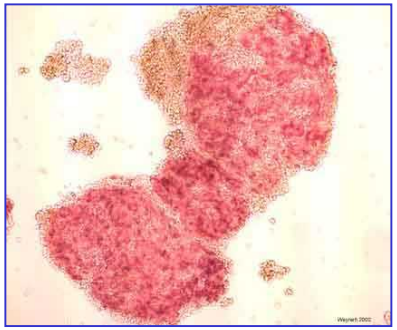


Novel Approaches to identify Islet Criteria that Predict Clinical Outcome

Philip O'Connell

Australian Collaborative Islet Transplant Centre

Centre for Transplant & Renal Research

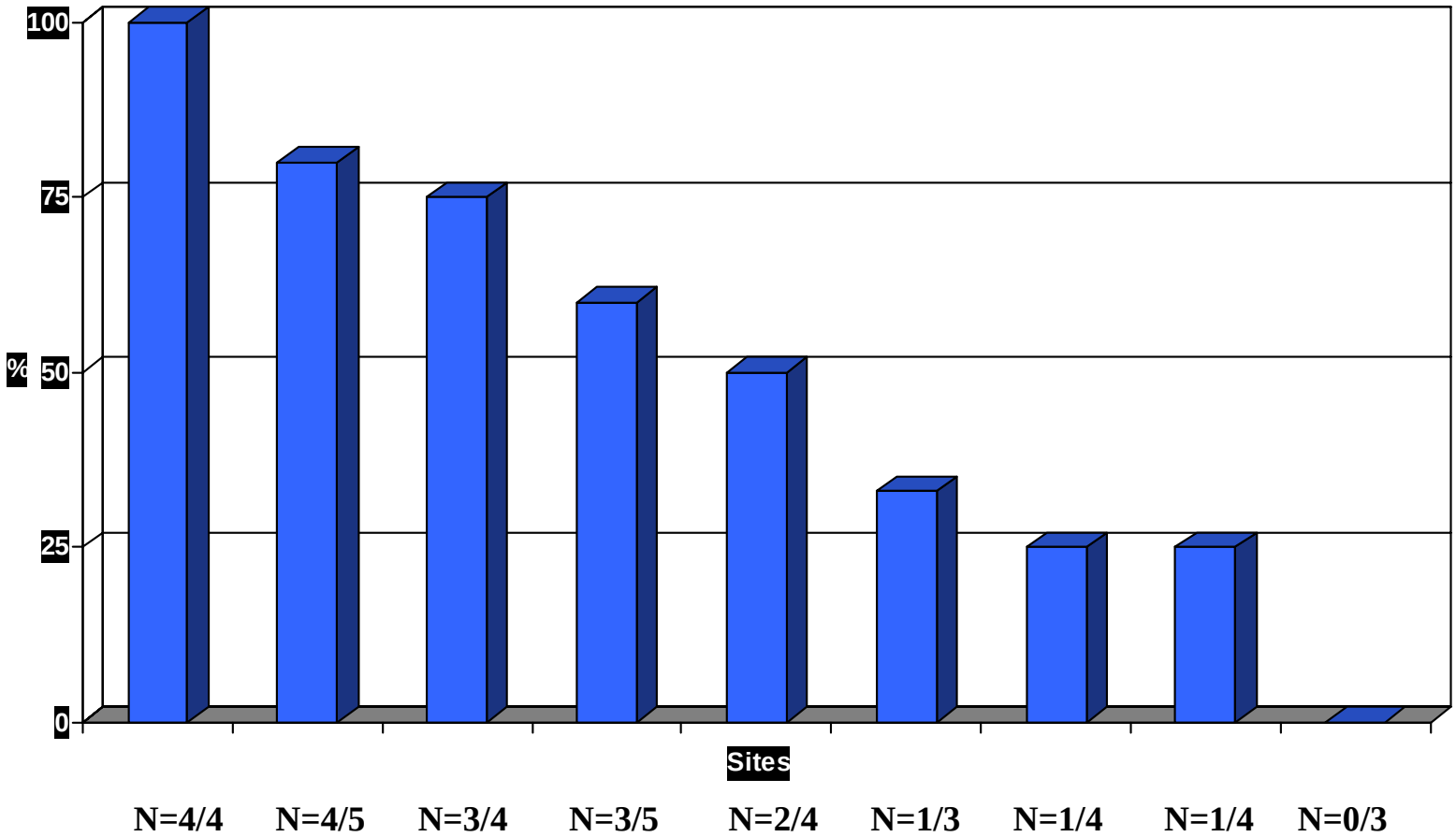
National Pancreas Transplant Unit

Westmead Millennium Institute

Westmead Hospital

The University of Sydney
AUSTRALIA

% Participants Insulin Independent



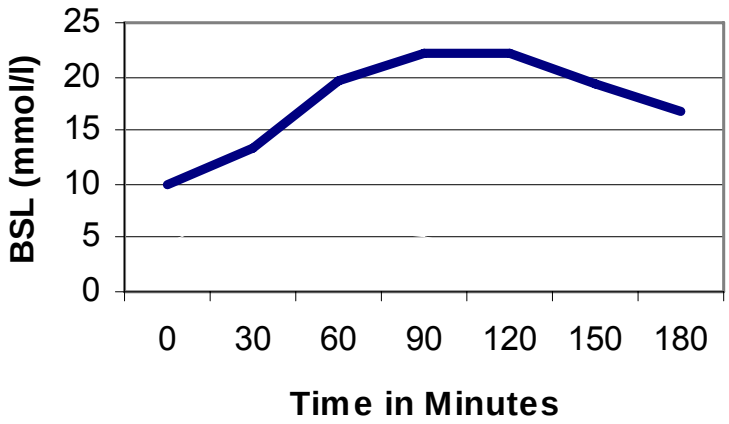
Centre Effect evident despite Experience of Centres Involved

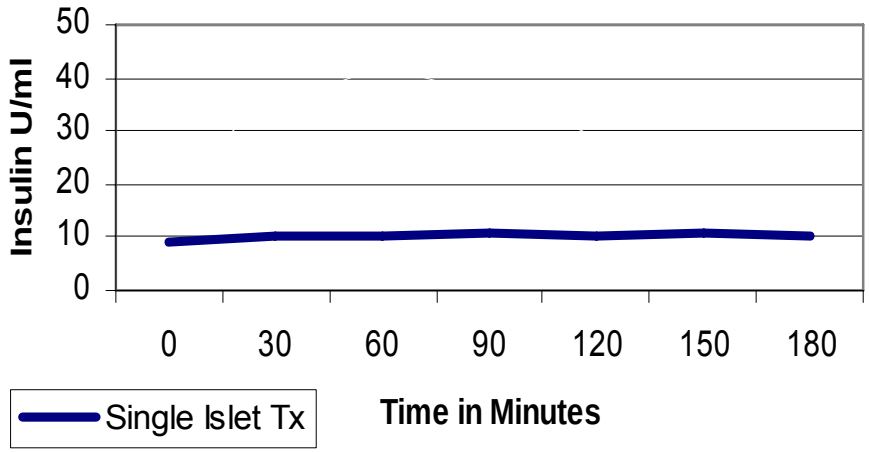
≤1 year post last transplant

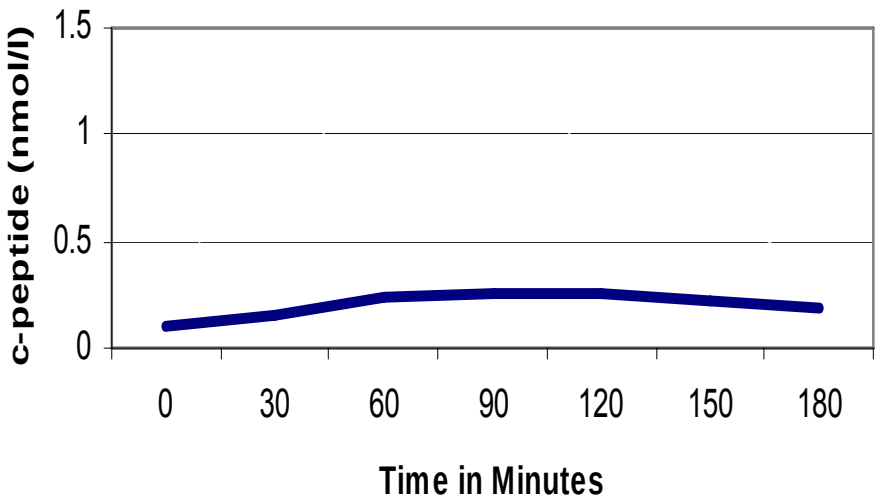
Islet Graft Outcome

Patient No.	No. of Tx	Unstim c-peptide	Insulin Dose Pre	Post
Patient 1	2	1.05 nmol/l	28	0
Patient 2	2	0.50 nmol/l	30	0
Patient 3	2	0.65 nmol/l	28	0
Patient 4	2	0.3 nmol/l	48	7
Patient 5	1	<0.1 nmol/l	35	35
Patient 6	2	0.4 nmol/l	40	6

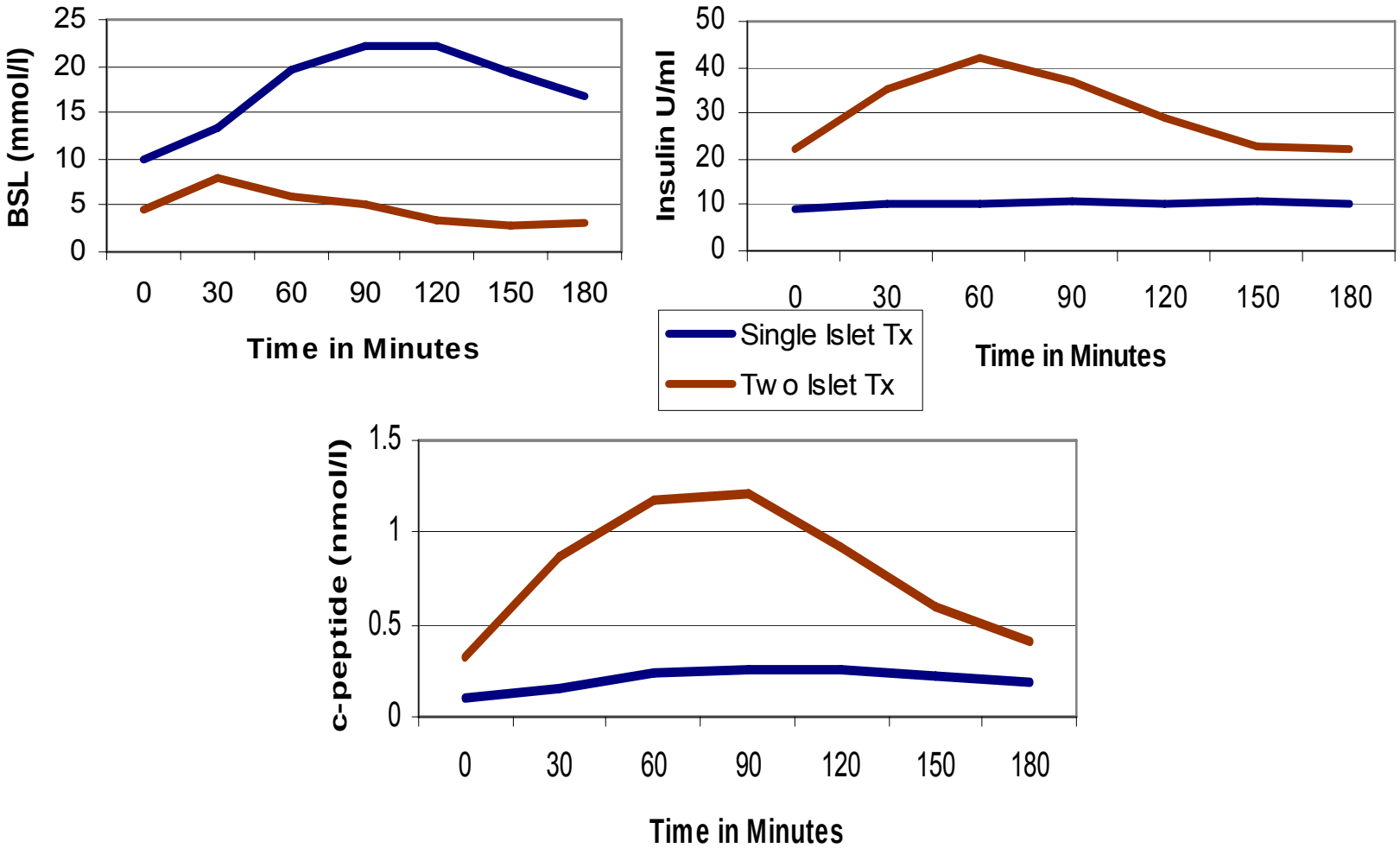
Effect of 2nd Islet Tx on Glucose Control







Effect of 2nd Islet Tx on Glucose Control



Details of Islet Preparations

Table 1. Summary of islet isolation data used in clinical islet transplantation.

Patient No.	Donor BMI kg/m2	Cold Ischaemia (mins)	Total IEQ	IEQ/kg body weight	Total Packed vol. (mLs)	Total Islet No.	Stimulation Ratio
Patient 1	31.84	120	717 037	11 117	6	262 400	-
Patient 1	39.84	120	570 766	8 849	5	207 333	9.7
Patient 2	27.77	258	494 690	6 776	9	202 500	-
Patient 2	23.37	150	537 345	7 361	10	163 000	1.04
Patient 3	32.84	150	712 960	16 580	7	184 000	7.75
Patient 3	32.65	315	425 715	9 900	10	174 000	-
Patient 4	29.29	335	1 108 216	14 976	10	170 125	5
Patient 4	33.96	435	774,230	11,385	6	96,500	1.3
Patient 5 *	20.9	360	468 685	6995	5	147 700	4.95
Patient 6**	27.75	200	759 519	12 873	8	226 206	2.5
Patient 6	27.8	270	468080	7933	8	100800	5.77

R Portal Vein Thrombosis, ** = Partial L Portal vein Thrombosis

Mean # Islets Transplanted = 17,958 IEQ/Kg

Current Predictors of β–Cell Function

- Wide variation in islet Tx outcome suggests wide variation in quality of islet preparations
- Current QA tests poorly predictive of Islet Graft function in vivo
- Better predictors if β-cell function will improve early islet graft function and, due to positive feedback, improve islet isolation across centres

Lessons learnt from Vascularised Pancreas Transplantation

- Prolonged pancreas anastomosis time correlated with impaired fasting C-peptide (r=0.371, p=0.034) and AUC C-peptide (r=0.385 p=0.028)
- No effect of cold ischaemic time or pancreas rejection
- “the susceptibility of islets to ischemia-reperfusion injury may have implications for islet transplant programs”
  - *Nankivell et al. Transplantation 1996, 12: 1705.*

Hypothesis for better analysis of β-cell Function

- Transcriptome analysis has identified genes important in protection from β–cell death and genes important in insulin secretion
- Gene profiling with targeted Gene Arrays will provide better predictive power of early graft function after transplantation
- Combination of gene analysis with functional assay likely to be more predictive than either analysis performed independently

Example 1

- Use of **custom microarray** to map immediate early anti-apoptotic gene profile in cytokine activated islets
  - Aim to identify early response genes within islets following cytokine induced apoptosis

Creating a *'death-chip'*:

Determining the beta cells stress/inflammatory response

Anti-Apoptotic
TNFAIP-3/A20 Family
Bcl Family
BIRC/IAP Family

Free Radical/Anti-oxidants
SOD Family
Peroxidase related
Thioredoxin related
NO Synthases
Cyclo-oxygenases

Apoptosis
Caspases
PCD Family
Death Associated Family
DNA Fragmentation Factors
Apoptosis inhibitor/associated
Defender against

Glucose sensing/secretion
Glut2/4
Glucokinase
Insulin
IR
IRS-1/2
UCP's

Cell cycle/Stress response
Myc Family
P53
CDK Family
Ryanodine R
Regenerating islet derived Family
HO Family
HSP Family

Cell Signaling I
Chemokines (&R)
CXC Family
CXC Family
CXC Family
Interleukins (&R)
TNF Family
TLR Family

Cell Signaling II
Map kinases
NF-κB Family
TRAF/TRADD Family
SOCS Family

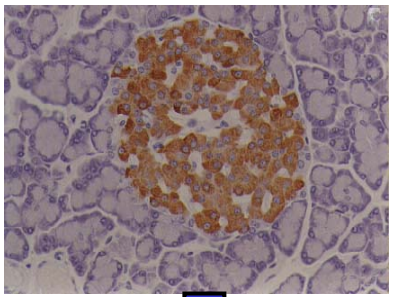
Protein Kinases
Fos/Jun Families
ATF Family

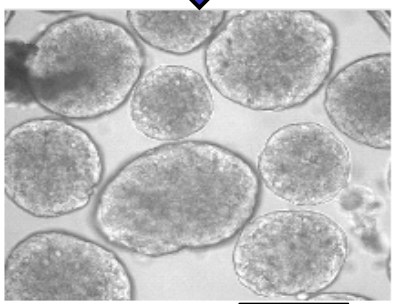
Identification of candidate genes regulating beta cell survival & death:*study design*

I. Isolate islets

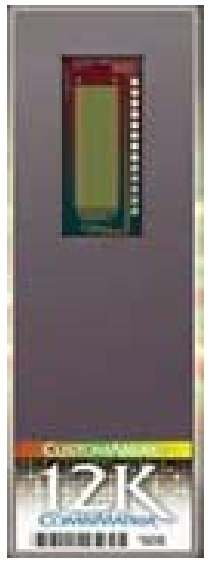
- **BALB/c & NOD mice**
- **10 mice per group**
- **islets pooled**
- **treatment=media, IL-1β, TNF-α 1h**

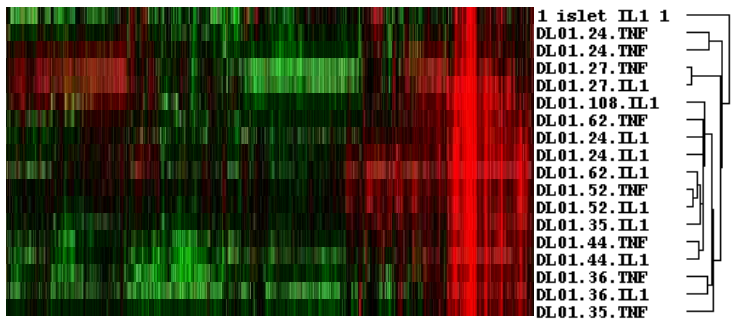
Repeat X3 NOD; X4 BALB/c





II. Micro-array analysis

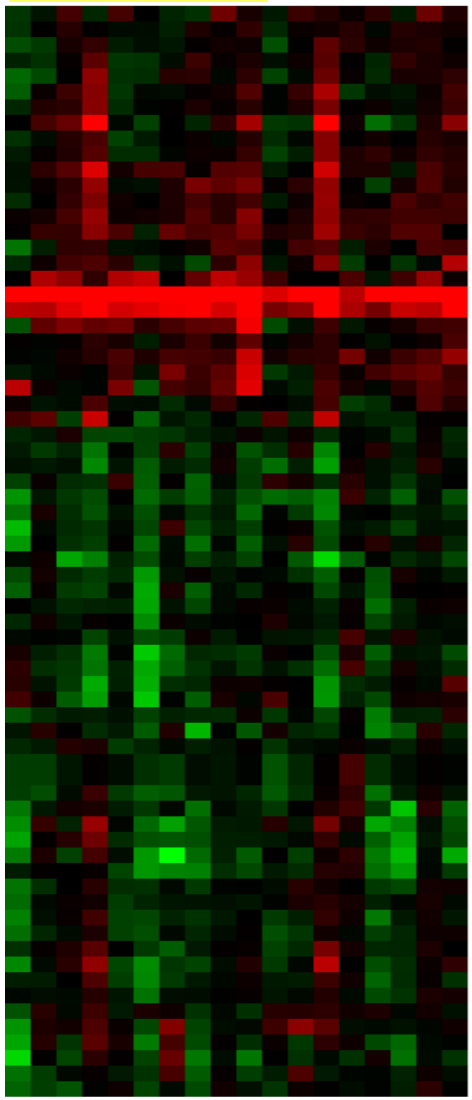




III. Identify Candidate Genes

D. Liuwantara et al. Diabetes in press

1-D Cluster Analysis Anti-apoptotic Genes



DL01.23.IL1
DL01.24.IL1
DL01.24.IL1
DL01.27.IL1
DL01.35.IL1
DL01.36.IL1
DL01.108.IL1
DL01.44.IL1
DL01.52.IL1
DL01.62.IL1
DL01.24.TNF
DL01.24.TNF
DL01.27.TNF
DL01.35.TNF
DL01.36.TNF
DL01.44.TNF
DL01.52.TNF
DL01.62.TNF

IL1 treated mice (n=9 animals, 10 experiments)

Blue- Balb-c mice, Black - NOD mice

Log_2(Ratio)

-2 0 2

–4 0 4

Fold

Hspb1	heat shock protein 1
Tnfaip1	tumor necrosis factor alpha-induced protein 1 (endothelial)
Txn1	thioredoxin 1
Txnrd1	thioredoxin reductase 1
Hspa2	heat shock protein 2 NM_008301
Hspca	heat shock protein 1 alpha
Hspa8	heat shock protein 8
Hspa1a	heat shock protein 1A
Hspa4	heat shock protein 4 NM_008300
Hsp70-4	heat shock protein 4
Sod2	superoxide dismutase 2 mitochondrial
Txnip	thioredoxin interacting protein
Gpx1	glutathione peroxidase 1
Birc3	baculoviral IAP repeat-containing 3
Bcl2a1c	B-cell leukemia/lymphoma 2 related protein A1c
Hsp105	heat shock protein 105
Hspa1b	heat shock protein 1B
Tnfaip2	tumor necrosis factor alpha-induced protein 2
Tnfaip3	tumor necrosis factor alpha-induced protein 3
Birc2	baculoviral IAP repeat-containing 2
Tnfaip6	tumor necrosis factor alpha induced protein 6
Hmox1	heme oxygenase (decycling) 1
Bcl3	B-cell leukemia/lymphoma 3
Bcl2a1a	B-cell leukemia/lymphoma 2 related protein A1a
Bcl2a1d	B-cell leukemia/lymphoma 2 related protein A1d
Hebp1	heme binding protein 1
Birc1a	baculoviral IAP repeat-containing 1a
Hspa9a	heat shock protein A
Txnrd2	thioredoxin reductase 2
Birc6	baculoviral IAP repeat-containing 6
Hspb2	heat shock protein 2
Sod3	superoxide dismutase 3 extracellular
Hspa1l	heat shock protein 1-like
Birc4	baculoviral IAP repeat-containing 4
Birc1e	baculoviral IAP repeat-containing 1e
Hspa12b	heat shock protein 12B
Txnl	thioredoxin-like
Txnl2	thioredoxin-like 2
Hmox2	heme oxygenase (decycling) 2
Bcl2l	Bcl2-like
Hspd1	heat shock protein 1 (chaperonin)
Hras1	Harvey rat sarcoma virus oncogene 1
Bag4	BCL2-associated athanogene 4
Hspb7	heat shock protein family member 7 (cardiovascular)
Birc1f	baculoviral IAP repeat-containing 1f
Hebp2	heme binding protein 2
Gpx5	glutathione peroxidase 5
Txndc4	thioredoxin domain containing 4 (endoplasmic reticulum)
Dad1	defender against cell death 1
Dad1	defender against cell death 1
Gpx2	glutathione peroxidase 2
Txndc5	thioredoxin domain containing 5
Hspcb	heat shock protein 1 beta
Hspa5	heat shock 70kD protein 5 (glucose-regulated protein)
Gpx4	glutathione peroxidase 4
Bcl2l2	Bcl2-like 2
Txn2	thioredoxin 2
Hnf4	Hepatic nuclear factor 4
Txndc1	thioredoxin domain containing 1
Bfar	bifunctional apoptosis regulator
Hspe1	heat shock protein 1 (chaperonin 10)
Aatf	apoptosis antagonizing transcription factor
Birc1b	baculoviral IAP repeat-containing 1b
Aven	apoptosis caspase activation inhibitor
Txnrd3	thioredoxin reductase 3
Birc5	baculoviral IAP repeat-containing 5
Sod1	superoxide dismutase 1 soluble
Hspb3	heat shock protein 3
Bcl2l10	Bcl2-like 10
Bcl2	B-cell leukemia/lymphoma 2

TNFAIP3/A20

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A20 is the Most Highly Regulated Anti-apoptotic Gene in Islets

Figure 2b.

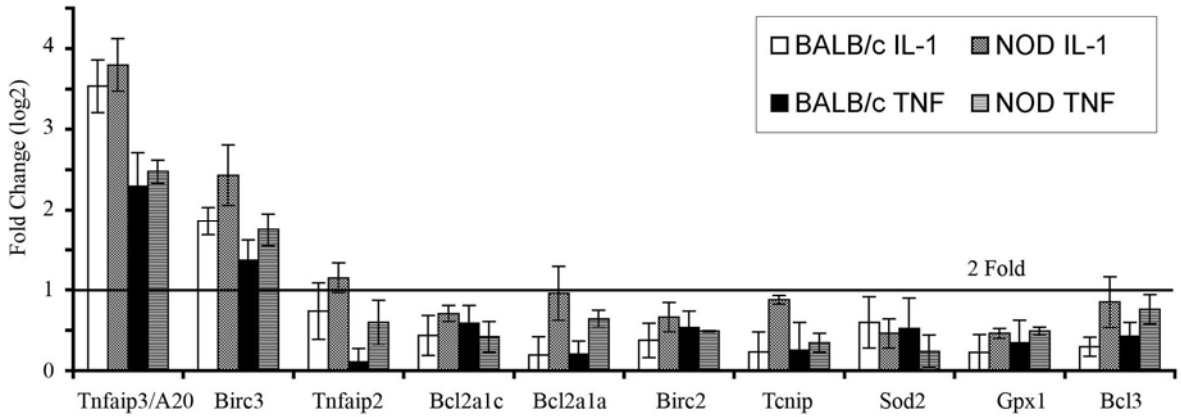
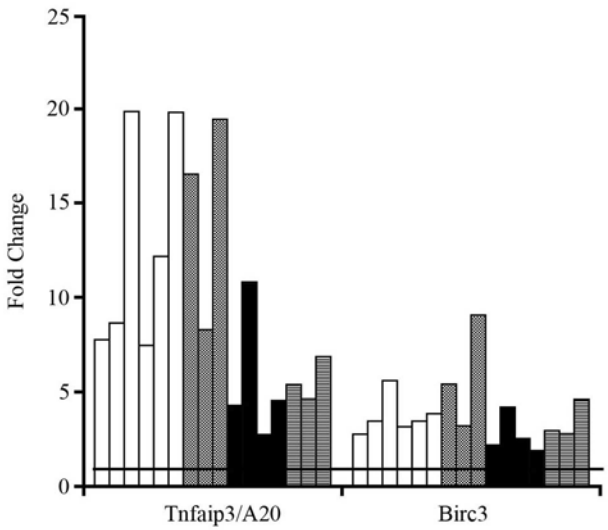


Figure 2c.



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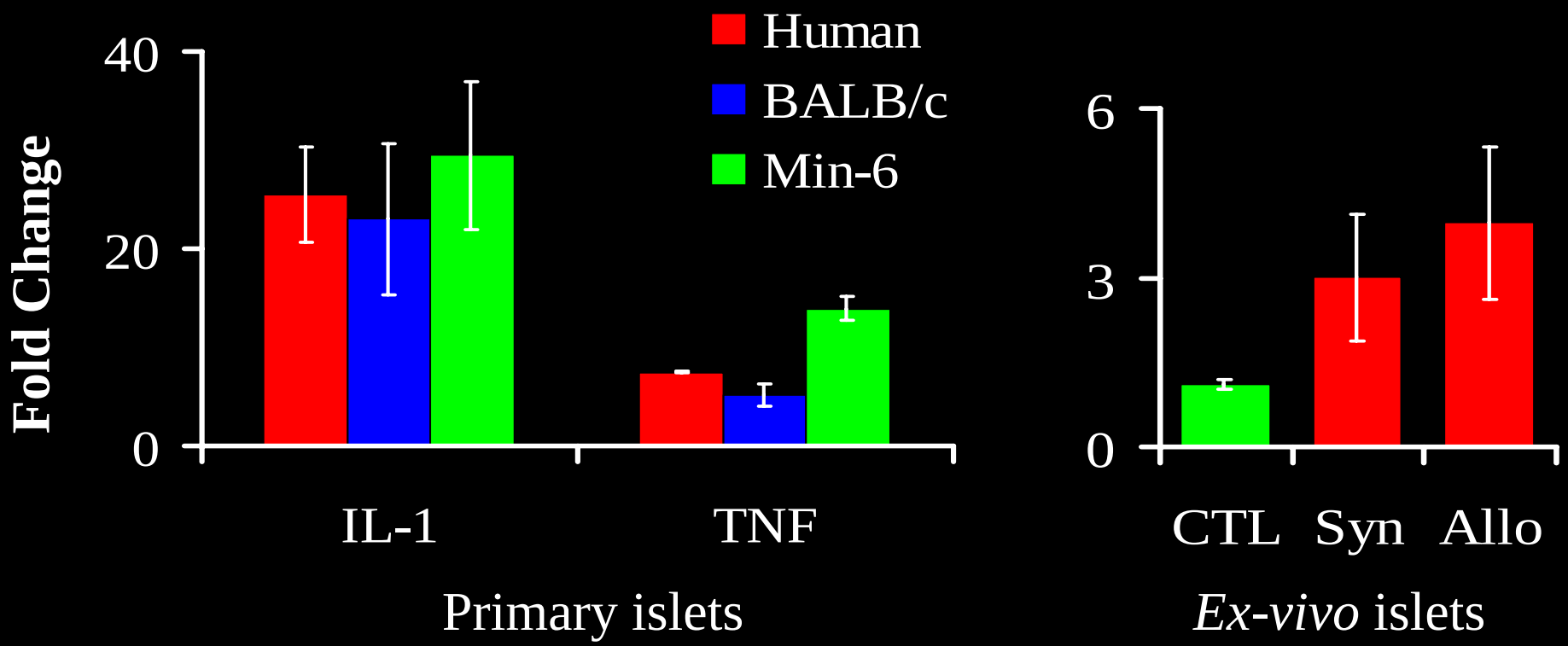
A20 expression in islets *in vitro* & *in vivo*



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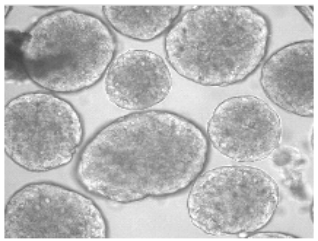
A20 Expression in Primary Beta-cells



A. FACS sorted IL-1β stimulated primary islets

Figure 4a.

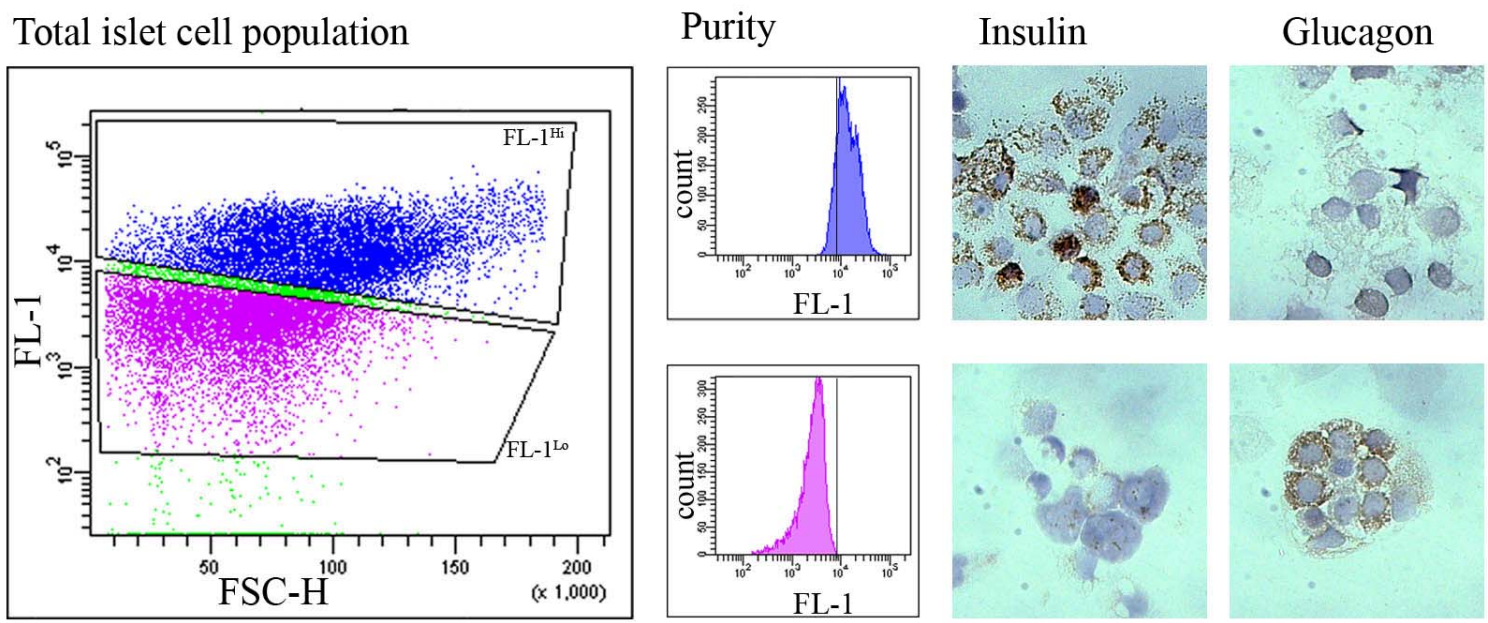
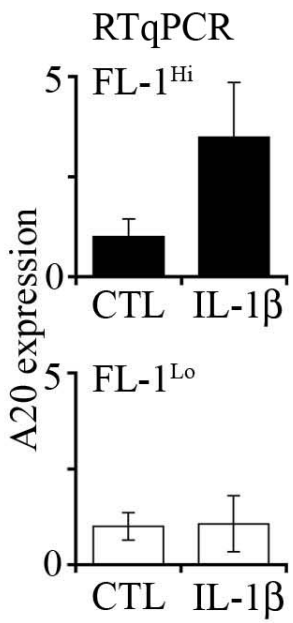


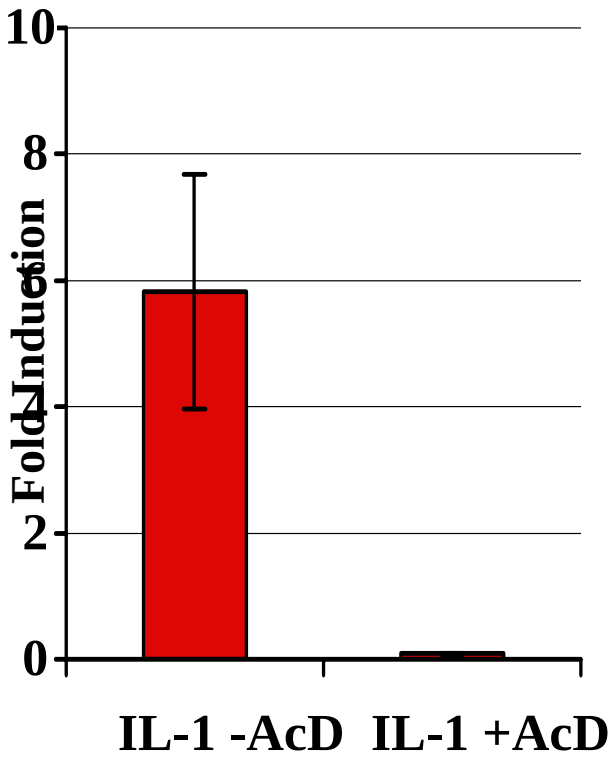
Figure 4b.



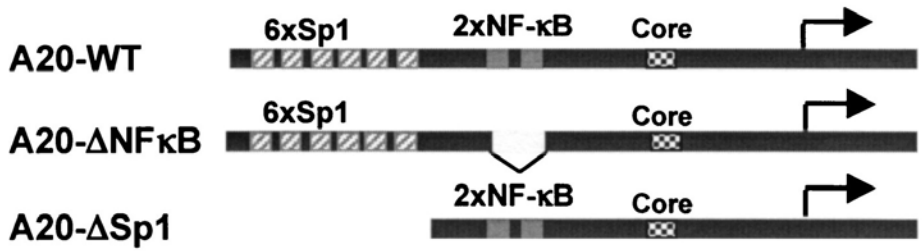
D. Liuwantara et al. Diabetes <u>in press</u>

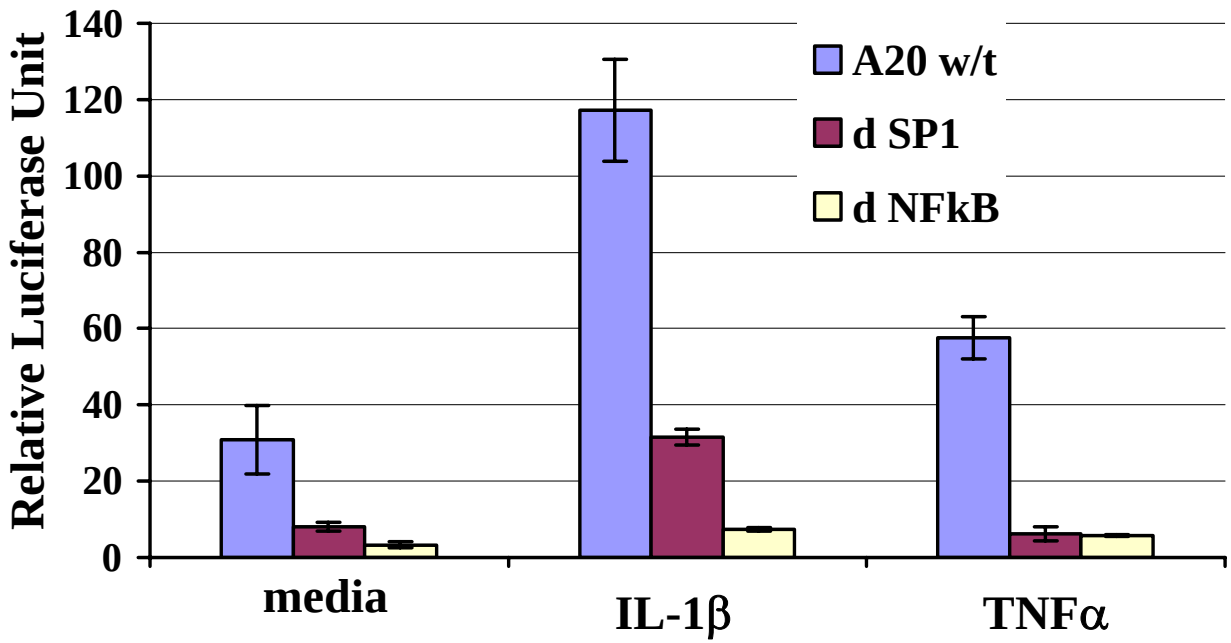
A20 is regulated at the level of transcription

A. Actinomycin D treatment



B. Reporter analysis





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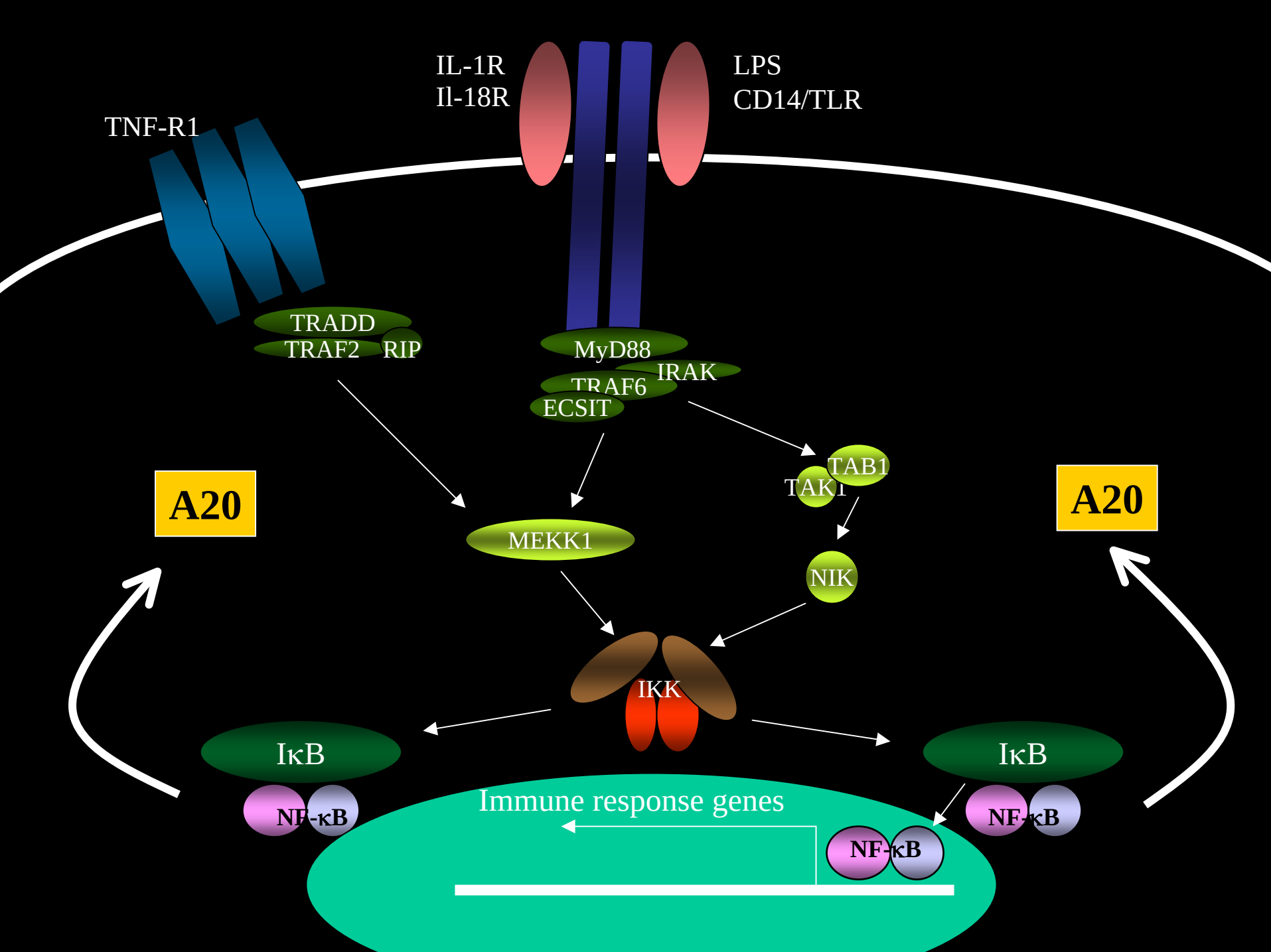

IL-1R
Il-18R
LPS
CD14/TLR
TNF-R1
TRADD
TRAF2
RIP
MyD88
IRAK
TRAF6
ECSIT
TAB1
TAK1
A20
A20
MEKK1
NIK
IKK
IκB
IκB
NF-κB
NF-κB
Immune response genes

Example 1

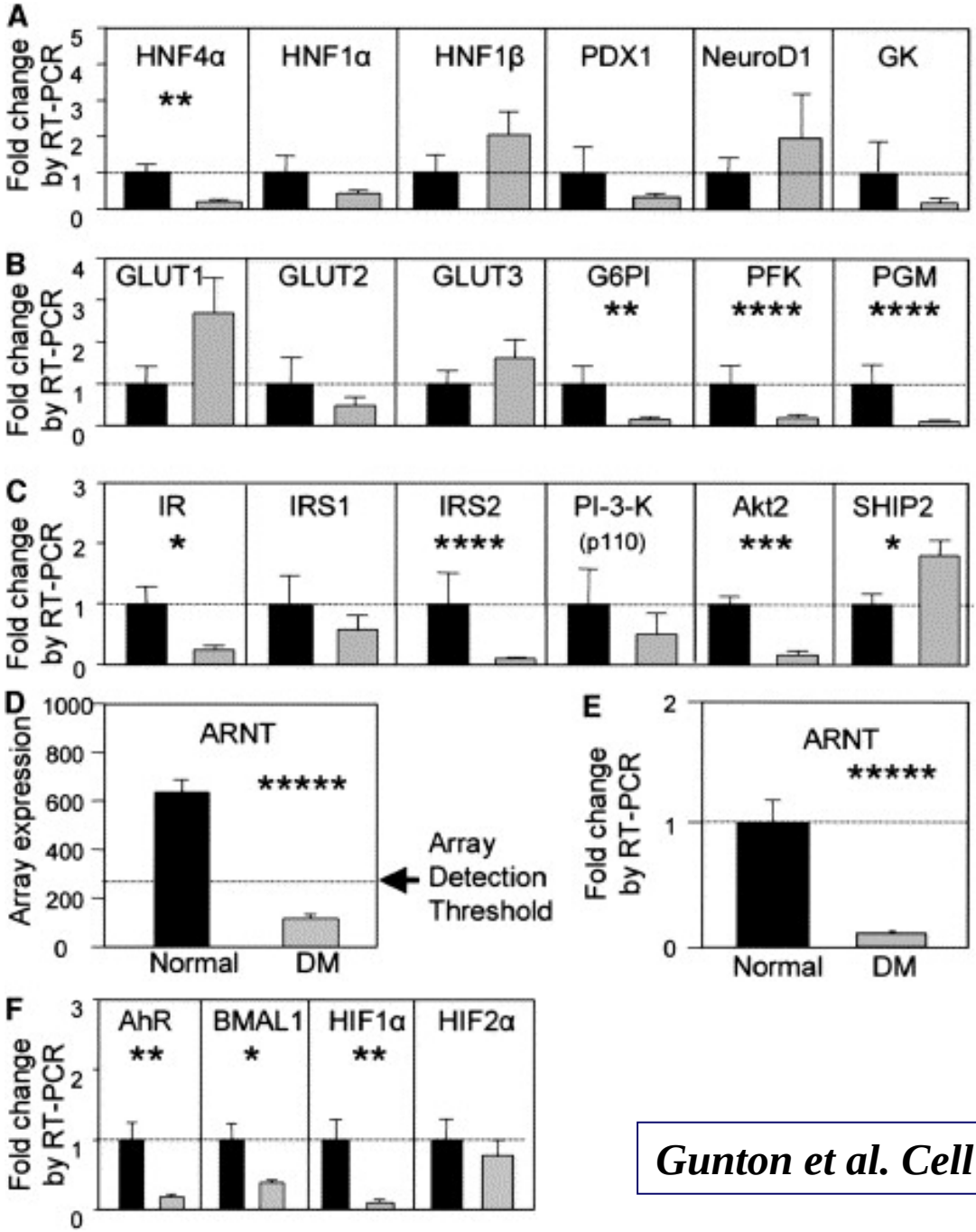
- Use of **custom microarray** to map immediate early anti-apoptotic gene profile in cytokine activated islets
  - Identifies **A20** as immediate early response gene in β–cells
  - Regulation via **NF-κB**
  - Outline possible strategies to promote expression

Example 2.

- Identification of Novel Genes involved in β– Cell Dysfunction in Human Islets

- Islets were isolated from whole pancreata of human subjects either with normal glucose tolerance (7) or type 2 diabetes (5).
- All of the subjects were organ donors due to catastrophic intracerebral events (CVA or hemorrhage).
- 6 samples were from the NPTU in Australia, and 6 from the USA (Boston and Miami).
- Duration of final illness was <3 days in all cases.
- Used Microarrays to identify changes in gene expression in genes important in β–cell function
- Transcription factor ARNT/HIF1β reduced in type 2 diabetes and responsible for impaired islet function

Gunton et al. Cell 2005, 122; 337



MODY Genes

Glucose Sensing Genes

Insulin Signaling Genes

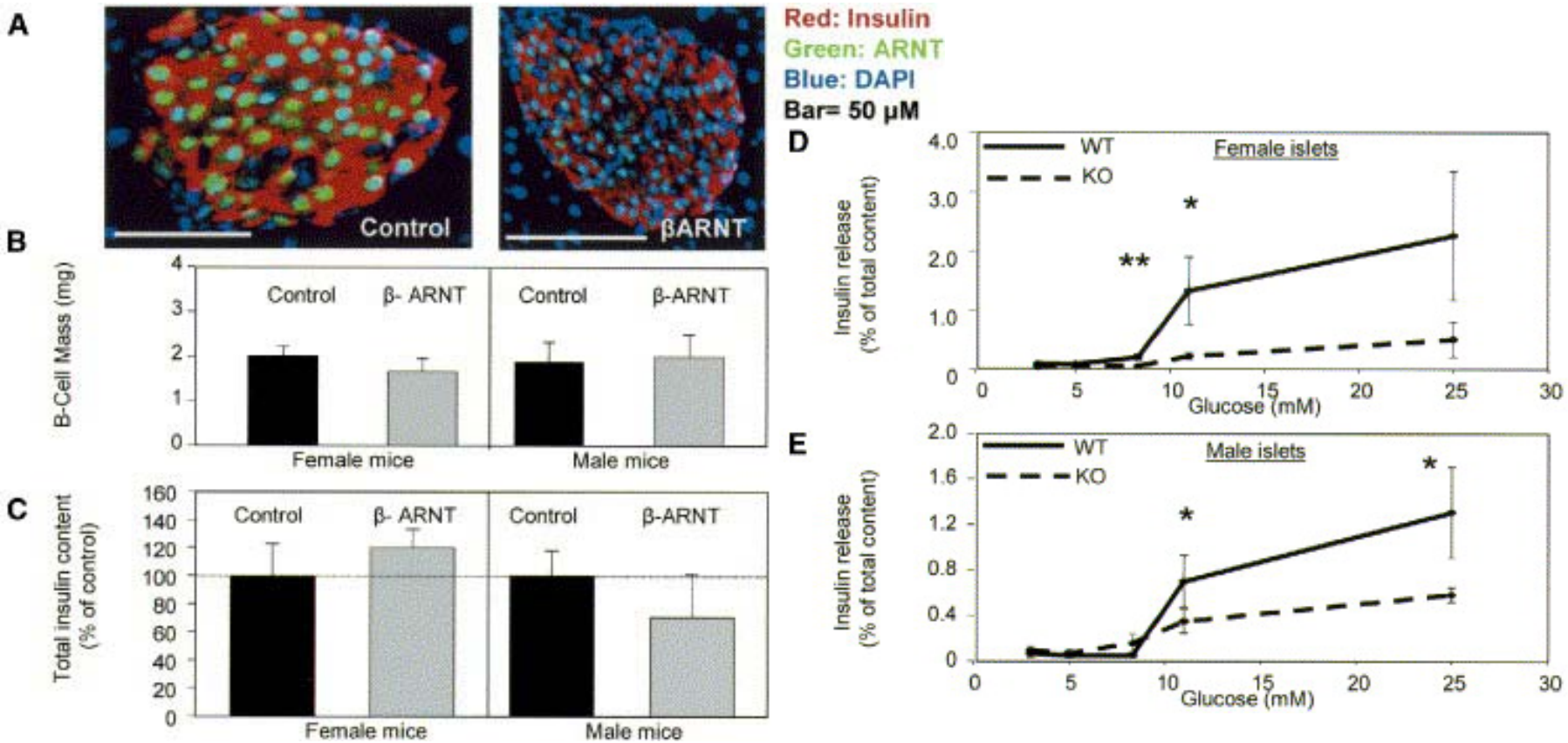
MICROARRAY ANALYSIS

Altered expression of:

- MODY
- Glucose Sensing
- Insulin Signaling Genes

Gunton et al. Cell 2005, 122, 337-349

Insulin release abnormal in Islets from β–ARNT KO mice



Gunton et al. Cell 2005, 122, 337-349

Summary 1 – Human Islets

- Pancreatic islets isolated from patients with type 2 diabetes exhibited multiple alterations in gene expression.
- Many interesting and significant abnormalities in expression were found
  - Decreased HNF4α
  - Decreased IR, IRS2, and Akt2,
  - Decreased G6PI, PFK, PGM, and Aldolase
  - Decreased Kir6.2
- ARNT was decreased by 90% in Type 2 diabetes.
- ARNT deficiency associated with impaired glucose secretion

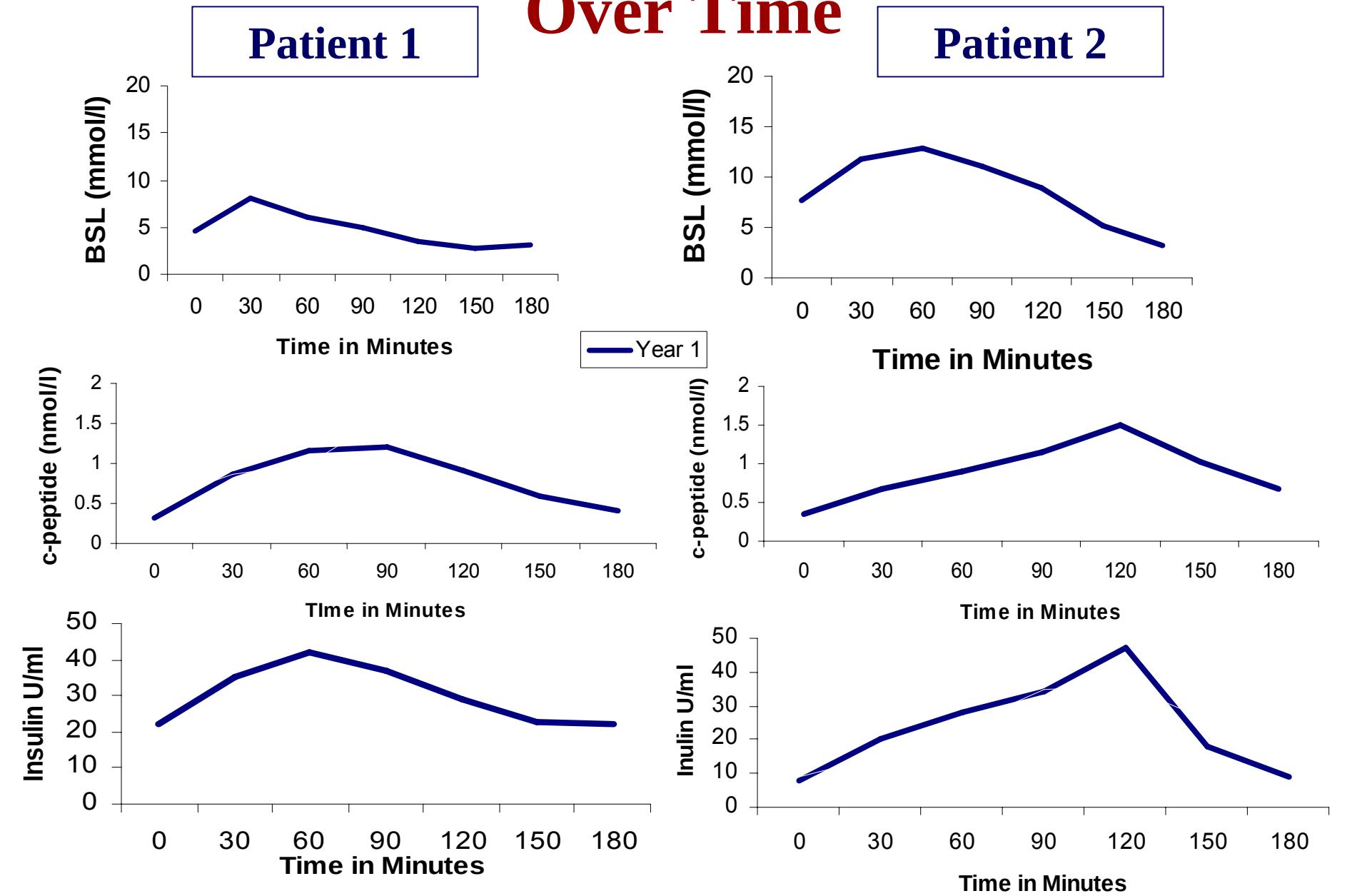
Identifying Islet Criteria predictive of Transplant outcomes using microarrays

- RNA isolated at the time of isolation (pre-Tx) for Affimetrix microarray
- Expression profiles correlated with in vitro and in vivo function post Tx
- Aim is to identify Gene “footprint” predictive of Graft Function post-Transplant

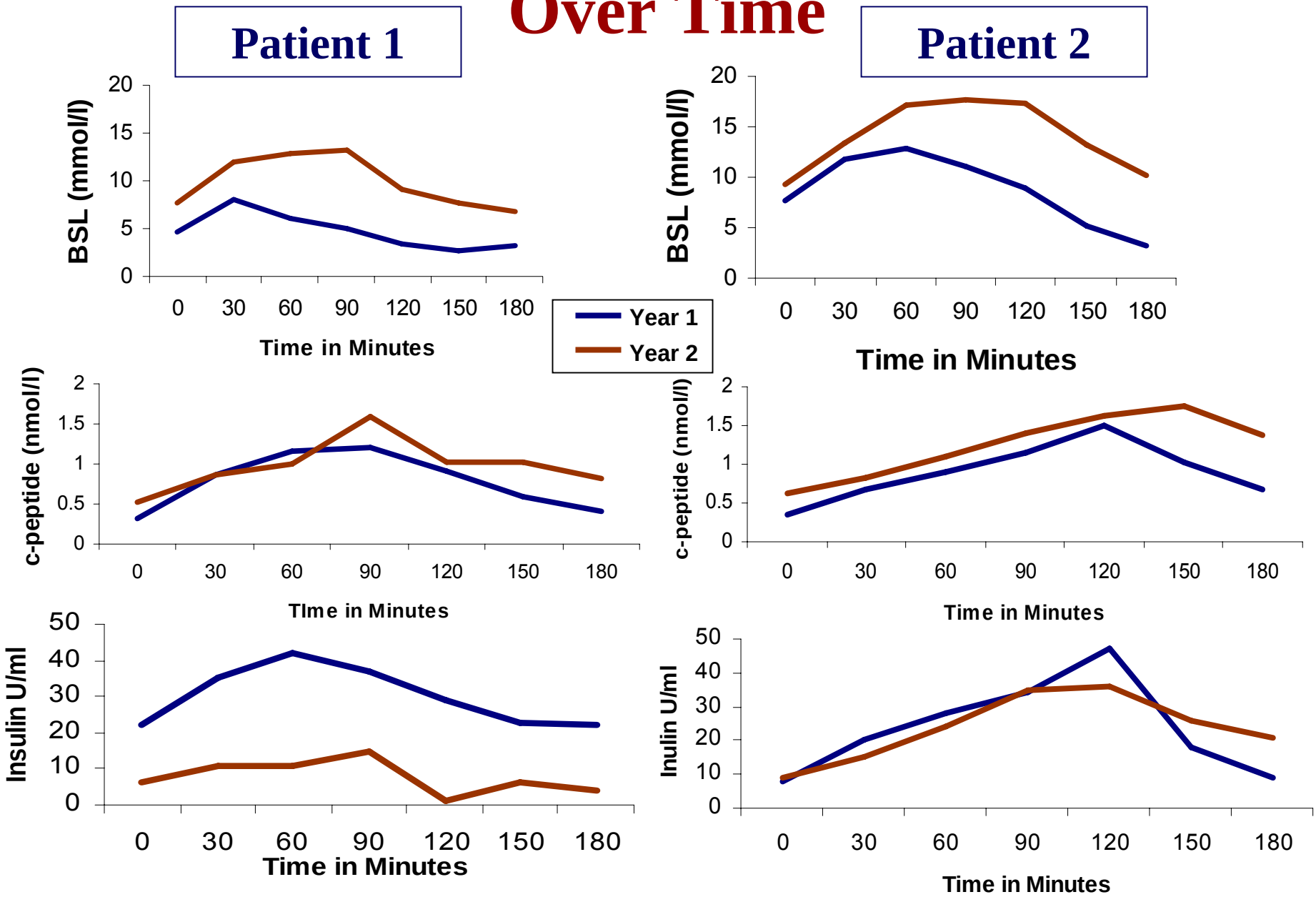
Crucial Steps for Success

- Identify a predictive panel of Genes
- Evaluate on “Test Cohort” to confirm that panel is predictive
- Confirm findings on large patient sample
- Sample size an important issue

Progressive Loss of Islet Function Over Time



Progressive Loss of Islet Function Over Time



Progressive Loss of Islet Function Over Time

Edmonton Experience - 5 Year Follow up

Number Transplanted	65 patients
# Insulin Independent	44 recipients
5 YEAR FOLLOW-UP	
C-Peptide positive	80%
Insulin Independent	10%
Median duration Insulin Ind.	15 months (6.2 - 25.5)

Ryan et al. Diabetes 2005; 54: 2060

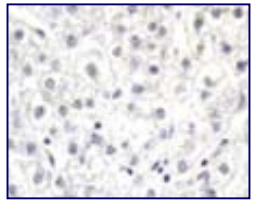
Postulated Causes of Progressive Graft Loss

- Poor engraftment / re-vascularization
- Transplantation of a marginal islet mass with “islet exhaustion”
- Chronic subclinical rejection
- Autoimmune recurrence
- Early loss of islets peritransplant as a result of IBMIR and other mediators of inflammation
- Islet induced fatty change within the liver and resultant increase in insulin resistance
- Accumulative toxic effects of the immunosuppression (Tacrolimus and Sirolimus).

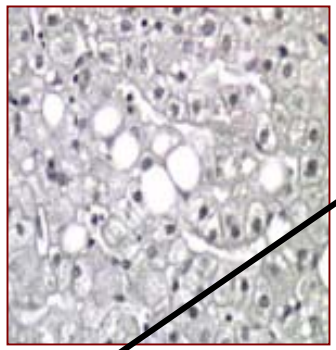
HYPOTHESIS

- Islet loss multifactorial
- An interaction between changed Liver Microenvironment and multiple pro-inflammatory signals

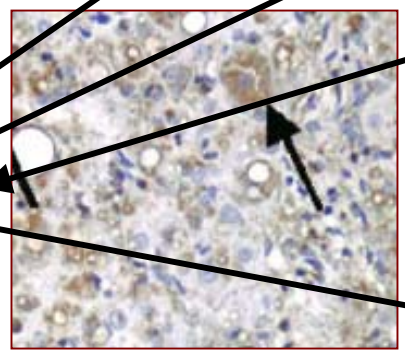
Intrahepatic Islet Tx



Intrahepatic Hyperinsulinemia
+/- Insulin Resistance
Steatosis



Oxidative Stress
Lipid Peroxidation



Inflammation

Cirrhosis &
Progressive Islet Destruction

IBMIR

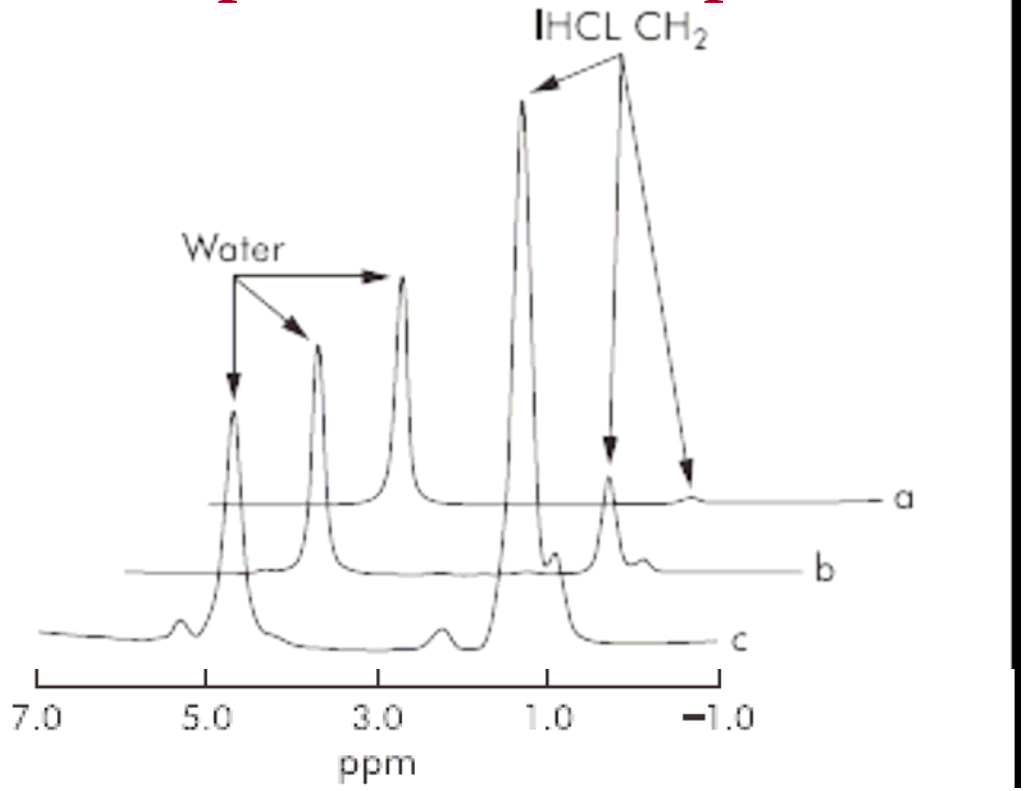
Rejection

Autoimmune Recurrence

Hepatotoxic Side Effects
Of Immunosuppressives

^{1}H-Magnetic Resonance Spectroscopy

Intra hepatocellular lipid: IHCL



Typical proton magnetic resonance liver spectra from three volunteers showing progressive degrees of fatty infiltration.

Spectrum (a) shows a liver with minimal fatty infiltration (1.0%), (b) a liver with moderate fatty infiltration (10.2%), and spectrum (c) shows a liver with severe fatty infiltration (74.9%).

Use of Protocol Liver Biopsy to Identify Causes of Progressive Loss of β–Cell Function

- Liver Biopsy at time of Second Islet Infusion
- 2nd Biopsy at 6 months post Tx
- Analysis by Histology
- LCM and Gene Array for
  - Pro-inflammatory and
  - Steatotic Gene Regulation (Compare with NASH cohort)
  - Apoptosis and cell cycle
  - β–Cell function gene expression
- Patient evaluated for development of insulin resistance

Australian Islet Transplant Consortium

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Jeremy Chapman
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Brian Nankivell
Chris Little
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Tom Loudovaris
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David Goodman
Helen Thomas
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Shane Grey
David Liuwantara
Jenny Gunton

Childrens Hospital Westmead

Steve Alexander

Australian Government
Dept. of Health