RESEARCH PAPER

Islets 6:5-6, e1017226; September–December 2014; © 2014 Taylor & Francis Group, LLC

Effects of activin A on survival, function and gene expression of pancreatic islets from non-diabetic and diabetic human donors

Melissa L Brown[1,2,3,*], Nathan Ungerleider[1,2], Lara Bonomi[1,2], Danielle Andrzejewski[1,2,4], Amy Burnside[1,2,4], and Alan Schneyer[1,2,4]

[1]University of Massachusetts Amherst; Amherst, MA USA; [2]Pioneer Valley Life Sciences Institute; Springfield, MA USA; [3]Department of Nutrition; University of Massachusetts; Amherst, MA USA; [4]Department of Veterinary and Animal Sciences; University of Massachusetts; Amherst, MA USA

Keywords: activin, diabetes, human islets

Emerging evidence suggests that activin with its associated receptors, second messengers, and antagonists would be excellent targets for therapeutic drug development in the treatment of diabetes. We undertook the current study to investigate the ability to extrapolate findings from rodent studies to human islets in which data thus far has been scarce. We tested the hypothesis that human islets synthesize activin and that activin participates in the regulation of islet β-cells. Human islets from 33 separate isolations were categorized based on functional status, culture status and diabetic status. Statistical comparisons were made by ANOVA with Tukey post-hoc adjustment for multiple comparisons. Experiments investigating activin utilized qPCR, FACS cell sorting, immunofluorescent antibody staining, functionality assays, viability assays and protein secretion assays. We have defined the transcript expression patterns of activin and the TGFβ superfamily in human islets. We found *INHBA (the gene encoding activin A)* to be the most highly expressed of the superfamily in normal, cultured islets. We elucidated a link between the islet microenvironment and activin A. We found differential ligand expression based on diabetic, culture and functional status. Further, this is also the first report that links direct effects of activin A with the ability to restore glucose-stimulated insulin secretion in human islets from type 2 diabetic donors thereby establishing the relevance of targeting activin for therapeutic drug development.

Downloaded by [alan schneyer] at 07:35 04 April 2015

Diabetes is characterized by the loss of functional β-cell mass. A clear understanding of normal β-cell maturation and the mechanisms underlying the functional integrity of islets is warranted. This will enable early detection of β-cell dysfunction and aid in the development of targeted therapeutics. Activin, along with other members of the transforming growth factor β (TGFβ) superfamily of hormones, has been implicated in the development of the pancreas.[1] Further, a potential role for activin in regulation of the maturation and functional states of the β-cell is emerging.[2-5]

The presence of several TGFβ superfamily members, including activins A and B, along with their receptors and Smad second messengers, has been established in islets and/or β-cells.[1,6,7] Genetically altered mice have been used to elucidate roles for these ligands in regulating adult islet function and/or β-cell proliferation.[1] Hypoplastic islets and hyperglycemia are the end result when the activin/TGFβ signaling pathway is blocked by overexpression of a dominant-negative receptor,[7,8] by reducing activin receptor expression,[9] or by conditionally overexpressing the inhibitory Smad7 in adult islets.[10] The Smad signaling network has recently been found to be a link between the 2 pathways of β-cell dedifferentiation and β-cell proliferation in mice. Smad7 in particular was found to mediate β-cell proliferation after a loss of β-cells by first inducing dedifferentiation.[11] Further, another member of the TGFβ superfamily, Nodal, has recently been identified in human islets and found to promote β-cell proliferation while maintaining differentiation status and cell viability.[12] This finding is an important contribution to the field through the rare use of human islets which further strengthens the clinical relevance and importance of studying the role of this superfamily in β-cell homeostasis.

Regulation of certain members of the TGFβ superfamily, namely activins A and B, myostatin and growth and differentiation factor 11 (GDF11), is carried out by the soluble antagonists follistatin (FST) and follistatin like-3 (FSTL3).[13,14] We previously reported that *Fstl3* knock-out (KO) mice have larger islets, β-cell hyperplasia, enhanced glucose tolerance and improved insulin sensitivity.[15] This suggests that removal of the antagonist increased the bioavailability of endogenous activin, myostatin, and/or GDF11, resulting in enhanced β-cell function and proliferation. What is not clear is whether this activity is direct on islet cells or indirect via other tissues or secreted factors. Moreover,

*Correspondence to: Melissa L Brown; Email: melissab@nutrition.umass.edu
Submitted: 02/03/2015; Accepted: 02/03/2015
http://dx.doi.org/10.1080/19382014.2015.1017226

the challenge of certain genetic models can be differentiating between alterations occurring during development that alter islet function in adults from actions directly on adult islets. Thus, substantial gaps remain in our understanding of how *Fstl3* deletion, and the resulting increase in activin, myostatin and GDF11 bioactivity leads to β-cell hyperplasia and enhanced glucose tolerance in adults.

Using a systematic comparison of synthesis and action of TGFβ superfamily ligands in mouse and rat islets, we recently determined that there is species-specificity that complicates extrapolating rodent results to human islets.[16] For example, we previously found a suppressive action of activin A on glucose-stimulated insulin secretion (GSIS) in mouse islets while it had an opposite, stimulatory effect on rat islets.[16] Further, we reported that expression patterns for TGFβ superfamily ligand mRNAs in rat and mouse islets were distinct. Myostatin was found to be abundant in mouse islets but not detectable in rat islets. Moreover, activin A and B proteins were localized primarily in α-cells, suggesting that their action on insulin secretion may be indirect or paracrine in nature. This finding is in agreement with Szabet et al.[3] who elucidated the suppressive effects of the TGFβ superfamily member, activin A, on adult β-cell maturation and function through paracrine and/or autocrine signaling. The few studies that have been published investigating the potential role for TGFβ superfamily members to directly modulate islet cell function have at times found conflicting results. There has been relative agreement as to the ability of activin A to increase GSIS in rat islets[4,5,16-18] but studies performed in mice have found contradicting results. Some studies have found activin lacks a stimulatory effect in mouse islets.[3,16] Another study has found opposing actions of activin A and activin B. Wu et al.[19] found that activin A stimulates and activin B inhibits insulin secretion in mice. The results also vary for human islets with some results finding increased GSIS after activin A treatment[20] while in other studies, activin A decreased GSIS.[3] Our previous results suggest that species differences in response to TGFβ superfamily ligands such as activin *in vitro* might be due, at least in part, to differences in endogenous ligand production.

Given the lack of data on the role of the activin and the TGFβ superfamily in human islets and the emerging data on species specificity, we undertook the current study to elucidate the roles of activin on human islets. We tested the hypothesis that human islets synthesize targets of FST and FSTL3, specifically activin. We also tested the hypothesis that activin participates in the regulation of islet β-cells which would support our previous findings in *Fstl3* KO mice. Here we compared the gene expression levels of the TGFβ superfamily in human islets obtained from non-diabetic and type 2 diabetic donors subdivided by functionality and effect of culture. Islets from the non-diabetic and diabetic donors were also tested for direct effects of activin A on functionality, viability and expression of islet-specific genes.

We found that the most prominently expressed member of the TGFβ superfamily in cultured, functional islets was *INHBA (gene encoding activin A). INHBA* was detected along with its corresponding protein expression in both α- and β-cells whereas *INHBB (gene encoding activin B)* and *FSTL3* were primarily in α-cells. We also uncovered interesting differences in the gene expression patterns of the *TGF*β superfamily in correlation with the condition of the islets and their functional integrity. Lastly, and quite interestingly, we reveal a restorative effect of activin A on stimulated insulin secretion in islets from T2D donors. The combination of results presented herein provide a strong argument in support of activin as part of a critical islet regulatory mechanism and that increasing activin signaling in T2D might lead to beneficial new therapies.

Materials and Methods

Materials

This study was approved under the designation of "Not Human Subject Research" by the Institutional Review Board and approved for use of biohazardous materials (human cells/tissue) by the Institutional Biosafety Committee. Human islets were kindly provided by the Integrated Islet Distribution Program (IIDP) and from isolation centers at the University of Texas Medical Branch (UTMB) and the University of Virginia (UVA). The islets were isolated from the various centers following the same standard operating procedure with broadcast viability and purity of ≥90% and ≥80%, respectively. Clinical characteristics of the human islets are reported in **Table 1**. Immediately upon arrival at our Institute, islet samples were washed with CMRL-1066 with 10% (vol./vol.) heat-inactivated fetal bovine serum and 1% (vol./vol.) penicillin-streptomycin (all from Corning Cellgro, Manassas, VA) and cleaned of non-islet tissue under a stereomicroscope. Islets were then cultured overnight under an atmosphere of 5% CO_2 95% air at 37° degrees in fresh culture media to recover from shipping prior to any experiments.

Other materials utilized were as follows: human recombinant activin A, at doses of 1, 5 and 10 nM and human recombinant follistatin at a dose of 5 nM (R&D Systems Minneapolis, MN); RNeasy Plus kits (Qiagen, Germantown, MD); Superscript II and SYBR Green PCR kit (Applied Biosystems, Foster City, CA); RT-PCR reagents (Invitrogen, Carlsbad, CA); guinea pig anti-insulin antibody (Dako, Carpinteria, CA), mouse anti-glucagon antibody (Sigma Chemical, St. Louis, MO), rabbit anti-activin A and B antibodies (W. Vale, Salk Institute, San Diego, CA), anti-guinea pig Trit-C secondary antibody (Jackson Immunoresearch, West Grove, PA), anti-mouse Alexa647 secondary antibody (Life Technologies, Grand Island, NY) anti-rabbit Alexa405 secondary antibody (Life Technologies, Grand Island, NY); Trypsin-EDTA (Sigma Chemical, St. Louis, MO); activin A ELISA (Ansh Laboratories, Webster, TX); dapi (Sigma Chemical, St. Louis, MO); glass bottom dishes for immunostaining and microscopy (Mattek, Ashland, MA); 12 um Millicell Cell Culture Polycarbonate Filter (PCF) (Millipore Corp. Billerica, MA); insulin ELISA (Mercodia, Winston Salem, NC); other common reagents and media (Sigma Chemical, St. Louis, MO).

Human islet culture and treatment

After overnight culture, islets were tested for functional integrity with a static glucose-stimulated insulin secretion

Downloaded by [alan schneyer] at 07:35 04 April 2015

Table 1. Human Islet Donor And Isolation Characteristics

Isolation ID	#Days in Culture	Gender	Age	BMI[a]	Donor Type[b]	Stimulation Index[c]
1	0	F	62	28	Normal	SI > 1
2	6	F	51	32	Normal	SI < 1
3	2	F	47	29	Normal	SI < 1
4	4	M	55	19	Normal	SI < 1
5	7	M	42	32	T2D	SI < 1
6	2	M	57	34	Normal	SI < 1
7	3	F	28	33	Normal	SI > 1
8	2	M	39	33	Normal	SI < 1
9	4	M	41	34	T2D	SI < 1
10	0 and 1	F	43	29	Normal	SI > 1
11	2	M	54	29	Normal	SI > 1
12	5	M	56	35	T2D	SI < 1
13	3	M	37	29	Normal	SI > 1
14	2	M	45	25	Normal	SI > 1
15	3	M	48	36	Normal	SI > 1
16	3	M	20	17	Normal	SI > 1
17	3	M	51	29	Normal	SI > 1
18	2	F	37	32	Normal	SI > 1
19	2	F	47	38	Normal	SI > 1
20	2	M	45	32	Normal	SI > 1
21	2	F	46	40	Normal	SI < 1
22	5	F	62	37	T2D	SI < 1
23	2	M	31	29	Normal	SI > 1
24	2	F	38	23.5	T2D	SI < 1
25	3	M	20	21	Normal	SI > 1
26	3	F	69	35	T2D	SI < 1
27	3	M	60	24.5	Normal	SI > 1
28	4	M	43	24	Normal	SI > 1
29	4	M	30	33	Normal	SI > 1
30	2	M	40	35.4	Normal	SI > 1
31	2	F	32	39.4	Normal	SI > 1
32	3	M	52	36.7	Normal	SI > 1
33	2	M	52	32.2	T2D	SI < 1

[a]BMI = body mass index
[b]Normal = non-diabetic donor; T2D = Type 2 Diabetic Donor
[c]SI = insulin secretion in high glucose/insulin secretion in low glucose; SI > 1 indicates functional islets; SI < 1 indicates non-functional islets.

Downloaded by [alan schneyer] at 07:35 04 April 2015

(GSIS) assay as described previously.[16] Briefly, islets were washed of the culture media and equilibrated in low glucose solution (1.67 mM) for 1 h. Ten size-matched islets (in triplicate samples) were placed in 12 um Millicell Cell Culture Polycarbonate Filter (PCF) inserts in 24 well plates. Fresh, low glucose solution was added for 2 h followed by moving the insert with the islets into a new well with high glucose solution (16.7 mM) for an additional 2 h. Remaining solution in the well was collected and insulin secretion levels were determined by ELISA. Islet shipments were then categorized based on the resulting stimulation index (SI). Stimulation index was calculated by dividing the secreted insulin in high glucose by the secreted insulin in low glucose. A stimulation index of >1 represented functional integrity. A total of 33 islet shipments were received with one of the isolations providing both a freshly, isolated islet sample and a cultured sample. Eighteen of the islet shipments were categorized as cultured, functional islets; 2 as freshly isolated, functional islets; 6 as cultured, non-functional islets and 7 as non-functional islets from Type 2 Diabetic (T2D) donors.

RNA isolation and quantitative RT-PCR

RNA extraction and gene expression assays were performed as described previously.[16] Primer sequences are listed in **Table 2**. Human islets were washed free of culture media and then total RNA was extracted using RNeasy Plus kits according to manufacturer's protocols. RNA was reverse transcribed as previously described.[16] Gene expression was determined by SYBR qPCR as described.[16] For quantitation, a standard was created using pooled RNA from islets and serially diluted. This standard was run with all qPCR assays including the normalization target RPL19. Only assays in which efficiency was 100 +/− 10% were used so they were all parallel. Since all PCR results are calculated in terms of the same standard they can be compared for relative expression levels. Data are presented as mRNA expression normalized to the ribosomal housekeeping gene RPL19 to control for variation in RNA or RT quality. Samples of human islets and the islet depleted fraction (remaining non-islet tissue after completion of digestion, dilution and purification) were also immediately collected after isolation and processed for RNA extraction following the same protocol for comparison to cultured islets.

Table 2. qPCR Primers

Gene Name	Forward Primer	Reverse Primer
INHBA	tcatcacgtttgccgagtc	cacgctccaccactgaca
INHBB	ctctgcctggctcgatgt	aggccttgaagcacgaag
GDF11	gaccaagccgtgtgcaata	caaacccctcaccctcaat
TGF β1	gcagcacgtggagctgta	cagccggttgctgaggta
TGF β2	cacgtgtgcgcttctctg	tttaaagaaggagcggttcg
TGF β3	tcaggtcctggccctttac	ctctccccatgcatctcct
FST	tctgccagttcatggagga	tccttgctcagttcggtctt
FSTL3	ctacatctcctcgtgccaca	tcttctgcagactcaccacct
SMAD2	ttttttcctcaggacctgtcac	agctttcaagaaaaattaggcagat
SMAD3	gtctgcaagatcccaccag	agccctggttgaccgact
INS	gcagcctttgtgaaccaacac	cccgggacactaggtagaga
GCG	agcatttactttgtggctggat	cgcttgtcctcgttcatctgat
GLUT1	ggttgtgccatactcatgacc	cagataggacatccagggtagc
GLUT2	ccctgtctgtatccagctttg	tgtttgctactaacatggctttg
PC1/3	caagagcttgtgaaggacaaga	tctttcagccaagagcacag
PC2	cgtgcaggaccctgagaa	gcgttgaccgtgatgaca
GCK	gacgaaaacctgctcttcca	tcaggatgttgtagatctgcttg
HIF1α	tttttcaagcagtaggaattgga	ttccaagaaagtgatgtagtagctg
CACNA1D	agggtaactcgtccaacagc	tgtcaaatggtttccattcc
CACNA1C	ctccttcaggaaccatattctgtt	gcattgcctaggatcttcagag

Statistical comparisons were made between the following categories of islets: fresh versus cultured (as well as the islet depleted fraction); diabetic vs. non-diabetic; and functional versus non-functional (SI > 1 vs. SI < 1). We also determined the effect of culture on *INHBA* by extracting RNA on consecutive days along a time continuum (fresh/never been cultured, and days 1-6 of culture). Finally, normal, functioning islets (SI > 1) from non-diabetic donors and islets from T2D donors were treated with activin A (1 nM) for 24-72 h and the effects on the expression of several important islet-related genes were measured, as well as, any self-regulatory effects on *INHBA* (primer sequences listed in **Table 2**).

Fluorescence activated cell sorting (FACS)

To determine cell-type specific expression of *INHBA* and *INHBB*, a sub-set of human islets were dispersed into single cells using Trypsin-EDTA solution (450 mL, 37°C, 7–9 minutes), washed then placed in a low glucose sorting buffer (HBSS plus 2.5 mM glucose, 1% BSA, 100 units per ml penicillin G, 100 ug ml^{-1} streptomycin-sulfate and 25 mM HEPES, pH 7.4) and filtered through 0.2 micron mesh screen. The dispersed cells were then sorted into primary β-cells and α-cells by FACS (BD FACSAria, BD Biosciences, San Jose, CA) using auto-fluorescence induced by low glucose solution and gating strategies as previously described.[21] Cells were then processed for RNA isolation and RT-PCR as described above, as well as, immunostaining for cellular location of the activin A and activin B proteins.

Immunostaining in primary α- and β-cells

Primary α- and β-cells were obtained following the FACS procedure above and then allowed to attach to glass bottom dishes. The single cells were then fixed for 30 minutes in 4% paraformaldehyde, permeabilized for 30 minutes with Triton X-100 (0.5% Triton X-100 in PBS), washed with a combination of 0.5% normal donkey and goat serum followed by blocking with a combination of 5% donkey and goat serum for one hour. The cells were then incubated overnight at 4°C with the primary antibodies (1:100; guinea pig anti-insulin, mouse anti-glucagon, rabbit anti-activin A, or rabbit anti-activin B). The cells were then washed with PBS (2 × 5 min) and incubated with the secondary antibodies for one hour (1:200; anti-guinea pig Trit-C for insulin; anti-mouse Alexa647 for glucagon; anti-rabbit Alexa405 for activins A or B) and then washed with PBS (2 × 5 min). The stained cells were then covered with Vecta shield mounting medium for fluorescence. Fluorescence was recorded using a Nikon TE-2000-U (X-Cite Series 120, Avon, MA) and images were captured using Metavue Software (Molecular Devices, Sunnyvale, CA).

Glucose-stimulated insulin secretion assay

To obtain the effects of activin A on secretory capacity, the islets were tested by performing GSIS as described previously.[16] Briefly, after overnight culture, islets were washed of the culture media and equilibrated in low glucose solution (1.67 mM) for 1 h. Ten size-matched islets (in triplicate samples) were placed in 12 um Millicell Cell Culture Polycarbonate Filter (PCF) inserts in 24 well plates. An acute GSIS model and a chronic GSIS model were utilized in regards to length of treatment. The acute GSIS does not involve any pre-treatment with ligand but does have ligand in the glucose solution of the actual GSIS which should reveal any post-transcript effects. Fresh, low glucose solution with or without ligand treatment (0, 1, 5, 10 nM activin A or 5 nM activin A combined with 5 nM fst) was added for 2 h followed by moving the insert with the islets into a new well with high glucose solution (16.7 mM) with or without ligand treatments (0, 1, 5, 10 nM activin A or 5 nM activin A combined with 5 nM fst) for an additional 2 h. Remaining solution in the well was collected and insulin secretion levels were determined by ELISA. The stimulation index as described above was calculated for each treatment group. The chronic GSIS model involves a 24-48 hour pre-treatment with ligand allowing for ample time for alterations at the transcript level but does not include any treatment in the actual GSIS. Islets for the chronic GSIS were treated with 0, 5 nM activin A, a combination of 5 nM activin A plus 5 nM FST or just 5 nM FST (to block endogenous activin A) for 24-48 hours and then underwent GSIS as described above.

Activin secretion assay

To determine if secretion patterns of activin A are glucose responsive, levels of activin A in low and high glucose solutions were measured. After overnight culture, islets were washed of the culture media and equilibrated in low glucose solution (1.67 mM) for 1 h. Ten size-matched islets (in triplicate samples) were placed in 12 um Millicell Cell Culture Polycarbonate Filter (PCF) inserts in 24 well plates. Fresh, low glucose solution was added for 2 h followed by moving the insert with the islets into a new well with high glucose solution (16.7 mM) for an additional 2 h. Remaining solution in the well was collected and activin A secretion levels were determined by ELISA.

Downloaded by [alan schneyer] at 07:35 04 April 2015

Viability assay

To determine if the up-regulation of activin A in cultured islets is associated with survival, the viability of the islets was measured using a live/dead fluorescent dye method (PI/FDA). The suggestions provided by Boyd et al.[22] on decreasing the limitations of viability assays based on membrane integrity were followed. Islets that had been cultured for at least 4 days were treated with activin (or left untreated as control) for 48 hours, dispersed and then a live/dead assay performed.

Statistical analysis

Data are presented as means ± SEM. Differences between means were evaluated by One Way Analysis of Variance (ANOVA) with Tukey post-hoc adjustment for multiple comparisons, or Student T Test when appropriate using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A P value of ≤ 0.05 was considered significant.

Results

Human islet donor and isolation characteristics

Table 1 illustrates the donor and isolation characteristics. A total of 33 separate islet isolations were included in the study. One of the isolations provided both freshly isolated and cultured islets (isolation ID#10), one provided just freshly isolated islets (isolation ID#1) and the remainder provided only cultured islets. The average length of time in culture for all isolations combined was 2.9 ± 1.4 d. Overall, there were 12 female and 21 male donors with an average age of 45.2 ± 11.8 years and an average BMI of 30.9 ± 5.6. There were 26 isolations from normal (non-diabetic) donors and 7 isolations from T2D donors. The 26 isolations from the non-diabetic donors were further classified based on the results of the post-arrival GSIS revealing 20 as functional (SI > 1) and 6 as non-functional (SI < 1). Results of the post-arrival GSIS also revealed that all 7 of the isolations from T2D donors had a SI < 1. Further, the normal, functioning islets (SI > 1) had an average length of time in culture of 2.7 ± 0.7 d, the non-functioning (SI < 1) of 3 ± 1.6 days and the islets from T2D donors 4 ± 1.8 days. Statistical analysis of the length of time in culture between groups revealed no statistical differences between the groups. There were no statistically significant differences found for donor gender, age or BMI between groups (normal, functioning, SI > 1; 6 females/14 males, age 40.2 ± 11.2 years, BMI 30.1 ± 5.9), (normal, non-functioning, SI < 1; 3 females/3 males, age 49.2 ± 6.6 years, BMI 31.2 ± 6.9) (T2D, 3 females/4 males, age 51.4 ± 11.7 years, BMI 32.7 ± 4.4). Additional donor information of interest includes an average cold ischemia time of 550.27 minutes ± 296.31 for islets from non-diabetic donors and 641 minutes ± 313.4 for islets from diabetic donors (no statistical significance between the 2 groups). The main causes of death for all donors was cerebrovascular accident/stroke, head trauma, motor vehicle accident and gunshot wound. Specific information of interest regarding the T2D donors include an average HgbA1C of 9.7 ± 3 with an average duration of diabetes of 5.9 years ± 6.4 (ranged from 0.5-20 years).

Expression of TGFβ superfamily members in human islets

Previously, we reported that rodent islets (rat and mouse) both express a wide spectrum of the TGFβ superfamily members. The level of expression for certain members differs suggesting potential species-specific roles.[16] To establish the role of the TGFβ superfamily in human islets, we performed a systematic qPCR analysis of these superfamily members in normal, cultured, functioning islets (SI > 1) and confirmed the protein expression by cellular location for activin A, activin B and FSTL3, an inhibitor of the activins. We determined mRNA expression levels by qPCR in normal, functioning (SI > 1) human islets. Since results for all targets were expressed relative to the same internal standard run in each PCR experiment, it is appropriate to compare the relative expression levels among the TGFβ superfamily members. The most prominently expressed member of the TGFβ superfamily in cultured, functional islets was *INHBA*, the gene encoding activin A which was expressed at a level at least 5 times greater than any other family member (**Fig. 1A**) and was islet-specific (not detected in the islet depleted fraction; data not shown). The expression levels of *INHBA* peak within 2 days of culture and gradually fall to ~8% by day 6 (data not shown). To confirm the protein expression and the cellular location, we used sorted primary human α- and β-cells from a subset of the islet shipments. The sorting procedure was adapted from Kohler, et al[21] and produced highly enriched populations of α- and β-cells based on qPCR of recovered cells (**Fig. 2A-C**) and verified by immunostaining (**Fig. 3**). Immunostaining for insulin and glucagon revealed 95% insulin positive cells in the β-cell population and 93.75% glucagon positive cells in the α-cell population (**Fig. 3**). We detected *INHBA* in both α- and β-cells whereas *INHBB* and *FSTL3* were primarily in α-cells, both distinct from rodent islets[9] (**Fig. 2D**). We then confirmed the protein expression by cellular location for the activins. The sorted α- and β-cells were stained for glucagon or insulin, respectively, as well as, for activin A and B and the individual and merged/overlay images are presented in **Figure 4**. Immunostaining confirms the activin A protein is expressed in both α- and β-cells while activin B protein is expressed mainly in α-cells. Identical immunostaining in cells from T2D donors for activin A and B was undetectable. These findings suggest the likelihood that there is a functional role in islet cells for these TGFβ superfamily members.

Gene expression patterns for TGFβ superfamily differ based on diabetic, culture, and functional status

After establishing the TGFβ superfamily expression patterns in normal, cultured, functioning islets (SI > 1), we were then interested to see if there were any variations in the expression pattern based on the condition and functional integrity of the islets. When compared to cultured islets, *INHBA* and *FST* expression in freshly isolated (i.e., never cultured) islets was significantly lower *by ~80%* and *INHBB*, *TGFβ2, TGFβ3*

Downloaded by [alan schneyer] at 07:35 04 April 2015

Downloaded by [alan schneyer] at 07:35 04 April 2015

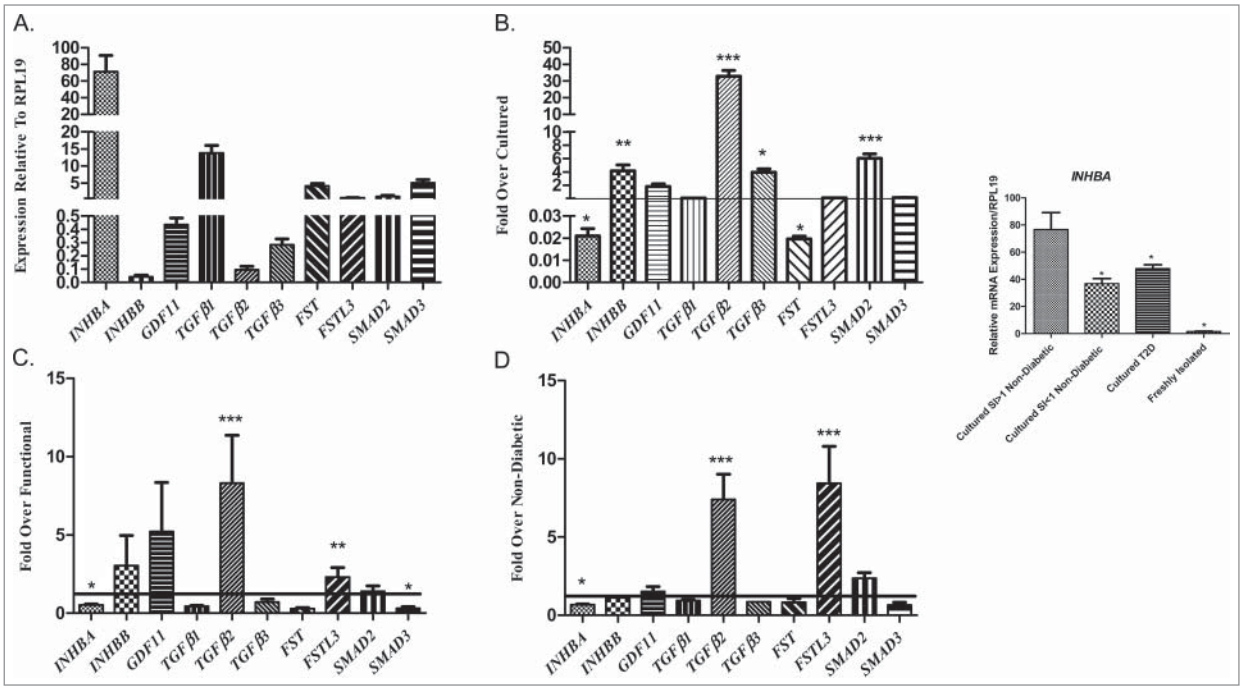

Figure 1. Overall gene expression of the TGFβ superfamily members in human islets. Expression of a broad range of TGFβ superfamily members was determined by qPCR. All qPCRs were performed with the same standard to allow comparison between targets. The line in B-D represent the level of expression in the comparative control. (**A**) Expression of TGFβ superfamily members in functional, cultured islets (N = 20). Results are expressed relative to RPL19 for normalization, all of which are interpolated from a standard curve produced from pooled islet RNA. The most prominently expressed member of the TGFβ superfamily was *INHBA*, the gene encoding activin A which was expressed at a level at least 5 times greater than any other family member and was islet-specific (not detected in the islet depleted fraction). (**B**) Expression of TGFβ superfamily members in freshly, isolated islets (N = 2). Results expressed as fold-over cultured islets for comparison. When compared to cultured islets, *INHBA* and *FST* expression in freshly isolated islets are significantly reduced *by ~80%* and *INHBB*, *TGFβ2, TGFβ3* and *SMAD2* expression is significantly increased by ~4, 33, 4 and 6 fold, respectively. (**C**) Expression of TGFβ superfamily members in cultured, non-functional islets (N = 6). Results expressed as fold-over functional islets for comparison. *INHBA* and *SMAD3* expression is significantly reduced by ~50% and 70%, respectively in non-functional islets and *TGFβ2* and *FSTL3* expression is increased by ~8 and 2 fold, respectively when compared to functional. (**D**) Expression of TGFβ superfamily members in islets from T2D donors (N = 6). Results are expressed as fold-over functional, non-diabetic for comparison. Islets from T2D donors have significantly reduced expression of *INHBA by ~30%* and significantly increased expression of *TGFβ2* and *FSTL3* by ~7.5 fold and 8.5 fold, respectively when compared to non-diabetic. *Inset:* graph separating out levels of expression of INHBA for ease of comparison between groups. Statistical analysis was performed with ANOVA with Tukey post-hoc adjustment for multiple comparisons. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

and *SMAD2* expression is significantly higher by ~4, 33, 4 and 6 fold, respectively (**Fig. 1B**). *INHBA* and *SMAD3* expression was significantly reduced by ~50% and 70%, respectively in non-functional islets (SI < 1) and *TGFβ2* and *FSTL3* expression was elevated by ~8 and 2 fold, respectively when compared to cultured functional islets (**Fig. 1C**). Islets from T2D donors had significantly reduced expression of *INHBA by ~30%* and also have significantly increased expression of *TGFβ2* and *FSTL3* by ~7.5 fold and 8.5 fold, respectively when compared to functional non-diabetic islets (**Fig. 1D**). These results reveal differences in the gene expression patterns in correlation with the condition of the islets and their functional integrity. The inset in **Figure 1** provides a view of just the expression level for *INHBA* for ease of comparison between groups. These results identify similarities between non-functional islets (SI < 1) and islets from T2D donors which also have limited functionality strengthening the likelihood of a role for this TGFβ superfamily in the function of human islets.

Effect of activin A on insulin secretion in normal, functioning human islets

We previously showed that treatment of mouse islets with exogenous activin A resulted in a decrease in insulin secretion while an increased secretion was found in rat islets.[16] This suggests the possibility of differing roles for this protein based on species. Further, based on our finding that the most prominently expressed TGFβ superfamily member in cultured, functional islets (SI > 1) is *INHBA*, we tested whether or not activin A modulates β-cell function using a GSIS assay. When functional islets from non-diabetic donors were treated with activin A acutely (treatment was in the glucose solution), the functionality was maintained with a stimulation index of >1 (SI = insulin secretion in high glucose/insulin secretion in low glucose); however, there was an overall decrease in insulin secretion when stimulated with elevated glucose concentration (**Fig. 5A**; $P \leq 0.05$, untreated control vs 10 nM activin A). The combination of the inhibitor FST with activin A in the glucose solution did not have the reverse effect (**Fig. 5A**). With the demonstrated high

Downloaded by [alan schneyer] at 07:35 04 April 2015

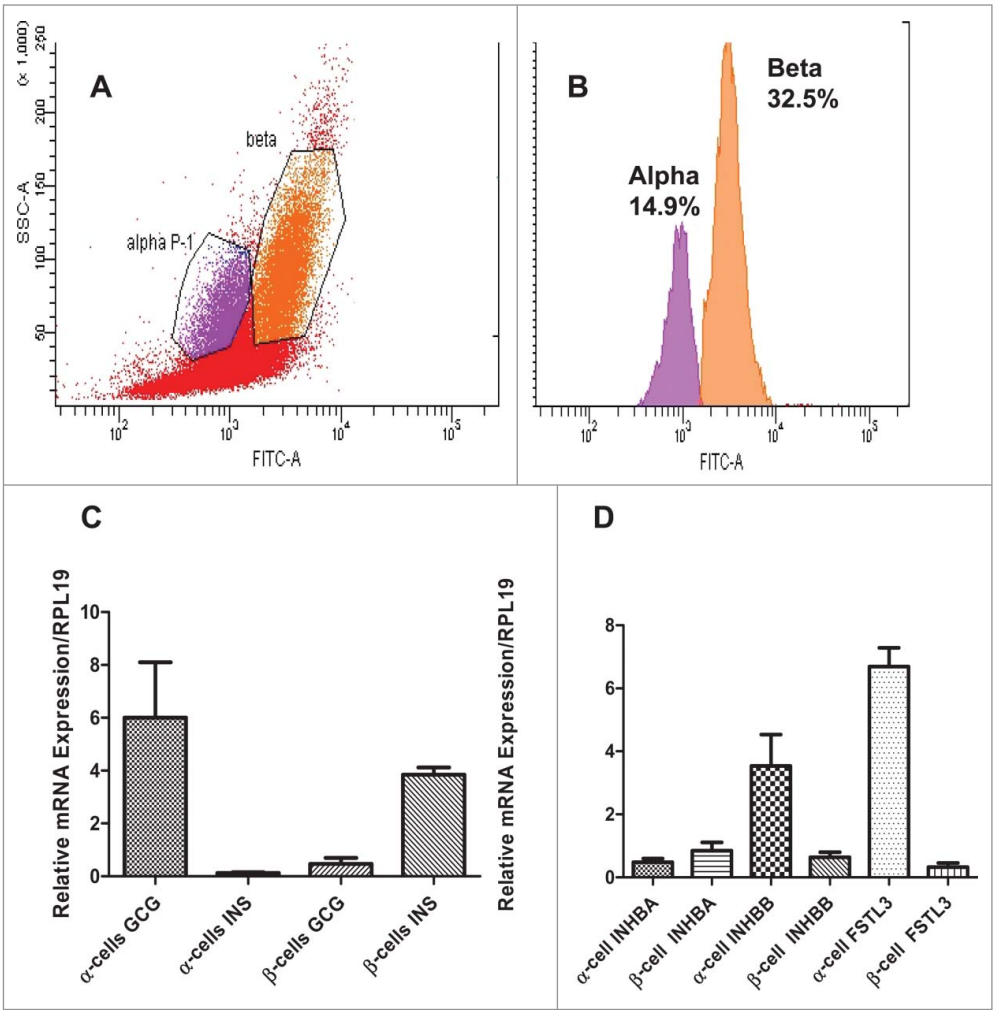

Figure 2. Cell sorting of dispersed human islet cells (N = 4). After sorting using the same parameters reported in Kohler et al,[21] the recovered α- and β-cell fractions (**A and B**) were similar to Kohler et al,[21] with a high purity and viability and low overall yield but sufficient for PCR (C) and ICC analysis (**Fig. 3**). PCR (C) results show α- and β-cell gene expression for *GCG* and *INS*. D) Note that *INHBA* (gene encoding activin A) was detected in both cell populations while *INHBB* (gene encoding activin B) and *FSTL3* appeared to be predominately in α-cells based on PCR.

endogenous activin A expression in human islets we speculated there is potential that any effect leading to an increased insulin secretion may be masked; therefore, we blocked endogenous activin A with the inhibitor FST for 24-48 hours. Following inhibition with FST we then treated the islets with 5 nM activin A while undergoing a GSIS (activin treatment was in the glucose solution; **Fig. 5A**). This unmasked the effects and the added exogenous activin A resulted in an increase in insulin secretion equivalent to the untreated controls. These results demonstrate a role for activin A in islet functionality, but only after inhibition of endogenous activin by pre-treatment with the FST inhibitor.

Treatment of islets from T2D donors with activin A restored GSIS

Data from the gene expression studies above revealed that islets from T2D donors have significantly reduced expression of INHBA (30% reduction). The islets from T2D donors were also found to be non-functional with an SI < 1 when tested for functional integrity upon arrival. After demonstrating a stimulatory effect of activin A on insulin secretion only after blocking the endogenous protein in normal, functioning islets (SI > 1), we speculated that adding exogenous activin A to islets from T2D donors (which express low levels of endogenous activin) would show an effect on insulin secretion. Untreated islets from T2D donors remained dysfunctional with an average SI ≤ 1. Interestingly, when islets from T2D donors were treated with activin A acutely (added to the glucose solution during GSIS; **Fig. 5A**) or treated with activin A chronically (for 24 hours prior to GSIS only with no treatment added to the glucose solution; **Fig. 5B**), glucose responsiveness and insulin secretion were restored (SI > 1). Doses of 1, 5 and 10 nM activin A all significantly stimulated insulin secretion in high glucose. We were able to reverse this restorative effect of activin A with the inhibitor, FST (**Fig. 5A and B**). These findings reveal a restorative effect of activin A on stimulated insulin secretion in islets from T2D donors.

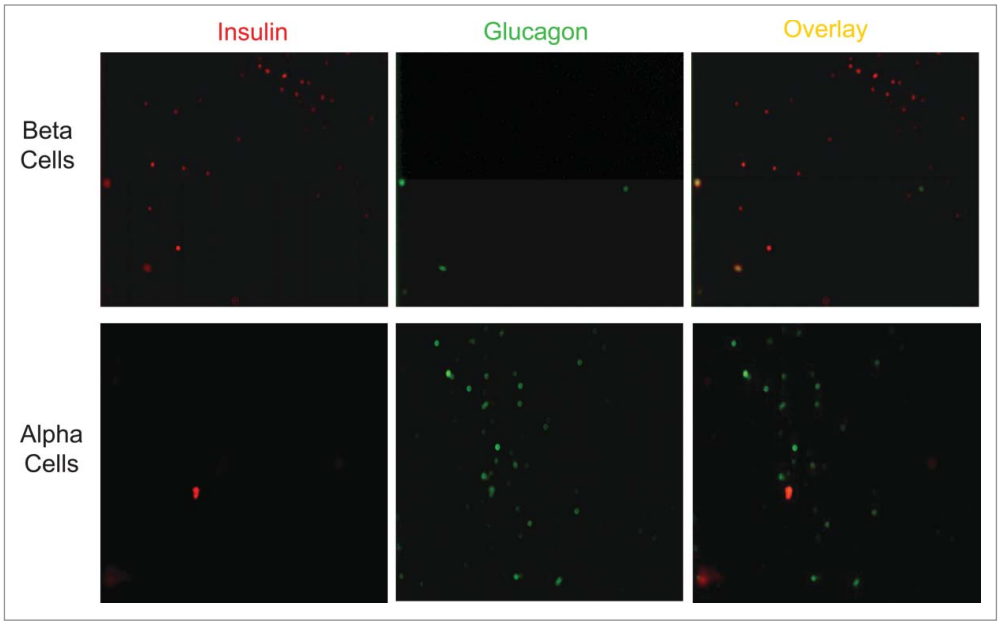

Figure 3. Immunostaining of sorted β- and α-cell populations to determine purity. Upper row represents β-cells. Bottom row represents α-cells. Immunostaining for insulin and glucagon reveal 95% insulin positive cells in the β-cell population and 93. 75% glucagon positive staining in the α-cell population. Note the presence of a few bihormonal cells in the β-cell population.

Downloaded by [alan schneyer] at 07:35 04 April 2015

Secretion patterns of activin A are glucose-responsive and differ based on functional and diabetic status

Activin A is a secretory protein and our data reveals the presence of activin A expression in both α- and β-cells. Combined with the effects of activin on the insulin secretory capacity of the β-cell, we next investigated whether the secretion patterns of activin A were glucose-responsive. **Figure 5C** illustrates that when functional islets were exposed to high glucose solution the secretion of activin A protein significantly increased compared to low glucose (high glucose/low glucose ratio = 2.4 ± 1.2). This secretion can be reversed by the addition of the inhibitor, FST. This ratio is significantly lower in islets that are non-functional (ratio = 0.97 ± 0.26; $P \leq 0.05$) and from T2D donors (ratio = 0.88 ± 0.22; $P \leq 0.05$) due to the lack of increased activin A secretion in response to elevated glucose. These findings illustrate the secretory patterns of activin A found in normal, functioning islets (SI > 1) are glucose-responsive. This normal secretory pattern is altered in non-functional islets and islets from T2D donors.

Activin A improves viability of cultured human islets

Human islets were cultured for at least 4 days and then activin A treatment was added for 48 hours followed by a viability assay (PI/FDA). The time point of 4 days in culture was selected based on the evidence that loss of islets increases as the length in culture increases. The optimal cultural length for viability of human islets prior to transplantation is 2-3 days.[23] Further impacting the decision for at least 4 days in culture was our finding that the endogenous levels of activin A peak at day 2 in culture and then decreases on subsequent days; therefore, most likely avoiding saturation effects. Results show a statistically significant increase in viability in the activin treated group when compared to the untreated control. (60.3% viability in control vs 74.2% in activin treated group; p = 0.03) (**Fig. 6**).

Activin A regulates expression of islet-related genes

In an attempt to understand the mechanism behind the ability of activin A to restore functionality to islets from T2D donors, several genes were investigated by qPCR and results compared between normal, functioning islets (SI > 1) and T2D (results displayed in **Fig. 7** and primers listed in **Table 2**). Several genes were found to be significantly reduced ($P \leq 0.05$) in untreated islets from T2D donors when compared to normal, functioning islets (SI > 1) including *INS (by 65%), GCK (by 57%), GLUT2 (by 92%), PC1/3 (by 74%)* and *INHBA (by 32%)* while *GCG* (3.2 fold), *GLUT1* (2.5 fold), *CACNA1C* (2.9 fold) and *HIF1*α (6 fold) were significantly increased ($P < 0.05$). *CACNA1D* was increased in untreated islets from T2D donors when compared to normal, functioning islets (SI > 1) but did not reach significance. In normal, functioning islets (SI > 1) treatment with activin A significantly increased *GCG* (2.4 fold) and *INHBA* (2.6 fold) ($P \leq 0.05$) and reduced the expression of *GLUT2* and *PC2* but neither reached significance. Further, activin A had no effect on *INS, GCK, GLUT1, PC1/3, CACNA1D, CACNA1C* or *HIF1*α in normal, functioning islets (SI > 1). Activin A significantly increased the levels of *INHBA* (2 fold) in the islets from T2D donors, as well as, *CACNA1D* (4.2 fold), *CACNA1C* (1.6 fold), *HIF1*α (1.5 fold) and *INS* (2 fold) ($P \leq 0.05$); however, the overall levels of *INS* gene expression were still significantly lower than those found in normal, functioning islets (SI > 1). Further, activin A decreased the expression of *GCG* in islets from T2D donors but did not quite reach significance (p = 0.055). Several of these gene changes stimulated by activin A treatment such as *INHBA, INS, GCG, CACNA1D* and CACNA1C may help explain the restorative effect of activin A on insulin secretion in the chronic GSIS when ample time was allowed for transcriptional changes; however, this does not explain the acute effects when activin exposure is limited to just 2 hours in the low and high glucose solutions. These results demonstrate a role of activin A in modulating several key islet functional (*INS, GCG, GLUT2, CACNA1C* and *CACNA1D*) and survival genes (*HIF1*α), as well as, self-regulating the expression of *INHBA*.

Discussion

The emerging role of activin and the TGFβ superfamily in β-cell regulation is of growing interest in the diabetes field. The structurally related ligands that comprise this superfamily have been found to be expressed in islets along with their associated second messengers and receptors and roles in cell differentiation have been defined.[1,6,7,24-30] Since both activins A and B are expressed in islet cells this is suggestive of a role in islet function and glucose homeostasis.

Regulation of the activity of activins A and B, is achieved through the soluble antagonists FST and FSTL3.[13,14] We previously reported that *Fstl3* knockout mice have larger islets, β-cell hyperplasia, enhanced glucose tolerance and improved insulin sensitivity. This suggests. that endogenous TGFβ superfamily ligands regulated by FSTL3 are important modulators of β-cell number and function in mice.[15] Thus it was critical to determine whether these observations in mice could be extended to human islets. We report that the most prominently expressed member of the TGFβ superfamily in cultured, functional human islets was *INHBA*. This was similar to our findings in rat islets but differed from mouse islets which expressed other TGFB superfamily members such as myostatin at a higher level. In human islets, the *INHBA* transcript along with its corresponding protein expression was detected in both α- and β-cells whereas *INHBB* and *FSTL3* were primarily in α-cells. This finding differed from our results in rodent islets which revealed α-cells are the primary location for both activin A and B.[16] This finding also differed from previous studies in human islets in which activin A was localized mainly to β-cells.[6] These differing findings may be accounted for by methodology. In the current study, cellular location was identified by utilizing FACS sorted purified cells while the previous studies,[6,16] including our rodent study, utilized pancreas sections.

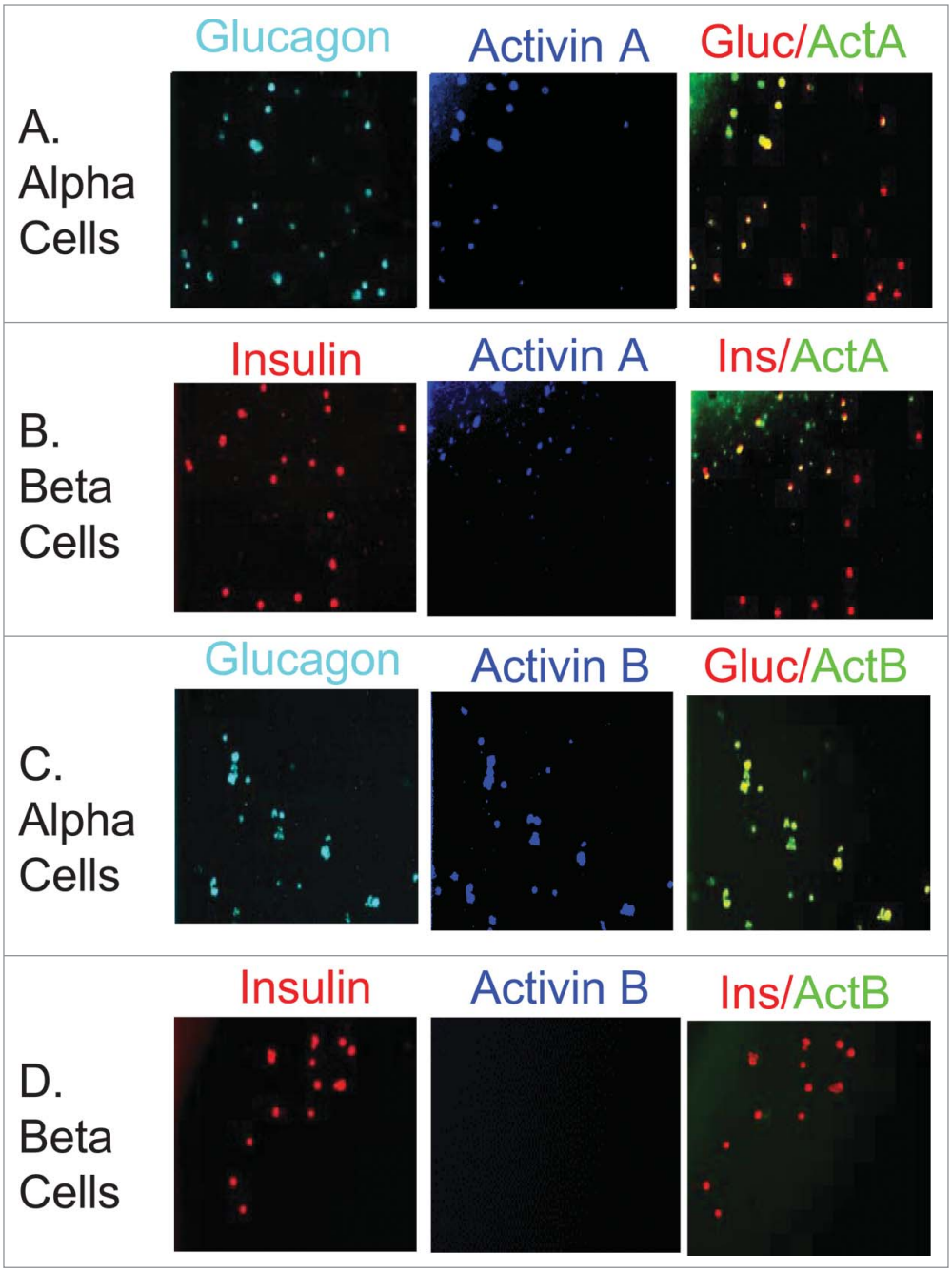

Figure 4. Activin immunostaining of human α- and β-cells (N = 4). Activin A is detectable in both α- and β-cells (overlays in row **A and B**) but activin B is only expressed in α -cells (overlays in row **C and D**; row C reveals the majority of α-cells are also activin B+ and therefore, the red color for glucagon does not show through on the overlay) which confirms the PCR results in **Figure 2**. Magnification 20x.

Activin A has been found to be a secretory protein with paracrine and autocrine effects on islet cells.[3] We can confirm these findings with the additional information that the secretion of activin A appears to be glucose responsive (increased secretion in high glucose). This secretion can be blocked by the addition of

Downloaded by [alan schneyer] at 07:35 04 April 2015

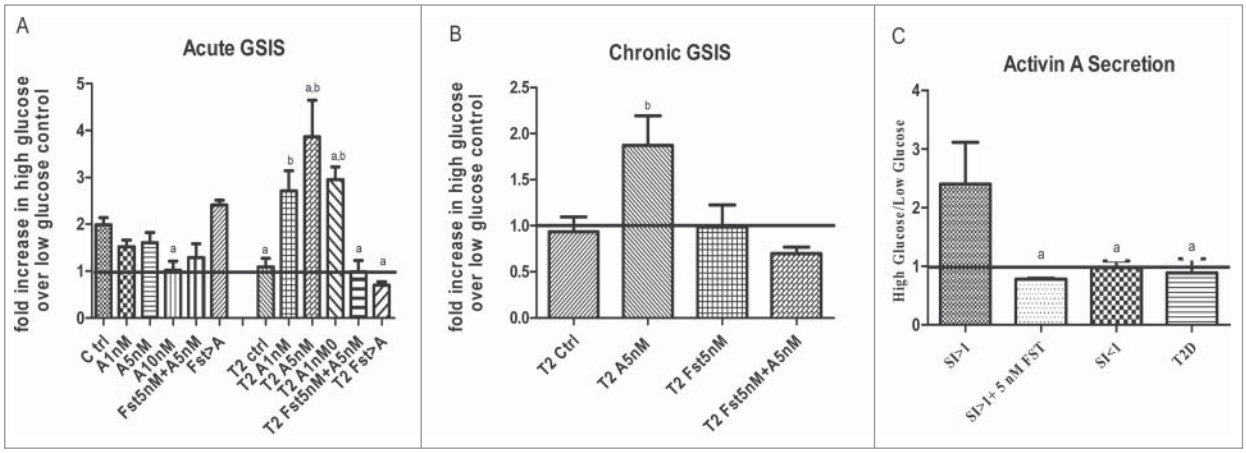

Figure 5. Activin A modulates function in islets from T2D donors and secretion of activin A protein is glucose-responsive. The line represents the level of insulin secretion by functional, non-diabetic islets in low glucose in A and B and the line in C represents a ratio of 1. (**A**) Acute GSIS: in the presence or absence of activin A at low and high glucose for 2 h. Results are presented as fold increase in high glucose over low glucose control. Functional, non-diabetic controls (left side of graph A; N = 20): All doses of activin A reduced insulin secretion but only 10 nM activin A reached significance. There was no effect of adding the inhibitor follistatin at the same time as activin A (fst5 nM + A5 nM) but when pre-treated with follistatin (fst > A) prior to the addition of activin A an increase in insulin secretion occurred. Diabetic: (right side of graph A; N = 6): Untreated islets from T2D donors on average had an SI $\leq$ 1 and were significantly different from the functional, non-diabetic controls. Doses of 1, 5 and 10 nM activin A all significantly stimulated insulin secretion in high glucose resulting in an SI > 1. This effect was reversed by both the addition of the inhibitor follistatin at the same time as activin A (fst5 nM + A5 nM), as well as, by pre-treatment with follistatin prior to the addition of activin A (fst > A) resulting in an SI $\leq$ 1. (**B**) Chronic GSIS: performed after 24 h pre-treatment +/- activin A without any activin A in the glucose solutions during the GSIS. Results are presented as fold increase in high glucose over low glucose control. Untreated islets from T2D donors had an SI $\leq$ 1. The 5 nM dose of activin A significantly stimulated insulin secretion in high glucose restoring the SI > 1. The restorative effect of activin A was reversed by the addition of the inhibitor, follistatin alone (T2 Fst5 nM), as well as, when added in combination with activin A (T2 Fst5 nM + A5 nM) yielding an SI $\leq$ 1. (**C**) Activin A protein secretion. Results expressed as a ratio of activin A secretion in high glucose/low glucose. When functional, non-diabetic islets (SI > 1; N = 20) were exposed to high glucose solution for 2 h the secretion of activin A protein significantly increased compared to low glucose (ratio = 2.4 $\pm$ 1.2) and this secretion can be reversed by the addition of FST. This ratio pattern is significantly lower in islets that are non-functional and non-diabetic (SI < 1; N = 6; ratio = 0.97 $\pm$ 0.26) and from T2D donors (N = 6; ratio = 0.88 $\pm$ 0.22) due to the lack of increased activin A secretion in response to elevated glucose. Statistical analysis was performed with ANOVA with Tukey post-hoc adjustment for multiple comparisons with a significance level set at $P \leq 0.05$. [a] significant vs non-diabetic, untreated control; [b] treated islets from T2D donors significantly different vs untreated T2D controls.

Downloaded by [alan schneyer] at 07:35 04 April 2015

the inhibitor FST. Further, activin A modulates several key islet functional and survival genes, as well as, self-regulates. Szabet et al[3] found that activin A negatively regulates the maturity state of the adult B-cell and found significant decreases in the transcript levels of *INS* and *GLUT2* in mouse and human islets. While we found a slight decrease in both of these transcripts the difference did not reach statistical significance. This may be in part due to the characteristics of the human islets used such as donor variables and length of time in culture. Further, we find that the addition of exogenous activin A results in an increase in insulin secretion in islets from non-diabetic donors; however, only after inhibition of endogenous activin by pre-treatment with the FST inhibiton. Activin also increases islet viability in culture. Of potential clinical significance is our finding that activin A has a restorative effect on stimulated insulin secretion in islets from T2D donors.

There has been relative agreement as to the ability of activin A to increase GSIS in rat islets[4,5,16-18] but studies performed in mice have found contradicting results. Some studies have found activin lacks a stimulatory effect in mouse islets.[3,16] Another has found opposing actions of activin A and activin B with activin A stimulating insulin secretion in mice and activin B inhibiting.[19] Previous studies demonstrated that exogenous activin A can increase the cytoplasmic free Ca^{+2} concentration[18] and stimulate insulin secretion in human and rat pancreatic islets, but information in human islets is still limited. Florio et al.[20] reported that activin A increased GSIS in human islets and cited activation of calcium voltage channels as a potential mechanism. Florio et al,[20] used a longer culture period of 7-10 d prior to determining a stimulatory effect of exogenous activin which may explain the contradiction with our results. By 7-10 d of culture, we found that *INHBA* expression falls to ~8% of peak levels. This suggests that stimulatory effects of exogenous activin A are masked when the endogenous levels are already elevated. Any stimulatory effects of activin are unmasked when these endogenous levels fall whether naturally by increased culture length or by blocking endogenous production with the inhibitor FST as seen in our current results.

This theory of a dynamic regulatory system based on the microenvironment is further supported by our finding that there are opposing effects of exogenous activin A on functional islets from non-diabetic donors and islets from diabetic donors. The former inherently have a very high level of endogenous *INHBA* and secrete activin A in response to high glucose. The latter have a significantly lower level of endogenous *INHBA* and no increase in activin A in response to high glucose. Inhibition of K_{ATP}

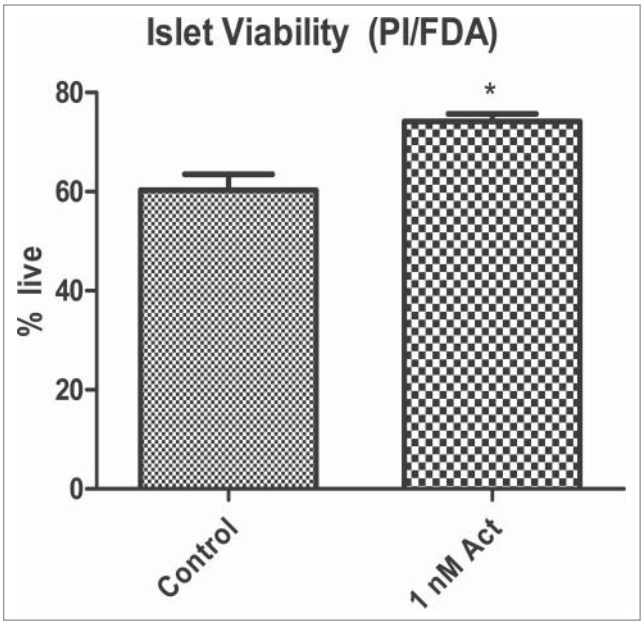

Figure 6. Activin A improves viability of cultured human islets. Functional, non-diabetic (N = 4) human islets were cultured for at least 4 days and then activin A treatment was added for 48 hours followed by a viability assay (PI/FDA). Results show a statistically significant increase in viability in the activin treated group when compared to the untreated control. (60.3% viability in control vs 74.2% in activin treated group; *p = 0.03).

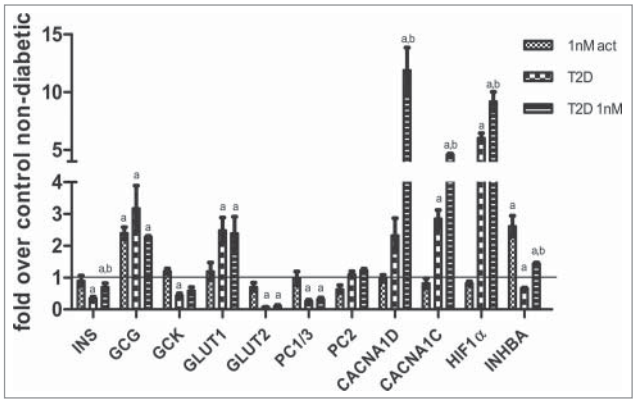

Figure 7. Activin A regulates expression of islet related genes. Expression of a broad range of islet related genes was determined by qPCR. All qPCRs were performed with the same standard to allow comparison between targets. The line represents the level of expression in the untreated, functional, non-diabetic control. In normal, functioning islets (SI > 1), treatment with activin A for 24-72 h significantly increased *GCG* (2.4 fold) and *INHBA* (2.6 fold) and reduced the expression of *GLUT2* and *PC2* but neither reached significance. Several genes were found to be significantly reduced in untreated islets from T2D donors (N = 6) when compared to normal, functioning islets (SI > 1) including *INS (by 65%), GCK (by 57%), GLUT2 (by 92%), PC1/3 (by 74%)* and *INHBA (by 32%)* while *GCG* (3.2 fold), *GLUT1* (2.5 fold), *CACNA1C* (2.9 fold) and *HIF1α* (6 fold) were significantly increased. Activin A significantly increased the levels of *INHBA* (2 fold) in the islets from T2D donors, as well as, *CACNA1D* (4.2 fold), *CACNA1C* (1.6 fold), *HIF1α* (1.5 fold) and *INS* (2 fold). Statistical analysis was performed with ANOVA with Tukey post-hoc adjustment for multiple comparisons with a significance level set at $P \leq 0.05$. [a] significant vs non-diabetic, untreated control; [b] treated islets from T2D donors significantly different vs untreated T2D controls.

Downloaded by [alan schneyer] at 07:35 04 April 2015

channels followed by activation of calcium channels are required for insulin secretion to occur and this process is glucose sensitive. The islets from T2D donors were found to be insensitive to glucose most likely due to the significantly reduced expression levels of *GLUT2* which is required for glucose sensing.[26] This decrease in *GLUT2* is often an early indicator of B-cell stress;[31] therefore, the islets from the T2D donors do not exhibit the subsequent increase in insulin secretion when exposed to high glucose. Mogami et al.[32] have shown in HIT-T15 cells that activin A can directly inhibit the activity of the K_{ATP} channel and modulate the voltage dependent calcium channel which subsequently leads to exocytosis of insulin-containing granules. Further, Nomura et al[2] have linked the activin second messenger, SMAD2, to modulation of ion channel activity required for insulin secretion in mouse islets. Our current results are in line with these findings and revealed that activin significantly increases the expression of both calcium channels studied (*CACNA1D* and *CACNA1C*). *CACNA1D* and *CACNA1C* code for the L-type calcium channels $Ca_v1.3$ and $Ca_v1.2$ respectively, and have both been found previously in human islets.[33] These L-type calcium channels have been found to regulate insulin release and polymorphisms of *CACNA1D* have been associated with T2D.[33]

Considering that both non-functioning islets and islets from T2D donors were found to have significantly increased expression levels of *FSTL3* in addition to the significantly decreased *INHBA*, there is a strong possibility that interaction between these proteins is important. For instance, *FSTL3* is an inhibitor of activin and the addition of exogenous activin A may compensate for the inhibition and lead to restoration of an intraislet regulatory loop controlling functionality. Taken together, these findings may elucidate the mechanism whereby activin stimulates insulin secretion in glucose-insensitive islets from T2D donors that express low endogenous levels of *INHBA* and lack glucose-stimulated activin A secretion.

It is likely that activin A acts in multiple capacities to maintain normal physiology within an islet. Islets in culture have undergone mechanical and enzymatic stress from the isolation procedure. These islets are further exposed to stress while under a hypoxic environment during culture. The isolation procedure and hypoxia are known to stimulate the stress-response and pro-survival mechanisms. These mechanisms include a switch to anaerobic metabolism and down-regulation of insulin in the basal state to conserve energy.[34] Andersen et al[35] were able to directly link activin A to the stress response when the harmful effects of pro-inflammatory cytokines in cultured islets were abrogated by the addition of exogenous activin A. We uncovered interesting differences in the gene expression patterns of the TGFβ superfamily in correlation with the condition of the islets and their functional integrity which may lend support to this role in stress-response. Similarities, such as significantly reduced levels of endogenous activin A, were found to exist between

non-functional islets and islets from T2D donors. The significantly elevated levels of *INHBA* gene expression in cultured, functional human islets, as well as, the ability of activin to enhance viability further link this hormone to the stress-response. It is also worth mentioning the longer length of culture time for the islets from the T2D donors. Given our current finding that activin A enhances viability and cell death increases with extended culture time, this may help to explain the effect on islets from T2D donors. Diminished levels of *INHBA* along with elevated *FSTL3* such as that found in islets from T2D donors and non-functional islets might suggest a defect in the pro-survival mechanisms. This defect may subsequently alter functional capacity that can be restored with exogenous activin A. By exposing islets from T2D donors to activin A, the expression of *HIF1a* was increased significantly indicating a possible alteration of the stress response pathway. HIF1α is an important transcription factor regulating cellular responses to hypoxia and stress. Diminished levels of HIF1α in human islets have been found to result in inadequate insulin secretion[36] and potentiate cell death.[37] Cheng et al.[36] have shown that by increasing HIF1α levels in islets from T2D donors, glucose stimulated insulin secretion was restored. Activin A may counteract the acute and chronic alterations that occur within an inflammatory environment. These alterations may include those associated with the development of T2D in humans, as well as, from the damaging inflammatory and hypoxic effects of islet isolation and culture.

However, our observation that the pattern of TGFβ superfamily mRNA is drastically different when cultured islets are compared to freshly isolated islets raises some concerns for analysis of human islets in culture. For example, synthesis of *INHBA* was drastically increased upon culture. Treatment of these islets with activin inhibited insulin secretion whereas islets producing much less INHBA, such as islets from T2D donors responded to the same dose of activin with increased insulin secretion. These results suggest that cultured human islets may not reflect islets *in vivo* in terms of TGFβ ligand synthesis or response. While islets from various animal models can be studied both *in vivo* and *in vitro* to identify potential *in vitro* artifacts, this is largely impossible with human islets. Therefore, results from human cultured islets need to be interpreted with care. Differences between human and animal models with respect to β-cell function or proliferation might need to account for the potential effects of altered microenvironment of human islet cultures as opposed to true species differences in islet gene function. Future studies should continue to seek a definitive mechanism of action.

We have found evidence that both acute and chronic exposure to activin A enhanced insulin secretion in islets from T2D donors. These findings suggest that not only does activin A likely act through transcriptional means such as by up-regulating *INS* but it can also act through post-transcriptional pathways. These pathways may involve modulation of ion channels and calcium signaling. Our results demonstrate a striking link between the islet microenvironment and TGFβ superfamily production. Our results reveal differential expression based on diabetic, culture and functional status. Further, we provide the first report of evidence of the ability of activin A to restore appropriate insulin secretion in human islets from T2D donors. Our results strongly support targeting activin and the TGFβ superfamily for therapeutic drug development in the treatment of diabetes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

Diabetes Action Research and Education Foundation #313; Juvenile Diabetes Research Foundation #17-2012-414; Integrated Islet Distribution Program Pilot Grant.

Author Contributions

M.L.B.: Initial conception and supervision of project, experimental design, data analysis, and preparation of manuscript.

N.U., L.B., D.A., A.B.: Performed experiments and reviewed manuscript.

A.L.S.: Contributed to design, performed experiments and reviewed manuscript.

References

1. Oliver-Krasinski JM, Stoffers DA. On the origin of the B cell. Genes Dev 2008; 22:1998-2021; PMID:18676806; http://dx.doi.org/10.1101/gad.1670808
2. Nomura M, Zhu H, Wang L, Morinaga H, Takayanagi R, Teramoto N. SMAD2 disruption in mouse pancreatic β-cells leads to islet hyperplasia and impaired insulin secretion due to the attenuation of ATP-sensitive K+ channel activity. Diabetologia 2014; 57(1):157-66; PMID:24068386; http://dx.doi.org/10.1007/s00125-013-3062-2
3. Szabat M, Johnson JD, Piret JM. Reciprocal modulation of adult β-cell maturity by activin A and follistatin. Diabetologia 2010; 53(8):1680-9; PMID:20440469; http://dx.doi.org/10.1007/s00125-010-1758-0
4. Totsuka Y, Tabuchi M, Kojima I, Shibai H, Ogata E. A novel action of activin A: stimulation of insulin secretion in rat pancreatic islets. Biochem Biophys Res Commun 1988; 156(1):335-9; PMID:3052451; http://dx.doi.org/10.1016/S0006-291X(88)80845-3
5. Verspohl EJ, Ammon HPT, Wahl MA. Activin A: its effects on rat pancreatic islets and the mechanism of action involved. Life Sci 1993; 53:1069-78; PMID:8366769; http://dx.doi.org/10.1016/0024-3205(93)90260-A
6. Wada M, Shintani Y, Kosaka M, Sano T, Hizawa K, Saito S. Immunohistochemical localization of activin A and follistatin in human tissues. Endocr J 1996; 43:375-85; PMID:8930525; http://dx.doi.org/10.1507/endocrj.43.375
7. Yamaoka T, Idehara C, Yano M, Matsushita T, Yamada T, Ii S, Moritani M, Hata J, Sugino H, Noji S, et al. Hypoplasia of pancreatic islets in transgenic mice expressing activin receptor mutants. J Clin Invest 1998; 102:294-301; PMID:9664070; http://dx.doi.org/10.1172/JCI2769
8. Shiozaki S, Tajima T, Zhang YQ, Furukawa M, Nakazato Y, Kojima I. Impaired differentiation of endocrine and exocrine cells of the pancreas in transgenic mouse expressing the truncated type II activin receptor. Biochim Biophys Acta 1999; 1450:1-11; PMID:10231550; http://dx.doi.org/10.1016/S0167-4889(99)00022-1
9. Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA. Activin receptor patterning of foregut organogenesis. Genes Dev 2000; 14:1866-71; PMID:10921901
10. Smart NG, Apelqvist AA, Gu X, Harmon EB, Topper JN, MacDonald RJ, Kim SK. Conditional expression of Smad7 in pancreatic β-cells disrupts TGF-β signaling and induces reversible diabetes mellitus. PLoS Biol 2006; 4:e39; PMID:16435884; http://dx.doi.org/10.1371/journal.pbio.0040039
11. El-Gohary Y, Tulachan S, Wiersch J, Guo P, Welsh C, Prasadan K, Paredes J, Shiota C, Xiao X, Wada Y, Diaz M, Gittes G. A Smad signaling network regulates islet cell proliferation. Diabetes 2014; 63:224-36; PMID:24089514; http://dx.doi.org/10.2337/db13-0432
12. Boerner B, George N, Targy N, Sarvetnick N. TGF-β superfamily member Nodal stimulates human β-cell proliferation while maintaining cellular viability. Endocrinology 2013; 154:4099-112;

Downloaded by [alan schneyer] at 07:35 04 April 2015

PMID:23970788; http://dx.doi.org/10.1210/en.2013-1197
13. Schneyer AL, Sidis Y, Gulati A, Sun JL, Keutmann H, Krasney PA. Differential antagonism of activin, myostatin and growth and differentiation factor 11 by wild-type and mutant follistatin. Endocrinology 2008; 149:4589-95; PMID:18535106; http://dx.doi.org/10.1210/en.2008-0259
14. Sidis Y, Mukherjee A, Keutmann H, Delbaere A, Sadatsuki M, Schneyer AL. Biological activity of follistatin isoforms and follistatin-like-3 is dependent on differential cell surface binding and specificity for activin, myostatin and bone morphogenetic proteins. Endocrinology 2006; 147:3586-7; PMID:16627583; http://dx.doi.org/10.1210/en.2006-0089
15. Mukherjee A, Sidis Y, Mahan A, Raher MJ, Xia Y, Rosen ED, Bloch KD, Thomas MK, Schneyer AL. FSTL3 deletion reveals roles for TGFβ family ligands in glucose and fat homeostasis in adults. Proc Natl Acad Sci USA 2007; 104(4):1348-53; PMID:17229845; http://dx.doi.org/10.1073/pnas.0607966104
16. Brown ML, Kumura F, Bonomi L, Ungerleider N, Schneyer AL. Differential synthesis and action of TGFβ superfamily ligands in mouse and rat islets. Islets 2011; 3(6):367-75; PMID:21964310; http://dx.doi.org/10.4161/isl.3.6.18013
17. Furukawa M, Nobusawa R, Shibata H, Eto Y, Kojima I. Initiation of insulin secretion in glucose-free medium by activin A. Mol Cell Endocrinol 1995; 113:83-7; PMID:8674816; http://dx.doi.org/10.1016/0303-7207(95)03617-G
18. Shibata H, Yasuda H, Sekine N, Mine T, Totsuka Y, Kojima I. Activin A increases intracellular free calcium concentrations in rat pancreatic islets. FEBS Lett 1993; 329(1,2):194-8; PMID:8394831; http://dx.doi.org/10.1016/0014-5793(93)80220-O
19. Wu H, Mezghenna K, Marmol P, Guo T, Moliner A, Shao-Nian Y, Berggren PO, Ib CF. Differential regulation of mouse pancreatic islet insulin secretion and Smad proteins by activin ligands. Diabetologia 2014; 57:148-56; PMID:24132781; http://dx.doi.org/10.1007/s00125-013-3079-6
20. Florio P, Luisi S, Marchetti P, Lupi R, Cobellis L, Falaschi C, Sugino H, Navalesi R, Genazzani AR, Petraglia F. Activin A stimulates insulin secretion in cultured human pancreatic islets. J Endocrinol Invest 2000; 23:231-4; PMID:10853708; http://dx.doi.org/10.1007/BF03343713
21. Kohler M, Dare E, Ali MY, Rajasekaran S, Moede T, Leibiger B, Leibiger I, Tibell A, Juntti-Berggren L, Berggren PO. One-step purification of functional human and rat pancreatic α-cells. Integr Biol 2012; 4:209-19; PMID:22267247; http://dx.doi.org/10.1039/c2ib00125j
22. Boyd V, Cholewa O, Papas K. Limitations in the use of fluorescein diacetate/propidium iodide (FDA/PI) and cell permeable nucleic acid stains for viability measurements of isolated islets of langerhans. Curr Trends Biotechnol Pharm 2008; 2:66-84; PMID:20814586
23. Kin T, Senior P, O'Gorman D, Richer B, Salam A, Shapiro AMJ. Risk factors for islet loss during culture prior to transplantation. Transpl Int 2008; 21:1029-35; PMID:18564983
24. Brown ML, Schneyer AL. Emerging roles for the TGFβ family in pancreatic β-cell homeostasis. Trends Endocrinol Metab 2010; 21(7):441-8; PMID:20382030; http://dx.doi.org/10.1016/j.tem.2010.02.008
25. Goulley J, Dahl U, Baeza N, Mishina Y, Edlund H. BMP4-BMPR1A signaling in β- cells is required for and augments glucose-stimulated insulin secretion. Cell Metab 2007; 5:207-19; PMID:17339028; http://dx.doi.org/10.1016/j.cmet.2007.01.009
26. Guillam MT, Hümmler E, Schaerer E, Yeh JI, Birnbaum MJ, Beermann F, Schmidt A, Dériaz N, Thorens B. Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. Nat Genet 1997; 17:327-30; PMID:9354799; http://dx.doi.org/10.1038/ng1197-327
27. Guo, T, Jou W, Chanturiya T, Portas J, Gavrilova O, McPherron AC. Myostatin inhibition in muscle, but not adipose tissue, decreases fat mass and improves insulin sensitivity. PLoS One 2009; 4:e4937
28. Harmon EB, Apelqvist AA, Smart NG, Gu X, Osborne DH, Kim SK. GDF11 modulates NGN3+ islet progenitor cell number and promotes β-cell differentiation in pancreas development. Development 2004; 131:6163-74; PMID:15548585; http://dx.doi.org/10.1242/dev.01535
29. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-β superfamily member. Nature 1997; 387:83-90; PMID:9139826; http://dx.doi.org/10.1038/387083a0
30. McPherron AC, Lee SJ. Suppression of body fat accumulation in myostatin-deficient mice. J Clin Invest 2002; 109:595-601; PMID:11877467; http://dx.doi.org/10.1172/JCI0213562
31. Reimer MK, Ahren B. Altered β-cell distribution of pdx1 and GLUT-2 after a short term challenge with a high fat diet in C57BL/6J mice. Diabetes 2002; 51 (suppl 1):S138-43; PMID:11815473; http://dx.doi.org/10.2337/diabetes.51.2007.S138
32. Mogami H, Kanzaki M, Nobusawa R, Zhang YQ. Modulation of adenosine triphosphate-sensitive potassium channel and voltage-dependent channel by activin A in HIT-T15 cells. Endocrinology 1995; 136:2960-6; PMID:7789321
33. Reinbothe TM, Alkayyali S, Abiqvist E, Tuomi T, Isomaa B, Lyssenko V, Renstrom E. The human L-type calcium channel $Ca_v1.3$ regulates insulin release and polymorphisms in CACNA1D associate with type 2 diabetes. Diabetologia 2013; 56:340-9; PMID:23229155; http://dx.doi.org/10.1007/s00125-012-2758-z
34. Dionne K, Colton C, Lyarmush M. Effect of hypoxia on insulin secretion by isolated rat and canine islets of Langerhans. Diabetes 1993; 42(1)12-21; PMID:8420809; http://dx.doi.org/10.2337/diab.42.1.12
35. Andersen G, Ueland T, Knudsen E, Scholz H, Yndestad A, Sahraoui A, Smith C, Lekva T, Otterdal K, Halvorsen B, et al. Activin A levels are associated with abnormal glucose regulation in patients with myocardial infarction. Diabetes 2011; 60(1544):1551.
36. Cheng K, Ho K, Stokes R, Scott C, Lau SM, Hawthorne W, O'Connell P, Loudovaris T, Kay T, Kulkarni R, et al. Hypoxia-inducible factor-1α regulates β cell function in mouse and human islets. J Clin Invest 2010; 120(6):2171-83; PMID:20440072; http://dx.doi.org/10.1172/JCI35846
37. Stokes RA, Cheng K, Deters N, Lau SM, Hawthorne WJ, O'Connell PJ, Stolp J, Grey S, Loudovaris T, Kay TW, et al. Hypoxia-inducible factor-1α (HIF-1α) potentiates β-cell survival after islet transplantation of human and mouse islets. Cell Transplant 2013; 22 (2):253-66; PMID:22710383; http://dx.doi.org/10.3727/096368912X647180

Downloaded by [alan schneyer] at 07:35 04 April 2015