Differentially regulated expression of neurokinin B (NKB)/NK₃ receptor system in uterine leiomyomata

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STUDY QUESTION: Are the vasoactive peptide neurokinin B (NKB) and its preferred NK₃ receptor (NK₃R) differentially expressed in leiomyomas compared with normal myometrium?

SUMMARY ANSWER: In leiomyomas, NKB is up-regulated and delocalized, while its preferred NK₃R is also differentially regulated.

WHAT IS KNOWN ALREADY: The expression of NKB/NK₃R in the central nervous system is essential for proper function of the human reproductive axis. Additionally, this system is also widely expressed throughout the female genital tract. Leiomyomas impair fertility and are a major source of abnormal uterine bleeding. The aberrant synthesis of local factors can contribute to the pathological symptoms observed in women with leiomyomata. NKB could be one of these factors, since a vasoactive role of this peptide at a peripheral level has been observed in different systems and species, including humans. NK₃R is strongly regulated by estrogens and its activation leads to nuclear translocation affecting chromatin structure and gene expression.

STUDY DESIGN, SIZE, DURATION: Samples were obtained between 2006 and 2012 from 28 women of reproductive age at different stages of the menstrual cycle by hysterectomy. Leiomyomas and matched macroscopically normal myometrium from each woman were analysed in vitro.

PARTICIPANTS/MATERIALS, SETTING, METHODS: RT–PCR, quantitative real time, immunohistochemistry and in situ hybridization were used to investigate the pattern of expression of NKB/NK₃R in tissue samples.

MAIN RESULTS AND THE ROLE OF CHANCE: Expression of the gene encoding NKB (TAC3) was up-regulated 20-fold in leiomyomas, compared with matched myometrium (P = 0.0008). In tumour tissue, not only connective cells, but also myometrial, endothelial and vascular smooth muscle cells express TAC3 mRNA. Immunoreactivity to NKB was preferentially located in the smooth muscle cell nuclei from normal myometrium in the secretory phase, unlike matched leiomyoma, which showed a predominant cytoplasmic expression pattern. In the normal myometrium, TAC3 mRNA showed variable expression throughout the menstrual phases, with samples showing strong, reduced or no amplification. In leiomyoma, TAC3 was significantly up-regulated compared with matched myometrium (P = 0.0349).

LIMITATIONS, REASONS FOR CAUTION: This study is descriptive and although we observed clear differential regulation of the NKB/NK₃R system at mRNA and immunohistochemical staining levels in leiomyoma, future functional studies are needed to determine the precise role of NKB in the myometrium in normal and pathological conditions. In addition, further analysis (e.g. in cell culture models) will be required to

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Neurokinin B (NKB) is a 10-amino acid peptide that belongs to the family of tachykinins. Although three tachykinin receptors are described, named NK₁, NK₂ and NK₃, NKB displays preferential affinity for the NK₃ receptor (NK₃R) and exerts its actions predominantly through this receptor (Almeida et al., 2004; Pennefather et al., 2004a,b; Satake and Kawada, 2006; Lasaga and Debeljuk, 2011). Until recently, the expression of the genes encoding NKB (TAC3) and NK₃R (TACR3) were considered to be restricted to the central nervous system (CNS) where they may, among other functions, modulate GnRH release at the hypothalamic–pituitary axis (Rance and Young, 1991; Goodman et al., 2007; Rance, 2009; Lehman et al., 2010; Pinilla et al., 2012). The important role of this system in human reproduction is illustrated by the fact that mutations in TAC3 and TACR3 are associated with human normosmic hypogonadotropic hypogonadism, a disease characterized by the failure of sexual maturation, impaired gametogenesis and infertility (Topaloglu et al., 2009; Semple and Topaloglu, 2010).

The function of NKB/NK₃R in the reproductive tract is less well understood. Several reports suggest that this system may play a vasoactive role at the peripheral level. Activation of the NK₃R leads to increased heart rate in the dog (Thompson, 1998) and rat (Lessard et al., 2003), contraction of the rat hepatic portal vein (Mastrangelo et al., 1987) and constriction of the mesenteric venous beds in the rat (D’Orleans-Juste et al., 1991). Alternatively, NKB induces vasodilatation in small arteries isolated from myometrium of pregnant women (Wareing et al., 2003) and in resistance vessels from human placenta (Laliberte et al., 2004). The addition of NKB to human preconstricted omental arteries and veins produces a significant vasodilatation of this vascular bed (Wareing et al., 2003). Similarly, Brownbill et al. (2003) showed that NKB is a dilator of the previously constrained fetal vasculature, suggesting an important role of NKB in the regulation of fetal placental vascular tone. This effect was also observed in rat mesenteric arteries, where a specific NK₃R agonist caused vasodilatation (Mizuta et al., 1995).

Uterine leiomyomas or fibroids are benign smooth muscle tumours that affect up to 60% of reproductive-age women and ~80% of women during their lifetime (Levy et al., 2012). These uterine fibroids are an important cause of infertility, particularly, in the case of submucosal or intramural leiomyomas that protrude into the uterine cavity, which can affect implantation and pregnancy (Horcajadas et al., 2008; Levy et al., 2012). Existing data suggest that leiomyomas are involved in ~10% of infertility cases and are the sole cause of infertility in 1–3% of patients (Levy et al., 2012). Despite this high prevalence and influence on fertility, little is still known about the pathophysiology of leiomyomas. Apart from their negative influence on fertility, uterine leiomyomas are a major source of abnormal uterine bleeding (Stewart and Nowak, 1996). Approximately 30% of women with leiomyomas experience menstrual abnormalities, with menorrhagia or excessively heavy menses being most common (Sankaran and Manyonda, 2008). Current evidence suggests that it is not the physical compression but the local action of vasoactive growth factors, which have altered synthesis, expression or sequestration in leiomyomas that account for these vascular abnormalities (Stewart and Nowak, 1996). Recently, it has been shown that the NKB/NK₃R system is expressed in human, mouse and rat uteri and their expression and function varies with age, menstrual cycle and during the course of pregnancy (Magraner et al., 1998; Pinto et al., 1999, 2000; Hamlin et al., 2000; Candenas et al., 2001; Cintado et al., 2001; Patak et al., 2003, 2005; Pinto et al., 2003; Löffler et al., 2004; Pennefather et al., 2004a, b). A more detailed histological analysis carried out in human myometrium showed the presence of NKB and NK₃R in smooth muscle cells, vascular smooth muscle cells and connective cells (Cejudo Roman et al., 2012). In order to find out whether the NKB/NK₃R system could be dysregulated in leiomyomas, we have analysed and compared their expression pattern in leiomyomata with the adjacent normal appearing-matched myometrium.

### Materials and Methods

#### Patients

Twenty-eight female patients between 36 and 49 years of age, admitted to the Hospital Universitario de Canarias (HUC) and the Hospital La Colina between 2006 and 2012 were enrolled into this study after giving informed consent. Ethical approval was granted by the Committee for Clinical Research Ethics of the HUC. Samples analysed included 26 intramural, submucous or subserous leiomyoma specimens from 26 women as well as the matched myometrial tissue, 2 tumours (intramural and submucous) obtained from one woman and her matched myometrial tissue and 2 tumours (intramural and subserous) obtained from another woman and her matched myometrial tissue. Myometrial samples were taken as far away as possible from leiomyoma. All patients underwent hysterectomy for menorrhagia without any previous treatment. Regarding the menstrual phase, participants enrolled into this study included 10 in the proliferative phase, 17 in the secretory phase and 1 in the menstrual phase. The proliferative and secretory phases were assigned based on the date of the last menstrual period and confirmed by histological assessment.
**TAC3 and TACR3 mRNA quantification**

**RNA extraction and reverse transcription**
TAC3 and TACR3 mRNA were analysed in paired samples of leiomyomas and adjacent myometrial tissue (30 tumours and 18 myometrium for TAC3 and 20 tumours and 18 myometrium for TACR3). To avoid degradation, tissue sections were immersed in RNA later (Sigma-Aldrich Co., Madrid, Spain) immediately after surgery, kept at 4°C overnight and stored at −80°C until processed.

Tissue was homogenized using Lysing matrix A tubes (MP biomedicals) containing 1 ml of RAL lysis buffer (GeHealthcare) in a FastPrep apparatus (MP biomedicals). After centrifugation at 18 000g for 20 min at 4°C, the supernatant was recovered and RNA purification was performed using the Illustra RNAspin Mini Kit (GeHealthcare) according to the manufacturer’s instructions.

Residual genomic DNA was removed by incubating the RNA samples with RNase-free DNase I and RNasin (Promega, Madison, WI, USA). The effectiveness of the DNase treatment was assessed in RT-negative samples. In our samples, TAC3 mRNA shows late cycle threshold (Ct), usually after 32 cycles, when analysed using quantitative PCR (qPCR). In order to obtain reliable data, 5 μg of RNA from 20 tumours and 18 matched leiomyomas (n = 38) were needed to obtain enough cDNA suitable for quantification. Regarding TAC3 and in order to compare this sample set with a previous one of 10 leiomyomas and 10 matched myometrium (Rodríguez et al., 2010), retrotranscription was carried out using 2 μg of RNA (n = 58). First-strand cDNA was synthesized from the total RNA using Moloney murine leukemia virus reverse transcriptase, RNase H Minus, Point Mutant (Promega) and a 1:1 mix of oligo(dT)23 primer (Sigma-Aldrich Co.) and Random Hexamers (Roche, Basel, Switzerland) according to manufacturer’s instructions (Promega).

**Real-Time PCR**
A Bio-Rad MyiQ Real-Time PCR detection system apparatus was used to perform the quantification of TAC3 and TACR3 transcripts. Each sample was analysed in triplicate in a total reaction volume of 20 μl consisting of a 1:2-fold dilution of cDNA, 10 μl of 2 × SensiMix Plus SYBR and Fluorescein kit (Bioline, Ecogen Barcelona, Spain) and 0.2 μM of each primer. The cycling conditions were 95°C for 10 min followed by 35–45 cycles of 95°C for 1 s, 60°C for 30 s and 72°C for 30 s. For each experiment, a non-template reaction was included as a negative control. The specificity of the PCR reactions was confirmed by melting curves analysis of the products as well as by size verification of the amplicon in a conventional agarose gel.

GeNorm program implemented in qBasePLUS (Biogazelle, Ghent, Belgium) was used to assess candidate reference genes that were stably expressed (Vandesompele et al., 2002). The sequence of the primer pairs used were as follows: TAC3, forward 5′-CCACAGGCACCATGAGGAT-3′ and reverse 5′-GGTTGATCTTTAGGAT-3′; TACR3, forward 5′-TGATGAGCAATGGGAAACAG-3′ and reverse 5′-TGATCCTTTGAAAACCCAGAC-3′. In addition, three housekeeping genes were used to normalize gene expression data: hypoxanthine phosphoribosyltransferase 1 was amplified using forward 5′-GAGTGTGGCCTTCTCCTCTG-3′ and reverse primer 5′-CATCTCTTCTGGCAGTAT-3′ and L32 ribosomal protein was amplified using forward 5′-GCCAGGAGGAGCAGCAT-3′ and reverse primer 5′-AACACCTCTCTGGGTTCTCTTCA-3′; guanine nucleotide-binding protein (G protein), beta polypeptide 2-like 1 (GNB2L1) was imported into qbasePLUS Data Analysis software, which employs the classic 2 -ΔΔCt method with PCR efficiency correction and multiple reference gene normalization to calculate the relative expression ratio (Hellemans et al., 2007).

**Statistical analysis**
The normalized relative quantitative data were obtained using qbasePLUS software (Biogazelle). TAC3 mRNA expression levels were compared between leiomyomas and matched myometrium using a two-tailed paired Student’s t-test after testing that our data passed the normality test using the method of Kolmogorov and Smirnov. Normalized Ct values for TACR3 did not pass the normality test and so the Wilcoxon matched-pairs signed-rank test was carried out to compare both groups. P-value of <0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 6 (GraphPad, San Diego, CA, USA).

**Histology**
Leiomyoma and adjacent uterine myometrium samples were fixed in 10% buffered formalin, then embedded in paraffin and cut in 3-μm thick slides. The sections were deparaffinized, hydrated, hematoxylin–eosin stained and evaluated for histopathology.

**In situ hybridization**
We used a short (30 mer) synthetic oligonucleotide probe complementary to TAC3 mRNA. The specificity of the designed oligoprobe was assessed by using a BLAST search of homologous sequences available in the GenBank. The probe was 3′-end labelled enzymatically with digoxigenin using terminal deoxynucleotidyl transferase according to the manufacturer’s instructions (Roche, Basel, Switzerland). The quality of the RNA in the section was assessed by using a probe complementary to beta-actin, which is constitutively expressed in the tissues. The following negative controls were performed: (i) without probe; (ii) with an excess of unlabelled probe and (iii) pre-treated with a solution of RNase A (10 mg/ml). Hybridization, washes and probe detection were carried out as described previously (Rodríguez et al., 2010), except that overnight hybridization with the TAC3 probe was performed at 30°C.

**Immunohistochemistry**
The 3-μm sections were deparaffinised and then re-hydrated in 0.05 M tris-buffered saline (TBS; 0.05 M Trizma base containing 0.9% of NaCl, pH 7.4), which was used for all further incubations and washes. The sections were incubated overnight at room temperature with a NKB antibody dilution 1:75 (Premise antibodies; Sigma-Aldrich Co.) or a NKB antibody diluted 1:500 (ab5772; Abcam, Cambridge, UK) in TBS buffer containing 0.2% Triton X-100. After rinsing, the sections were incubated with a biotinylated goat anti-rabbit (1:10000) followed by a streptavidin–peroxidase conjugate (1:1000; Jackson ImmunoResearch, West Grove, PA, USA), both for 60 min at room temperature. Peroxidase activity was detected using 4-chloro-1-naphthol (Sigma-Aldrich Co.) and 0.01% hydrogen peroxide. The specificity of the immunostaining was assessed by replacing the specific antiserum by normal serum, omitting one step of the reaction or following preadsorption of the antiserum with the corresponding antigens.

**Results**
**Detection and quantification of TAC3 mRNA by real-time PCR**
We compared the relative expression of TAC3 mRNA between leiomyomas and matched myometrium from 28 women using qPCR. RT-negative samples showed no amplification. Melt curve analysis of
positive samples displayed only a single peak corresponding to the TAC3 transcript. Normalized data using three validated housekeeping genes showed that TAC3 expression was significantly up-regulated in leiomyomas, 20-fold on average, compared with matched myometrium ($P = 0.0008$, Fig. 1, Supplementary data, Table S1). Subsequently, we analysed the samples according to menstrual cycle phases: 20 samples in the proliferative stage (10 leiomyomas and 10 matched myometrium) and 36 samples in the secretory stage (19 leiomyomas and 17 matched myometrium). In both cases, statistical analysis continued to show a significant increase in TAC3 transcript in tumour compared with normal tissue ($P = 0.0359$ and $P = 0.0133$ for samples in the proliferative and secretory phases, respectively). The only sample in the menstrual phase also showed a higher expression of TAC3 in leiomyoma compared with matched myometrium (Supplementary data, Table S1).

**TAC3 mRNA in situ hybridization**

In order to find out which cells synthesized TAC3 mRNA, we performed in situ hybridization of TAC3 using a digoxigenin-labelled oligonucleotide DNA probe. In the normal myometrium, the staining was observed mainly in connective tissue cells (Fig. 2A). Interestingly, leiomyomas not only showed greater number of connective cells (Fig. 2B), but also new cells such as endothelial, vascular smooth muscle cells and myometrial smooth muscle cells were positive for TAC3 mRNA (Fig. 2C and E). Co-localization of TAC3 mRNA with desmin immunostaining confirms the smooth muscle cell lineage observed in leiomyomas (Fig. 2D and F).

**NKB immunohistochemistry**

We have recently observed the presence of NKB peptide in smooth muscle cells, vascular smooth muscle cells and connective cells of myometrium (Cejudo Roman et al., 2012). These cells were also immunoreactive in leiomyomatous tissue (Fig. 3A). However, when comparing leiomyoma with adjacent myometrium, we observed that smooth muscle cells showed a differential localization of the NKB immunoreactivity (NKB-ir) in such a way that in the normal myometrium in the secretory phase NKB-ir was detected preferentially in the nucleus, while in the matched leiomyomas was observed mainly in the cytoplasm (Fig. 3B and D). In order to confirm the presence of NKB in the nucleus of smooth muscle cells, NKB immunostained slides were counterstaining with 4',6-diamidino-2-phenylindole, dilactate (DAPI) (Fig. 3C and E). In the myometrium, since nuclei are previously immunostained with NKB, the fluorescent DAPI is seen as a bright halo. On the contrary, in leiomyoma, the entire nucleus shows fluorescence, while the cytoplasm stains positively for NKB.

**Detection of TACR3 mRNA**

We compared the relative expression of TACR3 mRNA between 20 leiomyomas and 18 matched myometrium. RT negative samples showed no amplification. Melt curve analysis of positive samples displayed only a single peak corresponding to TAC3 transcript. Normalization with three housekeeping genes showed that TACR3 expression was significantly up-regulated in leiomyomas, 2-fold on average, compared with matched myometrium ($P = 0.0349$, Fig. 4). When samples were analysed according to the menstrual phase, we observed that four of the 6 samples in the proliferative phase showed weak or no amplification in the myometrium but TACR3 transcript was strongly detected in matched leiomyomas. Furthermore, although two samples showed TACR3 expression in myometrium, higher expression was observed in the matched leiomyomas (Supplementary data, Table S2). The statistical analysis carried out in this group showed a significant up-regulation of TACR3 in tumour compared with normal tissue ($P = 0.0313$, Fig. 4). The analysis of normal myometrium samples in secretory cycle phase (12) showed great heterogeneity regarding transcript amplification with samples showing strong (3), reduced (6) or no amplification (6; data not shown). Analysis of TACR3 by qPCR showed higher (6) lower (2) or similar (6) relative expression in leiomyomatous tissue when compared with matched myometrium (data not shown). No statistically significant differences were observed when tumour and normal samples in the secretory phase were analysed ($P = 0.4263$).

**NK3R immunohistochemistry**

The pattern of NK3R immunostaining varies during the ovarian cycle, and so normal myometrium obtained from patients in the proliferative phase, show weak immunostaining compared with those in the secretory phase (Fig. 5A and C). However, we observed that, in leiomyomatous tissue, immunoreactivity to NK3R varies in a different way to that observed in the myometrium. Thus, the intense and weak NK3R-ir is observed in the myometrium but not in matched leiomyomas (Supplementary data, Table S1). The statistical analysis of these samples carried out in this group showed a significant up-regulation of NK3R expression in tumour when compared with normal tissue ($P = 0.0349$, Fig. 4). When samples were analysed according to the menstrual phase, we observed that four of the 6 samples in the proliferative phase showed weak or no amplification in the myometrium but TACR3 transcript was strongly detected in matched leiomyomas. Furthermore, although two samples showed NK3R expression in myometrium, higher expression was observed in the matched leiomyomas (Supplementary data, Table S2). The statistical analysis carried out in this group showed a significant up-regulation of NK3R in tumour compared with normal tissue ($P = 0.0313$, Fig. 4). The analysis of normal myometrium samples in secretory cycle phase (12) showed great heterogeneity regarding transcript amplification with samples showing strong (3), reduced (6) or no amplification (6; data not shown). Analysis of TACR3 by qPCR showed higher (6) lower (2) or similar (6) relative expression in leiomyomatous tissue when compared with matched myometrium (data not shown). No statistically significant differences were observed when tumour and normal samples in the secretory phase were analysed ($P = 0.4263$).

**Discussion**

In this study, we analysed the expression at the molecular and cellular levels of the NKB and its high-affinity NK3R in leiomyomas and adjacent myometrial tissue. Our main findings are as follows: (i) TAC3 expression...
was increased 20-fold in leiomyomas compared with myometrium; (ii) in leiomyomas, not only connective cells, but vascular and myometrial smooth muscle cells expressed TAC3 mRNA as demonstrated by in situ hybridization; (iii) immunodetection of NKB showed a preferential nuclear localization of this peptide in normal uterine smooth muscle cells during the secretory phase, unlike fibroids, where its location was essentially cytoplasmic and (iv) TACR3 was significantly up-regulated in leiomyomas compared with normal myometrium ($P = 0.0349$).

It is increasingly evident that the role of the tachykinin peptide family and their receptors in the regulation of the reproductive function is not only at

Figure 2 In situ hybridization using TAC3 oligonucleotide probe. Bright-field microscopy, using an anti-digoxigenin antibody directed against 3′-digoxigenin-labelled TAC3 oligonucleotide probe to detect TAC3 mRNA expression in myometrium (A) and leiomyoma cells (B). In both cases, connective cells show positive reaction (round blue staining pointed by arrows), although more abundantly in cells of leiomyoma. Furthermore, leiomyoma stained positively for endothelial (C, red arrows) and vascular smooth muscle cells (C, black arrows) from blood vessels as well as myometrial smooth muscle cells (E). Asterisk denotes blood vessel lumen. The same sections observed in C and E were subsequently analysed by immunofluorescence using an antibody directed against desmin and a rhodamine-labelled secondary antibody (D and F). Fluorescent microscopy confirmed the myogenic origin of the vascular smooth muscle cells (D) and myometrial smooth muscle cells (F).
the CNS level, but also in peripheral organs. Thus, in a recent study (Cejudo Roman et al., 2012), we observed the presence of NKB and NK3R in smooth muscle cells (asterisk), myometrial smooth muscle cells (arrowheads) and connective cells (arrows) of the leiomyomas (A). A detailed analysis of smooth muscle cells showed that, in normal myometrium, in the secretory phase most of the immune reaction was localized in the cell nucleus (B, arrow) unlike the matched leiomyomas, which showed positive staining mainly in the cytoplasm (D, arrows). Panels (C and E) show the same NKB immunostained slides of panels (B and D) but now counterstained with blue fluorescent DAPI, a DNA-specific fluorescent probe. In normal myometrium (C) a blue-violet nuclear immunostaining with fluorescent halo was observed, while in matched leiomyoma (E) nucleus (DAPI) and cytoplasm (blue-violet) stained separately.

**Figure 3** NKB immunostaining in myometrium and matched leiomyoma. Blue-violet positive staining showing NKB-ir in vascular smooth muscle cells (asterisk), myometrial smooth muscle cells (arrowheads) and connective cells (arrows) of the leiomyomas (A). A detailed analysis of smooth muscle cells showed that, in normal myometrium, in the secretory phase most of the immune reaction was localized in the cell nucleus (B, arrow) unlike the matched leiomyomas, which showed positive staining mainly in the cytoplasm (D, arrows). Panels (C and E) show the same NKB immunostained slides of panels (B and D) but now counterstained with blue fluorescent DAPI, a DNA-specific fluorescent probe. In normal myometrium (C) a blue-violet nuclear immunostaining with fluorescent halo was observed, while in matched leiomyoma (E) nucleus (DAPI) and cytoplasm (blue-violet) stained separately.

The precise physiological role of NKB in the uterus remains unclear. As described in the section ‘Introduction’, given the presence of TAC3 in
Dysregulation of NKB/NK₃R in leiomyomas

The expression of the NK₃R seems to be tightly controlled by estrogens at both central and peripheral levels (Pinto et al., 1999, 2009; Navarro et al., 2012). In ovariectomized rat and mice, the treatment with estrogens caused a strong decrease of uterine TAC3 mRNA (Pinto et al., 1999, 2009). In the present study, we observed that samples in the proliferative phase showed absence or weak amplification of TACR3 mRNA and weak immunostaining for NK₃R. However, in the secretory phase, normal myometrium showed great heterogeneity regarding transcript amplification with samples showing strong, reduced or no amplification. In general, intense NK3R-ir was observed in the myometrium in the secretory phase. These differences in transcript amplification probably reflect different levels of estrogens during each cycle phase. Further studies analysing sex steroid levels from patient’s serum may establish a correlation between hormonal level and TACR3 mRNA expression in the human myometrium.

In leiomyomas, all six samples studied in the proliferative phase showed strong amplification of TACR3 mRNA and intense NK3R-ir. When this sample group was compared with matched myometrium, the significant up-regulation of TACR3 was observed. In addition, almost half of the samples in the secretory phase showed higher expression levels of TACR3 mRNA in leiomyomas compared with matched myometrium. Furthermore, when all samples were analysed, TACR3 was significantly up-regulated in leiomyomas compared with normal myometrium. Ovarian steroids play a key role in the pathophysiology of uterine leiomyomas and high levels of estrogen and progesterone receptors are found in leiomyomas, compared with adjacent myometrium (Brandon et al., 1993; Rodríguez et al., 2011). In this sense, our data argue for differential regulation or reprogramming of the effects of E₂ on NKB and NK₃R expression, such as occurs with other estrogen-responsive genes expressed in uterine leiomyomas (Rodríguez et al., 2010, 2011).

The presence of the NK₃R in smooth muscle cells of myometrium is especially intriguing, since NK₃R-selective agonists were ineffective in producing contraction in the myometrium from non-pregnant women (Patak et al., 2003). On the other hand, the presence of NKB in the nuclei of smooth muscle cells raises the question as to what mechanisms are involved in targeting NKB to the nucleus. It has been previously reported that rat NK₃R presents two putative nuclear localization sequences (NLSs) in the cytoplasmic C-terminus (Howe et al., 2004; Lee et al., 2004). Moreover, injections of senktide in rat brain induced a nuclear translocation of NK₃R in neurons, with NK₃R-ir localized in chromatin-rich regions of the nucleus (Howe et al., 2004; Haley and Flynn, 2006; Jensen et al., 2008). The studies by Howe et al., Haley et al. and Jensen et al. utilized two different antibodies, both directed at the COOH-terminal region of the NK₃R. Since mammalian tachykinin receptors are highly conserved, we have observed that human NK₃R also

![Figure 4](image-url) Expression of TACR3 mRNA in myometrium and matched leiomyoma. Relative quantification of TACR3 mRNA in 20 leiomyoma and 18 matched myometrium samples from 18 women (All). Samples were also analysed based on the stage of the menstrual cycle: 6 leiomyomas and 6 matched myometrium in the proliferative phase (Prol) and 14 leiomyomas and 12 matched myometrium in the secretory phase (Sec). Statistical analysis was performed using the Wilcoxon matched-pairs signed-rank test. Bars show the mean fold change and error bars indicate SEM. The sample that showed the lowest expression level was used as calibrator. Relative expression data are visualized on a Log₁₀ scale. *P < 0.05.
present identical NLS signals to that observed in rats. Therefore, it seems reasonable to suggest that, in uterine smooth muscle cells, NKB coupled to NK3R could be internalized to the cytoplasm and then translocated to the nucleus. The reason why we did not detect immunoreactivity to NK3R in the nucleus may be that only fragments of the receptor are translocated to the nucleus. This has been postulated as a mechanism for freeing active portions of membrane-bound receptors for moving into the nucleus (Wells and Marti, 2002) and is a possible reason for the variable detection of nuclear translocation of NK3R in different tissues (Howe et al., 2004). Under this scenario, the detection of nuclear NK3R would be dependent on the utilization of an antibody directed to the portion of the molecule that enters the nucleus. In the current study, the antibody assayed was directed at the N-terminal region of the NK3R. Alternatively, other mechanism(s) independent of the receptor may explain the presence of NKB in the nucleus of smooth muscle cells (Pederson, 1998).

Although the mechanism of nuclear translocation of NKB and its role in the nuclei of smooth muscle cells of normal myometrium are unknown, it is clear that, in leiomyomas, NKB is overexpressed, it shows a subcellular location preferably cytoplasmatic during the secretory phase and the expression of its high-affinity NK3R is also up-regulated, supporting a role for this system in leiomyoma pathophysiology. Given the vasoactive role of NKB in blood vessels, it seems tempting to speculate that excess of NKB released by tumour cells may act paracrinally on tachykinin receptors present in vascular cells contributing to the menorrhagia clinically seen in women with leiomyomata. In addition, whether NK3R dysregulated expression in leiomyoma may contribute to this vasoactive effect and/or may be related to tumour progression, merits further investigation.

**Figure 5** NK3R immunostaining in myometrium and matched leiomyoma in different stages of menstrual phase. Panels (A and C) show the differential NK3R immunoreactions (blue-violet) observed in two samples of myometrium during the proliferative phase (A, weak) and secretory phase (C, intense). The contrary is observed in matched leiomyomas showing an intense staining in the proliferative phase (B) but weak immunostaining in the secretory phase (D). During menses, leiomyoma showed a strong signal for NK3R in blood vessels (F) compared with matched myometrium (E). Asterisk denotes blood vessel lumen.
Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles
H.C. collected data, performed RNA extraction, cDNA amplification, real-time quantification and also contributed to the manuscript drafting. I.D. carried out immunohistochemistry, in situ hybridization and also contributed to the manuscript drafting. M.H. performed statistical analysis and drafted the manuscript. A.C.R., F.M.P. and L.C. participated in study design, conducted preliminary analyses to evaluate the feasibility of this study and they also revised the manuscript critically. F.V. processed and evaluated samples for histopathology and also participated in the design of the study. D.B. and F.M.O collected tumour and myometrium samples for molecular and histological analysis, provided patient data and also participated in study design. A.R.B. supervised and analysed the cytological data (immunohistochemistry and in situ hybridization) and revised the manuscript critically. L.C. and T.A.A. conceived the study and revised the manuscript critically. All authors have read and approved the final manuscript.

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Conflict of interest
None declared.

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