Reduction of Activin A gives rise to comparable expression of key definitive endoderm and mature beta cell markers

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Aim: Cell therapies for diabetes rely on differentiation of stem cells into insulin-producing cells, which is complex and expensive. Our goal was to evaluate production costs and test ways to reduce it. **Methods:** Cost of Goods (COGs) analysis for differentiation was completed and the effects of replacement or reduction of the most expensive item was tested using qRT-PCR, immunohistochemistry, flow cytometry along with glucose-stimulated insulin release. **Results:** Activin A (AA) was responsible for significant cost. Replacement with small molecules failed to form definitive endoderm (DE). Reducing AA by 50% did not negatively affect expression of beta cell markers. **Conclusion:** Reduction of AA concentration is feasible without adversely affecting DE and islet-like cell differentiation, leading to significant cost savings in manufacturing.

Diabetes is an increasingly important metabolic endocrine disorder affecting humans and many domestic species including dogs and cats [1–3]. Human diabetes is mainly subclassified as Type I, which occurs following autoimmune destruction of pancreatic beta cells, or Type 2, which occurs due to dysfunction of beta cells or insulin resistance [4], although other less common forms of diabetes, for example, gestational diabetes are also recognized [4,5]. Although Type I diabetes is primarily controlled by using exogenous insulin, proper management of diabetes is often difficult to achieve, and is accompanied by frequent high blood sugar excursions and which often leads to various complications [6,7]. For patients with Type I diabetes, there is increasing evidence that beta cell replacement, in the form of islet transplants, offers the best approach in controlling high blood glucose excursions and a potential cure [8–11]. One of the greatest challenges for this approach has been the limited availability of usable donor islet tissue [12], which has spurred the search for alternative and scalable sources of transplantable islet cells.

Arguably, the best source of scalable cells for diabetes treatment is pluripotent stem cells, which are sourced either from embryonic stem cells or from induced pluripotent stem cells (iPSCs) [13–15]. Due to ethical and other limiting considerations, iPSCs are preferred as a source of stem cells over embryonic stem cells as they are readily available and have been successfully differentiated into transplantable, islet-like cells for the treatment of diabetes [16,17]. Using a variety of similar protocols, numerous groups have successfully differentiated iPSCs into islet-like cells that have been shown to correct diabetes in experimental models [18,19]. Currently, there are US FDA-approved clinical trials for the cure of diabetes using stem cell derived beta cells [11,20,21].

One of the major considerations in developing cell therapies for diabetes is the high cost of goods (COGs) that affects manufacturing [22–26]. This is important for both human and the veterinary health markets where price limitations are critical [25]. Veterinary pharmaceutical markets have built in price pressures based on the average pet owner's spending limits. In the human pharmaceutical and biotechnology markets, the passing of the Inflation Reduction Act brought new price pressures on existing and emerging therapeutics. Thus, the cost of a new product must be considered earlier in the development process along with steps to reduce costs.

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A survey conducted by PhRMA summarizes the anticipated effects of the Inflation Reduction Act showing that 78% of pharmaceutical companies are expected to cancel early-stage pipeline projects and 95% said they expect to develop fewer new uses for medicines because of the limited time available before the government price setting takes effect [27]. Some companies are considering whether to offer certain medicines in the United States because they may never recoup their R&D costs. The Act is also causing pharmaceutical companies to reconsider which disease areas to pursue for pipeline projects [27,28]. Thus, it is imperative that the cost of complex cell therapies be considered early in the development phase, if the therapeutic is to have a successful market impact.

Some of the most expensive components in the manufacturing of cell therapy products are growth factors and other biological molecules used in cell expansion and differentiation protocols. Reduction of COGs in manufacturing includes the use of less expensive alternatives and/or reduction in usage either in concentration or amount of time utilized in the protocol. Small changes can ultimately lead to a reduction of final price points that will promote the development and commercialization of cell therapy products leading to easier reimbursement and broader adoption by a wider patient base, both in human and veterinary practices [22–24].

Based on our own unique protocol to differentiate iPSCs into stem cell-derived islet like cells (SC-ILCs), we calculated the percentage that each reagent and consumable contributed to the total manufacturing COGS. The calculations revealed that Activin A (AA), used in the first step of making definitive endoderm (DE), alone comprised a high percentage of the total cost of the differentiation reagents and goods in manufacturing SC-ILCs. Once the impact of AA on cost was determined, we examined the effect of using replacement reagents as well as decreasing the AA concentration on both the efficiency of the differentiation protocol and the cost of the final product. In addition to the cell line NH50191 reported in this study, other iPS cell lines were tested for their efficiency to generate DE and mature beta cell lines with varying degrees of success in earlier studies. The NH50191 cell line provided the best and reproducible differentiation outcome and was thus chosen for additional studies. Our results illustrate the significant impact that simple changes to a protocol can have on the final cost of a product.

Materials & methods

Cost of Goods analysis

COGs analysis was completed using manufacturer's suggested retail price (MSRP) for all products used from iPSC thaw and expansion to differentiation of SC-ILCs. Individual reagents and consumable were analyzed as a % of total cost of the product for a standard batch size ($625 \times 10^6 \pm 58 \times 10^6$ cells), excluding labor costs.

Cell culture & expansion

The human iPSC line designated ID NH50191 was obtained from RUCDR Infinite Biologics (NN005200) and is part of the NIH/NINDS Human Cell and Data Repository with a Drug Master File created by Lonza. The research grade version of the cell line was created by additional passages of the clinical GMP grade line under non-GMP conditions. Cells were seeded onto Matrigel (354277, Corning)-coated flasks and enzymatically passaged at 85–90% confluency with Dispase (07923, StemCell Technologies). At initial passaging, cells were seeded in mTeSR™ Plus Medium (100–0276, StemCell Technologies) supplemented with 5 μM Y-27632 Rock Inhibitor (1254, Tocris). The Rock inhibitor was removed after 24 hours and cells were fed once every 48 hours as per the manufacturer's instructions.

In addition to the cell line ID NH50191 iPS cell line reported in this study, other iPS cell lines were tested for their efficiency to generate DE and mature beta cell lines with varying degrees of success. Table 1 lists all relevant information regarding previous cell lines tested in studies leading up to the current study.

Differentiation of IPS cells to stem cell-derived islet-like cells SC-ILCs

Differentiation of cultured iPSCs was started when cells reached 95–100% confluency. Cells were rinsed two-times with Dulbecco's phosphate-buffered saline (DPBS), 1X (-/-) (21-030-CV, Corning) and started on a six-stage differentiation protocol. The first stage for the formation of definitive endoderm was comprised of the following: DE Medium comprised of MCDB131 (15-100-CV, Corning) supplemented with 9 mM Glucose (G8769, Sigma), 14 mM sodium bicarbonate (25-035-Cl, Corning), 0.1% BSA (68700, Proliant Biologicals), and 1% GlutaGro (25-015-Cl, Corning). The DE Medium was supplemented with either 100 ng/ml, 75 ng/ml, or 50 ng/ml Activin A (800-01, Shenandoah Biotechnology) and CHIR99021 at 3 μM (2520691, PeproTech) for 1 day only. DE medium contained AA at the same concentration for the next 2 days but, without the GSK3 Inhibitor. Alternatively, the same DE Medium from above was supplemented with either 100 nM IDE1 (72514, StemCell

| Table 1. Cell lines tested for conversion to definitive endoderm and differentiation into SC-ILCs.			
Cell line	Catalog #	Vendor	Derived tissue source
Episomal hIPSC line	A18945	Gibco	Cord-blood derived CD34+ progenitors
StemRNA™ human iPSC 771-3G	RCRP005N	Reprocell	Peripheral blood
Human CD34+ cord blood stem cell	CET.IPS.FFCD34-500	Cellular Engineering Technologies	Normal hematopoietic stem cell from umbilical cord blood CD34+ progenitors
Human multipotent stem cell	CET.IPS.FFMP-500	Cellular Engineering Technologies	Umbilical cord blood
Human foreskin fibroblast	CET.IPS.FFHFF-500	Cellular Engineering Technologies	Normal human foreskin fibroblast
Human amniotic membrane mesenchymal stem cell	CET.IPS.FFAM-500	Cellular Engineering Technologies	Normal human amniotic-derived
NH50191 human iPS stem cell	NN0005200	NINDS Human Cell and Data Repository	CD34+ cord-blood derived
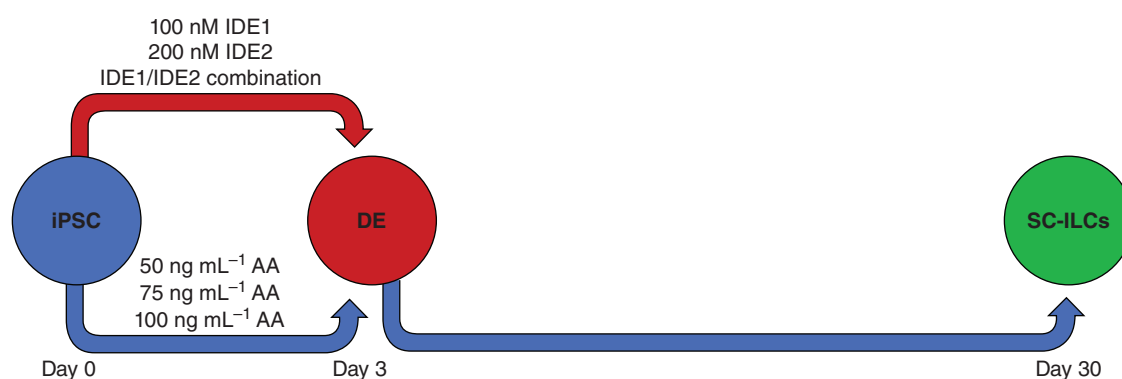
Figure 1.   iPSC differentiation to definitive endoderm. iPSCs cultured to 90–95% confluency were differentiated into Definitive Endoderm using a 3-day protocol described in the text. 6 groups were analyzed, control cells at 100 ng/ml Activin A (AA) concentration and test cells using 50 ng/ml AA, 75 ng/ml AA, 100 nM IDE1, 200 nM IDE2, and a combination of IDE1/IDE2.
DE: Definitive endoderm; iPSCs: Induced pluripotent stem cells; SC-ILCs: Stem cell-derived islet like cells.

Technologies), 200 nM IDE2 (72524, StemCell Technologies), or a combination of 100 nM IDE1/200 nM IDE2 and 3 μM CHIR99021 and 5 μM Y-27632 Rock Inhibitor for 1 day only. DE medium contained IDE1, IDE2 and the combination IDE1/IDE2 at the same concentration for the next 2 days but, without the GSK3 Inhibitor and Rock Inhibitor. Figure 1 illustrates the six groups examined in this study by replacing AA with IDEs or reducing the concentration of AA.

For downstream differentiation into SC-ILCs, cells were differentiated in a six stage protocol comprised of stages corresponding to definitive endoderm, primitive gut, posterior foregut, pancreatic endocrine progenitors, immature beta cells and islet-like cells (SC-ILCs). All groups underwent the same differentiation protocol with no alterations after the DE stage. The major growth factors and small molecules used at each stage are described in previously published methods [29,30].

Cell counts & viability

Cells at various stages (iPSC, DE, and SC-ILCs) were single-cell dispersed using TrypLE™ Express Enzyme (12604-021, ThermoFisher Scientific) followed by quenching of the enzyme with a 2% fetal bovine serum (FBS)/DPBS (-/-) solution. Each cell population was assessed for a live cell count and expressed per unit surface area. The cell viability (%) was determined using an EVE™ Automated Cell Counter (NanoEnTek). Cell counts at both DE stage and SC-ILCs were compared with the starting iPSC count to assess cell proliferation and cell death during the differentiation period.

Gene expression analysis by qRT-PCR

The progress of differentiation from iPSCs to insulin producing cells SC-ILCs was monitored by analysis of key biomarkers expression at the various stages. For gene expression analysis, cells harvested from the different stages were analyzed by qRT-PCR. Cells were lysed directly on the plate using TRI Reagent (R2050-1-200, Zymo

Table 2. Biomarkers for differentiation stage with TaqMan probes utilized.		
Stage	Gene name	Assay ID
Housekeeping gene	GUSB	Hs00939627_m1
Induced pluripotent stem cells	OCT4 (POU5F1)	Hs04260367_gH
	SOX2	Hs04234836_s1
	NANOG	Hs02387400_g1
Definitive endoderm	FOXA2	Hs00232764_m1
	SOX17	Hs00751752_s1
Stem cell-derived islet like cells	INS	Hs00355773_m1
	GCG	Hs00174967_m1
	NEUROD1	Hs00159598_m1
	NKX6.1	Hs00232355_m1
	PAX4	Hs00173014_m1
	SST	Hs00356144_m1
	PDX-1	Hs00236830_m1

Research) and lysates stored at -20°C until RNA extraction was done. RNA was extracted from the lysates with Direct-zol RNA MiniPrep Plus Kit (R2072, Zymo Research) using the manufacturer provided protocol. qRT-PCR was carried out using AgPath-ID One-Step RT-PCR Reagents (4387391, ThermoFisher Scientific) and TaqMan Probes for gene expression on a QuantStudio 3 Thermal Cycler. Results were analyzed by RNA quantification (RQ) using a $\Delta\Delta$Ct method with glucuronidase beta (GUSB) as the housekeeping gene. The product numbers for sequences used as TaqMan Probes (ThermoFisher Scientific) are provided in Table 2.

Immunohistochemistry

For transcription and surface marker expression analysis, cells were immunostained in the wells either using a single antibody or combination of antibodies. When indicated, staining for surface markers expression was done prior to cell fixation. Cells were rinsed in DPBS (-/-) and then fixed with 4% paraformaldehyde (J61899, Alfa Aesar) at room temperature for 30 minutes. Subsequently, cells were permeabilized with 0.1% Triton X (BP151-100, Fisher Scientific) solution at room temperature for 20 min and then blocked with 10% donkey serum (017-000-121, Jackson ImmunoResearch Laboratories) in DPBS at room temperature for 30 min. Samples were then incubated with primary antibodies diluted 1:200 in 10% donkey serum solution overnight at 4°C. The primary antibodies utilized in this study are provided in Table 3. The samples were washed twice with DPBS (-/-) and then incubated with secondary antibodies diluted 1:500 in 10% donkey serum for 2 h at room temperature. Samples were then washed once with DPBS (-/-) and stained with Hoechst diluted 1:1000 in DPBS (-/-). Fluorescent images were captured using a BioTek Cytation 5 Imaging Reader.

Flow cytometry

Flow cytometric procedures were used to analyze cells when cell numbers recoverable from assays were sufficient. Briefly, cells were released into single cell suspension by incubation in TrypLE Express (12604, Invitrogen) at 37°C for 3–5 min. Cells were then washed in staining buffer (2% FBS in DPBS (-/-) (26140, Invitrogen). For surface marker staining, approximately 1.0–1.5×10^6 cells were suspended in 100 µl staining buffer and stained with conjugated primary antibodies as per vendors instructions. Cells were incubated for 30 min at 4°C followed by two washes in staining buffer before FACS analysis.

For intracellular staining, cells were first incubated in protein-free PBS(-/-) with Ghost Dye Red 780 Viability Dye (13-0865, Tonbo Biosciences) for 20 min at room temperatures to allow for live-dead cell discrimination during analysis, followed by a single wash in DPBS (-/-). Cells were then fixed in 0.5–1.0 ml of Fixation Buffer (22015, Biotium) for 20 min at room temperature followed by a single wash in blocking buffer containing 5% donkey serum. Cells were resuspended in 100 ul staining/blocking buffer and incubated for 30 in at 4°C with primary antibodies at empirically determined dilutions, followed by two washes in staining/blocking buffer. Cells were then incubated with secondary antibodies at the appropriate dilutions for 30 min at 4°C followed by two washes prior to analysis on BD FACS Canto II. Appropriately labeled fluorescent beads were used for compensation. Data was analyzed using the FlowJo software (FlowJo 10.8.1). Antibodies used for flow cytometric analysis are shown in Table 3.

Table 3. Antibodies used for protein biomarkers in immunocytochemistry and flow cytometry analysis.

Stage of cells	Protein marker	Antibody species	Vendor	Catalogue number
Induced pluripotent stem cells	OCT3/4 (POU5F1)	Mouse	BD	561555
	OCT3/4 (POU5F1)	Mouse	BD	560186
	SOX2	Mouse	BD	561593
	NANOG	Mouse	BD	560873
Definitive endoderm	FOXA2	Mouse	BD	561580
	FOXA2	Mouse	BD	561589
	SOX17	Mouse	BD	561590
	SOX17	Mouse	BD	562205
	CD184	Mouse	BD	555976
Stem cell-derived islet like cells	INSULIN	Mouse	BD	565688
	INSULIN	Mouse	BD	565689
	C-PEPTIDE	Rat	DSHB University of Iowa	GN-ID4
	GLUCAGON	Mouse	BD	565859
	GLUCAGON	Mouse	BD	565860
	NKX6.1	Mouse	DSHB University of Iowa	F55A10
	NKX6.1	Mouse	BD	567609
	PDX-1	Goat	R&D	AF2419
	PDX-1	Mouse	BD	562161

Table 4. Cost of Goods summary.

Material	Percentage of Cost of Goods (excluding labor) (%)
Base media	35
Growth factors	33
Consumables	17
Media supplements and reagents	11
Small molecules	4

Glucose-stimulated insulin secretion

SC-ILCs in planar culture were exposed to the three different AA concentrations incubated with 1X Earle's Balanced Salt Solution (EBSS) without glucose for 1 h, followed by low (5.5 mM) glucose in EBSS for 1 h, followed by high (22.8 mM) glucose in EBSS for 1 h, followed by low (5.5 mM) glucose in EBSS for 1 h, followed by secretagogue stimulation (28 mM glucose + 30 mM KCl) in EBSS for 30 min. Supernatants were collected for all concentrations above and stored at -20°C. The insulin concentration was assayed using a standard insulin ELISA kit (10-1113-01, Mercodia) and stimulation index was calculated by dividing the value of insulin with stimulation by the value when incubated in low glucose (5.5 mM).

Statistical analysis

One way ANOVA was performed on all quantitative p-values are identified in the figure legends.

Results

Cost of reagents & consumables analysis

The percentage of each reagent and consumable toward the cost of the final product (excluding labor) was calculated. The base media category, comprised of several different constituent components, made up the most expensive group of materials used in the manufacturing process for both the cell expansion and differentiation phases of manufacturing. Next was the growth factors, which made up a large portion of the total cost of reagents and consumables, followed by the consumables, media supplements and reagents, and small molecules in that order (Table 4). When each individual growth factor/reagent was analyzed, it was revealed that AA comprised the single most expensive growth factor and it accounted for a surprising 12% of the total cost for a single batch, even though it is only used for 3 days in the differentiation protocol. Figure 2A illustrates the expense associated with AA at the standard 100 ng/ml concentration compared with the other major categories involved in the differentiation of
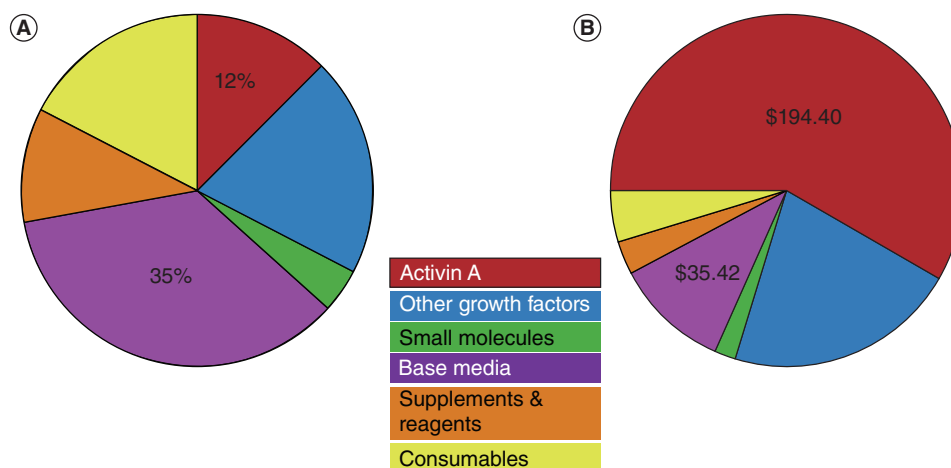
Figure 2.   Cost of reagents and consumables analysis for differentiation of induced pluripotent stem cells to stem cell-derived islet like cells. Analysis of the cost of reagents and consumables for full differentiation of iPSCs to SC-ILCs demonstrated that a single growth factor Activin A (AA) when utilized at the standard concentration of 100 ng/ml had a significant impact on the total cost. Analyses were conducted using AA at 100 ng/ml and compared with the total cost breakdown of all relevant categories. **(A)** Individual reagents and consumables were analyzed as a % of total cost for a standard batch. **(B)** Individual reagents and consumables were analyzed as a total cost per day of usage over the 50-day differentiation period.

Table 5. Viability and cell counts for differentiation to definitive endoderm.		
Groups	**Cell counts (live cells/cm²)**	**Viability (%)**
Starting iPSCs	5.22×10^5	90.5
AA	2.81×10^5	90
IDE1	8.50×10^5	93.5
IDE2	7.00×10^5	97.5
IDE1/IDE2	9.25×10^5	98

cells, including cell culture media, other reagents, and consumables. This is especially significant as illustrated in Figure 2B where the cost of all categories listed are expressed as a cost per day. With knowledge that a single reagent could have such an oversized impact on the final COGs, we examined the effect of using replacement reagents for AA as well as decreasing the AA concentration.

Small molecules IDE1 & IDE2 are weak inducers of definitive endoderm

AA has been widely used as a potent inducer of DE when used in conjunction with a GSK3 inhibitor. Efforts to convert iPSCs to DE using other less expensive components like small molecules that preclude the use of biologic growth factors, have been investigated and reported by numerous teams with varying degrees of success [18–20]. Specifically, the small molecules, IDE1 and IDE2, singly and in combination have been reported to successfully generate DE [18]. The cost of these molecules is far less than AA, thus we attempted to replicate earlier studies showing their ability to create DE from iPSCs.

At the completion of DE stage, there were differences in cell numbers between the 3 IDE treatment groups (IDE1, IDE2 or a combination of the two) and the standard protocol using 100 ng/ml AA (Table 5). The formation of high-quality DE cells is normally accompanied by significant cell death over the three-day period. There was a relative lack of cell death observed in the three IDE groups which is predictive of poor DE formation. Interestingly, the majority of the cells counted at this stage were detached from the surface; another sign that they were not progressing appropriately to DE. That conclusion was confirmed by the subsequent comparative qualitative analysis between the AA and IDE treated groups shown next.

The formation of DE is identified by expression of high levels of the DE biomarkers; SOX17 and FOXA2(Hnf3b) among other markers, and the diminishing expression of pluripotent stem cell markers, such as OCT4, Nanog, and SOX2. Figure 3A shows the quantitative expression of biomarkers for pluripotency and DE, expressed as RQ
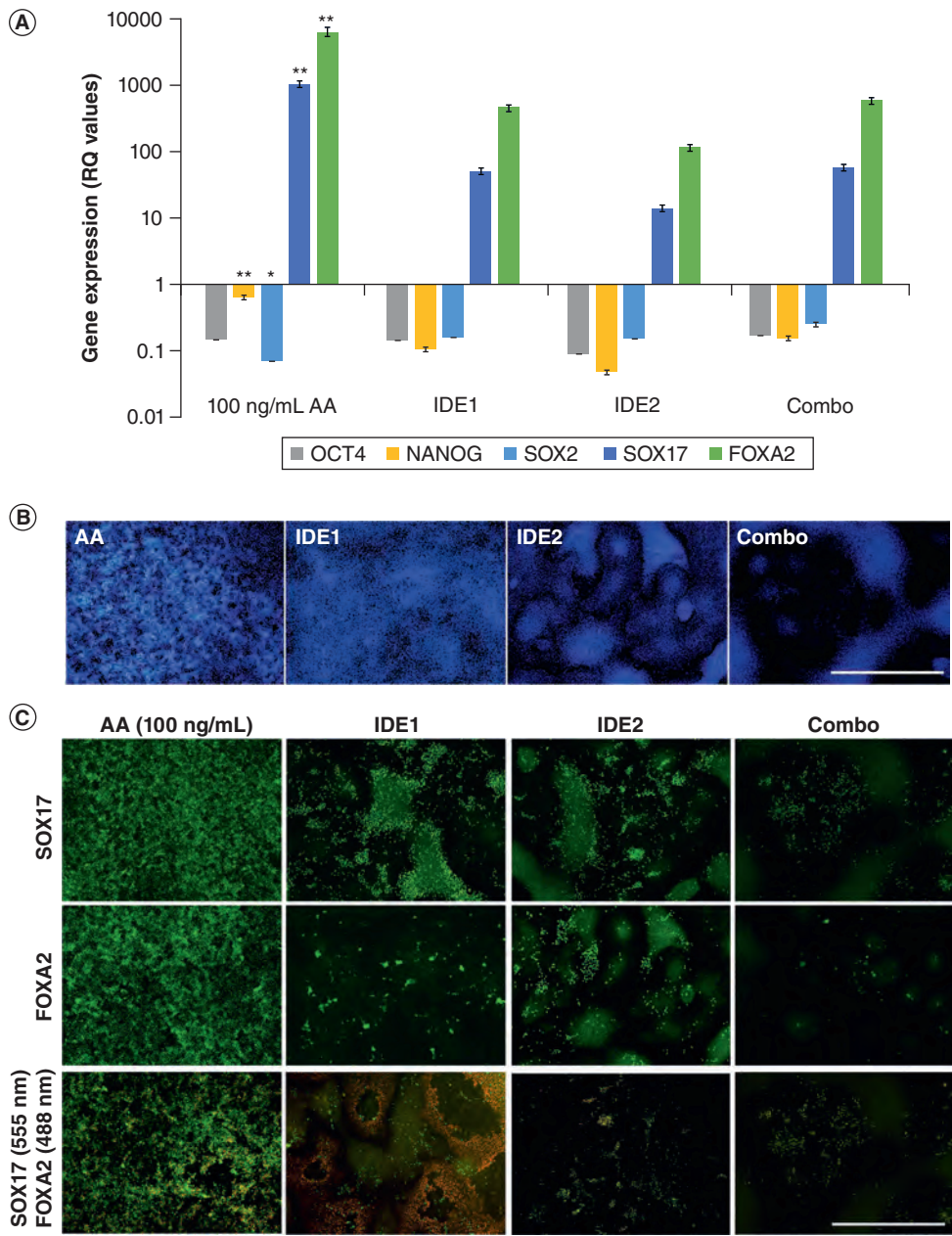
Figure 3. Expression of definitive endoderm markers on Day 3. The small molecules IDE1 and IDE2, used singly or in combination were tested for their ability to generate definitive endoderm compared with Activin A (AA) at 100 ng/ml. **(A)** Comparative quantitation of analysis of markers of definitive endoderm (DE) are shown as qRT-PCR and compared with 100 ng/ml AA and using the iPSCs as comparator for RQ and GUSB as the housekeeping gene. **(B)** Cell nuclei staining by Hoechst showed massive cell detachment associated with the IDE compounds compared with AA. The scale bar is 1000 μm. **(C)** The DE cells were fixed and stained using primary antibodies, FOXA2 and SOX17, biomarkers of DE individually and also stained for co-expression (bottom row). The scale bar for all images is 1000 μm. The results show that the replacements for AA (IDE1 and IDE2) did not produce cells with the same level of DE biomarkers as the standard protocol with AA.
* indicates p < 0.05, ** indicates p < 0.01.

normalized to the starting iPSC levels. The results showed reduced expression of the pluripotency markers (OCT4, Nanog, SOX2) in all DE preparations and increased expression of DE markers (SOX17, FOXA2). The largest increase in DE marker expression was observed in the cells exposed to AA compared with cells exposed to IDE1, IDE2, or the combination IDE1/IDE2 (Figure 3A, Combo). In fact, the gene expression levels for the group using

100 ng/ml AA were statistically different from all IDE groups for all genes tested, except OCT4. Generally, for each of the DE markers SOX17 and FOXA2, there was about ten-times less expression for the IDE methods compared with the standard AA method.

An explanation for the basis of the loss of gene expression could be identified when examining the groups under light microscopy. There was massive cell detachment in each of the individual IDE and combination groups during the DE differentiation process, resulting in poor cell harvests for analysis, as shown in the Hoechst cell nuclei dye staining (Figure 3B). Unfortunately, the loss of cells due to detachment, described previously, meant that flow cytometry could not be conducted, as there were not sufficient cells to conduct the procedure. Thus, the attached cells that are necessary for further differentiation, were dramatically less for the IDE groups than the AA group.

The protein levels of the DE biomarkers (SOX17 and FOXA2) were analyzed using immunohistochemistry. Again, the greatest levels of DE biomarkers were noted in the standard AA treated group and not in the single IDE treatments or in the combination IDE group (Figure 3C). In fact, the combination group had the lowest expression level of the two DE markers compared with each IDE treatment alone. The poor outcomes with the small molecules, IDE1 and IDE2 was surprising and highlights the lack of applicability to other cell lines. Due to the poor DE differentiation in response to IDE1 or IDE2, subsequent studies focused on the reduction of AA rather than its replacement with IDE small molecules.

Reduced concentration of activin a as an inducer of definitive endoderm

As stated earlier, the initiation of DE formation from iPSCs is accompanied by a significant loss in the cell number over the 3-day period. In this study, the anticipated cell loss was experienced at all concentrations of AA tested and is shown in Figure 4A. The cell numbers were normalized per unit area of culture vessel. The cell loss that occurred at the lower concentrations of AA was statistically less than at the standard concentration. There was only about 30–40% cell loss noted with 50 or 75 ng/ml AA treatments compared with the greatest loss of over 50% observed in 100 ng/ml AA treatment. While the standard AA concentration (100 ng/ml) was the only group with viability below 90%, there were no statistical differences between groups (Table 5).

Analysis of DE markers FOXA2 and SOX17 as well as the pluripotent marker OCT4 is shown as RQ analysis compared with iPS gene expression levels. The data show very high expression of the DE biomarkers, FOXA2 and SOX17, and poor to no expression of the pluripotent biomarker, OCT4, in all three concentrations of AA tested (50, 75 and 100 ng/ml) (Figure 4B). There were no statistically significant differences in expression of each biomarker analyzed between the three test conditions.

In addition to qRT-PCR analysis, cells were analyzed by immunostaining for protein expression of the two DE markers. Like qRT-PCR analysis, the cells showed expression of marker proteins FOXA2 and SOX17 at very high levels in all three conditions tested (Figure 4C). In addition, there was excellent co-expression of both biomarkers, as shown by the overlapping staining in Figure 4C. Taken together, these results show that there were no significant differences in the level of expression of the protein markers between the three conditions tested.

Effect of AA reduction in downstream differentiation

The successful formation of DE is followed by a further downstream, stepwise differentiation process to create insulin producing, SC-ILCs for the treatment of diabetes. We sought to determine if the use of a lower concentration of AA during DE formation affected the downstream differentiation process.

The cell loss noted previously with the conversion to DE (Figure 4A) was recovered with continued differentiation of cells through the endocrine progenitor phases with near full recovery of cell loss by the final differentiation stage as shown in Figure 5A. Although the cell recovery was similar and not statistically different from each other, it is notable that the biggest recovery occurred at the 100 ng/ml cell treatment test condition, the group that had also experienced the largest cell loss at the completion of the DE stage. Viability of the cells in the final stage of differentiation remained high with no statistically significant differences between the 3 groups of AA concentrations (Figure 5B).

To illustrate the differences in the gene expression between the 3 groups, RQ values were calculated for the SC-ILC cells and plotted in Figure 6A. In general, there was little difference between groups in the expression profile of the final stage SC-ILCs. The lowest AA concentration resulted in a slightly lower pluripotency biomarker (OCT4) and higher insulin expression, but with less PDX1 expression. There was no statistical difference between groups.
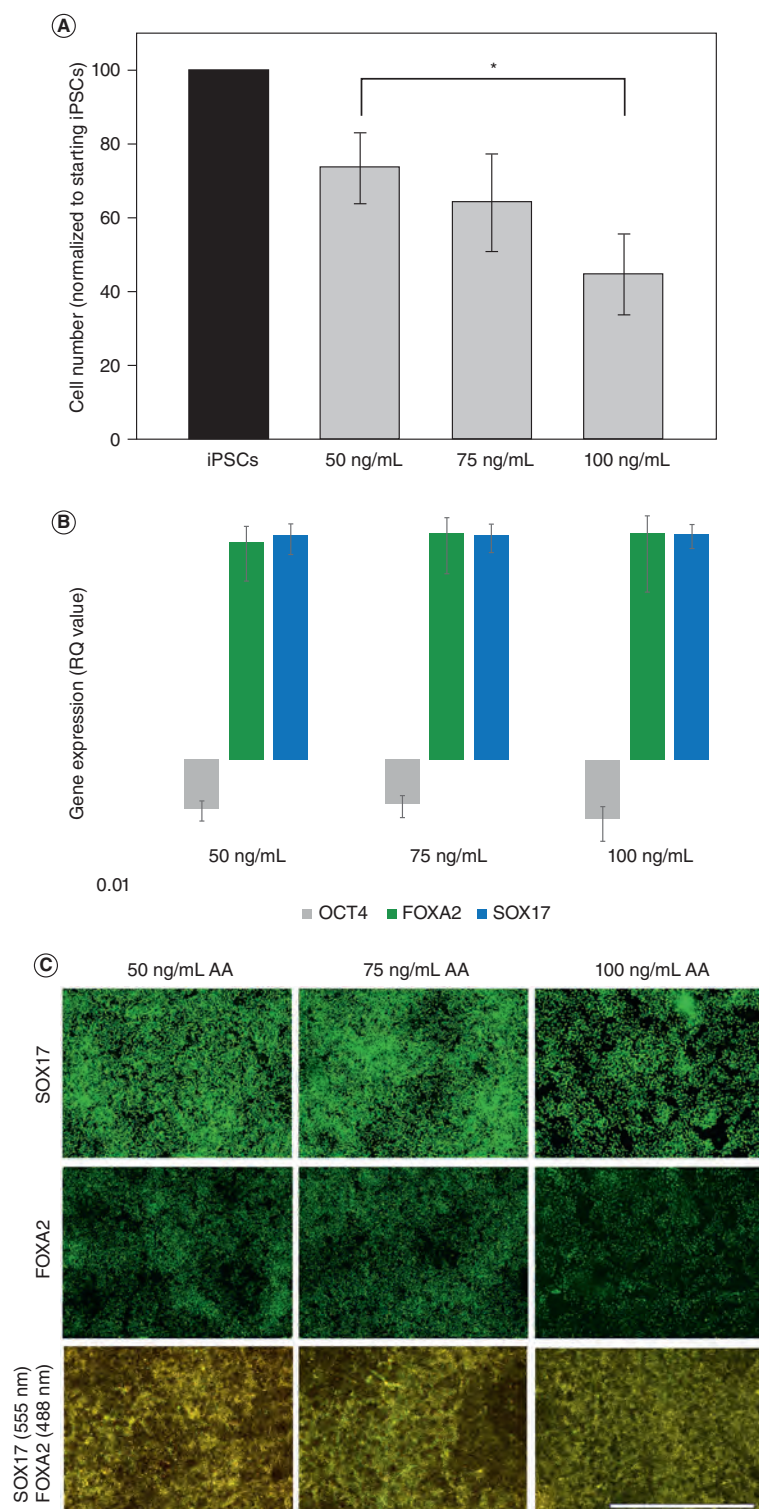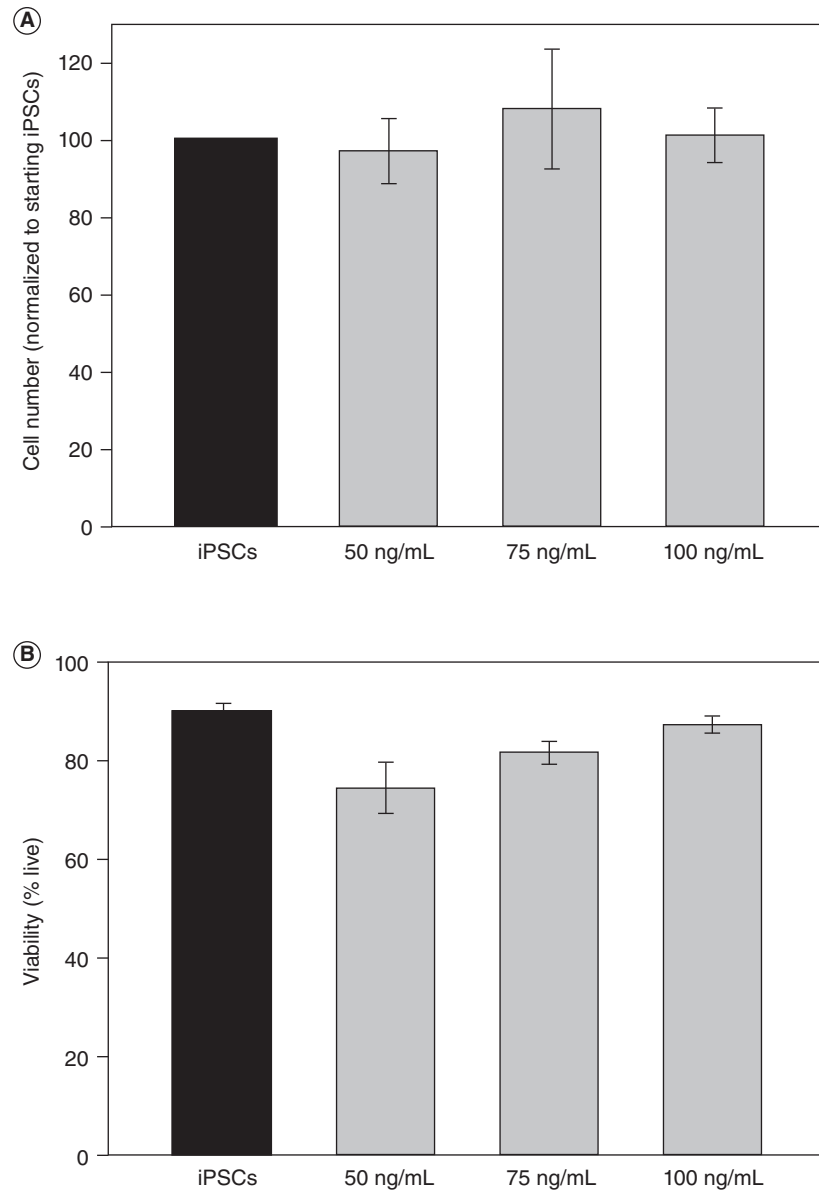
Figure 4.   Effect of lower AA concentrations on definitive endoderm formation. (A) Cells were counted and analyzed per surface area then normalized to starting iPSC count to assess the % of cell loss during the 3-day definitive endoderm (DE) differentiation. The greatest cell loss was noted in the 100 ng/ml AA group, which was statistically lower than the 50 ng/ml concentration. **(B)** Comparative quantitation of analysis of markers of DE are shown as qRT-PCR analysis using the iPSCs as comparator for RQ and GUSB as the housekeeping gene. There was no statistical difference between groups. **(C)** The DE cells were fixed and stained using primary antibodies to FOXA2 and SOX17 according to described methods. The scale bar indicates 1000 μm * indicates $p < 0.05$.

Figure 5.   Effect of lower AA concentrations on stem cell-derived islet like cells formation. (A) Cells were counted and analyzed per surface area then normalized to starting iPSC count to assess for % cell gain during 6-stage differentiation protocol. There was no statistical difference noted between groups. **(B)** Viability values also showed no statistical differences between groups.

The SC-ILCs were immunostained for analysis of proteins associated with islets, C-peptide, NKX6.1, Islet-1, and Insulin. The results are shown in Figure 6B. Very similar staining patterns and intensities for these markers were observed across groups, again indicating that the lowered concentrations of AA used during DE formation did not impact the downstream differentiation patterns of the SC-ILCs. Specifically, insulin, C-peptide, NKX6.1 and islet-1 are all key markers of mature beta cells in islets. Figure 6B shows that all stained brightly with no noticeable difference between the 3 groups. Further, overlapping images of insulin and PDX-1 showed strong co-expression, with only a few PDX-1 cells not also expressing insulin. In agreement, co-expression of C-peptide and islet-1 was also strong (Figure 6B).

In addition to immunostaining, flow cytometric analysis was completed on fully differentiated cells at the SC-ILC stage. The flow cytometric data plots for each of the AA concentration tested is shown in Figure 6C for co-expression patterns of insulin/ PDX-1 and insulin/NKX6.1. The percentage of insulin/PDX-1 co-expression was very similar
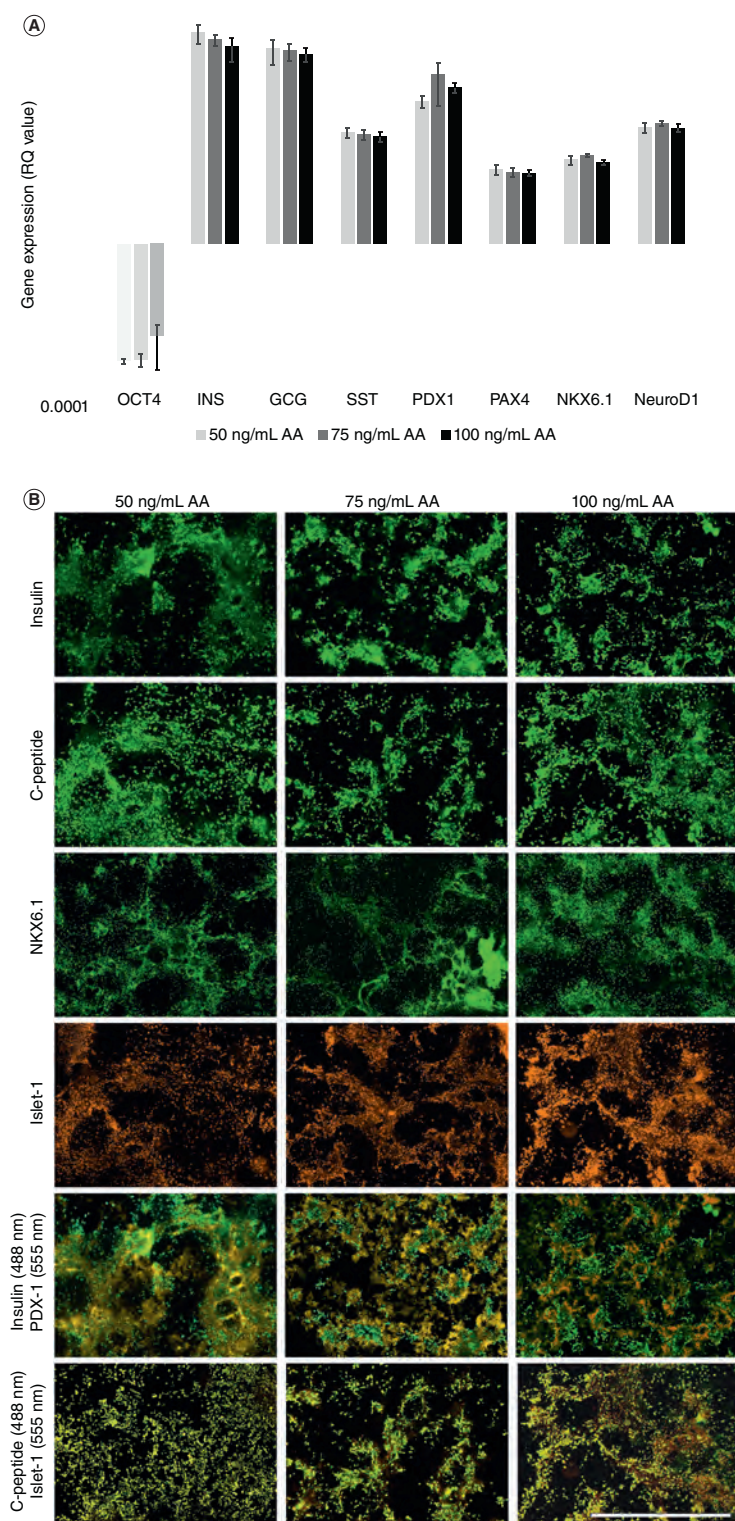
Figure 6.   Effect of lower AA concentrations on stem cell-derived islet like cells biomarkers and function. (A) Comparative quantitation of analysis of mature β-cell markers are shown as qRT-PCR analysis using the iPSCs as comparator for RQ and *GUSB* as the housekeeping gene. There were no statistical differences between groups. **(B)** The SC-ILCs were fixed and stained using antibodies to insulin, C-peptide, NKX6.1 and Islet-1 and PDX-1. Secondary antibodies were conjugated to Alexa-Fluor 488 or Alexa-Fluor 555 for co-localization. **(C)** Flow cytometric analysis of mature SC-ILCs shows co-expression of i) Insulin/ PDX-1 and ii) Insulin/NKX6.1. There were no differences between groups. The scale bar indicates 1000 μm. **(D)** The SC-ILCs derived from varying AA concentrations at DE were evaluated for insulin secretion following GSIS as described in the methods. The values reported are a stimulation index comparing the insulin secreted in initial low glucose incubation compared with the secretagogue incubation. *p < 0.05.
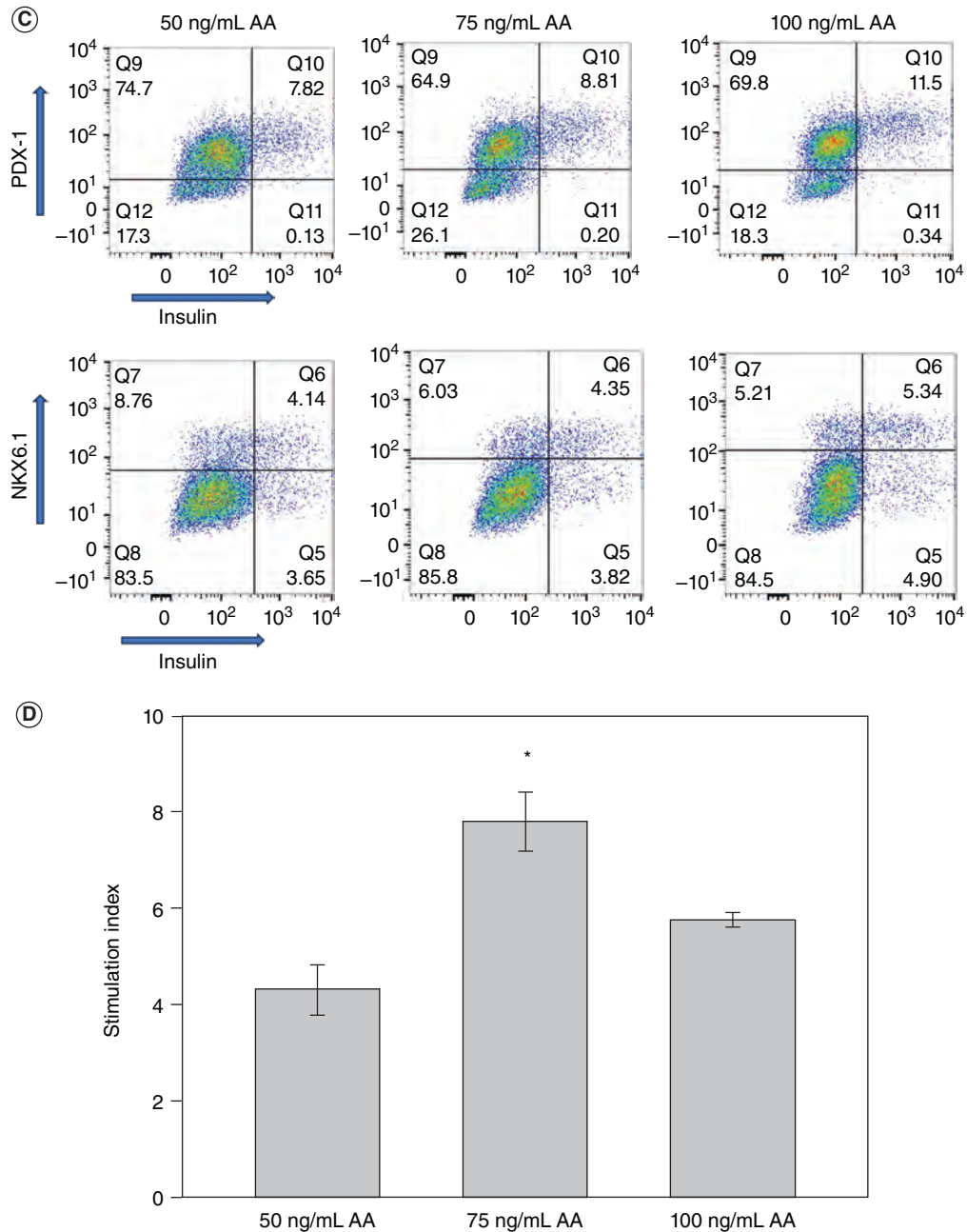
Figure 6.   Effect of lower AA concentrations on stem cell-derived islet like cells biomarkers and function (cont.). (A) Comparative quantitation of analysis of mature β-cell markers are shown as qRT-PCR analysis using the iPSCs as comparator for RQ and *GUSB* as the housekeeping gene. There were no statistical differences between groups. **(B)** The SC-ILCs were fixed and stained using antibodies to insulin, C-peptide, NKX6.1 and Islet-1 and PDX-1. Secondary antibodies were conjugated to Alexa-Fluor 488 or Alexa-Fluor 555 for co-localization. **(C)** Flow cytometric analysis of mature SC-ILCs shows co-expression of i) Insulin/ PDX-1 and ii) Insulin/NKX6.1. There were no differences between groups. The scale bar indicates 1000 μm. **(D)** The SC-ILCs derived from varying AA concentrations at DE were evaluated for insulin secretion following GSIS as described in the methods. The values reported are a stimulation index comparing the insulin secreted in initial low glucose incubation compared with the secretagogue incubation. *p < 0.05.

Table 6. Effects of AA reduction on definitive endoderm cell viability.	
Groups	Viability
Starting cells (iPSCs)	90.2 + 1.6
DE 50 ng/ml AA	94.3 + 1.1
DE 75 ng/ml AA	93.3 + 1.7
DE 100 ng/ml AA	89.0 + 4.7

in all test conditions and ranged from approximately 7–12%, while the insulin/NKX6.1 co-positive cells ranged between 4.0–5.5%. The differences in single expression or double co-expression was not statistically significant, again demonstrating that the range of concentration of AA tested was equally effective.

The implication of lower AA concentrations on SC-ILC function was analyzed with a static insulin secretion assay. Groups of SC-ILCs were exposed to secretagogues and the supernatant analyzed for insulin secretion which was normalized to the length of the static incubation. The stimulation index was calculated by dividing the stimulated insulin secretion/minute by the basal secretion/minute. The cells exposed to 75 ng/ml had a statistically greater amount of insulin secretion compared with the other groups (Figure 6D), again indicating that Activin A concentrations below the 100 ng/ml were effective in creating functional insulin producing cells (Table 6).

Discussion

The potential to create new therapies for endodermal derived tissues and organs in regenerative medicine, such as pancreatic cells, hepatic cells, gastrointestinal and lung tissues from pluripotent stem cells has necessitated the designing of protocols that are able to pass the first step of differentiation, the formation of definitive endoderm at very high efficiencies [31,32]. DE is the first major stage of differentiation and the ability to create robust DE can greatly influence the success achieved in downstream differentiation steps. In this regard most protocols described to date include use of the TGFβ-family, Activin A, in a 3- or 4-day protocol to convert pluripotent stem cells into DE [19,33].

Since the initial publication by D'rmour [33] numerous groups have described the formation of DE from many iPSC lines using Activin A at the concentration of 100 ng/ml as initially described [18,19]. In a recent article, Ghornani-Dalini et al. [34] examined the use of different concentrations of AA for DE formation under serum free and knock-out serum conditions. Their data showed that decreasing levels of Activin A to 30 ng/ml provided the best outcomes for the increase in expression of DE genes SOX17 and FOXA2, using the R1-hiPSC1 cell line [34]. It is particularly salient though at this point to indicate the unique nature of the Activin/Nodal signaling in the bifurcation or causation of cell fate decisions based on the dosage applied, as well as other components in the media environments, for example, FBS, insulin. For example, in mouse pluripotent stem cells, increased levels of Nodal signaling causes mesoendoderm differentiation, while lower levels cause differentiation to trophectoderm [35]. In other studies, Activin A at varying concentrations has also been shown to play a role in the maintenance of the pluripotency state [36,37], indicating somewhat conflicting outcomes in cell fate decisions based on dosages applied. Yet, Jiang et al. reported they could induce DE in the presence of small molecule GSK-3 inhibitors only and in the absence of Activin A, although in very specific media conditions that used RPMI with B-27 but not with insulin supplement [38]. Thus, the lack of acceptable differentiation into DE by IDE1, IDE2 or the combination, was an unexpected result. The negative results may be due to the unique clinically appropriate human cell line used in this study, which was not used previously for testing. Interestingly the combination of small molecules like the IDEs along with low concentrations of AA has not been reported in the literature and was not tested here. While the combination may work, when the goal is to reduce the overall production costs, adding small molecules to the protocol when lowering the AA concentration worked sufficiently alone, defeats the purpose of cost containment, unless the AA concentration could be reduced to significantly below 50 ng/ml.

Although we have not tested the whole possible range of Activin A dosages in our studies, we have demonstrated that reducing the dosages of Activin A does not negatively impact differentiation into definitive endoderm. Our findings that 50 ng/ml provided similar results in DE formation and in the final SC-ILC cells, compared with the 100 ng/ml traditionally used, confirms that alternative differentiation protocols that provide significant savings could be adopted or even optimized further. For instance, in a previous report, Kubo [39] demonstrated that using lower concentrations of AA of 10 ng/ml or 30 ng/ml under serum free conditions was sufficient to induce definitive endoderm in mouse embryonic stem cells. This suggests that lower levels of AA under 50 ng/ml might work in

derivation of definitive endoderm from iPS cells also, although under specific culture conditions, as reviewed by Sulzbacher [40].

Although the mechanistic action of AA is beyond the scope of this study, AA has been previously reported to act over a wide range of concentrations in the context of definitive endoderm differentiation [40]). AA belongs to the TGF-β super family of extracellular signaling protein, acting by binding to Type I and II surface receptors that in turn lead to activation of phosphorylation of specific Smad proteins, which are translocated into the nucleus and recognized by specific transcription factors. It remains to be determined if the pathways activated by the different reagent concentrations are similar or different.

While AA is a useful molecule to derive DE, the growth factor is quite expensive and limits industrial scale production and widespread application. The cost of novel therapies must be considered in the early stages of protocol development, especially with the introduction of drug price controls established in the Inflation Reduction Act for human therapeutics [41]. For this reason, strategies that avoid AA or use other more economical methods to derive DE, including small molecules, are actively being investigated.

In this report, we investigated whether the use of lower concentrations of AA during DE formation affected the efficiency of the process. We also investigated the use of small molecules that have been described in the literature to replace AA [32,42,43] and compared DE formation efficiency. Small molecules such as the IDEs help to lower the cost, and are very stable with simplified quality control due to a defined manufacturing process. Unfortunately, with the iPSC line tested in this study, the IDE replacements for AA failed to result in satisfactory DE formation. The result was a surprise given previous publications [32,42]. However, it may be due to the starting iPSC line, or differentiation protocol, as it has been amply demonstrated that different iPSC lines respond differently to the same growth factors using the same differentiation conditions [44,45]. It is therefore important to establish optimal differentiation conditions for each developing therapies that require DE formation including pancreatic (diabetes), hepatic, lung tissues, or another endodermal prodigy.

In the human healthcare space, the Inflation Reduction Act has been called the most significant healthcare reform since the Affordable Care Act. For advanced therapies, including cell therapies, the Act puts downward pressures on pricing especially for certain high-spend medications covered under Medicare, which may discourage development of some new therapies, especially if they require a complex manufacturing process. Thus, it is essential that those involved in any new small molecule or biologic program development begin to consider the cost of their eventual product as early as the discovery and development phases.

While the cost of base media did comprise the highest percentage of the total cost for a single category, it is important to note that that this cost is spread across the entire process of making SC-ILCs including cell thawing, expansion, and differentiation ($\sim$50 days). Comparatively, AA is only used for a 3-day period making it the best suited target of a COGs reduction effort. Since AA is one of the most expensive components in the beta-cell differentiation protocol, comprising about 12 percent of the total cost of the differentiation reagents and consumables in our specific protocol, the need to optimize the use of AA was obvious.

Our results demonstrated that use of AA at the lower concentrations did not negatively affect downstream differentiation into SC-ILCs. Yet, there was a 6% reduction in the total price of the product by using 50 ng/ml and a 3% reduction with 75 ng/ml compared with the standard protocol. This resulted in a reduction in the total cost of reagents and consumables from over $5000 to $4750. These values do not include labor and it is based on the use of research grade reagents, which are less expensive. Obviously moving to Good Manufacturing Practices (GMP) production will greatly increase the cost. However, the scale that will be achieved with automated large-batch production will help to mitigate some of that increase. At our current small scale manufacturing size and dosage of 30×10^6 cells/kg, to make a batch for an adult in the United States (assuming 80.7 kg), this would require four-times our standard batch size. With a reduction of 50% concentration of AA that is a cost savings of $1000 in just reagents and consumables. The overall COGs are assumed to decrease in a large-scale manufacturing setting by simply cutting down on labor [46]. If emphasis is placed early on cost savings during the discovery and development savings at small scale, that will translate into a huge savings at large scale assuming a standard 20% savings in COGs [46]. Even if the cost of GMP manufacturing is ten-times greater than the current research grade production, it would still result in a cell therapy to treat Type I diabetes with cost of reagents and consumables less than $50,000, which is significantly lower than current manufacturing, with cell therapies priced in the $500,000 and higher [47]. Additional efforts are now under way to compare and better understand the functional capabilities of these cells including their engraftment potential and functional capacity to regulate high blood glucose levels in diabetic rodents. Our data confirm that carefully selected and targeted changes to differentiation protocols aimed

at specific components can result in a meaningful cost savings, thus greatly improving on the overall availability and benefits of cell-based therapies to wider audiences.

Conclusion

The collective data support the conclusion that use of a lower concentration of AA during DE formation does not negatively impact the expression of key markers including insulin in the final product nor did it negatively affect insulin secretion from the cells, suggesting that lowering the concentration of AA could be used in differentiation protocols. The results illustrate the continued need for optimization and assessment of various reagents not only for their impact on the function of the final product, but also on its cost.

Summary points

- Cell therapies promise to provide a cure for many diseases in human and veterinary medicine.
- Cost of research, manufacturing, and delivery of cell therapies is extremely high, limiting the widespread adoption and benefits of such treatments.
- Recent legislation in the USA via the Inflation Reduction Act has added more pressure on companies working with advanced therapies such as cell and biologic therapies to reduce their costs.
- Our work highlights the need to explore and adopt cost cutting measures early in process development to fully realize the benefits of cell therapies.

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