Chapter 3
Molecular studies of human endometrial tissues

3.0 Quantitative real time polymerase chain reaction (qRT-PCR) in human endometrial tissues

3.1 Introduction

A previous study from our group showed that the ubiquitin protein was up regulated in endometriotic cells and that this was correlated with increased cell survival (Ilad *et al,* 2004) At the time, we did not speculate what pathway was potentially responsible and now aim to investigate the temporal pattern of key mRNA expression of transcripts within the NFκβ pathway, as ubiquitin is a known regulator of this system (Sun and Chen 2004) We examined the differential mRNA expression of PA28 (equivalent to the 19S proteasome), IKKα and NFκβ from women with and without endometriosis, as a starting point to our investigation of the ubiquitin mediated NFκβ pathway. These genes were selected to ascertain whether the non-canonical mediated pathway, which is solely activated by IKKα, was present within the ectopic endometrium. The activation of IKKα may allow its recognition by the 19S proteasome and subsequently provide a trigger for NFκβ activation that may aid in the survival of endometriotic tissues.

3.2 Materials and methods

3.2.1 Human tissues for qRT-PCR

We collected tissues for RT-PCR on women undergoing laparoscopy for non-endometrial pathologies such as leiomyomata and benign ovarian cysts and kept the samples in liquid nitrogen until use as described in Section 2.3.2. We then extracted RNA from these tissues using an Invitrogen, Micro-to-Midi Total RNA purification system isolation kit as in Section 2.3.3 and proceeded by measuring its RNA quality and quantity as in Section 2.3.4 with the use of the Agilent Technologies RNA_{6000} nano chip. Representative electropherogram summaries can be seen for the ladder markers Figure 3.1, human RNA extraction Figure 3.2, as well as the gel output for samples unsuitable for mRNA analysis Figure 3.3. The amplification of cDNA sequence was completed by following the instructions in Section 2.3.5, by using primers listed in Table 2.3, designed according to the methodology in Section 2.3.6. qRT-PCR was then

performed to measure the number of amplicon products generated using an Invitrogen Platinum® SYBR® Green qPCR Super Mix-UDG kit and a Corbett Rotor-Gene™ 6000 real-time analyser as noted in Section 2.3.7. Finally, statistical analysis was conducted, whereby standard curves for the relevant genes were generated and normalised against the 18S housekeeping gene, Figure 3.4, as described in Section 2.3.8.

3.3 Results

Table 3.1. Human mRNA expression of transcripts within the eutopic and ectopic endometrium normalized for 18S

Gene	Eutopic (n=3)	Ectopic (n=4)	Fold Change (P-value)
NFκβ/18S	0.0004 ± 0.0004	0.093 ± 0.059	257.88 (P = 0.057)
PA28/18S	0.102 ± 0.061	3.105 ± 1.970	30.50 (P = 0.064)
IKKα/18S	2043 ± 1818.14	420.10 ±420.10	0.206 (P = 0.186)

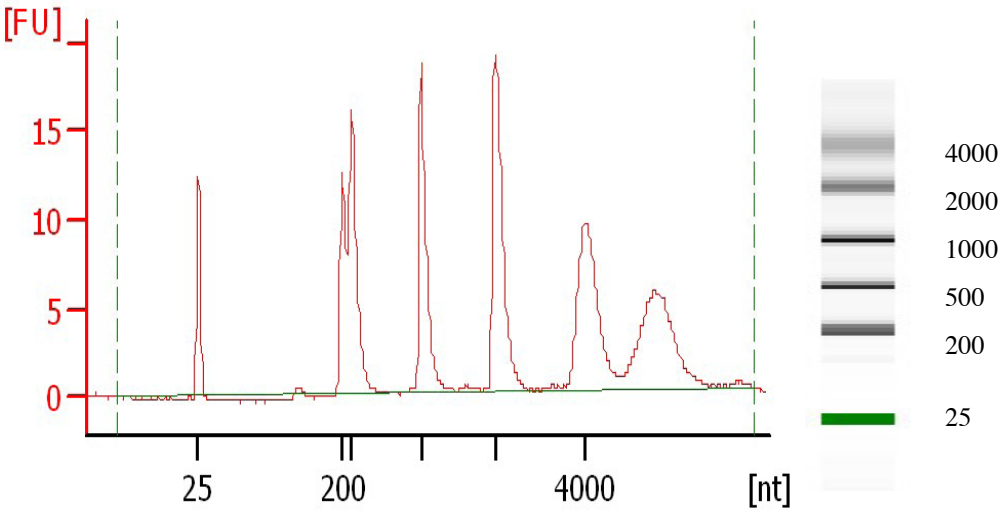

Figure 3.1: Representative electropherogram summary of the ladder marker supplied by Agilent Technologies RNA_{6000} nano chip. Height threshold [FU] and ladder peaks [nt]. Adjacent gel output represents ladder peak markers. Measured RNA concentration = 150 ng/µl.

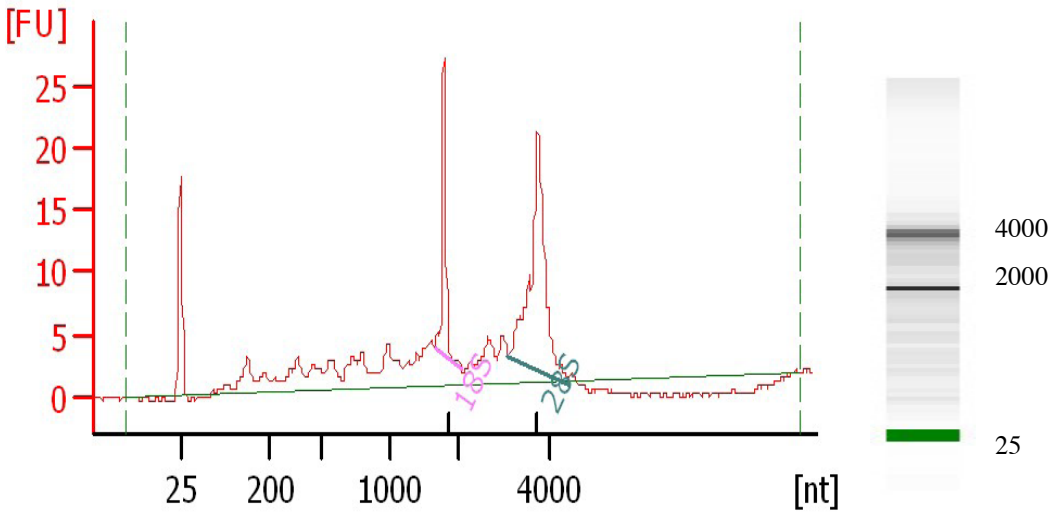

Figure 3.2: Representative electropherogram summary of a human RNA extraction. Height threshold [FU] and ladder peaks [nt]. Adjacent gel output represents ladder peak markers. Measured RNA concentration = 77 ng/µl; rRNA Ratio [28s/18s] = 2.3 and RNA Integrity Number (RIN) = 7.1.

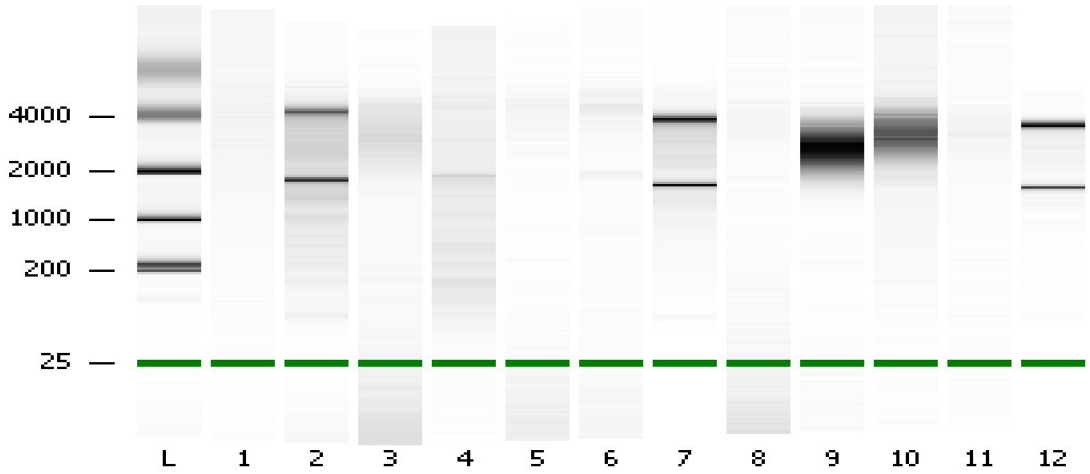

Figure 3.3: Representative gel output of a human RNA extraction whereby samples were unsuitable for further mRNA analysis. Lanes 1, 3, 4, 5, 6, 8, 9, 10 and 11 were excluded in the study. Ladder markers are represented on the y-axis, whilst the x-axis represents individual extracted human RNA sample.

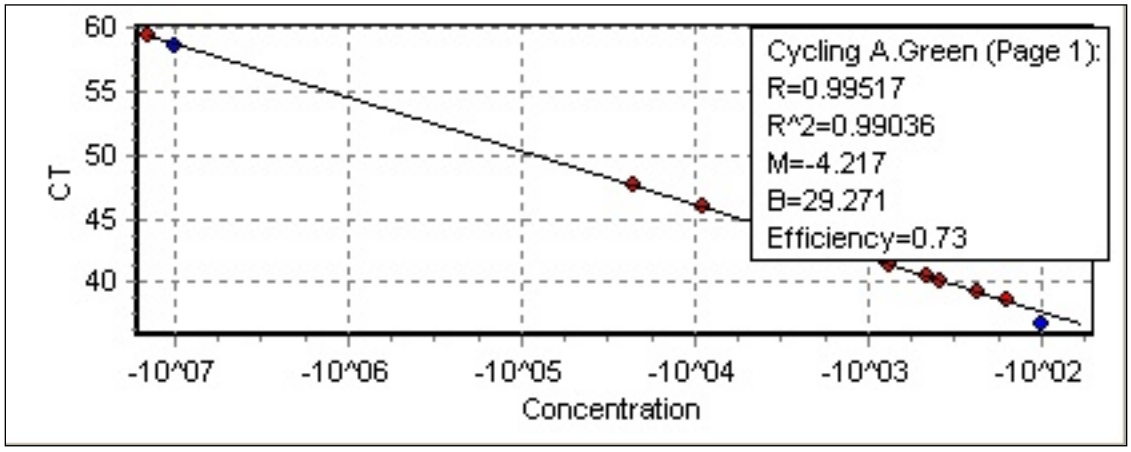

Figure 3.4: Representative standard curve for NFκβ in RNA extracted from women with and without endometriosis, normalized against 18S. y-axis represents the cycle threshold, whilst the x-axis represents the concentration of extracted human RNA sample.

3.4 Discussion

Many tissue samples were collected from different patients however only some of the tissues were suitable for analysis because samples either contained extremely low RNA concentrations or a low RIN output. Since our aim for this section is to determine whether the NFκβ is present in the ectopic endometrium and whether its upstream activators PA28 (equivalent to the 19S proteasome) and the non-canonical pathway mediator IKKα are also expressed, we decided to use the remaining samples suitable for mRNA expression analysis.

Table 3.1 reveals that a trend toward a high fold difference in NFκβ between the eutopic and ectopic endometrium exists however this result was not statistically significant, and may reflect the variability of the small sample size. A similar trend was also observed for the PA28 transcript for the two different cell types. IKKα does not seem to be the kinase responsible for NFκβ mediated endometrial cell survival in endometriosis as the fold change within this tissue was not statistically significant.

These results should be interpreted with caution, as the patient numbers are low enough as to potentially cause an inaccurate result.

The results in this study prompted us to look at whether the presence of NFκβ mRNA in the baboon. We also conducted protein studies using immunohistochemistry to verify the above findings, so as to definitively determine that another pathway and not NFκβ is involved in ectopic cell survival in endometriosis. The results for the human protein study can be seen in Chapters 7 and 8.