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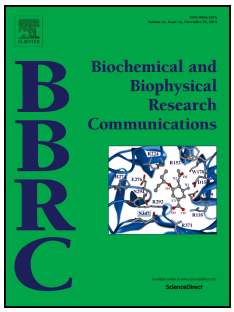

Wnt4 antagonises Wnt3a mediated increases in growth and glucose stimulated insulin secretion in the pancreatic beta-cell line, INS-1

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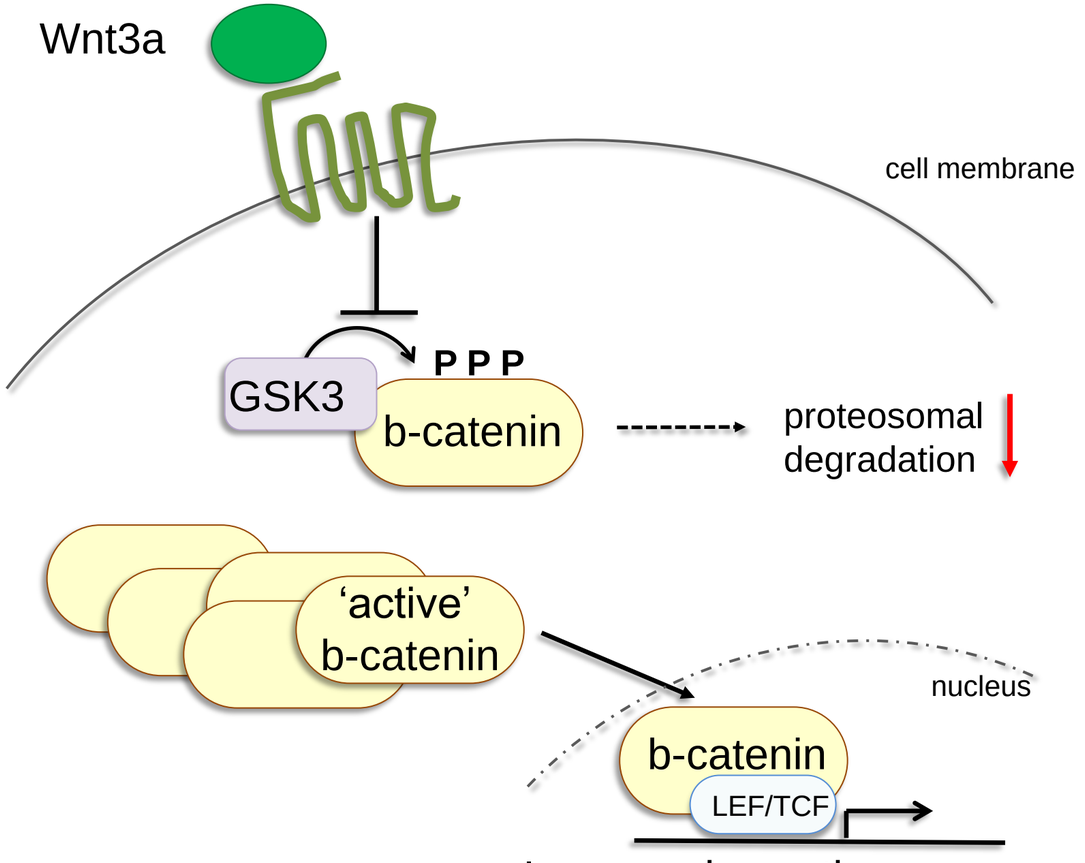
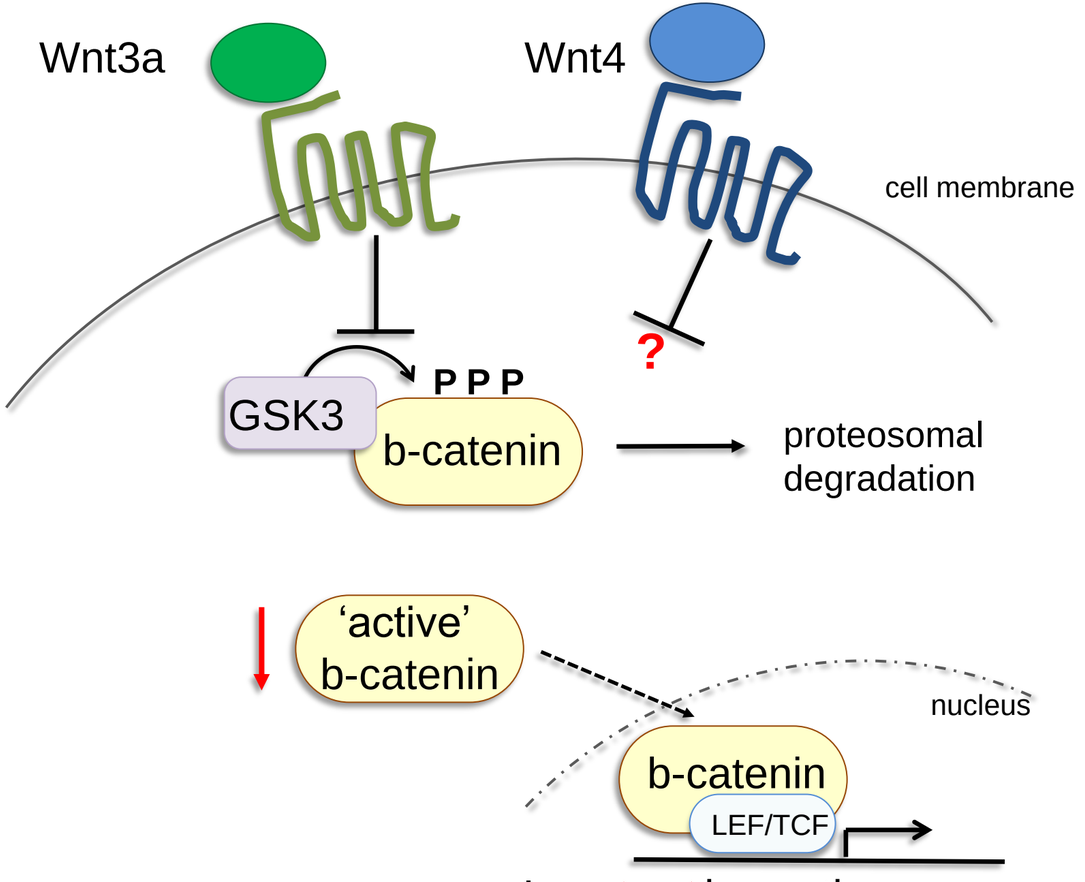


Increased growth

Increased glucose stimulated insulin secretion



Increased growth

Increased glucose stimulated insulin secretion

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Title: Wnt4 antagonises Wnt3a mediated increases in growth and glucose stimulated insulin secretion in the pancreatic beta-cell line, INS-1.

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Abstract:

The Wnt signalling pathway in beta-cells has been linked to the development of type 2 diabetes. Investigating the impact of a non-canonical Wnt ligand, Wnt4, on beta-cell function we found that in INS-1 cells, Wnt4 was able to completely block Wnt3a stimulated cell growth and insulin secretion. However, despite high levels of Wnt4 protein being detected in INS-1 cells, reducing the expression of Wnt4 had no impact on cell growth or Wnt3a signalling. As such, the role of the endogenously expressed Wnt4 in beta-cells is unclear, but the data showing that Wnt4 can act as a negative regulator of canonical Wnt signalling in beta-cells suggests that this pathway could be a potential target for modulating beta-cell function.

Key words: Wnt signalling, Wnt4, islet cells, insulin secretion, cell growth control

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Introduction

Wnt signalling was first proposed to be important in the pathogenesis of type 2 diabetes when variants in the transcription factor TCF7L2, a key component of the Wnt pathway, were discovered as strong risk factors for this illness [1]. Subsequent studies implicated β-cell dysfunction as the underlying cause of diabetes in patients with the TCF7L2 risk variant [2-5] and have suggested that Wnt signalling plays an important role in both pancreatic development and in the function of mature pancreatic islets [6]. The activation of the canonical Wnt pathway, by ligands such as Wnt3a, has been of particular interest as it can increase β-cell proliferation, decrease apoptosis and improve glucose stimulated insulin secretion [6-12]. However there is now increasing evidence that non-canonical Wnt ligands such as Wnt4 can antagonise the signalling mediated by Wnt3a and may play an equally important role in β-cell function [13-15].

The Wnt signalling pathway is mediated by Wnt ligands binding to cell surface receptors known as Frizzled (Fzd) molecules. Downstream signalling from these receptors appears to follow distinct pathways [16, 17]. The best characterised of these is the “canonical pathway” which regulates gene transcription by controlling the protein levels and cellular localisation of β-catenin [18]. In the nucleus β-catenin can act as a transcription factor (along with its co-factors TCF/LEF) to promote gene transcription of effectors of Wnt signalling, such as c-myc and cyclin D2. In contrast, the non-canonical pathways, such as those stimulated by Wnt4 are β-catenin independent and have been shown to antagonise the canonical Wnt pathway in several cell types [13, 14, 19-21].

Our previously published data [22] showed that most Wnt ligands were either not expressed (including the canonical Wnt ligand, Wnt3a) or were present at only very low levels in a β-cell line. The exception was Wnt4 which was expressed at levels more than 10 fold higher than any other Wnt ligand [22]. In addition Wnt4 protein has been detected in adult mouse islets but not in several other tissues including liver and kidney [14]. Wnt4 has also been found to be upregulated in the islets of two different insulin resistant mouse strains [14] and

in the islets of patients with type 2 diabetes [23] suggesting that Wnt4 may have islet specific functions in adults and a possible role in the development of diabetes.

In this paper we set out to determine the function of Wnt4 in pancreatic β-cells. Our data shows that the endogenous Wnt4 present in β-cells is not secreted and has no impact on β-cell growth or β-catenin levels. In contrast treatment of β-cells with exogenous Wnt4 is able to antagonise canonical Wnt signalling leading to inhibition of Wnt3a stimulated β-cell proliferation and insulin secretion.

Material and Methods:

Immunohistochemistry

Paraffin embedded human pancreatic sections were obtained from two different healthy non diabetic cases (60 year old male (202/75) and 76 year old female (329/66)) from the Exeter Archival Diabetes Biobank. All samples were studied with ethical approval.

Immunohistochemisty was carried out as previously described [24]. Sections were incubated with primary Wnt4 antibody (AF745; RnD systems; 1/25) before incubation with flurochrome-conjugated secondary antibody (Goat anti-rabbit Fluor488) for 1hr (room temperature). Tissue sections were then incubated with insulin (A0564; Dako; 1/600) and glucagon (AB10988; Abcam; 1/2000) primary antibodies for 1hr followed by flurochrome-conjugated secondary antibody (Goat anti-guinea pig Fluor647 and Goat anti-mouse Fluor568 1/400) and DAPI (1/1000) for 1 hour. Immunofluorescence image collection and processing was achieved using a Leica DM4000 B LED upright fluorescence microscope and Leica Image analysis software (LAS AF).

Cell culture:

INS-1 cells were grown in RPMI 1640 medium containing 11mM glucose, supplemented with 10% (v/v) foetal bovine serum, 2mM L-glutamine, 100µg/ml penicillin, 100µg/ml streptomycin and 50mM β-mercaptoethanol.

To determine cell growth, cells were treated with test ligands in 1% (v/v) serum containing media and counted using a haemocytometer.

Wnt4 siRNA of INS-1 cells

INS-1 cells were transfected with 10nM silencer select siRNA Wnt4 s136596 (Lifetech) using lipofectamine RNAi MAX (Lifetech) diluted in optimem (Gibco) media. A scrambled siRNA was used as a negative control.

Wnt4 ELISA

INS-1 cells were cultured for 72hrs before lysis in PBS by repeated freeze thawing. The media from the cultured cells was concentrated approximately ten fold using an Amico Ultra-15 centrifugal device. Levels of Wnt4 were measured using a Cusabio Wnt4 rat ELISA according to the manufacturer's instructions.

Western blotting

Whole cell lysates from INS-1 cells were prepared as previously described [25]. Nuclear and cytoplasmic protein fractions were separated using a nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. The membrane protein fraction was separated using differential centrifugation. INS-1 cells were lysed in ice cold homogenising buffer (HB; 20mM HEPES pH 7.4, 1µM EDTA, 250mM sucrose with the addition of protease and phosphatase inhibitors) and homogenised before two consecutive centrifugation steps at 1000g for 10 minutes at 4℃ to sediment the nuclei and cell debris. The remaining supernatant was centrifuged at 21,000g for 10 minutes at 4℃ and the supernatant containing the cytoplasmic fraction was removed. The remaining pellet containing enriched membrane fraction was resuspended in HB and centrifuged at 21,000g for 10 minutes at 4℃. The purified enriched pellet was resuspended in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1% (v/v) Triton-X-100, 1% sodium deoxycholate (w/v), 0.1% SDS (v/v), 1mM EDTA with the addition of protease inhibitor and phosphatase inhibitors).

Protein lysates were used in western blotting (previously described [25]) using the following primary antibodies and concentrations: Wnt4 (M70; Santa Cruz) 1 in 1000, Total β-catenin (05-613; Millipore) 1 in 1000, Active β-catenin (3024DP; Symansis) 1 in 1000, Histone H3

(D1H2; NEB) 1 in 5000, Tubulin (Ab4074; Abcam) 1 in 5000, Sodium Potassium ATPase (Ab7671; Abcam) 1 in 2500. Protein levels were quantified using image J (http://imagej.nih.gov/ij/).

PCR

RNA was isolated from INS-1 cells using trizol reagent. 1µg of RNA was used in a one-step RT reaction (Applied Biosciences) to produce cDNA for use in qRT-PCR; amplified by SYBR Green PCR master mix (Applied Biosystems) and analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). Primer sequences are available on request.

Glucose stimulated insulin secretion

To measure insulin secretion, INS-1 cells were incubated in Krebs-Ringer Bicarbonate buffer (KRB; 125mM NaCl, 4.74mM KCl, 1mM $CaCl_2$, 1.2mM KH_2PO4, 1.2mM $MgSO_4$, 5mM $NaHCO_3$, 25mM Hepes, pH 7.4 + 0.1% (w/v) BSA) containing 2.8mM glucose for 2 hours to reduce insulin secretion to basal glucose levels. This was followed by stimulation of the cells with the required treatments (in KRB) for 1 hour. Media was sampled for measurement of insulin by radioimmunoassay (Linco). Insulin secretion was normalised to the protein content of each well.

Statistical analysis

Data is presented as mean±SEM of treated samples compared to the untreated control. Significant differences between treatment and control values were determined by ANOVA followed by a Mann-Whitney test and Kruskal-wallis test. P values <0.05 were considered significant.

Results:

Functional impact of Wnt4 on β-cell growth and insulin secretion

To investigate the functional impact of Wnt4 on beta-cells, INS-1 cells were treated with Wnt4 alone or in combination with Wnt3a and the impact on cell growth and insulin secretion measured. As expected, Wnt3a caused an increase in cell proliferation (Fig 1A) and

increased insulin secretion stimulated by high glucose levels (Fig 1B). Wnt4 alone however had no impact on these parameters, but was able to completely block Wnt3a stimulated increases in cell growth and insulin secretion (Fig 1A+B), showing that Wnt4 is able to functionally inhibit the actions of Wnt3a.

Wnt4 mediated modulation of the canonical Wnt signalling pathway

We next sought to determine the mechanism by which Wnt4 can inhibit Wnt3a actions in β-cells. Stimulation of canonical Wnt signalling is characterised by the stabilisation of β-catenin protein due to inhibition of phosphorylation on two key sites (Ser^{37} and Thr^{41}) by GSK3β. This hypo-phosphorylated form of β-catenin is referred to as 'active' β-catenin and is used as a marker of activation of the canonical pathway [6, 18]. In these experiments we have used two antibodies, one which recognises the hypo-phosphorylated form (referred to as 'active') and another that will detect all β-catenin protein regardless of its phosphorylated state (referred to as 'total').

As expected, treatment of INS-1 cells with Wnt3a caused an increase in the levels of both total and active β-catenin protein in the cytoplasmic and nuclear cell protein fractions (Fig 1C+D). In contrast, treatment of INS-1 cells with Wnt4 didn't increase protein levels of active β-catenin, but surprisingly did increase protein levels of total β-catenin in both cytoplasmic and nuclear fractions (Fig 1C+D). When INS-1 cells were treated with a combination of Wnt3a and Wnt4, Wnt3a mediated increases in cytoplasmic and nuclear active β-catenin were prevented by Wnt4 (Fig 1C+D) despite the continued presence of elevated total β-catenin protein levels (Fig 1C+D).

mRNA levels of β-catenin were also measured in INS-1 cells treated with Wnt3a and Wnt4. No changes in β-catenin mRNA expression were detected with either ligand alone or in combination (Fig 2A), suggesting that, as expected from the mechanism of the Wnt signalling pathway, any changes in protein levels of β-catenin caused by Wnt3a or Wnt4 were not due to changes in gene expression.

To test the idea that Wnt4 acts as a receptor antagonist of Wnt3a [14], cells were treated with the specific GSK3β inhibitor, 1-AKP to activate the Wnt signalling pathway downstream

of the Wnt3a receptor. Wnt4 was able to inhibit the increase in active β-catenin protein stimulated by 1-AKP (Fig 2B), suggesting that inhibition of Wnt3a mediated signalling by Wnt4 is not due to antagonism at the receptor level.

Wnt4 has previously been shown to increase levels of β-catenin in the plasma membrane, suggesting that this might be a mechanism by which it could antagonise canonical Wnt signalling [21]. We investigated this theory and found that both Wnt3a and Wnt4 increased incorporation of β-catenin into the cell membranes, with no additive effect when both ligands were used in combination (Fig 2C).

To determine if Wnt4 prevents Wnt3a stimulated cell growth through inhibition of β-catenin mediated gene transcription, mRNA levels of cyclin D2, a key target gene of canonical Wnt signalling, was measured. As expected treatment of INS-1 cells with Wnt3a increased levels of cyclin D2 mRNA. Unexpectedly however treatment with Wnt4 alone had a similar effect and did not prevent the Wnt3a stimulated increases in cyclin D2 expression (Fig 2D).

Wnt4 expression and localisation:

Having shown that β-cells can respond to exogenous treatment with Wnt4 we sought to determine the potential source of this ligand using immunohistochemistry in human islet sections. In agreement with Lee et al [23] we found strong expression of Wnt4 in α-cells (Fig 3). However there was also staining for Wnt4 detectable at a lower intensity throughout the islet in insulin positive cells. These data suggest that in islets Wnt4 is expressed in both α and β-cells, with higher levels detected in α-cells.

To determine if the Wnt4 detected in the β-cells has a functional role we reduced levels of Wnt4 in INS-1 cells using Wnt4 specific siRNA (Fig 4A). This reduction of Wnt4 protein didn't cause any change in the basal rate of cell growth (Fig 4B). In addition β-cells with reduced Wnt4 expression didn't show any enhancement in Wnt3a mediated increases in active β-catenin levels when treated with Wnt ligands (Fig 4C), suggesting that endogenous Wnt4 doesn't moderate the signalling activity stimulated by exogenous sources of Wnt3a.

To establish whether Wnt4 is secreted from β-cells we measured Wnt4 in the culture media of INS-1 cells. Despite Wnt4 protein being detectable in the cell lysates, only very low levels

of Wnt4 were detectable in the cell media, suggesting that Wnt4 is not secreted by INS-1 cells (Fig 4D).

Discussion:

There is considerable interest in the action of Wnt signalling in modulating β-cell growth and function, with the implication that changes in this pathway may be causative in the on-set of β-cell dysfunction that occurs in type 2 diabetes. In this paper we present unique functional data showing that Wnt4 is able to inhibit Wnt3a stimulated increases in β-cell growth and glucose stimulated insulin secretion.

Interestingly previous studies have suggested that canonical Wnt signalling is not active in mature islets [14, 23]. It seems plausible that the lack of activity may be due to high levels of Wnt4 present in pancreatic islets inhibiting canonical Wnt signalling. Expression of Wnt4 could potentially be important to maintain β-cell phenotype and prevent dysregulated increases in proliferation and insulin secretion by suppressing the activation of canonical Wnt signalling. This correlates with a recent publication in which an effector of a non-canonical Wnt signalling pathway was found to be a marker of mature non-proliferating β-cells [26].

Experiments in this paper show that Wnt4 is highly expressed in human α-cells, with lower levels detected in β-cells, raising the possibility that β-cells may be exposed to Wnt4 from both exogenous and endogenous sources in the islets. Our data shows that β-cells are capable of responding to exogenously applied purified recombinant Wnt4 protein as treatment of INS-1 cells with recombinant Wnt4 protein was able to inhibit increases in cell growth and insulin secretion mediated by Wnt3a. However when endogenous levels of Wnt4 were reduced in the INS-1 cells lines, no impact on cell growth or Wnt signalling activation was measurable. There was also no change in cell growth rates of INS-1 cells with reduced Wnt4 expression. This finding differs from data presented by Heller et al (2011) [13] who also used Wnt4 siRNA in INS-1 cells, but found a decrease in cell growth. This difference

may be due to differing levels of knockdown or Wnt4 or different detection methods of cell proliferation.

In addition we found that cells with reduced levels of Wnt4 protein did not have an increased response to treatment with Wnt3a when measuring increases in active β-catenin levels. This suggests that endogenously expressed Wnt4 in β-cells does not have a role in regulating β-cell function and correlates with the data showing that Wnt4 is not secreted by INS-1 cells. Evidence suggests that Wnt4 would need to be secreted from the cells to activate cell signalling as the frizzled receptors are cell surface receptors and there are no reports to suggest an intracellular role of any Wnt ligands. However an *in vivo* exogenous source of Wnt4 acting on β-cells has yet to be proven. The high levels of Wnt4 expressed in α-cells, reported here and by others [23] points to an intra islet source of Wnt4. However a recent paper suggests that Wnt ligands secreted by adipose and muscle tissue can impact β-cell function [15].

Previous reports have proposed various mechanisms by which Wnt4 may antagonise the canonical Wnt signalling pathway. One of these is that Wnt4 may act as a receptor antagonist to Wnt3a preventing Wnt3a activation of target cell surface receptors [14]. The results presented here show that Wnt4 is able to inhibit increases in active β-catenin levels mediated by the specific GSK3β inhibitor 1-AKP. This would suggest that Wnt4 is able to inhibit the canonical Wnt pathway at or downstream of GSK3β, rather than acting as a receptor antagonist. Another possibility is that Wnt4 can increase β-catenin incorporation in adhesion junctions in the cell membrane [21] thus inhibiting canonical Wnt signalling by reducing the pool of β-catenin available for mediating gene transcription. We find that both Wnt3a and Wnt4 increase levels of β-catenin protein in the membrane fraction in INS-1 cells, with no further increase when cells are treated with both ligands together. This suggests increased incorporation of β-catenin at the adhesion junctions is not the mechanism by which Wnt4 inhibits Wnt3a mediated signalling.

As expected from an inhibitor of the canonical Wnt signalling pathway we find that Wnt4 is able to inhibit the Wnt3a mediated increase in the hypo-phosphorylated (‘active’) form of β-

catenin increased levels. Unexpectedly though for a proposed 'non-canonical' Wnt ligand Wnt4 itself lead to increased levels of 'total' β-catenin throughout the cell including the nucleus, cytoplasm and membrane. Under normal physiological conditions GSK3β phosphorylated β-catenin is rapidly degraded in the proteasome ensuring that cytoplasmic β-catenin proteins levels remain low [27]. Wnt4 treatment did not increase the expression of the β-catenin mRNA suggesting that increased β-catenin protein levels are due to reduced proteasomal degradation rather than increased gene transcription. As Wnt4 did not increase levels of 'active' β-catenin, the increased β-catenin seen with Wnt4 is presumably still phosphorylated by GSK3β, suggest that Wnt4 may disrupt the ubiquitin proteasome pathway or stabilise β-catenin by phosphorylating the protein on a different residue. This GSK3β independent stabilisation of β-catenin has recently been described in tumour cells in response to unsaturated fatty acids in [28].

In the nucleus β-catenin acts, in combination with tcf/lef, as a transcription factor leading to increased transcription of β-catenin sensitive genes, such as cyclin D2. Interestingly we found that Wnt4, despite leading to decreases in active β-catenin in the nucleus, wasn't able to prevent Wnt3a activation of cyclin D2. More surprisingly, Wnt4 itself was able to increase expression of cyclin D2 in β-cells, presumably due to its ability to increase levels of total β-catenin in the nucleus. These observations imply that in addition to Wnt4 preventing increases in active β-catenin levels, it may also be able to act downstream of β-catenin mediated gene transcription to prevent Wnt3a mediated cell growth and insulin secretion.

In conclusion the present results provide strong evidence that Wnt4 is able to functionally inhibit the actions of canonical Wnt signalling in pancreatic β-cells, leading to the inhibition of Wnt3a mediated increases in cell growth and insulin secretion. As such given the strong link of canonical Wnt signalling with diabetes on-set we suggest that modulation of Wnt4 protein expression in islets or targeting Wnt4 signalling pathways may represent a potential new target for the treatment of diabetes.

Declaration of interest:

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions: AB designed and performed experiments, analysed the data, and wrote the paper. JW and KK supervised the work. SR supervised the work and made available facilities and resources. HJW conceived the study, designed and performed experiments, supervised the work, and wrote the paper. All authors assisted in editing of the manuscript and approved the final version.

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Figure Legends:

Figure 1. Functional effects of Wnt4 on INS-1 cells.

A: INS-1 cells were treated with 10ng/ml of Wnt3, Wnt4 or Wnt3a + Wnt4. Cells were retreated every 24hrs. After 72hrs of treatment cell numbers were determined by vital dye staining. **B:** INS-1 cells were pretreated for 24hrs with 10ng/ml of Wnt3a, Wnt4 or Wnt3a+Wnt4. Cells were then incubated for 2hrs at 2.8mM glucose before 1hr stimulation with 5.6 or 16.7mM glucose media, all in the continued presence of the Wnt ligands. The media was then sampled for use in a radioimmunoassay to measure insulin secretion.

INS-1 cells were treated with 10ng/ml of Wnt3, Wnt4 or Wnt3a + Wnt4 for 24hrs before extraction of: **C;** cytoplasmic or **D;** nuclear protein for use in western blotting. Antibodies that recognise either total levels (total) or only the hypo-phosphorylated (active) form of beta-catenin were used. Expression levels were quantified and normalised to tubulin (C) or histone H3 (D) expression levels.

Figure 2: *Wnt4 regulation of β-catenin expression and localisation.*

A INS-1 cells were treated with 10ng/ml of Wnt3, Wnt4 or Wnt3a + Wnt4 for 24hrs before extraction of mRNA for PCR. The mRNA was converted to cDNA and used in qRT-PCR to measure β-catenin mRNA levels. The results are normalised to two housekeeping genes (GAPDH and HPRT). Results are expressed as mean ± SEM.

B: INS-1 cells were treated with either 5μM 1-AKP, 10ng/ml Wnt4 or 5μM 1-AKP and 10ng/ml Wnt4 for 24hrs and the whole cell protein extracted. Active and total β-catenin levels were measured by western blotting and normalised to tubulin. Data shown as mean values ± SEM, n=3 * $p<0.05$ compared to the control.

C: INS-1 cells were treated with Wnt3a, Wnt4 or Wnt3a and Wnt4 (10ng/ml) for 24hrs and the membrane fraction extracted. Total β-catenin levels were measured by western blotting and normalised to sodium potassium ATPase. Data shown as mean values ± SEM, n=3 * $p<0.05$ compared to the control.

D: INS-1 cells were treated with Wnt3a, Wnt4 or Wnt3a and Wnt4 (10ng/ml) for 24hrs and the mRNA extracted. The mRNA was converted to cDNA and used in qRT-PCR to detect

cyclin D2 mRNA levels. The results are normalised to two housekeeping genes (GAPDH and HPRT) and expressed relative to the control. Data presented as the mean values ± SEM. n=3. **p<0.01 compared to the control

Figure 3: *Expression of Wnt4 in human islets*

Human pancreatic sections were incubated with antibodies against **A:** insulin 1/600 for 1hr (light blue) **B:** Wnt4 (AF475) overnight 1/25 (red) and **C:** glucagon 1/2000 for 1hr (green). Nuclei were stained with DAPI 1/1000 (blue). Following primary antibody incubation the tissue was incubated with the appropriate secondary antibody 1/400 for 1hr. E: Wnt4 (AF475) Glucagon, insulin and dapi signals are overlaid. Scale bars = 25µm

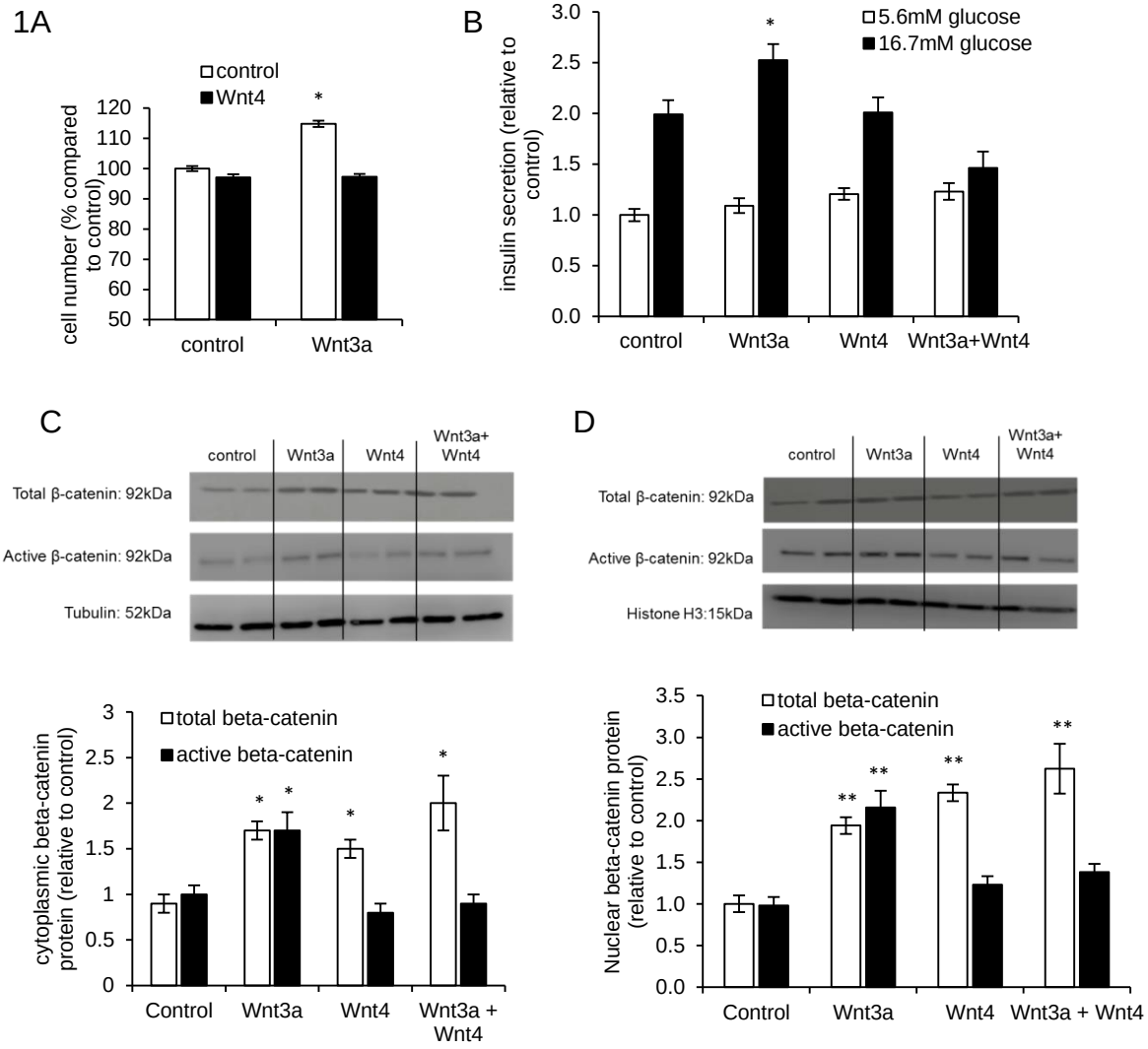
Figure 4. *Function of exogenously expressed Wnt4 in INS-1 cells.*

A: Protein was extracted from INS-1 cells transfected with siRNA specific to Wnt4 or a scrambled control. Wnt4 protein expression was measured by western blotting and normalised to tubulin. Data shown as mean values ± SEM. ** p<0.001 compared to the scrambled control, n=3

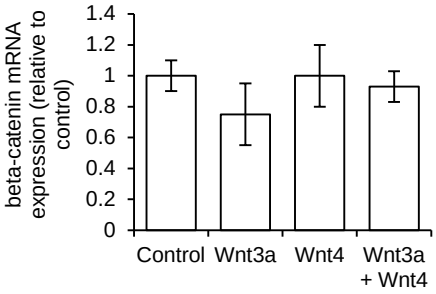
B: siRNA Wnt4 and scrambled control INS-1 cells were grown in 6 well plates for 72hrs and the total cell number counted and expressed as a fold increase over the number of cells seeded. Data shown as mean values ± SEM. n=3

C: Scrambled siRNA and Wnt4 siRNA INS-1 cells were treated for 24hrs with Wnt3a (10ng/ml) and the whole cell protein extracted. Active and total β-catenin protein levels were measured by western blotting and normalised to tubulin. Data shown as mean values ± SEM. * p<0.01 n=3

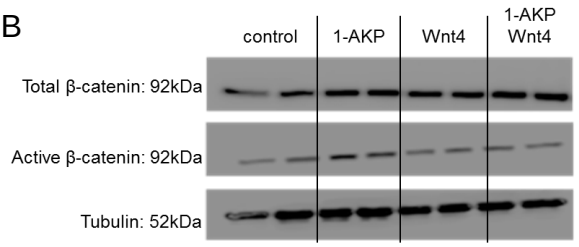
D: INS-1 cells were cultured for 72hrs before the protein was extracted and cell media removed for measurement of Wnt4 by ELISA. Data shown as mean values ± SEM. n=3.

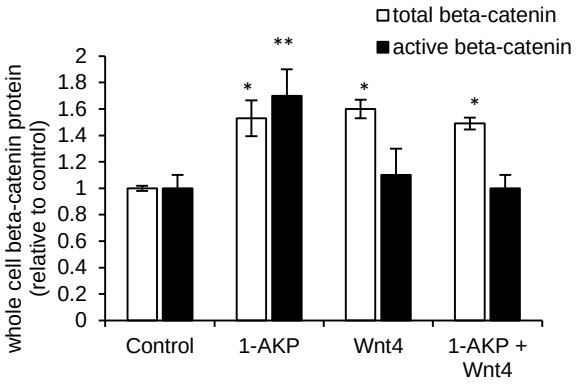

1A
control
Wnt4
cell number (% compared to control)
120
110
100
90
80
70
60
50
control
Wnt3a
B
5.6mM glucose
16.7mM glucose
insulin secretion (relative to control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
Wnt3a
Wnt4
Wnt3a+Wnt4
C
control
Wnt3a
Wnt4
Wnt3a+ Wnt4
Total β-catenin: 92kDa
Active β-catenin: 92kDa
Tubulin: 52kDa
total beta-catenin
active beta-catenin
cytoplasmic beta-catenin protein (relative to control)
3
2.5
2
1.5
1
0.5
0
Control
Wnt3a
Wnt4
Wnt3a + Wnt4
D
control
Wnt3a
Wnt4
Wnt3a+ Wnt4
Total β-catenin: 92kDa
Active β-catenin: 92kDa
Histone H3:15kDa
total beta-catenin
active beta-catenin
Nuclear beta-catenin protein (relative to control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Wnt3a
Wnt4
Wnt3a + Wnt4

2A

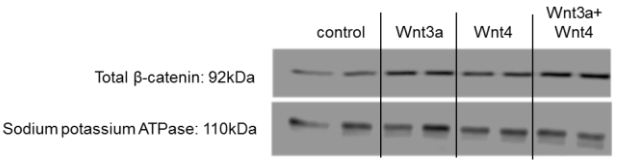

beta-catenin mRNA expression (relative to control)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Wnt3a
Wnt4
Wnt3a + Wnt4

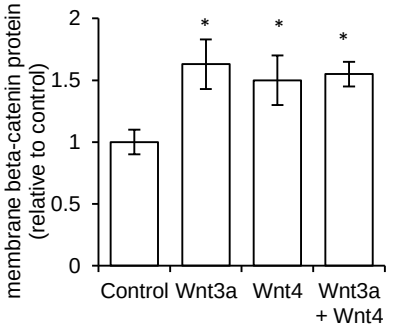
B


control
1-AKP
Wnt4
1-AKP Wnt4
Total β-catenin: 92kDa
Active β-catenin: 92kDa
Tubulin: 52kDa

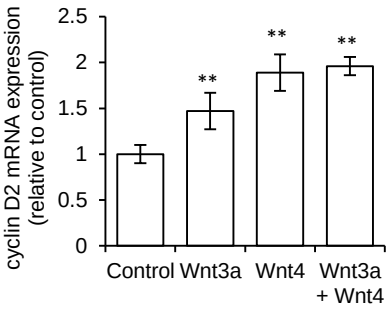

total beta-catenin
active beta-catenin
whole cell beta-catenin protein (relative to control)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
*
*
Control
1-AKP
Wnt4
1-AKP + Wnt4

C

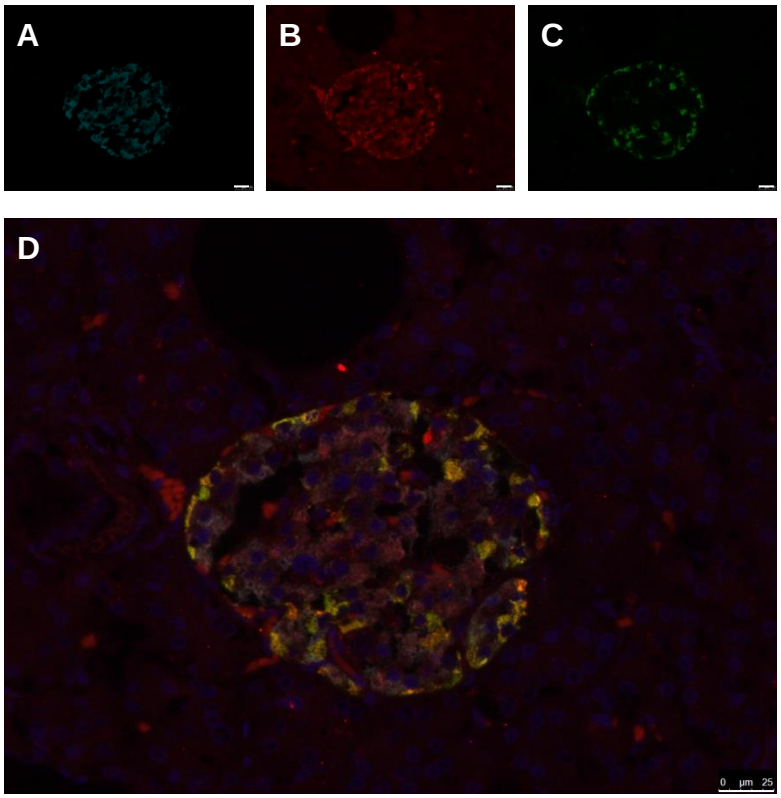

control
Wnt3a
Wnt4
Wnt3a+ Wnt4
Total β-catenin: 92kDa
Sodium potassium ATPase: 110kDa


membrane beta-catenin protein (relative to control)
2
1.5
1
0.5
0
*
*
*
Control
Wnt3a
Wnt4
Wnt3a + Wnt4

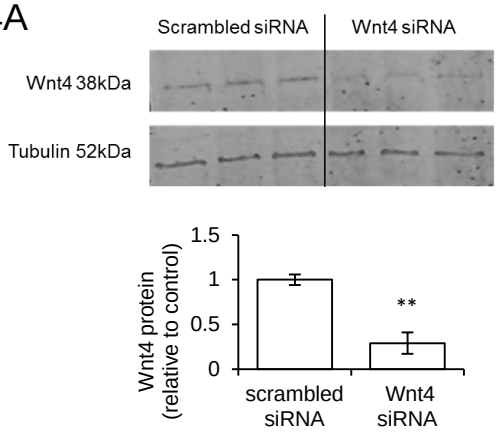
D


cyclin D2 mRNA expression (relative to control)
2.5
2
1.5
1
0.5
0
**
**
**
Control
Wnt3a
Wnt4
Wnt3a + Wnt4

3

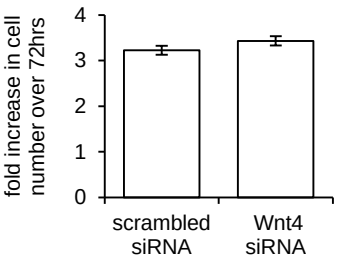


4A

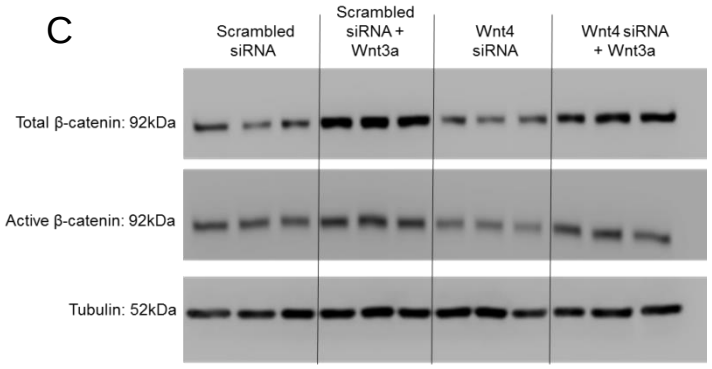

Scrambled siRNA
Wnt4 siRNA
Wnt4 38kDa
Tubulin 52kDa

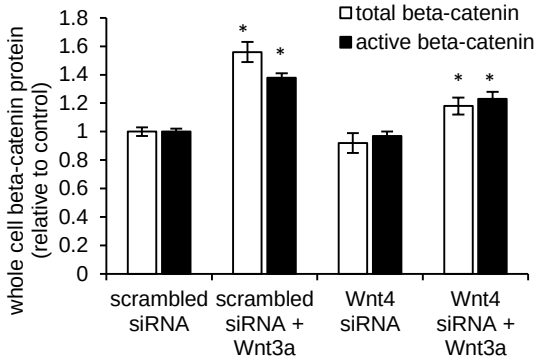
Wnt4 protein (relative to control)
1.5
1
0.5
0
**
scrambled siRNA
Wnt4 siRNA

B

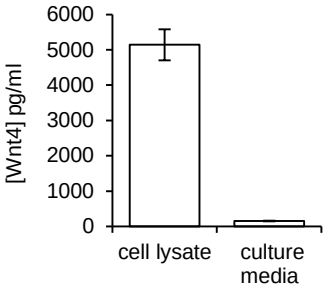

fold increase in cell number over 72hrs
4
3
2
1
0
scrambled siRNA
Wnt4 siRNA

C


Scrambled siRNA
Scrambled siRNA + Wnt3a
Wnt4 siRNA
Wnt4 siRNA + Wnt3a
Total β-catenin: 92kDa
Active β-catenin: 92kDa
Tubulin: 52kDa


whole cell beta-catenin protein (relative to control)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
total beta-catenin
active beta-catenin
*
*
*
*
scrambled siRNA
scrambled siRNA + Wnt3a
Wnt4 siRNA
Wnt4 siRNA + Wnt3a

D


[Wnt4] pg/ml
6000
5000
4000
3000
2000
1000
0
cell lysate
culture media

ACCEPTED MANUSCRIPT

- Wnt4 prevents Wnt3a from increasing growth and insulin secretion in β-cells
- Wnt4 inhibits canonical Wnt signalling despite increasing total β-catenin levels
- Exogenously expressed Wnt4 in β-cells has no impact on canonical Wnt signalling

ACCEPTED MANUSCRIPT