Chapter four: Stimulation of exogenous β-catenin expression in WHCO1 cells

4.1 Introduction

In the cellular environment, the expression of β-catenin is controlled endogenously at stages such as the transcriptional and post-translational level. At the transcriptional level, factors such as NF-κB, and AP-1, are involved in the expression of *CTNNB1* (Li *et al*., 2004; Nollet *et al*., 1996). The expression of β-catenin at the post-translational level is controlled by negative or positive regulators such as GSK-3β and wnt signalling, respectively (Moon *et al.,* 2004).

In order to get a handle on the effects of gene expression, it is possible to regulate genes such as β-catenin via exogenous means. This may be accomplished by the use of drugs (e.g. lithium), antisense mRNA technology, RNA interference and gene expression systems (Ni *et al*., 2003). Gene expression systems entail the transfection of a vector containing the desired gene into cells and subsequently stimulating its expression. The Complete Control ® Inducible Mammalian Expression System applied in this project is based on research done by No *et al*., (1996). This expression system involves a double vector system: one contains specific unique receptor and another specific gene of interest. Once cells are stably transfected with these vectors, the expression of the gene can be induced with ponasterone A (ponA), an analogue of ecdysone (appendix 6.18).

The mechanism employed by this system to stimulate expression of a chosen gene, is as follows: the basal transcriptional machinery binds to a minimal promoter, which is upstream of the MCS region of pEGSH vector (see vector map, section 3.2.2). Upstream of the minimal promoter is the synthetic ecdysone/glucocorticoid responsive element recognition sequence (E/GRE) AGTGCA N_1 TGTTCT. The pERV3 vector gene products: synthetic ecdysone binding receptor (EcBR) and Retenoid-x-Receptor (RxR) are constitutively

expressed nuclear receptors, which form a heterodimer. This heterodimer recognises and binds to the E/GRE sequence (Figure 4.1).

In the absence of ponA, the receptor heterodimer interacts with repressors of the basal transcriptional machinery to suppress transcription of chosen gene (Figure 4.1.a). PonA binds to EcBR initiating the interaction of the receptor heterodimer with activators of the basal transcriptional machinery and leading to transcription of a chosen gene (Figure 4.1.b). The advantage of using this expression system is that only ponA can activate transcription of exogenous (pEGSH contained) gene and not hormones or growth factors secreted by transfected cells.

Aim:

Following the transfection of pEGSH-β-catenin into WHCO1, the aim was to: 1) analyse the presence of pEGSH-β-catenin vector in tWHCO1; 2) confirm the presence of β-catenin protein in tWHCO1 with appropriate controls and 3) stimulate expression of β-catenin in tWHCO1 to increase cellular β-catenin levels.

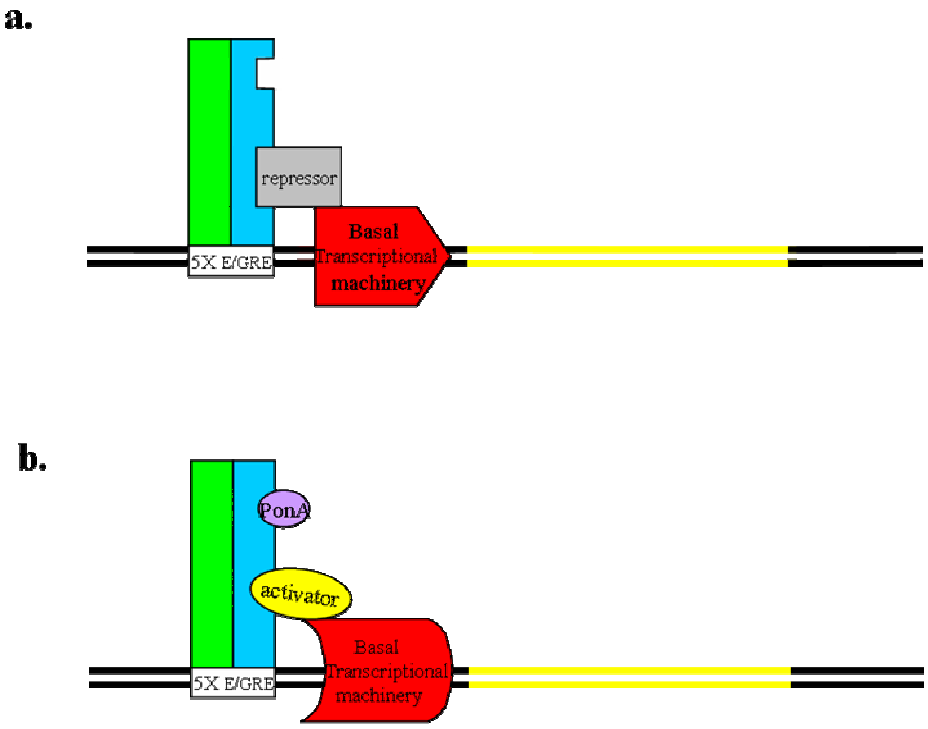


Figure 4.1 Activation of gene expression via ponA. The EcBR (blue rectangle) RXR (green) heterodimer binds to the E/GRE sequence. The basal transcriptional machinery (red), binds to the minimal promoter. **(a)** In the absence of ponA, the heterodimer binds to repressors (grey) and inhibit transcription. **(b)** In the presence of ponA (purple), the heterodimer binds to activators (yellow) and activate transcription. This leads to the transcription of the chosen gene (double yellow line). This figure is based on the diagram of the Complete Control® Inducible Mammalian Expression System.

4.2 Materials and methods

4.2.1 Tissue culture

Tissue culture was performed according to the procedure described in materials and methods, section 2.2.1.

4.2.2 DNA extraction from tWHCO1

DNA extraction was performed using the DNeasy Tissue Kit (Qiagen). The procedure was performed according to the manufacturer's protocol. The tWHCO1 cells were rinsed 3 times with PBS, scraped into the PBS and centrifuged for 5 min in Tomy HF-120. The resultant pellet was suspended in 200 µl of PBS. Proteinase K (20 µl) and 200 µl of buffer AL were added to the pellet. The contents were mixed via vortex (Genie-2TM) and incubated at 70^{o}C for 10 min. Subsequently 200 µl of 99.99% ethanol was added to the mixture and vortexed for 2 sec.

The mixture was added to a DNeasy Mini spin column placed in a 2 ml collection tube (Qiagen) and centrifuged at 6000 x *g*, for 1 min. Buffer AW2 (500 µl) was added to DNeasy Mini spin column, which was placed in a new 2 ml collection tube, and centrifuged at 8000 x *g* for 3 min. Buffer AE (200 µl) was added to the DNeasy Mini spin column, which was placed in an Eppendorf tube. The DNeasy Mini spin column in the Eppendorf tube was incubated at T_R for 1 min and centrifuged at 6000 x *g* for 1 min. Eluate was collected and comprised the DNA fraction. A further 200 µl of buffer AE was added to the DNeasy Mini spin column. The column was placed in a new Eppendorf tube, incubated at T_R for 1 min and centrifuged at 6000 x *g* for 1 min. Eluate was collected and stored in a sterile Eppendorf at -20^{o}C.

4.2.3 Primers

5′-MCS-primers:

FP_P and RP_β (for more detail, see section 3.2.10) used to amplify a region of 609 bp.

3′-MCS-primers:

β-catenin forward primer (FP_β), 5′ GAAACGGCTTTCAGTTGAGC 3′
(Inqaba Biotech)

FLAG reverse primer (RP_f), 5′ ATATTTCTTCCGGGGACACC 3′
(Inqaba Biotech)

These primers were used to amplify a region of 783 bp from the β-catenin insert and pEGSH within the pEGSH-β-catenin vector.

For position of the above primers with respect to the vector see appendix 6.15.1.

GAPDH primers:

Forward GAPDH primer, (FP_{GAPDH}), 5′ TTAGATTTGGTCGTATTGGG 3′ (Qiagen) (Lawinger *et al*., 1999).

Reverse GAPDH primer, (RP_{GAPDH}), 5′ TGATTTTGGAGGGATCTCGC 3′ (Qiagen) (Lawinger *et al*., 1999).

These primers were used to amplify a region of 431 bp from the genomic glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

4.2.4 Polymerase chain reaction (PCR)

PCR was performed according to the procedure described in materials and methods, section 3.2.11, with the following exceptions: primers that were used in the PCR were the ones described in section 4.2.3. The annealing temperature was set at 50^{o}C for the GAPDH primers and at 55^{o}C for primer set 1 and 2. The DNA fraction (1:50) (section 4.2.2.) was used as a template for all sets of primers. As a positive control, primer set 1 and 2 were used to amplify their respective regions with 0.14 μg of the pEGSH-β-catenin vector as a template. Following the PCR reaction, the PCR products were electrophoresed on a 0.8% agarose gel.

4.2.5 Transient transfection of WHCO1-pERV3 cells with pEGSH

WHCO1-pERV3 cells were transfected with pEGSH vector according to the transfection procedure described in section 3.2.12, with the following exceptions: to transfect the cells, 12 μl of FuGENE6 reagent was added to 85 μl of medium and subsequently 3 μg of pEGSH vector was added to form a transfection mixture. The WHCO1-pERV3 cells transiently transfected with pEGSH were passaged 24 hours following transfection. The resultant cells designated as WHCO1-pERV3-pEGSH, were used to carry out the experiments following 48 hours of transfection.

4.2.6 Treatment of tWHCO1 and WHCO1-pERV3-pEGSH cells with ponA

PonA (Stratagene) solution was made by dissolving 1 mg of ponA in 1 ml of 99.99% ethanol. The WHCO1-pERV3-pEGSH cells were treated with 1 μM ponA for 8 hours. The tWHCO1 cells were treated with 1 μM of ponA for 3, 8 and 72 hours (Jiang *et al*., 2003). The tWHCO1 cells were also treated with 5 μM of ponA for 8 hours. The tWHCO1 clones were treated with 1 μM ponA for 8 hours and one of the clones was also treated with 1 μM ponA for 12 hours. As a control, 99.99% ethanol, without ponA, was added to the cells.

4.2.7 Whole cell extraction

Whole cell extractions were performed on ponA treated or non-treated (control) WHCO1-pERV3-pEGSH, tWHCO1 and tWHCO1 clones. The cells were rinsed 3 times in PBS. Cells were scraped into PBS and centrifuged in the Tomy HF-120 for 10 min. Excess PBS was removed and a volume of single lysis buffer (appendix 6.19.1), of approximately 5 times the size of the pellet, was added. The contents were vortexed (10 sec), centrifuged in the Tomy HF-120 (10 sec) and immersed in boiling water for 5 min. This was followed by a 20 min centrifugation at 6000 x *g* at 4^{o}C. The whole cell extracts are referred to as cell samples in the following text.

4.2.8 Protein estimation of cell samples

Protein estimation of the cell samples was performed according to the procedure described in section 2.2.4, with the following exceptions: the BSA used to create the standard curve, was dissolved in single lysis buffer.

4.2.9 SDS-PAGE gel

A 10% SDS-PAGE gel was performed according to the procedure described in section 2.2.6, with the following exceptions: only 3.5 μg and 0.5 μg of cell samples of WHCO1-pERV3-pEGSH control and treated cells respectively were loaded onto the gel, due to availability. The cell samples of tWHCO1 and tWHCO1 clones were loaded (8 μg) onto the gel.

4.2.10 Western blot

A western blot was performed according to the procedure described in section 2.2.8. The densitometric analysis of β-catenin expression level in the western blots was calculated by computer program *Labworks version 4.5.* **Note:** since a 7:1 volume of WHCO1-pERV3-pEGSH control vs. treated cells was loaded, the densitometry data thus obtained, of the β-catenin expression level in the western blot of WHCO1-pERV3-pEGSH treated cells, needed to be multiplied by 7.

4.2.11 Treatment of WHCO1 with DMSO

WHCO1 were treated with 1% DMSO. Whole cell extraction, protein estimation, SDS-PAGE and western blot of control vs. treated cells were performed according to the procedure described in sections 4.2.7 to 4.2.10.

4.2.12 Computer analysis programs

Labworks ^{TM} Image Acquisition and Analysis software (Labworks version 4.5). MIT Primer3 program (section 3.2.14) was used to design FP_{β} and RP_{f} primers. mFold 3.0. program (section 3.2.14) was used to determine the temperature at which the primers RP_{β} and RP_{f} form secondary structures.

4.3 Results

4.3.1 The presence of pEGSH-β-Catenin in tWHCO1

Although according to the antibiotic resistance criteria the tWHCO1 line stably contains pERV3 and pEGSH-β-catenin (Chapter 3, section 3.3.7 and 3.4), it would reinforce the result to additionally demonstrate the presence of pEGSH-β-catenin in tWHCO1. Primers specific for either pEGSH or β-catenin insert, were used for the amplification of a region containing both pEGSH and β-catenin insert sequences. If amplification with these primers from DNA extract of tWHCO1 was successful, then the presence of pEGSH-β-catenin within tWHCO1 would be unequivocal.

Primers were designed for the specific amplification of the 3′ flanking region of β-catenin insert within pEGSH. This region extends from β-catenin insert (401 bp) to pEGSH (382 bp). A fragment between 650 and 850 bp was produced after amplification of this region from pEGSH-β-catenin (Figure 4.2, grey arrow). This corresponds to the size of the desired fragment (783 bp). In Chapter 3, it was mentioned that the 5′ flanking region of β-catenin insert within pEGSH-β-catenin, was successfully amplified with primers specific for the pEGSH vector and β-catenin insert (section 3.3.5). Therefore the primers used for amplification of the 5′ and 3′ end regions of β-catenin insert within pEGSH were suitable for the amplification of these regions from DNA extract of tWHCO1. However, amplification of DNA extracted from tWHCO1 was unsuccessful.

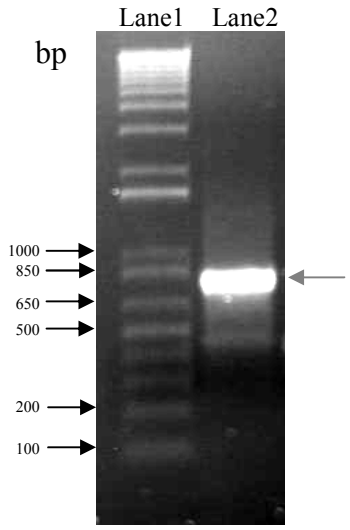


Figure 4.2 The 3′ region of β-catenin insert within pEGSH-β-catenin. An agarose gel separation of: 1 kb ladder (Lane1) and amplification of the region flanking the 3′ end of β-catenin insert from pEGSH-β-catenin sample (Lane2). Amplification product (grey arrow), was present at 783 bp. Gel = 0.8%

Consequently, it was necessary to examine the stability of the extracted DNA. This was achieved by the amplification of the housekeeping gene, *GAPDH* (Ercolani *et al*., 1988). Two fragments just below 450 bp and 220 bp (Figure 4.3, black and green arrows respectively) were produced after amplification with GAPDH specific primers, from the DNA extract of tWHCO1. The fragments correspond to the genomic *GAPDH* (431 bp) and *GAPDH* degradation product respectively. It is also possible that the fragment below 220 bp belongs to *GAPDH* mRNA (209 bp) which may have leaked into the DNA extraction.

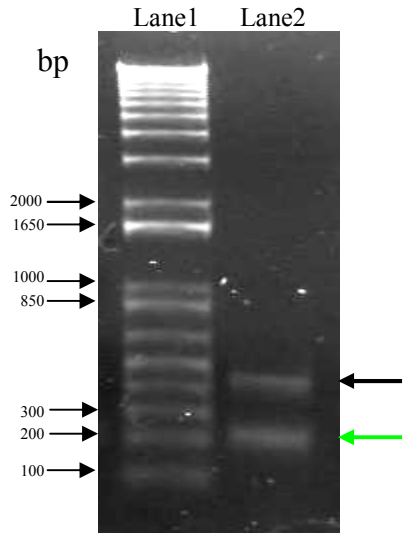


Figure 4.3 Amplification of the *GAPDH* gene from DNA fraction of tWHCO1. An agarose gel separation of 1 kb ladder (Lane1) and amplification of *GAPDH* from DNA extract (Lane2). The genomic *GAPDH* (black arrow) amplified fragment was present at 431 bp. Another amplified fragment (green arrow) was present below 220, which is possibly a degradation product or *GAPDH* mRNA (209 bp). Gel = 0.8%

4.3.2 The presence of β-catenin protein in tWHCO1

A 92 kDa band in the SDS-PAGE separation of the whole cell extract of tWHCO1 identifies a polypeptide with the correct molecular weight of β-catenin (Figure 4.4.a, blue arrow). The presence of β-catenin was confirmed by performing a western blot on this separation (Figure 4.4.b), with the application of the specific anti-β-catenin antibody used previously (Chapter 2, section 2.3.1). Degradation products of β-catenin were also present in the cell extract of tWHCO1 (Figure 4.4.b, green arrow). These results identified that the method of performing the whole cell extraction was successful for β-catenin and therefore used in all following procedures.

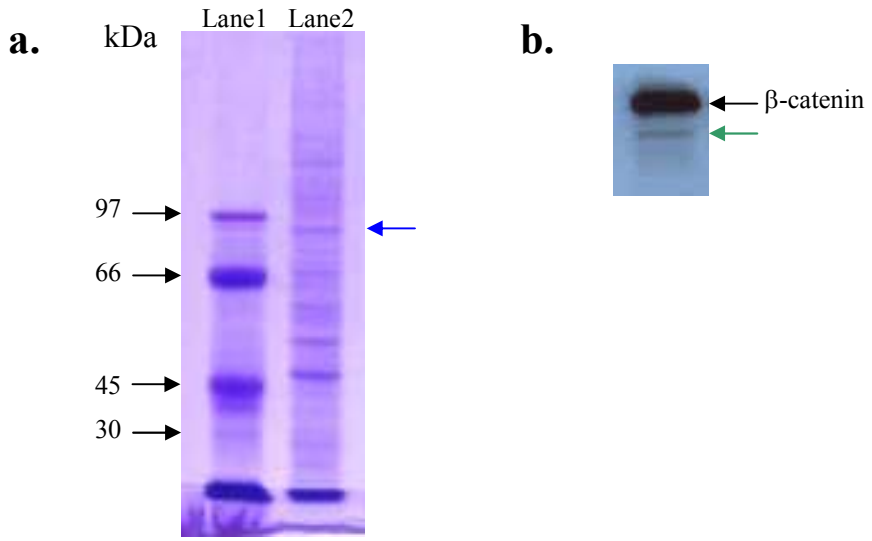


Figure 4.4 SDS-PAGE separation and western blot of β-catenin in the cell extract of tWHCO1. **(a)** A SDS-PAGE separation of molecular weight marker (Lane1) as well as whole cell extract (Lane2). A band at 92 kDa, (blue arrow) was present in the whole cell extract. **(b)** Western blot confirming the presence of β-catenin. Green arrow represent degradation product. All gels = 10%

4.3.3 Stimulation of WHCO1-pERV3-pEGSH with ponA

PonA was used for the stimulation of exogenous (pEGSH contained) β-catenin expression. It was therefore necessary to ensure that ponA stimulation does not have an effect on endogenous β-catenin. The WHCO1-pERV3-pEGSH (does not contain β-catenin insert) acting as a control line, was used for this purpose.

Western blots confirmed the presence of β-catenin in the cell extract of ponA treated vs. non-treated WHCO1-pERV3-pEGSH cells (Figure 4.5.a). Since extraction produced a low concentration of proteins, only 3.5 μg of non-treated and 0.5 μg of treated cell extracts were SDS-PAGE separated in preparation for western blot. Densitometric analysis of western blot (*Labworks version 4.5*) relative to the loading concentration, revealed that there was no difference of β-catenin expression in non-treated (97%) and treated cells (100%) (Figure 4.5.b, histogram). Stimulation with ponA was considered to have no specific effects on β-catenin expression in control cells (do not contain exogenous β-catenin) and hence used in following experiments.

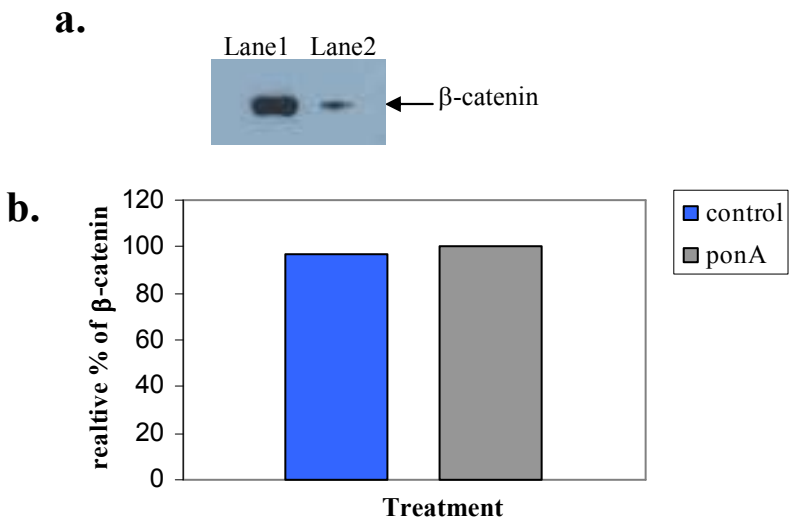


Figure 4.5 Treatment of WHCO1- pERV3-pEGSH with ponA. **(a)** Western blot confirming presence of β-catenin in non-treated (Lane1) and ponA treated (Lane2) cells. **(b)** Histogram comparing control and ponA treated cell. β-catenin is presented in the histogram as a relative percentage, with the maximum expression level of β-catenin set at 100%. **NB:** β-catenin expression level of the western blot was calculated relative to loading concentration.

4.3.4 β-catenin expression in total cell population of tWHCO1 in response to ponA stimulation

The tWHCO1 cells (contain pERV3 and pEGSH-β-catenin) were treated with 1 μM ponA at different time intervals (3, 8 and 72 hours) in an attempt to stimulate the expression of exogenous β-catenin and increase the overall concentration of β-catenin in the cells.

Western blots confirmed the presence of β-catenin in the cellular extract of the total cell population of tWHCO1, following PonA treatment at different time intervals (Figure 4.6). Uniform loading concentration of the cell extracts permitted densitometric analyses to be performed. These analyses of the β-catenin expression in response to the PonA treatment are presented in Table 4.1. β-catenin expression did not increase after 3 and 72 hours of treatment (Table 4.1). However, β-catenin expression showed a small increase of 1.2 fold after 8 hours of treatment (Table 4.1). Assuming this represented a real stimulation, it was necessary to ascertain if the result was ponA concentration dependent. The tWHCO1cells were subsequently treated with 5 μM ponA for 8 hours, however there was no increase in β-catenin expression (Table 4.1).

Treatment of tWHCO1 with 1 μM ponA for 8 hours was repeated twice more, and western blots were performed. Statistical analysis (student *t*-test) of the densitometric data revealed that the difference between the mean of β-catenin expression level in non-treated vs. treated cells was 1.2 fold, which was not significant (null hypothesis was rejected at the 95% level; Figure 4.7).

Although β-catenin expression in the total cell population of tWHCO1 upon treatment with ponA was non-significant, the parameters used to treat the tWHCO1 cells were thus established. These parameters were used in subsequent experiments to examine β-catenin expression in tWHCO1 population, which was single-cell cloned from the parent population.

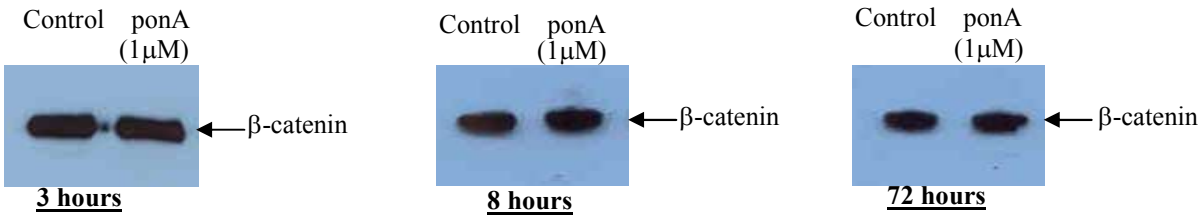


Figure 4.6 Western blots of β-catenin in total cell population of tWHCO1 following ponA treatment. The number of hours represent the time of treatment.

Table 4.1 Treatment of total cell population of tWHCO1 with ponA and its effect on β-catenin expression

Treatment with ponA (hours)	PonA concentration used	Increase of β-catenin expression level
3	1μM	no
8	1μM	1.2 fold increase
	5μM	no
72	1μM	no

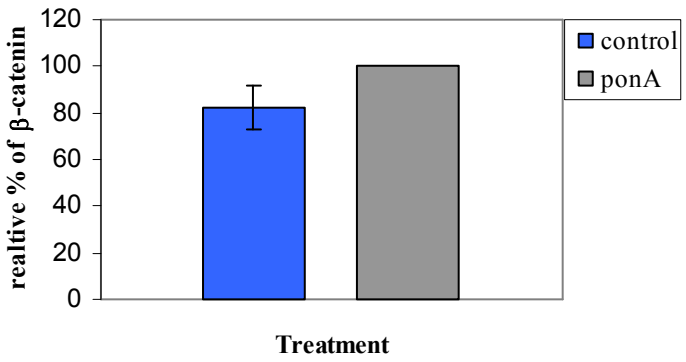


Figure 4.7 β-catenin expression in total cell population of tWHCO1 treated with ponA (8 hours). β-catenin is presented in the histogram as a relative percentage with maximum expression level of β-catenin set at 100%. The histograms represent the mean of β-catenin expression level, from 3 samples of non-treated (control) vs. ponA treated cells. The difference of the mean of β-catenin expression in non-treated vs. treated cells is 1.2 fold, which is insignificant. Bar represents standard error = 9.33

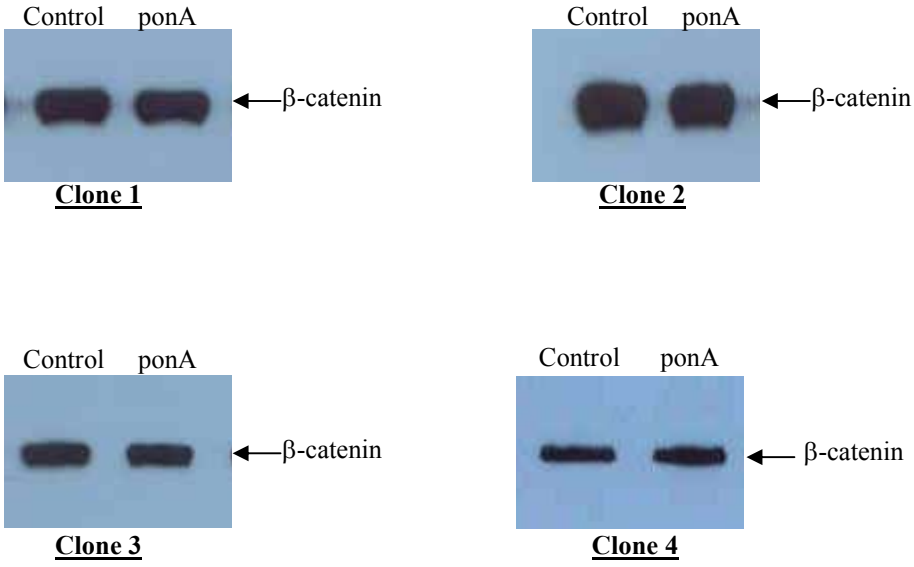
4.3.5 β-catenin expression in tWHCO1 clones

PonA stimulation of β-catenin expression was examined in 14 single cell initiated clones. It was necessary to find a tWHCO1-derived line in which stimulation leads to a uniform increase of β-catenin expression, and thus eliminate any possible variations when used in future experiments.

PonA treatment of the clones was followed by a western blot (Figure 4.8.a). Densitometric analysis of the western blot revealed that stimulation showed only small changes in β-catenin expression, ranging between a 1.1 fold decrease and a similar increase (Figure 4.8.b). In section 4.3.4, it was shown that an increase of 1.2 fold was insignificant, hence a smaller (1.1 fold) change was considered to be within the normal variation. Therefore, it was not possible to clone a population of cells that possessed a larger and uniform response to PonA stimulation of pEGSH-β-catenin expression.

Figure 4.8 illustrates 4 representatives of the clones. Treatment of clone 1 and clone 2 decreased β-catenin expression level by 1.1 fold, while treatment of clone 3 did not alter β-catenin expression (Figure 4.8.b). Treatment of clone 4 showed a small increase of 1.1 fold of β-catenin expression (Figure 4.8.b). Clone 4 was also ponA treated for 12 hours to examine whether a longer treatment time leads to an increase of β-catenin expression, however, no change was observed.

a.



b.

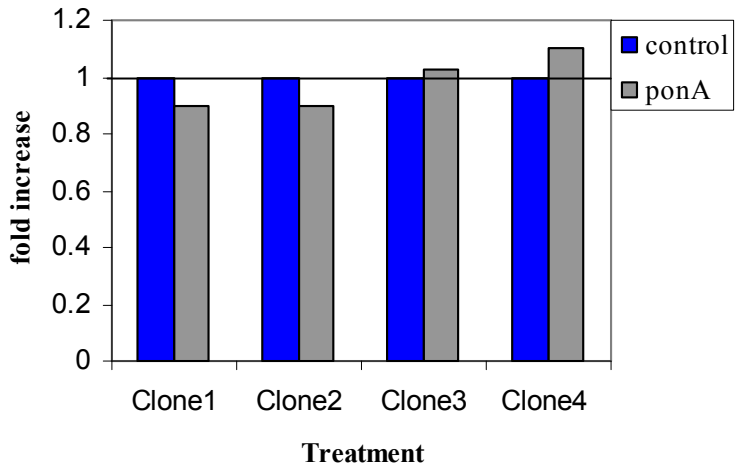


Figure 4.8 β-catenin expression levels in tWHCO1 clones treated with ponA. (a) Western blots of the various clones confirmed the presence of β-catenin. **(b)** Histogram representing the alteration of β-catenin expression in 4 representative tWHCO1 clones upon treatment with ponA. The non-treated control of each clone was set at 1, while treatments were set relative to the control. Variation observed not considered to be a product of ponA stimulation.

4.3.6 Stimulating expression of β-catenin in WHCO1 with DMSO

Since transfection with, and stimulation of, the transfected pEGSH-β-catenin vector did not increase its cellular concentration, and there not being sufficient time to retransfect to stable population, it was decided to exploit the report from Nakamura *et al*. (2003) who presented data suggesting that DMSO increases expression of β-catenin. The WHCO1 cells were treated with DMSO with the hope of producing an increase of β-catenin expression sufficient to affect nuclear translocation.

Western blots confirmed the presence of β-catenin in the cellular extract of non-treated WHCO1 and WHCO1 treated with 1% DMSO, for 24 and 72 hours (Figure 4.9.a). Uniform loading concentrations were used for the western blots. Densitometric analysis of the western blot revealed that β-catenin expression showed small changes, similar to those described for the vector-based stimulation i.e., 1.2 and 1.1 fold upon stimulation for 24 and 72 hours respectively (Figure 4.9.b).

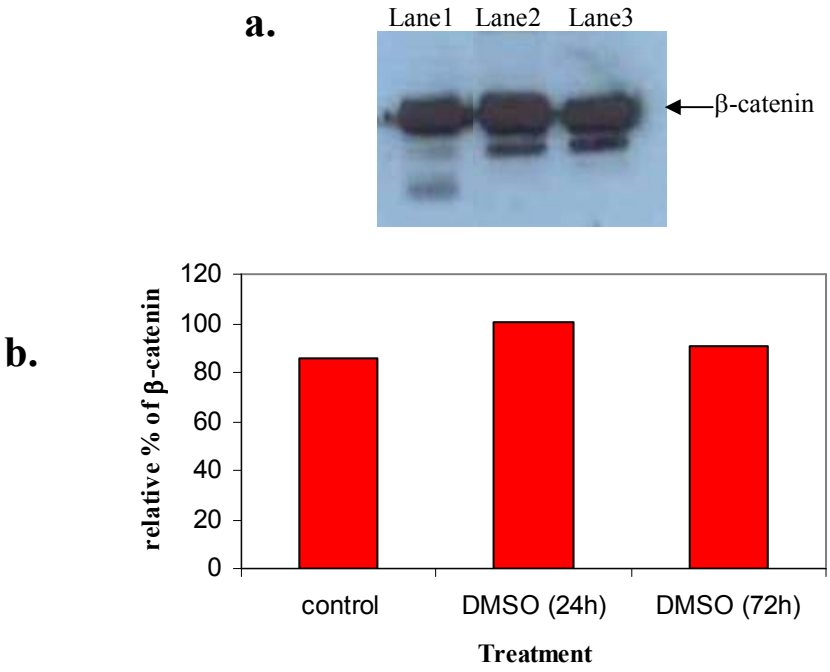


Figure 4.9 β-catenin expression in WHCO1 treated with 1% DMSO. **(a)** β-catenin was present in the western blot in non-treated WHCO1 (Lane1) vs. WHCO1 cells treated with DMSO for 24 hours (Lane2), or 72 hours (Lane3). **(b)** Histograms representing the relative expression level of β-catenin of the respective western blot. The β-catenin expression level of control, DMSO treatment of 24 and 72 hours was 85%, 100% and 90% respectively.

4.4 Discussion

Amplification of the 5′ and 3′ flanking region of the β-catenin insert within pEGSH was used to identify the presence of pEGSH-β-catenin in tWHCO1. These regions were successfully amplified from pEGSH-β-catenin, but not from DNA extracted from tWHCO1 (section 4.3.1). The fact that amplification from pEGSH-β-catenin was successful, suggests that the primers used, primer concentration, annealing temperature and concentration of other PCR constituents such as *Taq* polymerase and nucleotide concentration were suitable for the amplification procedure. Also, the specificity of the primers was such that they should not have been sequestered by other nucleic acids in the cell.

To dissect why the amplification from DNA extracts failed, it was necessary to determine whether the preparation of the DNA extracts produced an intact and stable DNA. The *GAPDH* gene is constitutively expressed in every cell, and it is therefore unlikely that the site of *GAPDH* gene in the DNA extracts should be concealed in such a way that amplification of this gene is hampered. The *GAPDH* gene was successfully amplified from the DNA extracts, signifying the stability of the DNA (section 4.3.1). Since the correct concentration of PCR reaction components and conditions (mentioned above) were used as well as the DNA being intact, the possibility remains that the integration of pEGSH-β-catenin into tWHCO1 cells' DNA results in the loss of one or two of the primer sites (this is explained in more detail in the following text, when discussing the chromatin position effect).

The ponA stimulation of the tWHCO1 total cell population and clones did not increase cellular β-catenin expression (section 4.3.4 and section 4.3.5). There are 2 possible explanations for the little or no increase of β-catenin expression upon stimulation with ponA. Firstly, the Complete Control® Inducible Mammalian Expression System manual states that "expression is integration site dependent". Hence, the stable transfection of pERV3 and pEGSH-β-catenin could have resulted in their integration into the chromosome in such a way that it leads to the

chromatin position effect (Recillas-Targa, 2004). In the chromatin position effect, the modification of the chromatin by the integrated gene, leads to suppression of gene expression (Henikoff, 1994; Recillas-Targa, 2004). This occurs by histone deacetylation and DNA methylation, which disrupts the packaging of the nucleic acids and transcription respectively (Lorincz *et al*., 2000; Recillas-Targa, 2004, Wade, 2001).

As a possible option to limit these problems in future experiments, green fluorescence protein (GFP) could be inserted into pEGSH to produce pEGSH-GFP. Subsequently, receptor activity is tested visually by observing GFP expression in cells stably transfected with pERV3, via confocal microscopy. Cells that were successfully able to express the receptors could than be transfected with pEGSH-β-catenin. The pEGSH vector is designed to have a FLAG-tag conjugated to gene inserted within the MCS region. To determine that chromatin position effect does not have an effect on pEGSH-β-catenin, an antibody specific for the FLAG-tag epitope, can be used. Upon stimulation with ponA, the expression of the FLAG-tag product is to be determined.

Secondly, the lack of an increase in β-catenin expression in the tWHCO1 cells could be due to mechanisms in these cells that compensate for an increase in β-catenin expression, such as the various ubiquitin proteosome pathways (discussed in more detail in Chapter 5). This phenomenon was also observed by Kratz *et al*. (2002) who showed that in transgenic lines transfected with wild type human β-catenin, there was an insignificant increase in β-catenin expression upon stimulation. Interestingly the exogenous β-catenin had a conjugated FLAG-tag-protein and a significant amount of the FLAG-tag was detected. This means that exogenous β-catenin was being expressed, however, the total cellular β-catenin was kept the same through cell regulated mechanisms.

In an attempt to circumvent this expression problem, it was decided to use DMSO to increase expression of β-catenin in non-transfected WHCO1 cells. The suggestion is that DMSO treatment results in an increase of β-catenin expression

via the wnt pathway in P19CL6 mouse embryonic carcinoma line (Nakamura *et al*., 2003). In contrast, the stimulation of WHCO1 cells with DMSO did not effect the expression of β-catenin. It is possible that the effect of DMSO depends on cell type and, while mouse embroyonic carcinoma cells are affected, human oesophageal squamous carcinoma cells are not. It is interesting that stimulation of β-catenin expression, with ponA and DMSO in tWHCO1 and WHCO1 respectively, led to a trivial increase of 1.2 fold in both lines. These results would seem to lend credence to the explanation that the inability to substantially increase β-catenin expression in the tWHCO1 cells is due to regulatory mechanisms in the WHCO1 cells, which compensate for the increase of β-catenin expression.