prediction of islet quality.

Islets were categorized into 3 groups based on their postisolation islet quality variables (purity, viability, β cell viability, and transplantability; Table 1). The functionality of all isolated human islets was determined using in vitro glucose-stimulated insulin secretion (GSIS) assay and in vivo islet transplantation in diabetic mice. The GSIS and in vivo mouse transplant outcomes were included to assess the function of islets from the 3 groups (Supplemental Figure 4, A–C). Intriguingly, the majority of the group 3 islet samples had a late response (>3 weeks) for diabetes reversal (Supplemental Figure 4, B and C). Although 71% of group 1 islet samples (prediction study set) were transplanted into individuals with T1D, the β scores (28) for these are not shown because most of our islet recipients had a second transplant within 3 months of receiving their first islet transplant. Thus, changes in HbA1c over 3 months after transplantation would be confounded because of the successive transplant. The current study was not designed or powered to predict islet transplantation outcome. This study presents the potential of *MALAT1* variants as a biomarker to help accurately predict the quality of islets before initiating human islet isolation from the pancreas. The prediction of the outcome of islet transplant procedure is a separate question, which will be the focus of future investigation.

A recent study demonstrated the potential of assessing insulin immunopositive area in predicting islet isolation outcomes with 89% sensitivity and 76% specificity (29). Our data demonstrate that *MALAT1* expression alone (AUC: 0.83, maximum specificity of 100% and a sensitivity of 80%) or along with Edmonton Donor Points, BMI, and NAIDS can accurately stratify a donor pancreas to group 1 (transplantable) or group 3 (not transplantable) before the initiation of islet isolation. In addition to *MALAT1*, lncRNAs such as *GAS5* may add better discriminatory value to identify the effect of glucocorticoids (30) and stratify group 2 versus

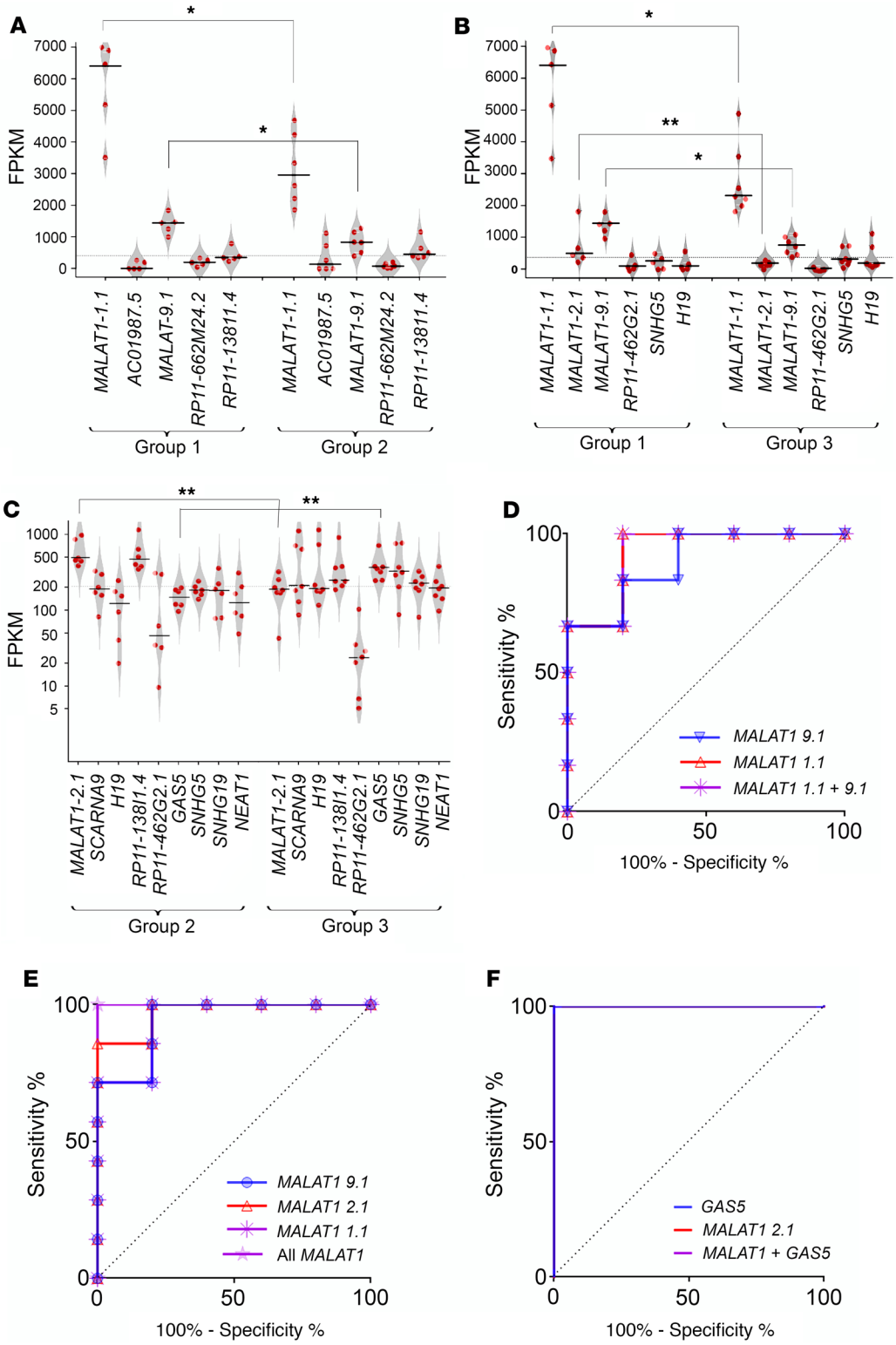

Figure 2. Key lncRNAs identified in islet quality stratified discovery sample set. Penalized regression analysis and bootstrapping were performed on the (6983) annotated lncRNAs and the identified lncRNAs across all group-wise comparisons (n = 18 human islet samples, categorized into 3 groups) as presented in **A–C**: (**A**) group 1 versus group 2, (**B**) group 1 versus group 3, and (**C**) group 2 versus group 3. (See Supplemental Table 4 for details; unequal-variance Student's t test was used. Note: The lncRNA AC010987.5 was present at very low FPKM and hence is not shown in **C**.) $^*P < 0.05$; $^{**}P < 0.01$. Each red dot represents individual islet preparation. The horizontal line represents the mean while the polygons represent the estimated density of data (scatter plot). Two-tailed distribution, with 2-sample unequal-variance Student's t test, was used to identify the difference for each lncRNA between each group-wise comparison. ROC curves: (**D**) group 1 versus group 2: *MALAT1*-1.1, *MALAT1*-9.1, and both *MALAT1* variants (9.1 + 1.1); (**E**) group 1 versus group 3: *MALAT1*-1.1, *MALAT1*-2.1, *MALAT1*-9.1, and all the above 3 *MALAT1* variants (1.1 + 2.1 + 9.1); (**F**) group 2 versus group 3: ENST00000450589.5 (*GAS5*), *MALAT1*-2.1, and both; *GAS5* + *MALAT1*-2.1.

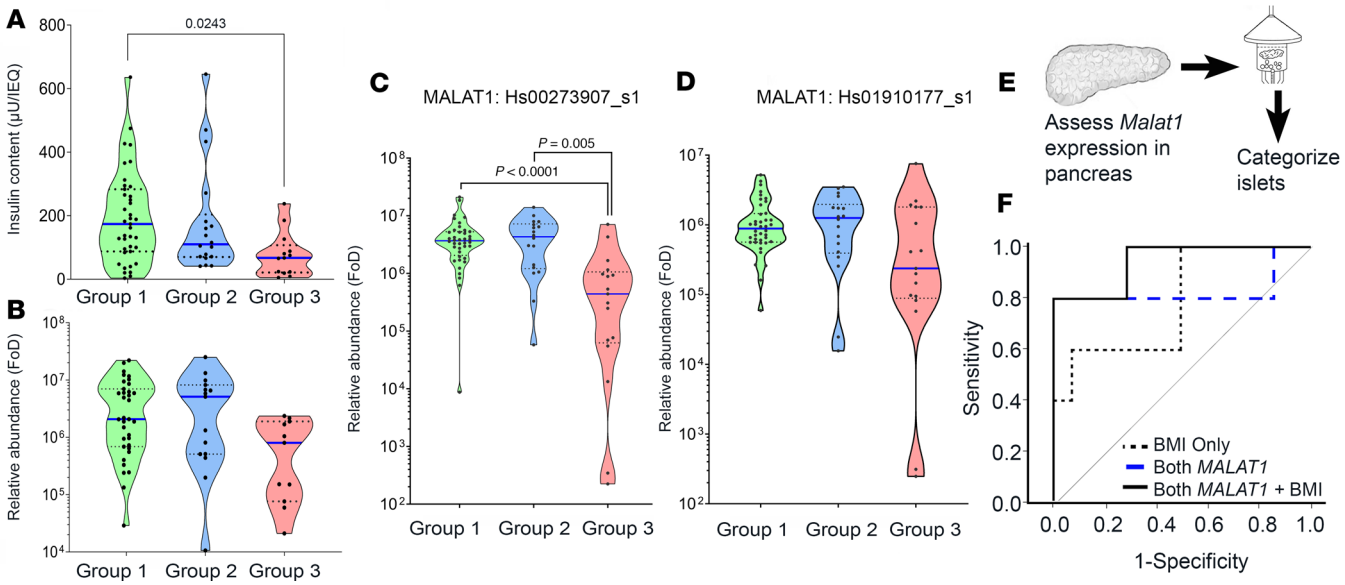

Figure 3. Assessing MALAT1 lncRNA expression in the validation (islet) and prediction (pancreas) sample sets. The 3 categorized human islet sample groups in the validation set (n = 75) were assessed for (**A**) insulin content levels, (**B**) (pro-) insulin transcript levels, and (**C** and **D**) *MALAT1* lncRNA expression (using qPCR primer/probe assay Hs_00273907 and Hs_01910177, respectively) targeting 2 variants of *MALAT1* lncRNA. To test the difference between the 3 categorized groups, nonparametric, 1-way ANOVA was used, and significant P values, adjusted for multiple comparisons, are reported. Each dot in the violin plot represents a different islet sample preparation. Horizontal solid blue line represents the median for each group, the horizontal dotted line represents quartiles, and the polygons represent the density of individual data points and extend to min/max values. FoD, fold over the detectable limit (55). (**E**) *MALAT1* lncRNA qPCR was carried out before islet isolation. (**F**) ROC curve for *MALAT1* lncRNA of pancreas tissue samples (n = 19) to stratify postisolation islet quality (group 1 vs. group 3).

group 3 human islet sample preparations. *MALAT1* was originally identified to be the most abundant lncRNA in the pancreas (31), and purified human islets (19), as well as being highly abundant in purified human β cells (19). Intriguingly, mouse knockout of *MALAT1* showed no differences in the histology of pancreases and islets (32, 33) and did not affect proliferation or viability in hepatocellular carcinoma cells (32). It is currently unclear why *MALAT1* is associated with high-quality (group 1) islet sample preparations. *MALAT1* is encoded within an active enhancer cluster offering multiple binding sites for pancreatic islet transcription factors (34). Thus, a possible role of *MALAT1* could be in facilitating (pro-) endocrine gene expression within human islets. Apart from the role of *MALAT1* in islet cells, it is intriguing to note that *MALAT1* is regulated by hypoxia (35–37). Indeed, hypoxia can be a major determinant of postisolation islet quality (38), and therefore the regulation of *MALAT1* under hypoxic conditions can influence islet survival. Knockdown of *MALAT1* leads to apoptosis (39), whereas increase in *MALAT1* expression has been shown to inhibit apoptosis (40). It can therefore be speculated that islets that do not demonstrate the capacity to upregulate *MALAT1* expression following exposure to hypoxic conditions would be more vulnerable to apoptotic cell death. Therefore, levels of *MALAT1* detected in human pancreas before islet isolation may provide a measure of the postisolation islet quality. Indeed, the abundance of 2 *MALAT1* variants (*MALAT1*-1.1 and -9.1) negatively correlated with postisolation islet cell death (Supplemental Figure 5).

Our study strengths are (a) the use of an unprejudiced high-depth sequencing platform for human islets from 3 groups (n = 18); (b) the use of sophisticated, unbiased data analysis methods with resampling validation (bootstrapping); (c) the independent replication of these findings in a separate set (n = 75) of human islet samples; and (d) the contribution of *MALAT1* variants to improving the predictive power of Edmonton Donor Points, BMI, and NAIDS in classifying islet samples from a set (n = 19) of preisolation pancreatic tissue samples. The study limitations are the lack of larger number of matching high-quality discovery samples (which we have attempted to address via resampling/bootstrapping strategies) and the need to replicate our findings in independent islet isolation centers. It is essential to note that *MALAT1* variants alone demonstrate better specificity to predict postisolation quality of islets than Edmonton Donor Points, BMI, and NAIDS. We do not know whether *MALAT1* can predict clinical outcomes or graft functions after transplant, which would be an interesting question to pursue.

Table 2. MALAT1 measurements offer higher predictive power alone or when combined with donor characteristics

	AUC	Specificity	Sensitivity
Edmonton Donor Points	0.41	0.79	0.40
Donor BMI kg/m^2	0.79	0.93	0.60
MALAT1 variants + Edmonton Donor Points	0.83	1.00	0.80
MALAT1 variants	0.83	1.00	0.80
NAIDS	0.84	0.86	0.80
MALAT1 variants + NAIDS	0.93	1.00	0.80
Edmonton Donor Points + NAIDS + BMI	0.93	0.79	1.00
MALAT1 variants + BMI	0.94	1.00	0.80
MALAT1 variants + BMI + NAIDS	0.96	1.00	0.80
MALAT1 variants + BMI + Edmonton Donor Points	1.00	1.00	1.00
MALAT1 variants + BMI + Edmonton Donor Points+ NAIDS	1.00	1.00	1.00

ROC curve analyses were carried out to assess the potential of MALAT1 variants alone or when modeled along with donor characteristics (BMI, Edmonton Donor Points, and/or NAIDS) of pancreas tissue samples (n = 19) to stratify postisolation islet quality (group 1 vs. group 3). Area under the curve (AUC), specificity, and sensitivity measurements for each of the ROC curve are presented.

The present study identifies lncRNA biomarkers such as *MALAT1*, which could facilitate the prediction of islet isolation quality, within minutes of receiving the cadaveric pancreas. Currently, commercially available kits have been demonstrated (41) to offer the capacity to isolate and directly measure RNA in minutes. Advances in nanotechnology further enable reliable as well as cost- and time-efficient assessment of RNA quantity (42) and can be used to measure biomarker levels (43). Cross-disciplinary technological advances can be easily used to develop a rapid detection kit, similar to a pregnancy test, for clinical biomarkers (such as *MALAT1* variants). Platforms to measure microRNAs and lncRNAs using nanosensor-based (44) and photonics-based technologies (45) can detect ultralow (subpicomolar to attomolar) levels of ncRNAs from cell lysates. Because *MALAT1* is a highly abundant lncRNA in the human pancreas, the development of such rapid detection tests for *MALAT1* variants is achievable and is indeed one of our research foci in the coming years. Nanotechnology- and photonics-based platforms offer a rapid (2–5 minutes) and low-cost (<$1) method that could improve donor selection and enhance the development of tests that ultimately help us stratify donors with high accuracy, so as to meet the release criteria for clinical islet transplantation. Because the time from cross-clamp at retrieval of a donor pancreas to initiating islet isolation is around 9.8 ± 0.179 (mean ± SEM) hours (46), the identification of *MALAT1* lncRNA variants allows us a sufficient window to use this test that could change current practice. Measurement of *MALAT1* variants will improve donor selection while offering the other (nonselected) pancreas for research-grade isolation workflows. Our study underscores the importance of existing donor pancreas selection criteria (Edmonton Donor Points, BMI, and NAIDS) and presents the added predictive power offered by the new lncRNA biomarkers. As discussed above, although existing criteria (such as donor BMI) may be good estimators of islet number, the measurement of *MALAT1* lncRNA variants in the pancreas provides a "readout" of their quality, substantially increasing the predictive power for selection of donor pancreases in clinical islet transplantation.

Study impact. The present study provides the capacity to make an informed choice for research or clinical islet isolation workflow. Clinical islet isolation costs using GMP-grade facilities and reagents and involving screening, organ recovery, transportation, and islet preparation are estimated around US$40,000 per preparation (1). Based on CITR data, around US$97,000,000 was spent on 2421 isolations (47), of which only 750 preparations (48) were deemed suitable for human islet transplantation. The current study provides the means to not only direct the best donor tissues for clinical transplants but also make appropriate decisions for the less cost-intensive research-grade islet isolation workflows. Our data corroborate previous findings indicating the high level of *MALAT1* lncRNA in islets. The stratification of islets based on the quality of islet preparations helps in understanding the role of *MALAT1* lncRNA variants as biomarkers for predicting islet isolation outcome. We also provide the first report to our knowledge of human islet lncRNA expression profiles in a quality-stratified set of islet samples to the research community through this publication.

Methods

Human pancreatic islets

Pancreatic tissue samples were taken from the middle of the pancreas at the time of transection of the pancreas for cannulation, usually close to the duct itself. Islets were isolated following the standard protocol carried out by the National Islet Transplantation Unit at the Westmead Hospital, University of Sydney (25). A standard set of donor characteristics were recorded by the team and were used in calculations of the islet quality score (Table 1), Edmonton Donor Points (2, 6), and the NAIDS (24). Islet samples were categorized into 3 groups based on their islet quality score and presented in this study as group 1 (high quality), group 2 (intermediary quality), and group 3 (inferior quality) islet sample preparations. To compare the predictive power of lncRNA biomarkers along with existing clinical and donor characteristics, we also compared the classification of islet quality based on Edmonton Donor Points as well as NAIDS.

Islet quality score

Islet quality score (presented in Supplemental Tables 1–3) was mathematically computed as the average score divided by the number of available criteria (Table 1) from the scoring of each criterion listed in Table 1. The methodology for each of these assessments is provided below.

Postisolation islet purity. Islet purity was assessed as described elsewhere (49). Briefly, postisolation islet purity was assessed by using Dithizone stain (MilliporeSigma) (3 mg/mL). Triplicate aliquots of known volumes were sampled from the final islet cell preparation. The total number of islets was counted using standard criteria and percentage of acinar tissue quantified and scored in each of the aliquots (IEQ > 4000 IEQ/kg recipient body weight) as detailed elsewhere (49).

Postisolation islet viability. Postisolation islet viability was determined using fluorescent labeling of cell preparations using DNA-binding dyes, so as to differentiate between live and dead cells. Fluorescein diacetate (FDA; 24 μM) and propidium iodide (PI; 750 μM) solutions (MilliporeSigma) were used to stain 10 replicates of cells with compromised membranes. Westmead Hospital utilizes a cutoff of 70% viability as a minimum for release of the product for clinical transplantation. FDA diffuses passively across the cell membrane and is converted to fluorescein by nonspecific esterases in the cytoplasm, causing live cells to fluoresce green under a 490-nm excitation wavelength. Dead or dying cells have compromised cell membranes and do not show cytoplasmic esterase activity and therefore do not fluoresce green. Counterstaining with PI allows better identification of these dead and dying cells because these cells (nuclei) will take up PI, fluorescing red at 545 nm. Triplicate aliquots of a known volume were sampled from the final islet cell preparation and stained with FDA and PI. A total of at least 100 islets were stained and cells counted and quantified as to the percentages of viable (green) versus dead (red) cells. A total accumulated score was then calculated and the mean value taken as the viability score.

Postisolation islet β cell viability index. Flow cytometric assessment was carried out on the human islet cells following an established method developed by Ichii et al. (50). This method simultaneously determines the β cell composition, viability, and apoptotic cell percentage in enzymatically dispersed single cells from islets. Zinc-binding dye Newport Green (NPG) (Thermo Fisher Scientific) was used to determine β cell composition (NPG-positive staining), while apoptotic cells were probed using tetramethylrhodamine ethyl ester and the membrane-impermeant 7-aminoactinomycin D staining (50). Our center at Westmead Hospital conducts flow cytometric analysis on islet cells after culture to determine this β cell viability index, defined as (% β cells × % viable β cells)/10,000, with indices of 0.5 or higher considered satisfactory for release for transplantation.

Transplantability. The analysis of the suitability of the islet preparation for human transplantation was carried out using the following criteria: the release criteria formally accepted for our program are based on islet count per recipient weight (5000–20,000 IEQ/kg for the first transplant and 3000–20,000 for following transplants), with purity at least 30%, viability at least 70%, endotoxin concentration less than 5 endotoxin units/kg recipient weight, and no detectable organisms in a Gram stain before transplant, in addition to a glucose stimulation index (ratio of stimulated insulin secretion/basal insulin secretion) greater than 1 and a β cell viability index of 0.5 or higher.

Islet insulin content

Insulin content was measured in 2 replicates for each postisolation islet preparation. One mL Azol was added to each replicate before sonication. Sonicated aliquots were then mixed with FSA solution and appropriately processed and diluted for total insulin content analysis using the manufacturer's method for immunoassay on the Architect (Abbott Diagnostics). Data were normalized to DNA content (Quant-IT Pico Green dsDNA Assay Kit, Life Technologies). Values are presented in total insulin content for the entire islet preparation, after normalizing for the amount of DNA.

Human islet transplantation into mice

Athymic mice (Animal Resources Centre) were rendered diabetic with streptozotocin, before transplantation of between 2000 and 3000 IEQ of (after culture) islets beneath the kidney capsule. Blood sugar levels were monitored after transplant to determine the success of the islet transplant procedure, defined as either a reduction in nonfasting blood glucose levels to under 11 mmol/L (200 mg/dL) or halving in nonfasting blood glucose levels from the diabetic state, in 2 successive measurements 48–72 hours apart. An intraperitoneal glucose tolerance test was conducted 3–4 weeks after transplant to assess glucose clearance and hence determine whether success in islet function was achieved after transplant.

Human islet stimulation index

The functional capacity of human islets was assessed by measuring insulin secretion in response to glucose. Briefly, (after culture) islets were incubated at 37°C and 5% CO_2 for 1 hour in RPMI 1640 (Thermo Fisher Scientific) with either 2.8 mM glucose or 25 mM glucose. For each islet preparation, 6 replicates were exposed to 2.8 mM glucose and 25 mM glucose. Supernatants were collected and their insulin content was measured using an Architect (Abbott Diagnostics). The stimulation index was calculated by dividing the insulin content in the 25-mM glucose incubation sample by the insulin content in the 2.8-mM glucose incubation sample. Higher values indicate a better insulin secretion response to glucose stimulation and therefore a higher functional capacity (26, 51).

RNA isolation and QC

Total RNA was isolated from 2000 to 5000 IEQ using the manufacturer's TRIzol RNA isolation (Thermo Fisher Scientific) protocol with minor modifications (52). Details and QC of the selected samples (discovery set, n = 18) for RNA-Seq are provided in supplemental material (Supplemental Table 7). RNA integrity number (RIN) was assessed for every sample, and only those with an RIN value of at least 7.4 were taken for discovery analysis.

High-depth RNA-Seq

High-depth RNA-Seq (rRNA depletion library construction) was carried out using the HiSeq4000, 150 paired-end (PE) reads platform (Novogene), on n = 18 human pancreatic islet preparations (Supplemental Table 7). An input of 3 μg RNA per sample was used for sequencing. Epicentre Ribo-Zero rRNA Removal Kit and ethanol precipitation were used to remove the rRNA and rRNA-free residues for each sample, respectively. Subsequently, sequencing libraries were generated using the rRNA-depleted RNA with the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB) following the manufacturer's protocol. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina) following the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq platform and PE reads were generated. FPKM sequenced were calculated for both lncRNAs and coding genes in each sample using Cuffdiff (v2.1.1). FPKM are the means mapped and calculated based on the length of the fragments and read count mapped to these fragments (53). Results are presented in normalized FPKM.

RNA-Seq analysis

In-house perl scripts were used to remove reads containing adapter, reads containing poly-N, and low-quality reads from the raw data (reads), leaving only the clean data (reads). The index of the reference human GRCh38 genome (http://ftp.ensembl.org/pub/release-82/fasta/homo_sapiens/dna/) and the GTF files (ftp://ftp.ensembl.org/pub/release-82/gtf/homo_sapiens/) were used for annotation (obtained from the genome website) and built using Bowtie v2.0.6. TopHat v2.0.9 was used to

align PE clean reads to the reference genome. On average 77,127,891 clean reads were obtained from each of the 18 samples that passed QC in this discovery set. Phylogenetic codon substitution frequency (phyloCSF; v20121028) was used to identify and distinguish the characteristics to align conserved coding regions (54). Multispecies genome sequence alignments were built and run on phyloCSF with default parameters. Transcripts predicted with coding potential (by all or either of the 4 tools CMCI, CPS, Pfam-scan, and phyloCSF) were removed, while transcripts without coding potential were identified to be the candidate set of lncRNAs.

Real-time qPCR

The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used followed by TaqMan real-time qPCR for assays. Briefly, synthesis of cDNA from RNA was carried out using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). TaqMan real-time qPCR was performed in 5-μL reactions using 96-well plates with 33.3 ng input cDNA with TaqMan Fast Universal PCR Master Mix (Thermo Fisher Scientific). Selected TaqMan primer/probe gene expression assays are provided in Supplemental Table 8. We used 2 *MALAT1* gene transcript assays (907 and 177), which span different regions of the human *MALAT1* gene. *MALAT1* gene transcript assay-907 captures variants *MALAT1*-9.1 and -2.1, while *MALAT1* gene transcript assay-177 covers only *MALAT1*-1.1. Results were normalized to 18s rRNA values. Real-time qPCR was carried using the ViiA7 platform (Thermo Fisher Scientific).

Data availability

We intend to replicate these findings at other islet isolation centers and welcome collaborative or independent assessment of our predictive algorithms. All data from our lncRNA-Seq studies were uploaded to the Gene Expression Omnibus database and are available through the study accession number GSE134068. We welcome future collaborations to analyze and validate these in other centers. Such studies through multiple islet isolation centers would help in confirming the potential of these *MALAT1* lncRNA variants for improving the prediction of pancreatic islet isolation procedure outcomes.

Statistics

All statistical analyses were performed using GraphPad Prism v7 (GraphPad Software), SPSS, R glmnet, penalized, or corrplot packages. A 2-tailed distribution, with 2-sample unequal-variance Student's *t* test, was used to compare specific lncRNAs between 2 groups for the RNA-Seq data. To identify a subset of lncRNAs presenting the strongest associations with the 3 categorized groups of islet preparations, L1-PLR (Lasso) techniques were used, as described elsewhere (27). The lncRNAs identified by Lasso analyses were confirmed by resampling validation/bootstrapping analyses (for 1000 iterations). During bootstrapping, about 37% of samples were randomly removed and the same number of different (randomly selected) samples duplicated so that the total number of samples remained the same in each of the 1000 iterations. Resampling validation was an important part of the process, so as to eliminate any sampling bias. A Wilcoxon *P* value cutoff of $P > 0.5$ was also applied in each iteration. Univariate logistic and linear regression was carried for each comparison to examine the association of each independent variable with the outcome. A 1-way ANOVA test with multiple-comparisons correction was used for all group comparisons for the qPCR data. R scripts for the above machine-learning algorithms will be made available following publication.

Study approval

Human tissue samples. Use of research-consented human tissues was approved through the human research ethics committee (HREC) approval X16-0289 (previously X12-0176) and the HREC/12/RPAH/282 at the University of Sydney, Sydney Local Health District, and tissues from donors who gave informed research consent were obtained through the National Islet Transplantation Program at the Westmead Hospital, University of Sydney, Australia (AU RED/HREC/15/WMEAD/284).

Animal studies. Animal studies were approved by the animal ethics committee at the Western Sydney Local Health District (protocols 4198.10.12 and 5146.10.17).

Author contributions

WKMW performed data analysis, experimentation, write-up, and revision. GJ, AES, RCM, and LTD were responsible for biostatistics, data analysis/validation, and data interpretation. CLM, YVC, DL, LW, PJO, and WJH performed data analysis, data interpretation, and islet isolation/banking. AAH and MVJ were responsible for study design, acted as guarantors of the work, and performed data analysis, data interpretation, and manuscript writing and revision. All authors agreed on the final revised draft.

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