Deficiency of ER beta and prostate tumorigenesis in FGF8b transgenic mice

Teresa Elo¹, Lan Yu¹, Eeva Valve², Sari Mäkelä³ and Pirkko Härkönen¹,⁴

¹Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku, Finland
²Department of Pharmacology, Drug Development and Therapeutics, Institute of Biomedicine, University of Turku, Finland
³Institute of Biomedicine, Turku Center for Disease Modeling & Functional Foods Forum, University of Turku, Finland
⁴Department of Laboratory Medicine, MAS University Hospital, Lund University, Sweden

Short title: FGF8b and ERb in prostate tumorigenesis

Key words: estrogen receptor beta, fibroblast growth factor 8b, transgenic mouse, prostate, tumorigenesis.

Abbreviations: ERb, estrogen receptor beta; FGF8, fibroblast growth factor 8; BERKO, ERb knockout; mPIN, mouse prostatic intraepithelial neoplasia; Tg, transgenic.

Word count: 5566

Corresponding author and to whom reprint request should be addressed: Teresa Elo,
Department of Cell Biology and Anatomy, Institute of Biomedicine, University of Turku,
Kiinamyllynkatu 10, 20520 Turku, Finland. E-mail:teresa.elo@utu.fi

Abstract

Estrogens contribute to the development and growth of the prostate and are implicated in prostate tumorigenesis. In their target tissues, estrogens mediate their effects via estrogen receptor alpha (ERα) and beta (ERβ). Hyperplasia and decreased differentiation of epithelial cells in the prostate have been reported in ERβ knockout (BERKO) mice. We studied here the effect of ERβ deficiency on prostate tumorigenesis by crossing BERKO<sub>FVB</sub> mice with prostate-targeted fibroblast growth factor 8b transgenic (FGF8b-Tg) mice. Consistent with our previous report, the prostates of one-year-old FGF8b-Tg mice displayed stromal aberrations, prostatic intraepithelial neoplasia (mPIN) lesions, inflammation and occasionally cancer. The prostates of BERKO<sub>FVB</sub> mice contained mild epithelial hypercellularity and inflammation. The prostate phenotypes of FGF8b-Tg-BERKO<sub>FVB</sub> mice closely resembled those of FGF8b-Tg mice. However, mucinous metaplasia, indicated by Goblet-like cells in the epithelium, was significantly more frequent in the prostates of FGF8b-Tg-BERKO<sub>FVB</sub> mice than FGF8b-Tg mice. Furthermore, compared to FGF8b-Tg mice, there was a tendency for increased frequency of inflammation but milder hyperplasias in the prostate stroma of FGF8b-Tg-BERKO<sub>FVB</sub> mice. The expression levels of mRNAs for FGF8b-regulated genes including osteopontin (Spp1), connective tissue growth factor (Ctgf), fibroblast growth factor receptors (Fgfrs), and for steroid hormone receptors and cytokines were similar in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice. Our results suggest that ERβ plays a role in differentiation of the prostatic epithelium and potentially in protection from inflammation but do not support a direct tumor suppressive function of ERβ in the prostate of FGF8b-Tg mice.
Introduction

Estrogens can affect the development of the prostate and prostate tumorigenesis. Exposure to high levels of circulating estrogens during *in utero* development and aging has been shown to impact the prostate and may contribute to the development of benign prostatic hyperplasia (BPH) and prostate cancer (Härkönen & Mäkelä 2004, Prins & Korach 2008, Ellem &Risbridger 2009, Hartman *et al.* 2012).

In target tissues, estrogens act via ERa (ESR1) and ERb (ESR2), which are nuclear receptors encoded by separate but structurally homologous genes (Greene *et al.* 1986, Kuiper *et al.* 1996, Tremblay *et al.* 1997). In the prostate, ERa and ERb are expressed in different compartments: ERa is expressed in the stroma, and ERb is expressed primarily in the epithelium (Schulze & Claus 1990, Prins & Birch 1997, Prins *et al.* 1998, Mäkelä *et al.* 2000). Moreover, evidence from several *in vitro* and *in vivo* studies indicates that the two ERs have opposing functions in the prostate; ERa promotes proliferation, inflammation and the development of dysplasia, whereas ERb promotes differentiation and has anti-proliferative and anti-inflammatory effects (Risbridger *et al.* 2001, Prins *et al.* 2001, McPherson *et al.* 2007, Savolainen *et al.* 2007, Ricke *et al.* 2008, Slusarz *et al.* 2012, Dey *et al.* 2013). Based on the prostate phenotypes of conditional Era-knockout (ERKO) mice, ERa is required for the normal branching morphogenesis of the prostate and for maintaining fibroblast proliferation in the prostatic stroma (Chen *et al.* 2009). Interestingly, the reports on the prostate phenotype of the BERKO mice have been conflicting. Some studies have reported epithelial hyperplasia, increased proliferation (Weihua *et al.* 2001), inflammation (Prins & Korach 2008) and an elevated number of basal cells in the prostate of BERKO mice (Imamov *et al.* 2004), but other studies did not find any prostate phenotype in the BERKO mouse models (Dupont *et al.* 2000, Prins *et al.* 2001, Antal *et al.* 2008).

Several functionally different isoforms of human ERb (hERb1-5) exist (Moore *et al.* 1998, Leung *et al.* 2006). Studies have reported a decreased level of hERb in PIN lesions or in prostate
cancer, supporting the tumor-suppressive role of ERb (Horvath et al. 2001, Leav et al. 2001, Pasquali et al. 2001, Fixemer et al. 2003, Muthusamy et al. 2011). According to Zhu et al. (2004) the loss of ERb expression during prostate tumorigenesis is epigenetically regulated by the hypermethylation of CpG islands in the ERb promoter. On the other hand, a high level of ERb expression has been reported in advanced prostate cancer (Horvath et al. 2001, Leav et al. 2001, Torlakovic et al. 2002, Walton et al. 2009) and in prostate cancer metastasis (Fixemer et al. 2003). The conflicting results regarding the expression of ERb during prostate tumorigenesis in different studies can be at least partly explained by the use of different antibodies, which were either specific for the hERb1 isoform or recognized all or several hERb isoforms, which have differential functions (Leung et al. 2006). Recent studies have shown that the different isoforms of ERb have different functions in prostate tumorigenesis. hERb1 has a tumor-suppressive role, while hERb2 and hERb5 are oncogenic and promote the proliferation, invasion and metastasis of prostate cancer cells (Leung et al. 2010, Dey et al. 2012). The results regarding the expression of ERa in human prostate cancer are also conflicting; some reports have shown an increase in the expression of ERa during prostate tumorigenesis (Bonkhoff et al. 1999), but others have found that ERa expression is silenced by promoter methylation in early prostate cancers (Lau et al. 2000, Li et al. 2000).

The purpose of this study was to evaluate the potential role of ERb in prostate tumorigenesis and the relationship between ERb and FGF signaling in a novel mouse model. We have previously reported that prostate-targeted FGF8b overexpression in Tg mice leads to the development of epithelial and stromal hypercellularity, which progresses with age to preneoplastic and neoplastic lesions such as mPIN, adenocarcinoma and sarcoma in the prostate (Elo et al. 2010). To study whether the deficiency of ERb accelerates the tumorigenesis observed in the FGF8b-Tg mice or provides a more severe prostate phenotype, FGF8b-Tg mice were crossed with BERKO FVB mice, and the prostate histology of and expression of genes in the prostate of the resultant hybrid mice were carefully examined.
Materials and methods

Animal experiments

FGF8b-Tg mice in the FVB/N strain were generated in our laboratory as previously reported (Elo et al. 2010). BERKO mice, generated by conventional knockout techniques in the C57BL/6J strain (Krege et al. 1998), obtained from Jan-Åke Gustafsson (Karolinska Institutet, Sweden), were crossbred with FVB/N mice for 7 generations to produce BERKO<sub>FVB</sub> mice. Generation of FGF8b-Tg<sup>−/−</sup> (FGF8b-Tg-BERKO<sub>FVB</sub>) mice by crossings in two generations is described in detail in Supplementary figure 1. The genotyping for both genomic modifications has been previously described (Windahl et al. 1999, Elo et al. 2010). Male mice with the FGF8b-Tg-BERKO<sub>FVB</sub>, FGF8b-Tg, BERKO<sub>FVB</sub> and WT genotypes were kept until the age of 12-16 months and housed under controlled environmental conditions (12 h light / 12 h darkness, temperature 21 ± 3 °C). Pelleted chow (RM1(E)SQC, 811002, SDS) and tap water were offered ad libitum. The mice were killed by CO<sub>2</sub> asphyxiation and cervical dislocation. Animal experiments were approved by the National Animal Experiment Board. For gene expression studies, prostate lobes (ventral, anterior and dorsolateral prostate; VP, AP and DLP, respectively) were dissected and frozen in liquid nitrogen. For histologic preparations, prostate blocks were removed and fixed in 10% neutral buffered formalin. The fixed tissues were embedded in paraffin, and 5-µm sections were prepared for standard hematoxylin and eosin (HE), Periodic Acid-Schiff (PAS), Masson’s trichrome and immunohistochemical (IHC) staining.

Histologic analysis

Histologic analysis was performed in a blinded manner from the HE–stained prostate sections of a total of 52 mice (8 WT, 12 BERKO<sub>FVB</sub>, 21 FGF8b-Tg and 11 FGF8b-Tg-BERKO<sub>FVB</sub>) between the ages of 10.5-14 months. Sections from several levels of the ventral and dorsolateral prostate (VP
and DLP) were evaluated. The degree of inflammation (on a scale of 0-3) was evaluated by analyzing the mean number of lymphocyte aggregates in the prostate stroma as previously described (Elo et al. 2010).

**Immunohistochemical staining**

The procedures for IHC staining of the AR and SMA have been previously reported (Elo et al. 2010). For the staining of p63, a mouse monoclonal anti-p63 antibody BD Pharmingen (4A4) was used at a 1:500 dilution. The antigens were retrieved in 10 mM citric acid buffer pH 6 in a microwave oven. The frequency of AR-positive cells was evaluated from sections stained by AR IHC (n = 6-8 / mouse group) by estimating the percentage of positively stained nuclei in the VP epithelium and stroma separately in the areas with normal, hypercellular, atypic, dysplastic or malignant histology. For each histologic phenotype, 4-6 microscopic fields (using a 20x objective) were analyzed if available; otherwise the total available area was analyzed. ERβ was detected from methanol-fixed frozen sections of anterior and dorsolateral prostates (AP and DLP) by a polyclonal anti-mouse ERβ antibody (Santa Cruz, Y19, sc-6821) recognizing a peptide from N-terminus of ERβ. Alexa Fluor 594 donkey anti-goat IgG (A-11058) was used as a secondary antibody.

**RNA extraction and quantitative RT-PCR**

RNA extraction from the VPs and DNAse treatment were performed as previously described (Elo et al. 2012). The reagents and machinery for quantitative RT-PCR (qRT-PCR) were described previously (Elo et al. 2012). The sequences of the primers and annealing temperatures used in qRT-PCR are presented in Table 1.
Statistical analysis

Statistical analyses were performed using SPSS 11.0 software for Windows (SPSS, Chicago, IL) and GraphPad Prism 6 (Graphpad Software, Inc.). Differences in the frequencies of the histological changes between $FGF8b$-Tg and $FGF8b$-Tg-BERKO$_{FVB}$ mice were tested using the chi$^2$ test. For qRT-PCR results, the normal distribution of the data was tested using the Shapiro-Wilk $W$-test. Then, either an analysis of variance (One-way ANOVA) corrected by Tukey’s multiple comparison test or a Kruskall-Wallis test corrected by Dunn’s multiple comparison test were applied. Values of $p < 0.05$ were considered to be statistically significant.

Results

$FGF8b$-Tg-BERKO$_{FVB}$-mice have abnormalities in their prostates and other urogenital organs

$FGF8b$-Tg and BERKO$_{FVB}$ mice were successfully bred, and the genotypic distribution of the offspring was as expected. The offspring of all the genotypes, including the $FGF8b$-Tg-BERKO$_{FVB}$ mice, were viable and phenotypically normal. The prostate and other urogenital organs of the BERKO$_{FVB}$ mice were macroscopically normal. The prostates and in some cases the epididymides and testes of the $FGF8b$-Tg-BERKO$_{FVB}$ mice were enlarged and/or had an abnormal appearance, as was previously described for the $FGF8b$-Tg mice (Elo et al. 2010, 2012).

Increased frequency of mucinous metaplasia in the prostates of $FGF8b$-TG-BERKO$_{FVB}$ mice

Histological evaluation revealed mild changes in the VP and DLP of one-year-old BERKO$_{FVB}$ mice. Compared to WT mice, the prostates of the BERKO$_{FVB}$ mice displayed a tendency to
increased frequency of focal epithelial hypercellularity (38% vs. 58%) and inflammation (13% vs. 33%), presented as aggregates of lymphocytes in the prostatic stroma (Fig. 1A-1D); the prostates were otherwise normal (Fig. 1A).

Consistent with our previous report (Elo et al. 2010), the VP and DLP of the one-year-old FGF8b-Tg mice contained a high frequency of epithelial and stromal abnormalities (Fig. 1A) such as epithelial and stromal hypercellularities with atypic cells, mPIN lesions and inflammation (Fig. 1E). Prostatic adenocarcinoma was present in one FGF8b-Tg mouse, and either sarcoma or carcinosarcoma was present in 10% of the FGF8b-Tg mice (Fig. 1A).

The frequency of histological changes and the presence of those changes in VP and DLP were mostly similar in the prostates of FGF8b-Tg-BERKO<sub>FVB</sub> and FGF8b-Tg mice (Fig. 1A, E, F). However, mucinous metaplasia foci, defined by Goblet-like cells in the prostate epithelium in the HE-stained sections, were significantly more frequent (Chi²-test, p = 0.028) in the FGF8b-Tg-BERKO<sub>FVB</sub> mice than in FGF8b-Tg mice (82% vs. 38%) (Fig. 1A). PAS staining, which stains neutral and acidic mucins, verified the presence of mucin-secreting cells in the prostate epithelium of the majority of FGF8b-Tg (71%) and FGF8b-Tg-BERKO<sub>FVB</sub> (89%) prostates that were defined as Goblet-cell positive in the initial analysis (Fig. 1I and 1J). In contrast, the majority of BERKO<sub>FVB</sub> (100%) and WT (67%) prostates that were defined as Goblet-like cell-positive did not contain PAS-positive cells in their epithelium (Fig. 1G and 1H). In addition, inflammation was slightly, but not in a statistically significant way, more frequent in the prostates of FGF8b-Tg-BERKO<sub>FVB</sub> mice than in the prostates of FGF8b-Tg mice (82% vs. 62%). The mean score for inflammation (on a scale of 0-3) was similar in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (0.9 vs. 1.0) (Fig. 1B). Epithelial and stromal hypercellularity were present at similar frequencies in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (90% vs. 100% and 57% vs. 64%, respectively).

However, the hypercellular stroma seemed to be more extensive in the prostates of FGF8b-Tg mice than in the FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fig. 1E, F, I and J). In addition, both epithelial and
stromal hypercellularities containing atypical cells were slightly increased in the prostates of 
*FGF8b*-Tg compared to *FGF8b*-Tg-BERKO_{FVB} mice (67% vs. 55% and 38% vs. 27%, 
respectively) but the differences between the groups were not statistically significant. In contrast to 
*FGF8b*-Tg mice, malignant changes were not found in the *FGF8b*-Tg-BERKO_{FVB} prostates 
(adenocarcinomas 5% vs. 0%, sarcomas or carcinosarcomas, 10% vs. 0%) (Fig. 1A).

**Alterations in the prostate stroma of *FGF8b*-TG-BERKO_{FVB} mice**

To study whether there were changes in the percentage of basal cells, indicative of cellular 
differentiation in the prostate epithelium, ICH staining for p63 was performed (Fig. 2). An increased percentage of p63-positive cells in the prostate epithelium of BERKO mice has been 
previously reported (Imamov *et al.* 2004). Our results showed that especially the hypercellular foci 
of the prostate epithelium in BERKO_{FVB} mice contained a high frequency of p63-positive cells (Fig. 
2B). In contrast, in the prostate epithelium of *FGF8b*-Tg mice, there was a trend toward a decreased 
number of p63-positive cells, especially in foci containing mPIN-lesions (Fig. 2C). The prostate 
epithelium of the *FGF8b*-TG-BERKO_{FVB} mice contained some areas with an increased frequency 
of p63-positive cells and others with a decreased frequency of p63-positive cells (Fig. 2D). 
However, when larger areas of the VP were analyzed, no statistically significant differences in the 
percentage of p63–positive cells in the prostate epithelium were found between any of the mouse 
groups (Fig. 2E).

Staining for the androgen receptor (AR) was performed to examine whether there were 
changes in the percentage of AR-positive cells in the prostates of the studied mice, as has 
previously been reported in the *FGF8b*-Tg mice (Elo *et al.* 2010). A total of 90-100% of the normal 
prostate epithelial cells was positive for the AR in all mouse groups (Fig. 3A-D). A similarly high 
percentage of AR-positive cells was also present in the hypercellular areas of BERKO_{FVB} mouse
prostate epithelium (Fig. 3B). Consistent with our previous report, (Elo et al. 2010) the percentage of AR-positive cells was often focally reduced (50-75% of epithelial cells) in the PIN-lesions of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fig. 3C). In the normal prostate stroma, the percentage of AR-positive cells varied between 35-50% in all mouse groups. Also consistent with our previous report (Elo et al. 2010.), the percentage of AR-positive cells was reduced in the hypercellular and atypic prostate stroma of FGF8b-Tg mice (10-40% AR-positive cells) (Fig. 3C) and increased in the sarcoma-like lesions (70% AR-positive cells). However, in contrast, the percentage of AR-positive cells was more variable (15-75% AR positive cells) in the hypercellular stroma of FGF8b-Tg-BERKO<sub>FVB</sub> mouse prostates (Fig. 3D).

The composition of prostate stroma was studied by IHC-staining for SMA (Fig. 3E-H) and by Masson’s Trichrome staining (Fig. 3 I-J). The normal prostate stroma of the WT and BERKO<sub>FVB</sub> mice contained SMA-positive cells in a ring-like structure surrounding the prostatic acini (Fig. E and F). The hypercellular stroma of both FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mouse prostates contained SMA-negative areas surrounding the prostatic acini, but in FGF8b-Tg mice, these areas were frequently more extensive and more irregular than those in FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fig. 3 G and H). The staining patterns were similar in the prostate of WT and BERKO<sub>FVB</sub> mice by Masson Trichrome staining, which stains collagen fibers blue-green and smooth muscle red (Fig. 3 I and J). The hypercellular stroma in both FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> prostates contained wider blue-green-staining areas (Fig. 3 K and L). However, in the prostate stroma of FGF8b-Tg mice, these areas were generally more extensive and the collagen fibers were less organized compared to the FGF8b-Tg-BERKO<sub>FVB</sub> mice.
Gene expression patterns in VPs of FGF8b-Tg-BERKO<sub>FVB</sub> and FGF8b-Tg mice

To study whether there were changes in the expression of genes that were previously found to be upregulated in the VP and epididymides of FGF8b-Tg mice (Elo et al. 2010, 2012), such as osteopontin (Spp1) and connective tissue growth factor (Ctgf), we performed qRT-PCR analysis on the VPs of 10-16 month-old WT, BERKO<sub>FVB</sub>, FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fig. 4). The expression of FGF8b mRNA, which is an indicator of the function of the transgene construct, was increased several hundred-fold in the prostate of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice compared to WT and BERKO<sub>FVB</sub> mice (Fig. 4). Consistent with our previous report (Elo et al. 2010), the expression of Spp1 and Ctgf mRNAs was significantly upregulated in the prostate of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice compared to WT mice (Kruskall-Wallis test, Spp1, p = 0.0035; p = 0.078, respectively and Ctgf, p = 0.0181 and 0.003, respectively). The level of Spp1 or Ctgf mRNA did not differ significantly between the VPs of BERKO<sub>FVB</sub> mice and WT mice.

Because we previously reported that there are changes in the expression levels of the c-isoforms of Fgfr1 and Fgfr3 in the epididymides of the FGF8b-TG mice (Elo et al. 2012), we studied the expression of the mRNAs encoding the b- and c-isoforms of Fgfr1, Fgfr2 and Fgfr3 in the prostates of all the mouse groups (Fig. 4). Compared to the VPs of WT mice, the expression of Fgfr1c was significantly upregulated and Fgfr2c was downregulated in the VPs of Fgf-8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fgfr1c, Kruskall-Wallis test, p = 0.0276 and p = 0.0123, respectively; Fgfr2c, one-way ANOVA, p = 0.0133 and p = 0.0029, respectively). In the VPs of BERKO<sub>FVB</sub> mice, the expression of Fgfr1c and Fgfr2c mRNA was similar to that of WT mice. There were no significant differences in the expression of Fgfr1b and Fgfr2b mRNA in the VPs of different mouse groups. The expression of Fgfr3b and Fgfr3c mRNA could not be quantified because the levels of these mRNAs were below the detection limit of qRT-PCR.
To study whether the deficiency of ERβ expression in BERKO<sub>FVB</sub> and FGF8b-TG- 
BERKO<sub>FVB</sub> mice affected the expression of other steroid hormone receptors in the prostate, the 
expression of ERα and AR was analyzed by qRT-PCR (Fig. 4). The upregulation of the AR in 
BERKOs was previously reported by Imamov et al. (2004). Compared to WT mice, the expression 
of Ar mRNA in the prostate was slightly upregulated in BERKO<sub>FVB</sub> mice and slightly 
downregulated in FGF8b-Tg mice, but these differences were not statistically significant. However, 
the difference between the level of Ar mRNA in the prostates of BERKO<sub>FVB</sub> and FGF8b-Tg mice 
was statistically significant (ANOVA, p=0.0032). No significant differences were detected in the 
mRNA levels of Era between any of the mouse groups.

Because prostatic inflammation was frequent among the mouse groups, the mRNA levels of 
several pro-inflammatory cytokines, such as tumor necrosis factor alpha (Tnfa) and interleukin 6 
(IL6) and 17 (IL17), were analyzed by qRT-PCR (Fig. 5). Compared to WT mice, the level of Tnfa 
mRNA was significantly upregulated in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> 
mice (Kruskal-Wallis test, p=0.0347 and p=0.044, respectively). The expression of IL17 mRNA 
was undetectable in most of the samples (19/26 samples, including all the WT prostates) but was 
upregulated in the prostate of some of the BERKO<sub>FVB</sub> and FGF8b-Tg-BERKO<sub>FVB</sub> mice and in one 
FGF8b-Tg mouse. However, no statistically significant differences between the groups were found 
in the IL6 or IL17 mRNA levels.

Finally, the expression of several markers associated with a mucinous phenotype and/or 
prostate cancer progression, such as mucin1 (Muc1) and mucin 2 (Muc2) and transforming growth 
factor beta 1 (Tgfb1), were analyzed using qRT-PCR (Fig. 4). The prostates of FGF8b-Tg- 
BERKO<sub>FVB</sub> mice showed a tendency towards higher levels of Tgfb1 compared to other groups, but 
due to large interindividual variation, the difference was not statistically significant (Kruskal-
Wallis-test p=0.12). The mean level of both Muc1 and Muc2 mRNA was highest in the prostate of
FGF8b-Tg mice, but there were no significant differences in the level of Muc1 or Muc2 mRNA between the mouse groups.

Discussion

Several studies have provided evidence for anti-proliferative and differentiation-promoting effects of ERb and tumorigenic effects of ERa in the prostate (Prins et al. 2001, Risbridger et al. 2001, McPherson et al. 2007, Savolainen et al. 2007, Ricke et al. 2008, Hartman et al. 2012, Kawashima & Nakatani 2012, Dey et al. 2013). Recently, the anti-tumorigenic effect of ERb was supported by a study, which compared the incidence of prostate tumorigenesis in genistein- and casein-fed TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate), BERKO-TRAMP and ERKO-TRAMP mice (Slusarz et al. 2012). In the current study, we examined how the deficiency of ERb affects the prostate tumorigenesis that had been previously observed in the FGF8b-Tg mice (Elo et al. 2010) by crossing FGF8b-Tg mice with BERKO_FVB mice. We hypothesized that two genomic, putative tumor-promoting modifications might generate a model with accelerated or more advanced prostate tumorigenesis, as has been previously reported for several mouse models bearing more than one genomic modification (Jeet et al. 2010, Ittmann et al. 2013).

Consistent with our previous report, a high frequency of the one-year-old FGF8b-Tg mouse prostates contained epithelial and stromal hypercellularity, inflammation and mPIN lesions. In addition, some FGF8b-Tg mouse prostates contained cancers (adenocarcinoma, sarcoma or carcinosarcoma) (Elo et al. 2010). The prostate of one-year-old BERKO_FVB mouse contained relatively mild changes, including epithelial hypercellularity and inflammation. This is in contrast with the study of Weihua et al. (2001), which reported a higher frequency of more pronounced hyperplasia in the prostate of their BERKO mice. This result is interesting because the BERKO_FVB mice used in the present study initially originated from the same colony as those studied by Weihua.
et al. (2001), but they were bred in a different genetic background. However, other research groups have not detected any changes in the prostate histology of different BERKO mouse models (Dupont et al. 2000, Prins et al. 2001, Antal et al. 2008), and therefore, the prostate phenotypes of BERKO mice have remained a subject of controversy.

In addition to hyperplasia, previous studies have shown increased proliferation, decreased apoptosis, increased AR expression and an increased number of p63-positive, incompletely differentiated epithelial cells in the prostate of the BERKO mice (in a C57BL background) (Weihua et al. 2001, Imamov et al. 2004). Our result on the expression pattern of p63 differs from that described by Imamov and coworkers (2004) because even though we noticed a tendency toward a higher frequency of p63-positive cells in the hypercellular foci of BERKO<sub>FVB</sub> prostate epithelium, the overall frequency of p63-positive cells in the epithelium was not significantly altered. This may be explained by the relatively sparsely located hypercellular foci in the prostate epithelium of our BERKO<sub>FVB</sub> mice. According to our data, including the immunohistochemical staining and qRT-PCR analysis of the AR, the level of AR mRNA or protein was not significantly increased in the prostates of BERKO<sub>FVB</sub> mice compared to those of WT mice, but interestingly, the level of Ar mRNA was significantly higher in the prostates of BERKO<sub>FVB</sub> mice compared to those of FGF8b-Tg mice; this may be due to previously reported decreased level of AR in the PIN lesions and in the hypercellular stroma of the FGF8b-Tg mice (Elo et al. 2010).

Contrary to the original hypothesis, the FGF8b-Tg-BERKO<sub>FVB</sub> mice did not contain more mPIN lesions or more advanced neoplastic changes than the FGF8b-Tg mice. The epithelial and stromal hypercellularity were, in fact, slightly more frequent in the prostates of FGF8b-Tg-BERKO<sub>FVB</sub> mice than in FGF8b-Tg mice, while the hypercellular changes, especially in the stroma, were less extensive in the FGF8b-Tg-BERKO<sub>FVB</sub> mice and tended to contain less frequently atypical cells. The cellular composition of hypercellular stroma also seemed to be different in these two mouse groups. In addition, unlike in the FGF8b-Tg mice, malignant changes were not found in the
prostates of FGF8b-Tg-BERKO_FVB mice. Taken together, these data suggest that ERb does not seem to protect against the development of atypical hypercellularity in the epithelium and stroma nor does it function as a tumor suppressor in the prostates of FGF8b-Tg mice. Our results are not consistent with previous results regarding the role of ERb, which have shown that ERb agonists can induce apoptosis in the epithelium and stroma of prostate cancer and BPH (McPherson et al. 2010).

It is interesting that mucinous metaplasia, as indicated by mucin-secreting, PAS-positive cells in the prostate epithelium, was clearly more frequent in FGF8b-Tg-BERKO_FVB mice than in any other mouse group studied. This suggests that even if the structural changes in the prostate histology of BERKO_FVB mice were mild, the lack of ERb combined with overexpression of FGF8b influences the differentiation of the epithelial cells. An increased frequency of mucinous metaplasia was previously reported in the prostates of old FGF8b-Tg mice (Elo et al. 2010), but a lack of ERb appears to make the epithelial cells more likely to adopt the mucin-secreting, Goblet cell-like phenotype. The significance of these mucin-secreting cells in the prostate is unclear, but in human prostate, benign lesions with mucin-secreting cells and mucinous adenocarcinoma, a rare form of prostate cancer, have both been described (Bohman & Osunkoya. 2012). Interestingly, mucinous metaplasia in association with prostatic adenocarcinoma has been reported in several genetically modified mouse models (Ittmann et al. 2013).

We have previously reported that prostatic inflammation, indicated by aggregates of several types of inflammatory cells such as T-cells, B-cells and macrophages in the stroma, was frequent in FGF8b-Tg mice (Elo et al. 2010). Our current results showed a tendency to increased frequency of inflammation in the prostates of FGF8b-Tg-BERKO_FVB mice than in those of the FGF8b-Tg mice, suggesting that a deficiency of ERb could facilitate the formation of inflammatory lesions in the FGF8b-Tg mice. This is in line with previous animal studies; it has been reported that ERa mediates neonatal estrogen treatment-induced prostatic inflammation (Prins et al. 2001), and the anti-inflammatory effects of ERb have been demonstrated in a rat model of inflammatory bowel
disease (Harris et al. 2003) and in luteinizing hormone receptor knockout (LuRKO) mice (Savolainen et al. 2007). Furthermore, Prins & Korach (2008) have reported massive T-cell infiltration in the prostates of BERKO mice. The present study did not find statistically significant differences in the mRNA levels of proinflammatory cytokines (IL6, IL17, Tnfa) between the prostates of WT and BERKO<sub>FVB</sub> mice or between FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice. However, a trend toward higher IL17 levels in BERKO<sub>FVB</sub> and Tg-BERKO<sub>FVB</sub> mice was observed. In addition, Tnfa was upregulated in both FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> prostates. This upregulation is most likely associated with the ongoing inflammatory process. TNFa is a multifunctional protein that can be secreted by several cell types, especially by macrophages, and, depending on conditions, it can promote inflammation, cell proliferation and tumorigenesis or induce apoptosis (Balkwill. 2009).

As expected, the expression of the mRNA for FGF8b and two genes (Spp1, Ctgf) previously found to be upregulated in the prostate and epididymis of FGF8b-Tg mice (Elo et al. 2010,Elo et al. 2012) was increased in the prostate of FGF8b-Tg-BERKO<sub>FVB</sub> mice. Interestingly, an upregulation of the mRNA for Fgfr1c and a downregulation of the mRNA for Fgfr2c were found in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice. This observation is in line with our previous results in S115 mouse mammary tumor cells, MCF7 breast cancer cells (Ruohola et al. 1995,Tarkkonen et al. 2012) and the epididymides of FGF8b-Tg mice (Elo et al. 2012), indicating that FGF signaling regulates the expression of FGFRs. A recent report showed that FGF8 upregulates the expression of Fgfr1 in the neuronal cells (Mott et al. 2010). Importantly, the upregulation of Fgfr1c expression provides a means for the enhancement of FGF signaling because FGF8b can efficiently bind and activate the c-isoforms, but not the b-isoforms, of FGFRs (Zhang et al. 2006). The induction of Fgfr1c in the prostate of FGF8b-Tg mice may also explain the similarities previously observed between the prostatic malignancies (mixed neoplasias of epithelium and stroma) of FGF8b-Tg and FGFR1-Tg mice (Acevedo et al. 2007, Elo et al. 2010). The downregulation of Fgfr2c mRNA in
the prostates of FGF8b-Tg and FGF8b-Tg-BERKO$_{FVB}$ mice is also consistent with the
development of prostatic hyperplasia and PIN lesions because previous studies have shown that,
unlike the activation of FGFR1c, the activation of FGFR2c does not induce the development of
hyperplasias or dysplasias in the prostate epithelium (Freeman et al. 2003).

It is probable that environmental and genetic factors have had a considerable influence on the
variable prostate phenotypes of BERKO mouse lines studied by different laboratories (Dupont et al.
2000, Weihua et al. 2001, Prins et al. 2001, Antal et al. 2008). Our BERKO$_{FVB}$-model is the first
one generated in the FVB/N mouse strain, whereas others were made in the C57BL strain. There is
evidence that different mouse strains have differential susceptibilities to prostate tumorigenesis
(Bianchi-Frias et al. 2007). It should also be noted that the BERKO$_{FVB}$ model used in the present
study is not a complete knockout because it was generated by insertion of NEO-cassette into exon 3
of the Erb gene (Krege et al. 1998). The presence of shorter forms of Erb mRNA in BERKO mice
that result from alternative splicing of Erb mRNA has been previously described in the ovaries of
BERKO mice (Krege et al. 1998, Dupont et al. 2000). Our experiments demonstrated similar
shorter forms of Erb mRNA and immunohistochemistry-based evidence for a protein product
(truncated ERb) for the first time in the prostates of BERKO mice (Supplementary figure 2).
According to Krege et al. (1998) and our results, two of these ErbKO mRNAs (ErbKO$_1$ and
ErbKO$_2$) lack the disrupted exon three and contain early stop codons in the exon four. They would
therefore be translated to protein products lacking the DNA-binding domain (DBD) and the ligand
binding domain (LBD) (Supplementary figure 2). The third ErbKO mRNA (ErbKO$_3$, Krege et al.
1998), which seemed to be present only occasionally at low levels in the prostate our BERKO$_{FVB}$
mice, lacks only the exons three and four. It would thus translate to a protein product devoid of the
DBD but containing the AF-1 and AF-2 (and the LDB) domains. These shortened ERbKO protein
products are not capable of exerting the classical functions the ERb but they may, especially the
ERbKO3 product possess some of the non-classical functions of ERb, such as those mediated by activation of AP1-sites (Price et al. 2001).

One feature that complicates our understanding of the function of ERb is the presence of functionally different isoforms of ERb in the prostate. In line with the previous studies on ERb expression in the rat prostate (Petersen et al. 1998, Hanstein et al. 1999), we observed the expression of Erb isoforms 1 (“mErb1, wt isoform”) and 2 (mErb2) in the prostate of WT mice (Supplementary Fig. 2a). The structure and nomenclature of ERb isoforms differs between mouse and human, and for example, hERb2 and mERb2 are not homologs (Lu et al. 1998, Moore et al. 1998). In fact, normal human tissues do not express an ERb isoform homologous to the mERb2 isoform (Lu et al. 1998), but a corresponding isoform (different from hERb1, 2, 3, 4 and 5) is expressed in some human cancer cell lines (Hanstein et al. 1999). The mERb2 has different ligand- and coactivator-binding properties than mERb1 (Zhao et al. 2005), but its physiological function is unclear. It has been suggested that mERb2 acts as a negative regulator of ERa (Zhao et al. 2005) and/or ERb1 (Lu et al. 2000). In humans, the different isoforms of ERb are functionally different and have differential, even opposite roles in prostate tumorigenesis (Leung et al. 2010, Kawashima & Nakatani 2012, Day et al. 2012), which may partly explain conflicting results of the role of ERb in the prostate.

In conclusion, our results from FGF8b-Tg-BERKOFVB mice suggest that ERb plays a role in the differentiation of prostatic epithelium and may protect from prostatic inflammation. These effects of ERb deficiency were more obvious when it was combined with the overexpression of FGF8b, whereas the prostates of BERKOFVB mice displayed only mild changes. Surprisingly, prostate tumorigenesis was not accelerated in the FGF8b-Tg-BERKOFVB mice compared to FGF8b-Tg mice, and our results do not support a tumor-suppressive role for ERb in this mouse model. The mild phenotype of BERKOFVB could be partly explained by the presence of shorter ERb
forms in the BERKO<sub>FVB</sub> prostates that may have some of the non-classical functions of intact ERβ
(Kushner <i>et al.</i> 2000, Price <i>et al.</i> 2001).

On the whole, ERβ may have different roles in the different phases of prostate development and the different phases of prostate tumorigenesis (Nelson <i>et al.</i> 2014). Therefore, a conditional inducible knockout model of ERβ would be required to fully dissect the effect of ERβ abrogation in prostate tumorigenesis and to eliminate the potential consequences of the lack of the functional receptor during prostate maturation. The function and expression of the different ERβ isoforms in prostate cancer should be examined in future studies because it is possible that altered combination of these isoforms influences tumorigenesis and tumor progression.

**Declaration of interest**

The authors declare that there are no conflicts of interest.

**Funding**

The study has been financially supported by the Finnish Cancer Foundation, the Sigrid Jusélius Foundation, the Finnish Cultural Foundation, the Turku University Foundation, the Finnish Concordia Fund, the Finnish-Norwegian Medicine foundation, the Cancer Association of South-Western Finland and the Faculty of Medicine, University of Turku, Finland.

**Acknowledgements**

We would like to thank Soili Jussila and Liudmila Shumskaya for their technical assistance and Tero Vahlberg for assistance with statistical tests.
References


Fixemer T, Remberger K & Bonkhoff H 2003 Differential expression of the estrogen receptor beta (ERbeta) in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. The Prostate 54 79-87.


Mott NN, Chung WC, Tsai PS & Pak TR 2010 Differential fibroblast growth factor 8 (FGF8)-mediated autoregulation of its cognate receptors, Fgfr1 and Fgfr3, in neuronal cell lines. *PloS one* **5** e10143.


Prins GS & Korach KS 2008 The role of estrogens and estrogen receptors in normal prostate growth and disease. *Steroids* **73** 233-244.


Tolnakovic E, Lilleby W, Tolnakovic G, Fossa SD & Chibbar R 2002 Prostate carcinoma expression of estrogen receptor-beta as detected by PPG5/10 antibody has positive association with primary Gleason grade and Gleason score. *Human pathology* **33** 646-651.


**Figure 1.** (A) Frequency of histological changes in the prostates of 10-14-month-old WT, BERKO<sub>FVB</sub>, FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice. Differences in frequencies between FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice were tested by chi²-test. Exact p-values were used. *p < 0.05. (B) The scores of inflammation in the mouse prostates evaluated on a scale of 0 to 3 as defined in the Materials and Methods section. The mean values and standard deviations are shown. (C) The normal histology of a 12.5-month-old WT mouse VP. (D) Inflammation displayed by an aggregate of lymphocytes in the VP stroma of a 14-month-old BERKO<sub>FVB</sub> mouse. (E) mPIN, stromal hypercellularity with atypia and inflammation in the VP of a 12-month-old FGF8b-Tg mouse. (F) mPIN, stromal hypercellularity and inflammation in the VP of a 12-month-old FGF8b-Tg-BERKO<sub>FVB</sub> mouse. (G) PAS staining of a 10.5-month-old WT mouse VP showing no positive signal in the epithelium. (H) PAS staining of VP of a 13-month-old BERKO<sub>FVB</sub> mouse showing no positive staining in the epithelium. (I) PAS staining of VP of a 10.5-month-old FGF8b-Tg mouse showing positive PAS staining in the epithelial cells of a mPIN lesion. (J) PAS staining of VP of a 12.5-month-old FGF8b-Tg-BERKO<sub>FVB</sub> mouse showing PAS-positivity in the epithelial cells. The images were obtained using 20x and 40x objectives. The scale bars represent 100 µm.

**Figure 2.** Immunohistochemical staining of p63 (basal cells) in the prostate epithelium. (A) A representative image of p63 staining in the normal VP epithelium of a 12-month-old WT mouse. (B) The hypercellular epithelium of a BERKO<sub>FVB</sub> mouse prostate with a focally increased frequency of p63-positive cells (arrow). (C, D) A decreased frequency of p63-positive cells in the mPIN-lesions (*) of FGF8b-Tg (C) and FGF8b-Tg-BERKO<sub>FVB</sub> mice (D). The images were obtained using a 40x objective, and the insets show 2x magnification of the images. The scale bars represent 100 µm. (E) The percentage of p63-positive cells in the VP epithelia of WT, BERKO<sub>FVB</sub>,...
**FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice.** A minimum of one thousand epithelial cells from multiple prostatic acini from sections stained by p63 IHC were analyzed (n = 5-7 mouse group). The mean values ± SDs are shown. There were no statistically significant differences between the mouse groups (one-way ANOVA and Tukey’s multiple comparison test).

**Figure 3.** Immunohistochemical (IHC) staining of AR (A-D) and SMA (E-H) and Masson’s trichrome staining (I-L) in the VPs of 10-15-month-old WT, BERKO<sub>FVB</sub>, FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice. (A) A representative figure of AR staining in the VP of a WT mouse in which 100% of the epithelial cells and 40% of the stromal cells are AR-positive. (B) VP of a BERKO<sub>FVB</sub> mouse in which epithelium and stroma showed similar staining patterns of AR as that of the WT mice. (C) The prostate of a FGF8b-TG mouse in which the PIN-lesion (arrow) and hypercellular stroma (*) contained a decreased frequency of AR-positive cells compared to WT mice. (D) An FGF8b-Tg BERKO<sub>FVB</sub> mouse VP with hypercellular epithelium and stroma (*) with approximately 100% and 50% of AR-positive cells, respectively. (E) A representative figure showing SMA staining in the VP of a WT mouse (F) and SMA staining in the VP of a BERKO mouse. (G) The VP of a FGF8b-Tg mouse with wide areas of SMA-negative hypercellular, atypic stroma surrounding the acini (*). (H) The VP of a FGF8b-Tg-BERKO<sub>FVB</sub> mouse showing the SMA-negative hypercellular stroma (*). (I) Masson’s trichrome staining for collagen in the VP of a WT mouse. (J) Masson’s trichrome staining in the VP of a BERKO<sub>FVB</sub> mouse showing similar a staining pattern as in the WT mouse. (K) The VP of a FGF8b-Tg mouse with hypercellular and atypic stroma displaying wide areas of blue-green-staining and collagen-rich stroma. (L) The VP of an FGF8b-Tg-BERKO<sub>FVB</sub> mouse, showing an increased amount of blue-green-staining and collagen-rich stroma around the prostatic acini. These images were obtained using a 20x objective, and the insets show a 2x magnification of these images. The scale bars represent 100 µm.
Figure 4. The expression of the indicated genes in the VPs of 12-16 month-old WT, BERKO, FGF8b-Tg and FGF8b-Tg-BERKO_{FVB} mice as analyzed by qRT-PCR. Beta-actin was used as a reference gene for data normalization, and the relative values were measured using the WT average as a reference artificially set to 1. The mean values and SDs are shown. The differences between groups were tested by one-way ANOVA corrected with Tukey’s multiple comparison test or by a Kruskall-Wallis test corrected with Dunn’s multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 5. The expression of genes for cytokines (Tnfa, IL6, and IL17), prostate cancer promoting factors (Tgfb1) and proteins related to the mucin-secreting phenotype (Muc1 and Muc2) as analyzed by qRT-PCR. Beta-actin was used as a reference gene for data normalization, and the relative values were calculated using the WT average as a reference value artificially set to 1. In case of IL17 qRT-PCR, the average of all the CT value data was used as a reference artificially set to 1 because no signal could be detected in any of the WT prostates. The mean values and SDs are shown. The differences between groups were tested by one-way ANOVA corrected with Tukey’s multiple comparison test or by a Kruskall-Wallis test corrected with Dunn’s multiple comparison test. * p < 0.05.
Figure 1. (A) Frequency of histological changes in the prostates of 10-14-month-old WT, BERKOFVB, FGF8b-Tg and FGF8b-Tg-BERKOFVB mice. Differences in frequencies between FGF8b-Tg and FGF8b-Tg-BERKOFVB mice were tested by chi2-test. Exact p-values were used. * p < 0.05. (B) The scores of inflammation in the mouse prostates evaluated on a scale of 0 to 3 as defined in the Materials and Methods section. The mean values and standard deviations are shown. (C) The normal histology of a 12.5-month-old WT mouse VP. (D) Inflammation displayed by an aggregate of lymphocytes in the VP stroma of a 14-month-old BERKOFVB mouse. (E) mPIN, stromal hypercellularity with atypia and inflammation in the VP of a 12-month-old FGF8b-Tg mouse. (F) mPIN, stromal hypercellularity and inflammation in the VP of a 12-month-old FGF8b-Tg-BERKOFVB mouse. (G) PAS staining of a 10.5-month-old WT mouse VP showing no positive signal in the epithelium. (H) PAS staining of VP of a 13-month-old BERKOFVB mouse showing no positive staining in the epithelium. (I) PAS staining of VP of a 10.5-month-old FGF8b-Tg mouse showing positive PAS staining in the epithelial cells of a mPIN lesion. (J) PAS staining of VP of a 12.5-month-old FGF8b-Tg-BERKOFVB mouse showing PAS-positivity in the epithelial cells. The images were obtained using 20x and 40x objectives. The scale bars represent 100 µm.
Figure 2. Immunohistochemical staining of p63 (basal cells) in the prostate epithelium. (A) A representative image of p63 staining in the normal VP epithelium of a 12-month-old WT mouse. (B) The hypercellular epithelium of a BERKOFVB mouse prostate with a focally increased frequency of p63-positive cells (arrow). (C, D) A decreased frequency of p63-positive cells in the mPIN-lesions (*) of FGF8b-Tg (C) and FGF8b-Tg-BERKOFVB mice (D). The images were obtained using a 40x objective, and the insets show 2x magnification of the images. The scale bars represent 100 µm. (E) The percentage of p63-positive cells in the VP epithelia of WT, BERKOFVB, FGF8b-Tg and FGF8b-Tg-BERKOFVB mice. A minimum of one thousand epithelial cells from multiple prostatic acini from sections stained by p63 IHC were analyzed (n = 5-7 mouse group). The mean values ± SDs are shown. There were no statistically significant differences between the mouse groups (one-way ANOVA and Tukey's multiple comparison test).
Figure 3. Immunohistochemical (IHC) staining of AR (A-D) and SMA (E-H) and Masson's trichrome staining (I-L) in the VPs of 10-15-month-old WT, BERKOFVB, FGF8b-Tg and FGF8b-Tg-BERKOFVB mice. (A) A representative figure of AR staining in the VP of a WT mouse in which 100% of the epithelial cells and 40% of the stromal cells are AR-positive. (B) VP of a BERKOFVB mouse in which epithelium and stroma showed similar staining patterns of AR as that of the WT mice. (C) The prostate of a FGF8b-TG mouse in which the PIN-lesion (arrow) and hypercellular stroma (*) contained a decreased frequency of AR-positive cells compared to WT mice. (D) An FGF8b-Tg BERKOFVB mouse VP with hypercellular epithelium and stroma (*) with approximately 100% and 50% of AR-positive cells, respectively. (E) A representative figure showing SMA staining in the VP of a WT mouse (F) and SMA staining in the VP of a BERKO mouse. (G) The VP of a FGF8b-Tg mouse with wide areas of SMA-negative hypercellular, atypic stroma surrounding the acini (*). (H) The VP of a FGF8b-Tg-BERKOFVB mouse showing the SMA-negative hypercellular stroma (*). (I) Masson’s trichrome staining for collagen in the VP of a WT mouse. (J) Masson’s trichrome staining in the VP of a BERKOFVB mouse showing similar a staining pattern as in the WT mouse. (K) The VP of a FGF8b-Tg mouse with hypercellular and atypic stroma displaying wide areas of blue-green-staining and collagen-rich stroma. (L) The VP of an FGF8b-Tg-BERKOFVB mouse, showing an increased amount of blue-green-staining and collagen-rich stroma around the prostatic acini. These images were obtained using a 20x objective, and the insets show a 2x magnification of these images. The scale bars represent 100 µm.

96x61mm (600 x 600 DPI)
Figure 4. The expression of the indicated genes in the VPs of 12-16 month-old WT, BERKO, FGF8b-Tg and FGF8b-Tg-BERKOFVB mice as analyzed by qRT-PCR. Beta-actin was used as a reference gene for data normalization, and the relative values were measured using the WT average as a reference artificially set to 1. The mean values and SDs are shown. The differences between groups were tested by one-way ANOVA corrected with Tukey’s multiple comparison test or by a Kruskall-Wallis test corrected with Dunn’s multiple comparison test. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 5. The expression of genes for cytokines (Tnfa, IL6, and IL17), prostate cancer promoting factors (Tgfβ1) and proteins related to the mucin-secreting phenotype (Muc1 and Muc2) as analyzed by qRT-PCR. Beta-actin was used as a reference gene for data normalization, and the relative values were calculated using the WT average as a reference value artificially set to 1. In case of IL17 qRT-PCR, the average of all the CT value data was used as a reference artificially set to 1 because no signal could be detected in any of the WT prostates. The mean values and SDs are shown. The differences between groups were tested by one-way ANOVA corrected with Tukey’s multiple comparison test or by a Kruskall-Wallis test corrected with Dunn’s multiple comparison test. * p < 0.05.
Table 1. Sequences of primers used in qRT-PCRs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
<th>Ann. *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar</td>
<td>GTCTCCGGAATGTATGAA</td>
<td>AAGCTGCCTCTCTCAAG</td>
<td>58 °C</td>
</tr>
<tr>
<td>Era</td>
<td>CCGTGCTGCAATGACTATGCC</td>
<td>GTGCTTCAACATTCTCCCTCCTC</td>
<td>58 °C</td>
</tr>
<tr>
<td>IL17</td>
<td>TCATCCCTCAAAGCTCAGCG</td>
<td>TTCATTGCGGGGAGAGTGTC</td>
<td>58 °C</td>
</tr>
<tr>
<td>IL6</td>
<td>CCGGAGAGGAGACTTCACAG</td>
<td>CAGAATTGCCATTGCACAAC</td>
<td>60 °C</td>
</tr>
<tr>
<td>Muc1</td>
<td>GTGCCAGTGGCCGCGAAAGA</td>
<td>TGCCGAAACCTCCTCAGGGGC</td>
<td>60 °C</td>
</tr>
<tr>
<td>Muc2</td>
<td>GCCAGATCCCGAAACAC</td>
<td>TGTAGGAGTCTCGGCAGTCA</td>
<td>60 °C</td>
</tr>
<tr>
<td>Tgfb</td>
<td>CAACATTCCTCGGCTACCTTGG</td>
<td>GAAAGCCCTGTATTCGTCTCCCTT</td>
<td>60 °C</td>
</tr>
<tr>
<td>Tnfa</td>
<td>CCCCCAGGGGATGAGAAGTT</td>
<td>CACTTGGTGGTTGCTACGA</td>
<td>60 °C</td>
</tr>
</tbody>
</table>

*Annealing temperature