Primary characterization of a prolactin receptor dominant negative mutant using PC12 cell line

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ABSTRACT

Prolactin is a multi-functional peptide hormone produced and secreted by the anterior pituitary gland. In addition to its traditional role, the action of prolaction has been implicated in many other physiological and pathological processes. The aim of this project was to investigate the effectiveness of a newly developed dominant negative prolactin receptor using phaeochromocytoma (PC12) cell line. The response of PC12 cells to prolactin stimulation was tested using immuno-staining for STAT1 and p-STAT3 which are proteins in the activation pathway of prolactin. Preliminary results indicate there was a difference between the prolactin stimulated PC12 cells and the control, thereby providing information for future investigations.

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INTRODUCTION

Prolactin (PRL) is a peptide hormone primarily associated with lactation and, is synthesised and secreted mainly by lactotrope cells in the adenohypophysis[1]. PRL has a range of physiological actions including the stimulation and maintenance of mammary tissue during lactation[1]. Furthermore, it is appreciated that PRL has diverse roles in many other physiological processes such as osmoregulation, behaviour, immunity and growth[2]. In addition, PRL has been shown to be involved in pathological conditions associated with cancer and sexual dysfunction[3]. A recent study demonstrated that the appearance of genetically-induced mammary tumours is delayed in mice with PRL deficiency; whereas PRL transgenic mice spontaneously develop mammary neoplasia indicating that inhibition of PRL may have a role in anti-tumour therapy.

Prolactin receptors (PRLr) are widely expressed on a range of tissues and cells including breast epithelia, prostate cells and lymphocytes[4]. The PRLr belongs to the class I cytokine receptor family and contains an extracellular binding domain, a single transmembrane domain, and an intracellular domain required for signal transduction, mainly via Janus Kinase2 / signal transducer and transcription activator 5 (JAK-2/STAT5). In addition, other pathways that includes STAT1/STAT3 pathway also play a role in prolactin mechanism cascade as illustrated in the diagram below (Figure 1). The PRLr in humans has been shown to have three isoforms (long, intermediate and short) . Prolactin exerts its effect through binding to the extracellular domain of the receptor which induces dimerisation[5], leading to the activation of a protein tyrosine kinase (Jak2), which is non-covalently associated with the cytoplasmic domain of the prolactin receptor. Jak2 phosphorylates Stat5 which dimerises and translocates to the nucleus where it specifically binds to sequence elements in the promoter regions of genes and initiate cellular responses accordingly.

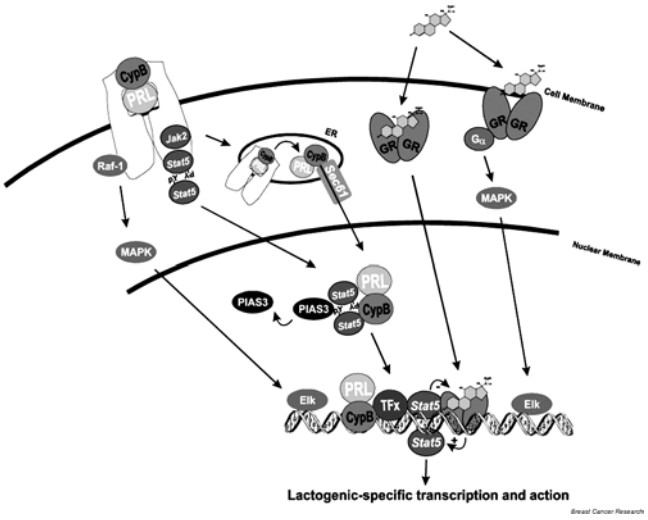


Figure 1: PRL hormone action and its transcription cascade (adopted from Breast Cancer Research Journal).

Understanding PRL's diverse actions has been limited by the lack of effective receptor antagonists and a model with a mutant PRLr (dominant-negative) which would be an ideal control model for future research of prolactin. This research investigates the use of cell cultures to test the effectiveness of this recently developed dominant-negative prolactin receptor. A dominant negative PRLr has been designed and inserted into a pEGFP-N2 vector. When transfected, this vector will express green fluorescent protein (GFP) and the mutant receptor. Phaeochromocytoma (PC12) cells are known to endogenously express PRLr[3]. In response to PRL stimulation these cells have been reported to exhibit an activation of Jak2/STAT3 pathway and undergo a proliferative response[3] which can be quantified. In the current project, PC12 cells were transiently transfected with dominant-negative PRLr and stimulated with PRL. The response to PRL stimulation was monitored by staining for STAT1 and phosphoraylated-STAT3 proteins. This study was the first attempt to characterize dominant negative PRLr in a cell culture.

METHODS

Bacterial transformation.

The vector was generously donated by Dr Paul Le Tissier (National Institute for Medical Research, UK). DNA was eluted from filter paper in 50 µl Milli-Q water. This was left for five minutes then centrifuged for four minutes at 14,000g. From the extracted DNA, 2µl was used to transform cells while the rest was frozen at -80 °C. Ultra-competent E.Coli cells (One Shot® TOP10 from Invitrogen) were thawed on ice, then, 2µl of DNA added and mixed gently. The cells were left on ice for 30 minutes and heat shocked at 42°C for 30 seconds. Next, 250µl of pre-warmed Super Optimal Broth (SOB) was added and incubated for one hour at 37°C in a shaking water bath. Aliquots of 20µl, 50µl and 100µl of cells were plated onto three Luria-Bertani (LB) neomycin-containing agar plates. The plates, and an empty plate (control), were incubated for 72 h at 37°C. After that, a single isolated bacterial colony was collected using a pipette tip, and transferred to LB broth containing neomycin and grown for 48h in 37°C incubator. The resultant bacterial culture was centrifuged for 10 minutes at 4,000g and DNA isolated using MAXI prep (PureLink™HiPure) according to the manufacture's protocol.

Restriction enzymes analysis

1.2% agarose gel was used in this analysis and formulated using a standard agarose preparation. Purified DNA samples were incubated with different restriction enzymes (EcoRI, BamHI, NcoI and HindIII). With a final volume of 10µl each combination consisted of 8µl DNA sample and 2µl of restriction enzyme. The 50bp and 1kbp DNA ladders were from Invitrogen and the Bench Top 1kbp DNA was from Promega. DNA ladders consisted of 2µl dilution buffer, 1µl DNA ladder and 9µl Tris-Borate-EDTA (TBE) buffer. 10µl of sample and DNA ladders were run on the agarose gel at 130 V and 60 mA for two hours. Bands then located under UV transilluminator and a Polaroid photograph taken.

PC12 cell culture

Plate collagen coating: plates were coated with 6-10 µg/cm of 0.01% collagen solution (Sigma C 8919) and then rinsed with Milli-Q water prior to cells plating. A frozen PC12 cell stock was quickly thawed in 37°C water bath. Pre-warmed complete RPMI medium was added and the cells centrifuged at 220g at room temperature for five minutes. Next, 5ml of RPMI was added and cell were re-suspended and plated at about 1×10^7cells per well. Cells were maintained at a 37°C and 5% CO2 environment. In this project PC12 cells were fixed using -20 °C methanol by removing the RPMI medium and gently adding -20 °C methanol for five minutes at room temperature. Methanol was then aspirated and the cells left to dry for one hour.

PC12 cell transfection.

Before the day of transfection, cells were deprived of complete medium and grown in serum-free medium. The density of cells was calculated to be around 8×10^3 cell per well in 96-well plates. DNA (40ng, 200ng and 500ng) was diluted in 20µl of serum-free (SF) medium. Lipofectamine ™LTX Reagent from Invitrogen (0.35µl) was added to the 20µl DNA-containing medium and left for 25 min at room temperature to form DNA-lipofectamine complexes. To each well, a 100µl of fresh SF medium was added and 20µl of DNA-Lipofectamine complex added and the cells incubated at 37°C, 5% CO2 for 48h. Transfection was observed under fluorescent microscope using appropriate optics for GFP.

PC12 cell stimulation with prolactin

Cells were plated at density of about 100,000 per well on collagen-coated cover slips contained in 24-well plates two days prior to stimulation. Cells were rinsed twice with HEPES buffer at 37°C. Some cells were treated with 10nM of ovine PRL (Sigma L6520) for 15 minutes whereas other cells were incubated with HEPES buffer only. Cells were fixed using -20°C methanol as described above. After fixation, cells were blocked with 4% goat serum/phosphate buffer saline (GS/PBS) for one hour and then incubated with primary antibody against STAT1 (Biosource™) or p-STAT3 (Biosource™) at a dilution of 1:200 and 1:100 in GS/PBS, respectively, at 4°C for 48h. Goat anti-mouse secondary antibody (Alexa Fluor 488) was used to label the primary antibodies. The secondary antibody was incubated for 90 minutes at room temperature under reduced light exposure. The cover-slips then rinsed with Milli-Q water, mounted with Vectashield and viewed under fluorescence microscopy.

Fixation of PC12 cells for immuno-localization was with -20°C methanol or 8% para-formaldehyde (used in preliminary experiments) which preserved more cells and reduced the washing effect on cells compared with room-temperature 70% ethanol(data not shown in this article). However, in this research -20°C methanol was superior to 8% para-formaldehyde because rapid fixation was required to rapidly preserve immunocytochemical events occurring as a result of PRLr activation.

RESULTS

Cell transformation and vector amplification

Ultra-competent cells transformed successfully following the protocol described in the Methods. On the third day of incubation with DNA, colonies of cells were growing in a volume-dependent fashion. The 100µl agar plates had 8 distinct colonies compared to 5 in 50µl and 2 in 20µl plate. The control plate grew no observable colonies. The average absorbance ratio at 260/280nm wavelengths was 1.82 which is consistent with reference range for pure DNA (1.8-2.0). The quantity of DNA extracts was 147ng/µl and 145ng/µl for the separate preparations.

Restriction enzymes analysis of purified DNA

Samples of DNA were tested to verify the presence of the construct of interest using restriction enzymes (EcoRI, BamHI, NcoI and HindIII). The uncut sample gave a fragment of 5.7kbp (Lane A, Figure 2) which was as expected from uncut vector containing the construct (Figure 3). The use of EcoRI and BamHI, which demarcated the insert region (Figure 3), gave two fragments of about 1kbp and 4.6kbp (Lane B, Figure 2). Similarly, the use of HindIII and BamHI enzymes, which also enclosed the insert region (Figure 3), gave two bands with 1kbp and 4.6kbp (Lane E, Figure 2). The two fragments from both combinations of restriction enzymes matched the expected values for the vector and the construct. Furthermore, using NcoI restriction enzyme which has three recognition sites in this vector, gave three bands of the expected size about 2kbp, 1kbp and 700bp (Lane C, Figure 2). In addition, XbaI restriction enzyme which has one recognition site in this vector was tried and gave one fragment which looks similar to the uncut DNA (Lane D, Figure 2).

Figure 2: Restriction enzymes analysis Polaroid photograph.

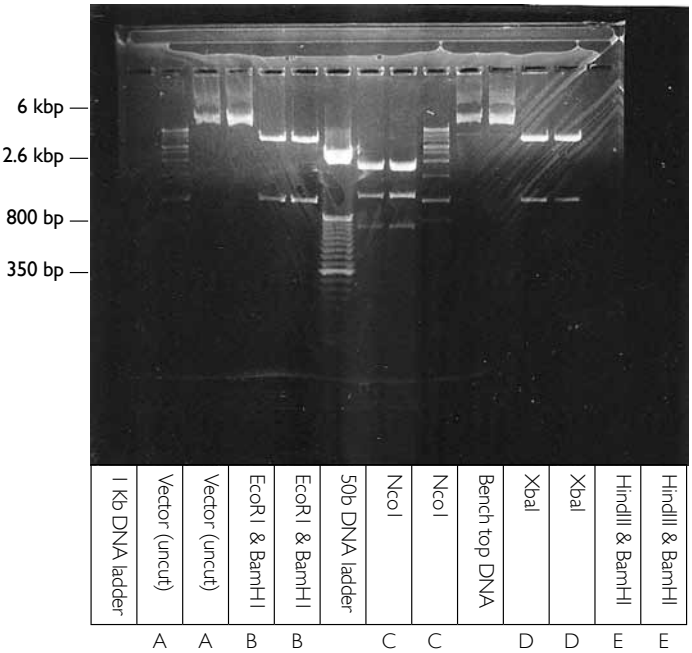


Figure 3: p EGFP-N2 vector.

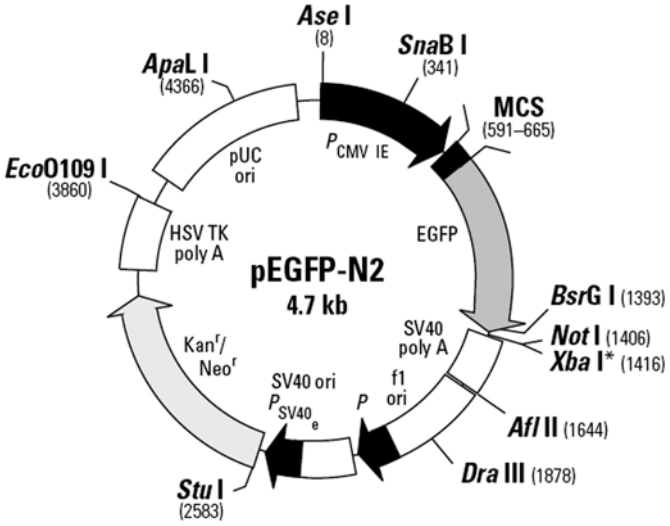


Figure 4: Two transfected PC12 cells showing green fluorescent due to p-EGFP-N2 after 48 hours.

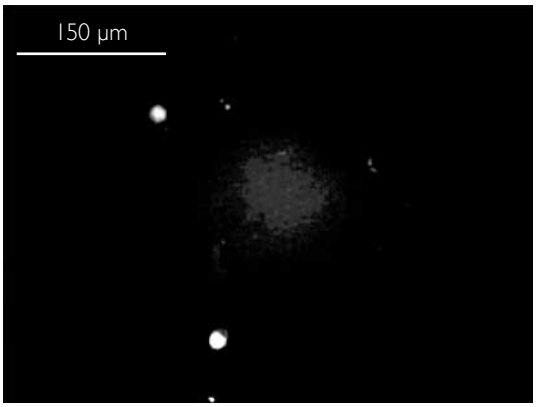


Figure 5: Basal cells stained for STAT1.
Uniformly cytoplasmic staining with difference in intensity between cells.

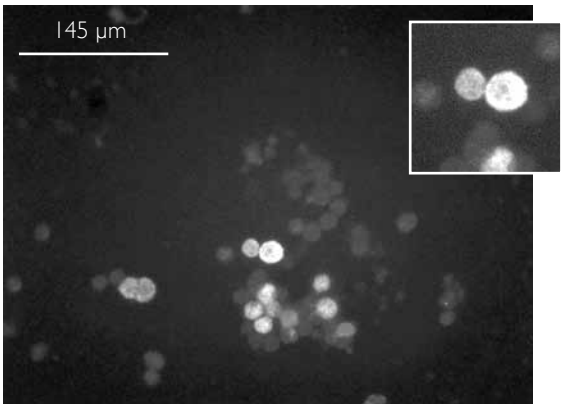


Figure 6: Prolactin-stimulated cells stained for STAT1.
The arrow indicate two cells with finely distributed stain.

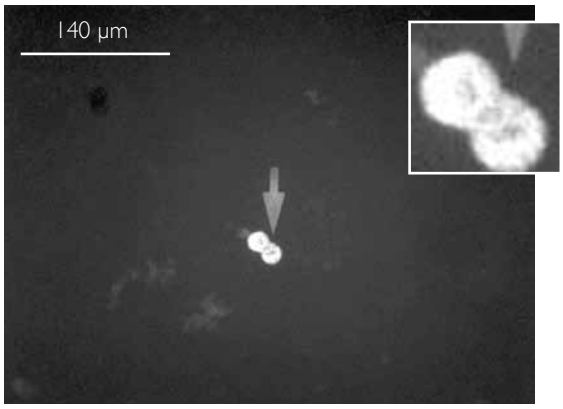


Figure 7: Basal cells stained for p-STAT3.
Nuclear&granular staining with difference between cells.

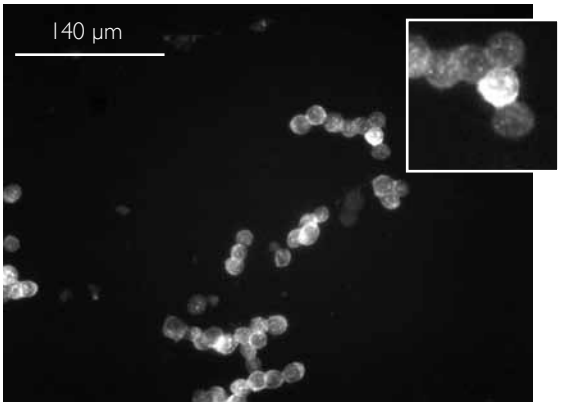
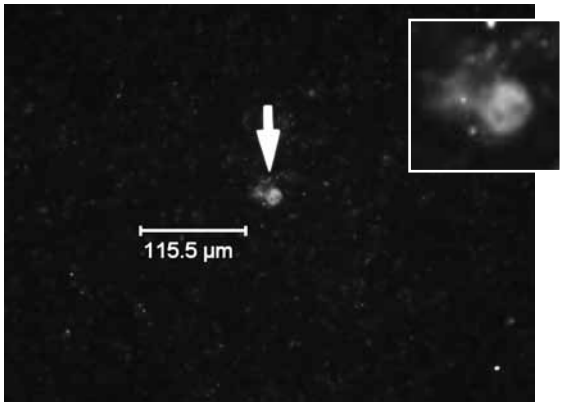


Figure 8: Prolactin-stimulated PC12 cell stained for p-STAT3.
The arrow points to a cell with stain mainly nucleus.



PC12 cell culture and transfection

PC12 cells were cultured with good adherence on 24-well plates containing collagen-coated cover-slips. Similarly, titrating the PC12 cells vigorously using a (0.5X25mm) needle gave a single cell suspension, which readily formed a monolayer distribution of cells when plated and thus reduced the lifting effect of non-adherent cells in larger clumps. PC12 cells transfected with the combination of 200ng/µl DNA and 0.35µl of Lipofectamine™ showed a few green fluorescent cells after 48h of transfection, indicating the incorporation of the p-EGFP-N2 vector into the cellular DNA (Figure 4). The cells that were not transfected were spherical and showed no signs of granulations indicative of Lipofectamine toxicity. Increased DNA concentration (500ng/µl) showed reduced transfection. The same was observed with reduced DNA concentration (100ng/µl). Longer duration of transfection showed no increase in the number of transfected cells; in that 72h transfection was similar to 48h. Cells grown in serum-containing medium for 24h prior to transfection were not transfected at higher rate compared with the cells grown in serum-free medium for 24h prior to transfection. Moreover, there were more cells transfected when the cells

were grown in 96-well plates compared to the larger wells in 24-well plates. This was consistent in three experiments.

mmuno-staining

Basal cells, grown in serum-containing medium with no PRL treatment, stained for STAT1 were strongly fluorescent. The stain was finely distributed in the cytoplasm but not the nucleus. There was a marked variation of brightness between groups of cells within the same well (Figure 5). In prolactin-stimulated cells, there was similar staining pattern to the basal cells but it may be less bright (Figure 6). Staining for p-STAT3 in the basal cells showed a different pattern compared to STAT1 staining. The stain was mainly nuclear and granular in nature. There was obvious variation in stain intensity between cells within the same well (Figure 7). PC12 cells that were stimulated with prolactin and stained for p-STAT3 were faintly stained but still with same staining distribution to the basal cells (Figure 8). The very low number of transfected cells resulted in no observable difference between transfected and non-transfected cells regarding prolactin response.

DISCUSSION

The increase in number of discrete colonies with increased bacterial amount indicates that there was a selection of transformed bacteria using neomycin containing dishes. Moreover, the control dish that did not grow any colonies ruled out contamination of neomycin resistance bacteria. Restriction enzymes, with their ability to cleave DNA sequences at specific sites, were a reliable test of identifying the presence of a specific DNA segment. According to the vector data sheet, the uncut vector is about 4.7kbp and that was confirmed in two sample of purified DNA. The region of the PRL dominant-negative receptor was demarcated by two combinations of restriction enzymes (EcoR1&BamH1/HindIII&BamH1) and they both gave two bands of the expected molecular weight of about 1kbp which represents the inserted construct and the 4.6kbp which is the carrying vector. The use of Xbal which has one recognition site gave similar picture to the uncut sample which means that cutting in one site only still create a one fragment of DNA. The restriction enzymes analysis confirmed that the purified DNA was the required construct. PC12 cells transfection in one study was reported to be about 14% efficient[5]. However, there were very few cells that were successfully transfected as indicated by GFP-fluorescence imaging. The observation of better transfection when the PC12 cells were deprived of serum-containing medium might be because serum contains biological molecules that interfere with the DNA/ Lipofectamine complexes. In addition, serum deprivation weakens cell membranes and facilitates the DNA/ Lipofectamine uptake[6]. For future projects, more easily transfected cell lines such as Chinese Hamner Ovary cells could be used. PC12 cells have been reported to respond to prolactin stimulation with a proliferative-type response[6]. This response is mediated mainly via the Jak2/ STAT pathway. In one study, STAT3 (p-STAT3) was measured using western blotting as marker for the PRL response in PC12 cells and demonstrated a significant increase after 15 minutes of stimulation[7,8]. In this project, immuno-staining for STAT1 and p-STAT3 was used as the measurement of PRL response. These were selected because STAT1 is mainly cytoplasmic and p-STAT3 mainly nuclear[8].

This project has demonstrated that there is a difference between the p-STAT3 and STAT1 staining. However, this difference could not be clearly related to prolactin stimulation. It was interesting to observe that basal cells stained more for STAT1 and p-STAT3 than prolactin-stimulated cells. At this moment, there are no clear explanations for this but questions of PC12 response to prolactin are still valid. In future research, it would be of great value to establish a cell line that is easily transfected with prolactin dominant negative receptors and are responsive for prolactin stimulation in a measured way. This will allow co-labelling and testing whether the dominant-negative receptor damps down the responsiveness of the cell line to prolactin. In general, it was valuable research project to address the difficulty that could be overcome next time in an attempt to understand the diverse functions of prolactin in biology.

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