17beta-oestradiol-induced gene expression in cattle prostate: biomarkers to detect illegal use of growth promoters

I 7p-estradiol-induced gene expression in prostate cell culture and tissue from cattle: biomarkers to detect illegal use of growth promoters

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The effects of 17p-oestradiol (E_2) on gene expression in cultures of bovine primary prostate stromal cells (BPSCs) and prostate gland tissue were studied. In the first part of the study, BPSCs were grown in the presence of E_2 from the first passage to the end of the experiment; a second group was treated in the same way but the treatment was suspended for 48 hours before the end of the experiment; a third group of BPSCs served as a control. In the second part of the study, five male veal calves, aged 130 days, were treated four times intramuscularly with 10 mg of E_2 at intervals of two weeks and then euthanased two weeks after the last treatment. Quantitative **PCR** and immunohistochemistry were used to evaluate the expression of fibroblast growth factor **(FGF)** receptors (FGFRs), FGFs, progesterone receptor, androgen receptor and oestrogen receptor in BPSCs and prostate tissue. E_2 induced a significant over-expression of progesterone receptor in both BPSCs and prostate tissue. There was also a marked up-regulation of **FGFR** types I, 2 and 3 genes observed in the BPSCs.

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THE illegal use of growth-promoting agents is a significant problem in animal production. In this context, sex steroids, β-agonists, thyreostatics and corticosteroids are often administered to cattle. Anabolic steroids increase growth rate, stimulate the growth of muscle and reduce fat tissue in calves (Groot and others 1998) by acting on lipid and protein metabolism. The use of natural and synthetic hormones as growth promoters in food producing animals is prohibited in the European Union and analytical methods are employed to prevent their illegal use (Anon 1996, Courtheyn and others 2002). However, these techniques appear to give unsatisfactory results (Groot and others 2007) and it is therefore essential to develop indirect biomarkers to reinforce veterinary controls.

In recent years, increasing attention has been paid to the study of the effects of growth promoters on target organs, most of all on the histo-pathology of the accessory sex glands (Groot and others 2007). Among the anabolic steroids, 17β-oestradiol (E_2) is one of the most efficient. Histologically, it induces hyperplasia and metaplasia of prostate cells and the urethral epithelium (Ruitenberg and others 1970, Kroes and others 1971, Groot and others 1998). Squamous cell metaplasia is a specific response to a variety of oestrogenic substances, including E,, diethylstilbestrol and zeranol, and it is considered to be a reliable histological marker to assess their oestrogenic action in cattle (Biolatti and others 2003). However, more accurate and specific methods are required to reinforce the methods described above; for this purpose the development of genomic methods could lead to a potentially reliable technique, easily transferable to veterinary public health.

Oestrogens and androgens regulate the growth and differentiation of the prostate gland in rats (Naslund and Coffey 1986) and mice (Gail and others 2001). In human beings, the homeostasis of normal prostate depends on a two-way communication between epithelial and stromal compartments, including smooth muscle cells, myofibroblasts, fibroblasts and blood vessel cells (International Prostate Health Council 2000). Stromal cells regulate the growth and function of the prostate, acting as epithelial inducers and participating in a close paracrine stromal-epithelial regulatory network in the adult gland (Franks and others 1970, Cunha and others 1980, 2004a). They have receptors for androgens, oestrogens, glucocorticoids and progesterone (Naslund and Coffey 1986). Oestrogens act through their specific receptors, oestrogen receptor xx, (ERxx) and oestrogen receptors (ERs) a and β (Kuiper and others 1996, Leav and others 2001), expressed in the prostatic stroma and epithelium, respectively. As a result of their interactions with oestrogens, stromal cells synthesise various growth factors: epidermal growth factor, keratinocyte growth factor, insulin-like growth factors I and II, fibroblast growth factors (FGFs) and transforming growth factors a and β (Wong and Tarn 2002, Bektic and others 2004). These substances mediate many functions, including cellular secretion, proliferation, differentiation and apoptosis (Steiner 1993). Among several growth factors and receptors implicated in the proliferation of the prostate, the family of FGFs and FGF receptors (FGFRs) plays a critical role (Kwabi-Addo and others 2004, Gowardhan and others 2005). The gene encoding for progesterone receptor (PR) is regulated by oestrogens and shows the typical oestrogen receptor element at the 5' end. This gene is widely accepted as a marker of oestrogenic action in different human and murine tissues (Graham and Clarke 1997, Kurita and others 2000, Williams and others 2000). The PR gene and its protein have been characterised in the reproductive system of female cattle where they behave and have a tissue distribution similar to that in women (Schams and others 2003, Taylor and others 2006), but there is no evidence of their presence in the reproductive system of male cattle.

The aim of this work was to study E,-related gene expression, first by challenging cultures of bovine primary prostate stromal cells (BPSCs) to better understand the biological role of E in prostate cell physiology, and secondly, in veal calves, to establish potential new biomarkers to be applied in the detection of calves treated illegally with hormones.

Materials and methods

Establishment of bovine prostate stromal cell cultures and experimental design

Samples of prostate tissue were obtained postmortem from six-month-old Holstein calves. The tissue was dissected under sterile conditions and the samples were minced into small pieces and spread on to the surface of 25 cm^2 culture flasks according to the methods described by Zhang and others (1997). The arising cells were routinely maintained in MCDB-131 medium (Sigma) supplemented with 15 per cent decalcified horse serum (Gibco), non-essential amino acids (Sigma), 5 µg/ml insulin (Sigma), 10 mg/ml transferrin (Sigma), 5 ng/ml sodium selenite (Sigma), 100nM dexamethasone (Sigma), 0-1 ng/ml epidermal growth factor (Gibco), 1 ng/ml basic FGF (bFGF) (Gibco), an antibiotic-antimycotic cocktail (Sigma) and glutamine (Sigma). After seven days, the medium was removed and then renewed every three days. When the primary culture reached two-thirds confluence, the cells were detached with 3 ml trypsin/EDTA solution and poured through a 100 pm mesh to remove pieces of tissue. The cells were used for the experiment at the fifth passage, when a higher density of BPSCs were available. The confluent cells were subdivided as follows: group C (control), cells grown in MCDB-131 medium with supplements and without phenol red and E_2; group T (treated), cells cultured in MCDB-131 medium with 100nM E_2 for five passages; group S (suspended), cells cultured in lOOnM E_2 for five passages and then grown without E_2 for 48 hours. All the experiments were conducted in triplicate.

Two groups of 130-day-old male Holstein veal calves weighing an average 178 kg were used. They were fed liquid milk replacer twice a day and after a month 0-5 kg/day of straw was added to their diet according to the recommendations of the European Commission (97/182/EC). The five calves in group T were injected intramuscularly with 10 mg E_2 on four occasions at intervals of 15 days, an anabolic dosage according to Vanderwal and others (1975) and Meyer Heinrich (2001). They were slaughtered 15 days after the last injection. The three calves in group C served as controls. Samples of prostate tissue from the treated and control calves were fixed in 10 per cent buffered formalin solution for histological examination and in RNA/later (Ambion) for molecular studies.

Authorisation to carry out the experiment was given by the Italian Ministry of Health and the Bioethics Committee of the University of Turin. After the experiment, the carcases of the treated animals were destroyed in accordance with European Council Directive 2003/74/ **EC.**

Total RNA extraction and quantitative PCR

Total RNA was extracted from the prostate tissue and the BPSCs by the method of Chomczynski and Sacchi (1987) and any residual genomic DNA was removed (DNA-free Kit; Ambion). The integrity of the RNA was confirmed by denaturing agarose gel electrophoresis, and the concentration was measured as the optical density at 260 nm by spectrophotometry. Complementary DNA (cDNA) was synthe-sised from 400 ng of total RNA using ImProm-II reverse transcriptase (Promega) and random primers (Promega). To determine the amount of the specific target genes (Table 1), cDNA was subjected to quantitative PCR, using the Syber Green method and the IQ 5 (Bio-Rad) detection system. The primer sequences were designed using Primer Express v 1.5 (Applied Biosystems).

The relative amount of gene expression was calculated by a relative quantification assay corresponding to the comparative cycle

threshold (Ct) method. The amount of target, normalised to an endogenous house-keeping gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and relative to the calibrator (control sample), was then given by $2\sim^{AACt}$ (power), where AACt = ACt(sample)—ACt(control), and ACt is the Ct of the target gene subtracted from the Ct of the housekeeping gene.

The data were analysed using GraphPad InStat version 3.00 for Win-dows (GraphPad Software). The Mann-Whitney U test (a two-sample non-parametric test) was applied to compare the data from the treated and control samples. Differences between data were considered significant at P<0-05.

Cytological and histological investigations

BPSCs were fixed in 100 per cent acetone for 10 minutes at room temperature. Samples of prostate tissue were fixed in 10 per cent neutral buffered formalin overnight at room temperature, embedded in paraffin by routine procedures, and representative sections of each sample were stained with haematoxylin and eosin. To evaluate the expression of the stromal cell markers, immunocytochemistry was applied to the BPSCs, using the following antibodies: anti-smooth muscle actin (clone 1A4; Sigma), anti-desmin (clone DE-U-10; Sigma), anti-smooth myosin (clone HCMV; Sigma) and anti-vimentin (clone AS02; Sigma).

Immunohistochemistry for anti-PR and anti-proliferating cell nuclear antigen (PCNA) was performed on sections of prostate tissue after inactivation of the endogenous peroxidase and antigen retrieval in 0-01M citrate buffer, pH 6-0 for 15 minutes at 700 W. The sections were subsequently incubated with anti-PR clone HPRA2 and HPRA3 (Sigma) and anti-PCNA (Dako) primary antibodies for one hour at room temperature. The immunostaining was visualised with the EnVi-sion kit (Dako) containing secondary antibody labelled with horseradish peroxidase.

Results

Maintenance and characterisation of BPSCs

After seven days in the stromal cell-selective medium, cells began to emerge from the tissue pieces (Fig la) and after two to three passages a homogeneous stromal cell culture was obtained (Fig lb). The cells expressed the following mesenchymal markers: α-actin, desmin, vimen-tin and smooth muscle myosin (Fig lc, d, e, f). The residual cells of epithelial origin disappeared after two to three passages, as revealed by negative cytokeratin staining.

Gene expression in BPSCs

The BPSCs challenged with E showed a significant gene over-expression for the FGFRs (power of 1-88 for FGFR1, 3-45 for FGFR2 and 8*41 for FGFR3) (Fig 2). The PR gene was also over-expressed up to 30-fold compared with controls (Fig 2). The gene over-expression was maintained in group S, suggesting that the biological effect persisted for 48 hours after treatment with E_2 had been suspended. The mRNA encoding for ERa was significantly down-regulated (3-45-fold) in the presence of E_2 and a non-significant decrease of transcript corresponding to bFGF was also observed.

The prostate tissue of the veal calves treated with E showed a significant 10'78-fold up-regulation of the PR gene and 1'34-fold up-regulation (P<0-05) of the ERa gene (Fig 3).

Morphological and histological investigations

There were no statistically significant differences between the growth rates of the experimental groups. No muscular lesions were detected at the sites of injection of the drug or placebo, and there were no macroscopic changes in the prostate glands of the treated animals; however, there were severe morphological changes in the form of hyperplasia and metaplasia of the urothelium and glandular epithelium, fibrosis and duct dilation (Fig 4b, d, f, h).

Immunohistochemistry

The epithelial proliferating cells of the treated animals, both in the gland and urethra, were strongly positive to PCNA antigen (Fig 2b, d) compared with the control animals (Fig 2a, c). The over-expression of PR in the prostate gland and urothelium of the treated animals was confirmed by immunohistochemical staining (Fig 2e, f, g, h). There was positive expression in both the nucleus and cytoplasm, suggesting the presence of the nuclear (A) and cytoplasmic (C) isoforms.

Discussion

Oestrogens are the most frequently used illegal growth promoters in ani-mal production. The aim of this study was to identify the genes regulated by oestrogens in the prostate gland, in order to identify a new molecular target to apply in veterinary inspection. By using a quantitative PCR assay, Toffolatti and others (2006) identified seven genes that are differentially expressed in the prostate gland of calves treated experimentally with two combinations of growth promoters (boldenone undecylenate and oestradiol benzoate, and testosterone enantate and oestradiol ben-zoate), suggesting further gene targets to apply as biomarkers in veteri-nary inspection.

In this study, the effects mediated by E_2 in bovine prostate tissues and in primary BPSCs were evaluated. As already described in mouse models (Gail and others 2001), the prostate and urethra of calves treated with oestrogens showed typical histological changes of epithelial metaplasia and hyperplasia (Groot and others 1998). Moreover, the epithelial pro-liferating cells of the prostate and urethra of the treated animals were strongly positive for PCNA antigen, one of the most important markers involved in mitogenic signals.

Because stromal cells express ERa and have a principal role in the regulation of prostate growth and function in human beings and mice (Cunha and others 2001, 2004b), BPSCs were established and characterised and the biological effects mediated by E_2 on gene expression in them were evaluated. Morphologically, the BPSCs were similar to those described in human beings and reacted to E_2 by modifying the expression of genes encoding for FGFRs and FGFs as already observed in human beings and mice (Kwabi-Addo and others 2004, Graham and Clarke 1997). The treatment of the BPSCs with E_2 induced a two-to three-fold increase in FGFR1, FGFR2 and FGFR3 genes, and this high level of expression remained unchanged for at least 48 hours after the suspension of the treatment with E_2. In addition, they expressed a 30-fold increase in the gene encoding for PR and this value remained unchanged for 48 hours after the withdrawal of E_2. The results are similar to those obtained in studies of the mouse prostate, in which E, induces a significant over-expression of PR protein and mRNA (Gail and others 2001). Moreover, in the BPSCs, there was only a small decrease in the transcripts corresponding to bFGF, adrogen receptor, ERa, vascular endothelial growth factor, transforming growth factor p and insulin-like growth factor. It is known that oestrogens induce a down-regulation of the androge receptor which is mediated by PR isotype A, but the decrease in mRNA encoding for ERa is not completely understood. In human beings, Smith and others (2002)

assume that, in vitro, a high concentration of E, saturates the ERa receptor, inducing a decline in its rate of synthesis.

The gene expression profile observed in the BPSCs was also investigated in the prostate glands of veal calves treated with E_2. It was found that 15 days after the last hormone administration, the prostate gland tissue expressed 10*8- and 1-34-fold increases in the genes encoding for PR and ERa, respectively, but the level of gene expression of the FGF system was unchanged. The involvement of the FGF system in hormone regulation has also been demonstrated in follicle maturation and in the mammary gland in veal calves (Schams and others 2003, Sinowatz andothers 2006).

The differences between the expression of the FGF system genes in vitro and in vivo may be related to the experimental models adopted in this study: BPSCs are a homogeneous cell type, whereas the prostate gland contains different cell types, for example, stromal cells and epi-thelial cells, which respond differently to E_2 treatment. On the other hand, the prostate tissue was examined 15 days after the last treatment, whereas the BPSCs were analysed 48 hours after the withdrawal of E_r The up-regulation of the FGF-system may not persist for as long as 15 days after the treatment has been discontinued. The results of this study demonstrate that PR isoform A is the most important gene regulated by oestrogen, both in the prostate gland and in BPSCs.

As in human beings, the bovine PR is composed of three distinct isoforms, A, B and C. Isoforms A and B are localised in the nucleus and isoform C is localised in the cytoplasm (Schams and others 2003, Taylor and others 2006). Using specific primers focused on a gene encoding for isoform A, it was shown that E_2 regulated the expression of isoform A, and isoform B was not involved. These data are in agreement with the results of studies in human beings in which it has been shown that the PR isotype A is regulated by oestrogens and acts as a potent activator of progesterone-responsive genes and as a strong trans-dominant repressor of PR isotype B, androgen receptor, mineralocorticoid receptor and ER transcriptional activity (Taylor and others 2006).

In cattle, the regulation of PR isotype A by oestrogens has previously been studied only in the female reproductive system (Van den Broeck and others 2002, D'Haeseleer and others 2007). In the present study, the high level of mRNA encoding for PR was confirmed by protein expression and its detection by immunohistochemistry in prostate tissue. Both the nucleus and the cytoplasm of urethral and epithelial prostate cells were positive. The positive cytoplasm suggests the presence of PR isoform C, already described in human amnichorion and bovine mammary gland.

This report demonstrates that oestrogens induce an over-expression of PR isotype A in the prostate of prepubertal male calves, and that it is hardly detectable in control calves. These results, and the absence of scientific data on the effects on the expression of PR in bovine prostate tissue of other physiological and pathological conditions, suggest that it might be used as a specific oestrogen-related biomarker to detect the illegal use of oestrogen as a growth promoter in livestock production, specifically in male prepubertal veal calves. These preliminary results suggest that the study of gene expression profiling could lead to the development of specific tests for the detection of the illegal treatment of cattle with anabolic steroids, and support the results of chemical analyses and histopathology. Gene over-expression after the administration of E, can be detected for up to 15 days after the treatment is discontinued, whereas chemical methods can detect residues for only a few hours.

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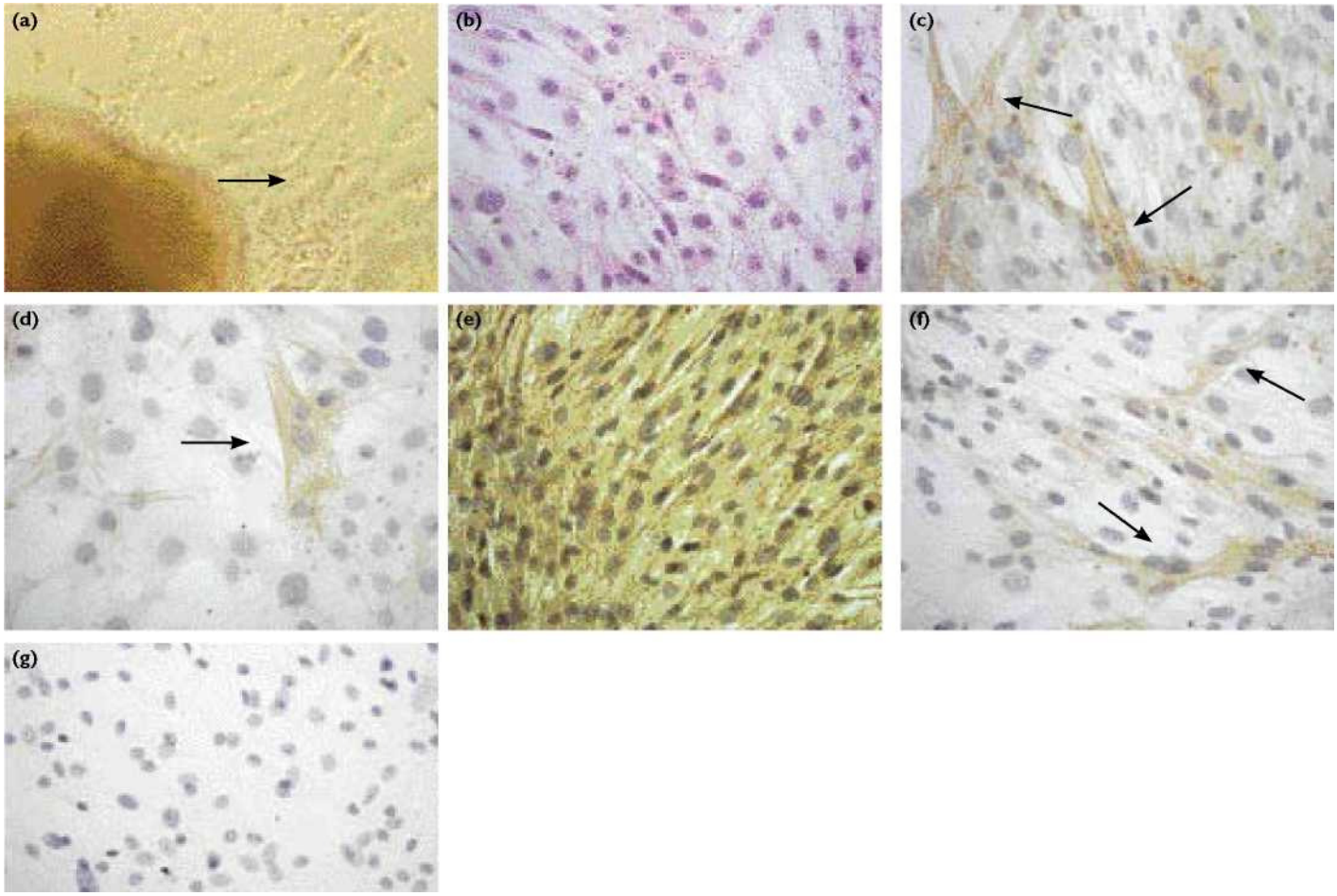
TABLE 1: Target genes selected in quantitative real-time PCR

GenBank accession number	Name	Forward primer	Reverse primer
NM 001001443.1	ER	ACCAACCAGTGCACGATTGA	TTCCGTATTCCGCCTTTCAT
AY862875.1	AR	TCCACATTGTCCCTGCTGG	TGAAGAAGTTGCATGGTGCC
XM 583951.3	PR	CCAGAGCCCACAGTACAGCTT	CAGCTTCCAC AGGTGAGGACA
AJ004952.1	FGFR1	CGAGACATCCACCACATCGA	TGGGTGTAGATCCGGTCAAAC
Z68150.1	FGFR2	TCCTACGACATCAACCGCG	TGGGAAGCCAAGTACTCCATG
NM 174318.2	FGFR3	AAGATGACGCCACGGATAAGG	GGCGCCTAGCAGGTTGATAAT
X54980.1	IGFR	GAGGGAGGTCATGCAGATCG	TCTCGAGCTCTTCCGGGTC
NM 174056.2	bFGF	GGCTTCTTCCTGCGCATC	ACCCCTCTCTCTTCTGCTTGAA
NM 174621.2	TGF	TGGGCTTTGCTTTGTTTCTGT	CAAATGGCCTGTCTCGTGG
NM 174216.1	VEGF	AGAAAATCCCTGTGGGCCTT	TGCAACGCGAGTCTGTGTTT
U85042.1	GAPDH	ACACCCTCAAGATTGTCAGCAA	TCATAAGTCCCTCCACGATGC

AR Androgen receptor, bFGF Basic fibroblast growth factor (FGF), ER Oestrogen receptor, FGFR FGF receptor, GAPDH Glyceraldehyde 3-phosphate dehydrogenase, IGFR Insulin-like growth factor receptor, PR Progesterone receptor, TGF Transforming growth factor, VEGF Vascular endothelial growth factor


(a)
(b)
(c)
(d)
(e)
(f)
(g)

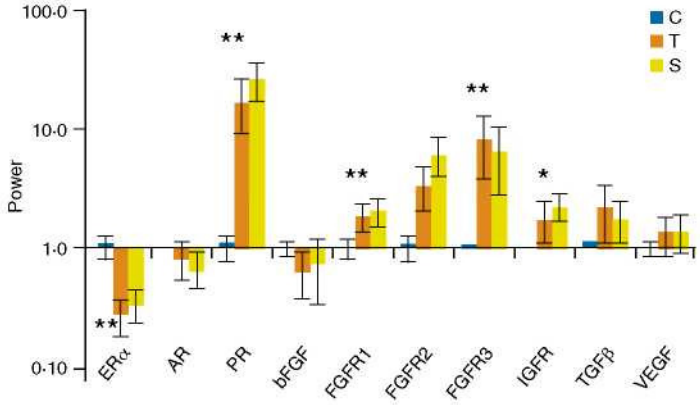


FIG 2: Analysis of gene expression under different growth conditions in bovine primary stromal cells (BPSCs) treated with 17β-oestradiol (E_2) (group T), in BPSCs treated with E_2 in which the treatment was suspended for 48 hours (group S) and in control BPSCs (group C). * P<0-05, ** P<0-01. AR Androgen receptor, bFGF Basic fibroblast growth factor (FGF), ER Oestrogen receptor, FGFR FGF receptor, IGFR Insulin-like growth factor receptor, PR Progesterone receptor, TGF Transforming growth factor, VEGF Vascular endothelial growth factor

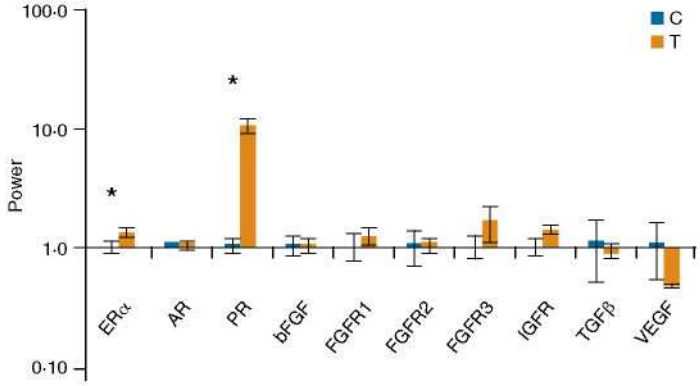
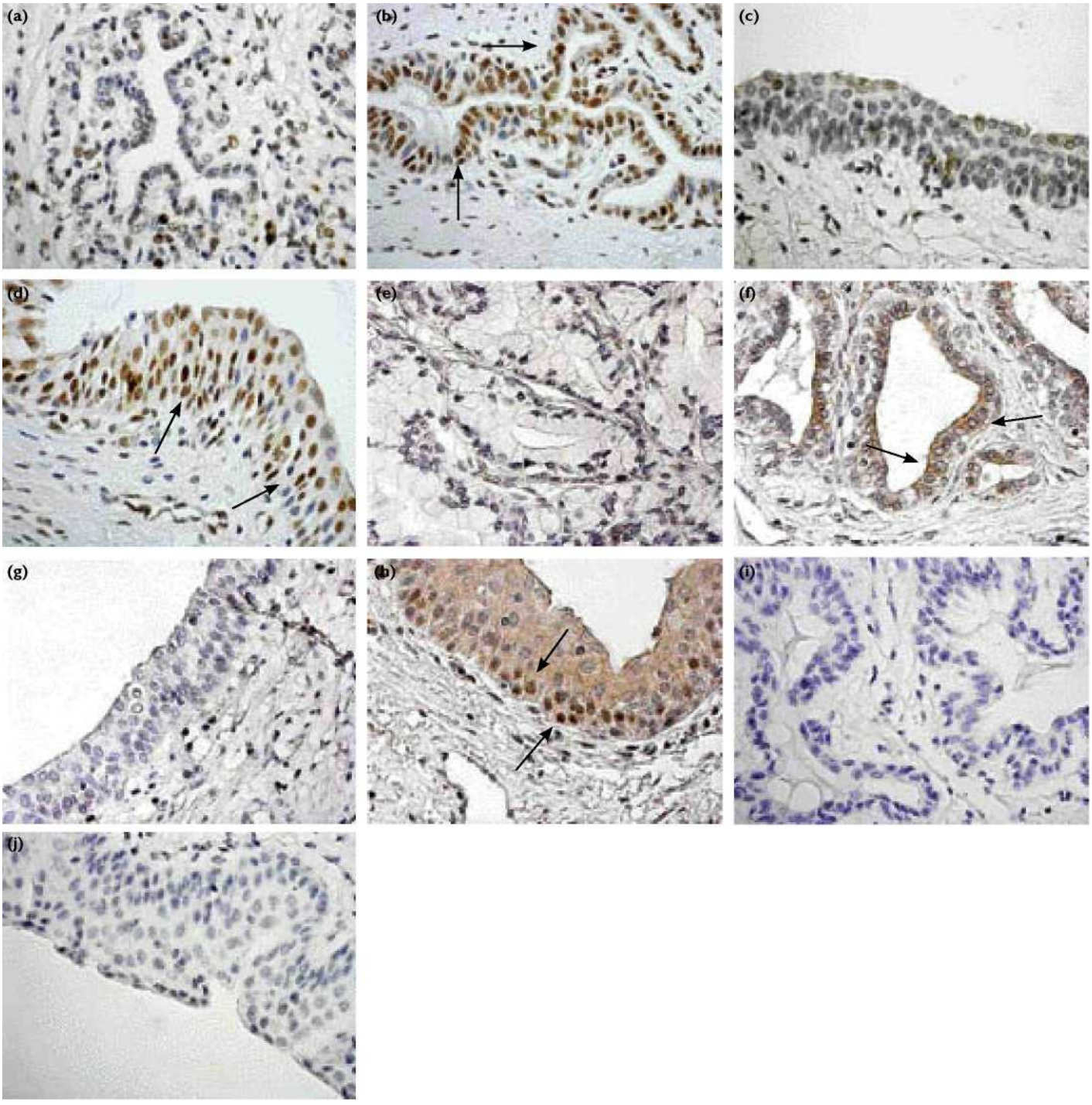


FIG 3: Analysis of gene expression in prostate tissue from calves treated with 17p-oestradiol (group T) or untreated control calves (group C). *P<0-05. AR Androgen receptor, bFGF Basic fibroblast growth factor (FGF), ER Oestrogen receptor, FGFR FGF receptor, IGFR Insulin-like growth factor receptor, PR Progesterone receptor, TGF Transforming growth factor, VEGF Vascular endothelial growth factor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