Deficiency of ERβ and prostate tumorigenesis in FGF8b transgenic mice

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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 α (ERα (ESR1)) and β (ERβ (ESR2)). Hyperplasia and decreased differentiation of epithelial cells in the prostate have been reported in ERβ knockout (BERKO) mice. Herein, we studied the effect of ERβ deficiency on prostate tumorigenesis by crossing BERKO-EVB mice with prostate-targeted human fibroblast growth factor 8b transgenic (FGF8b-Tg) mice. Consistent with results described in our previous report, the prostates of 1-year-old FGF8b-Tg mice displayed stromal aberrations, prostatic intraepithelial neoplasia (mPIN) lesions, inflammation, and occasionally cancer. The prostates of BERKO-EVB mice exhibited mild epithelial hypercellularity and inflammation. The prostate phenotypes of FGF8b-Tg-BERKO-EVB 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-EVB mice when compared with FGF8b-Tg mice. Furthermore, compared with FGF8b-Tg mice, there was a tendency for increased frequency of inflammation but milder hyperplasias in the prostate stroma of FGF8b-Tg-BERKO-EVB mice. The expression levels of mRNAs for FGF8b-regulated genes including osteopontin (Spp1), connective tissue growth factor (Ctgf), fibroblast growth factor receptors (Fgfrs), and steroid hormone receptors and cytokines were similar in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO-EVB mice. Our results indicate that ERβ plays a role in the differentiation of the prostatic epithelium and, potentially, in the defensive mechanism required for protection against inflammation but do not support a direct tumor-suppressive function of ERβ in the prostate of FGF8b-Tg mice.

Key Words

- estrogen receptor beta
- fibroblast growth factor 8b
- transgenic mouse
- prostate
- tumorigenesis

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 affect the prostate and may contribute to the development of benign prostatic hyperplasia (BPH) and prostate cancer (Härkönen & Mäkelä 2004, Prins & Korach 2008, Ellem & Ristbrider 2009, Hartman et al. 2012).

In target tissues, estrogens act via estrogen receptor α (ERα) (ESR1) and ERβ (ESR2), which are nuclear receptors encoded by separate but structurally homologous genes.
In the prostate, ERα and ERβ are expressed in different compartments: ERα is expressed in the stroma, and ERβ 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; ERα promotes proliferation, inflammation, and the development of dysplasia, whereas ERβ promotes differentiation and has anti-proliferative and anti-inflammatory effects (Prins et al. 2001, Risbridger et al. 2001, McPherson et al. 2007, Savolainen et al. 2007, Ricke et al. 2008, Slusarz et al. 2012, Dey et al. (In Press)). Based on the prostate phenotypes of conditional Erα-knockout (ERKO) mice, ERα 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 ERβ (hERβ1–5) exist (Moore et al. 1998, Leung et al. 2006). Studies have reported a decreased level of hERβ in prostatic intraepithelial neoplasia (PIN) lesions or in prostate cancer, supporting the tumor-suppressive role of ERβ (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 ERβ expression during prostate tumorigenesis is epigenetically regulated by the hypermethylation of CpG islands in the ERβ promoter. On the other hand, a high level of ERβ expression has been reported in advanced prostate cancer (Horvath et al. 2001, Leav et al. 2001, Tordalovic et al. 2002, Walton et al. 2009) and in prostate cancer metastasis (Fixemer et al. 2003). The conflicting results regarding the expression of ERβ during prostate tumorigenesis in different studies can be at least partly explained by the use of different antibodies, which were either specific for the hERβ1 isoform or recognized all or several hERβ isoforms, which have differential functions (Leung et al. 2006). Recent results have indicated that the different isoforms of ERβ have different functions in prostate tumorigenesis. hERβ1 has a tumor-suppressive role, while hERβ2 and hERβ5 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 ERα in human prostate cancer are also conflicting; some reports have indicated an increase in the expression of ERα during prostate tumorigenesis (Bonkhoef et al. 1999), but others have found that ERα 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 ERβ in prostate tumorigenesis and the relationship between ERβ and FGF signaling in a novel mouse model. We have reported previously that overexpression of prostate-targeted of human fibroblast growth factor 8b (FGF8b) 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 examine as to whether the deficiency of ERβ accelerates the process of 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 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 described previously (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 seven generations to produce BERKO/FVB mice. Generation of FGF8b-Tg-ERβ−/− (FGF8b-Tg-BERKOFVB) mice by crossings in two generations is described in detail in Supplementary Figure 1, see section on supplementary data given at the end of this article. The genotyping for both genomic modifications has been described previously (Windahl et al. 1999, Elo et al. 2010). Male mice with the FGF8b-Tg-BERKOFVB, FGF8b-Tg, BERKOFVB, and WT genotypes were kept until the age of 12–16 months and housed under controlled environmental conditions (12 h light:12 h darkness cycle at a temperature of 21 ± 3 °C). Pelleted chow (RM1(E) QC, 811002, SDS) and tap water were offered and animals were allowed to feed ad libitum. Mice were killed by CO2 asphyxiation and cervical dislocation.
Animal experiments performed in this study 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 histological preparations, prostate blocks were removed and fixed in 10% neutral buffered formalin. The fixed tissues were embedded in paraffin, and sections of 5 μm thickness were prepared for standard hematoxylin and eosin (HE), Periodic Acid–Schiff (PAS), Masson’s trichrome, and immunohistochemical (IHC) staining.

Histological analysis
Histological analysis was performed in a blinded manner from the HE-stained prostate sections of a total of 52 mice (eight WT, 12 BERKO_FVB, 21 FGF8b-Tg, and 11 FGF8b-Tg-BERKO_FVB) between the ages of 10 and 14.5 months. Sections from several levels of the 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 described previously (Elo et al., 2010).

IHC staining
The procedures for IHC staining for the androgen receptor (AR) and alpha smooth muscle actin (Acta2, hereafter referred to as SMA) have been described previously (Elo et al., 2010). The staining for 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, atypical, dysplastic, or malignant histology. For each histological phenotype, 4–6 microscopic fields (using a 20× objective) were analyzed if available; otherwise, the total available area was analyzed. ERβ was detected from methanol-fixed frozen sections of AP and DLP using a polyclonal anti-mouse ERβ antibody (Santa Cruz, Y19, sc-6821) recognizing a peptide from the 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 described previously (Elo et al., 2012). The reagents and machinery for quantitative RT-PCR (qRT-PCR) have been described previously (Elo et al., 2012). The sequences of the primers and annealing temperatures used in qRT-PCR are given in Table 1.

Statistical analysis
Statistical analyses were performed using the SPSS 11.0 software for Windows (SPSS) and GraphPad Prism 6 (Graphpad Software, Inc., San Diego, California, USA). Differences in the frequencies of the histological changes between FGF8b-Tg and FGF8b-Tg-BERKO_FVB mice were tested using the χ²-test. For qRT-PCR results, the normal distribution of the data was tested using the Shapiro–Wilk W-test. Then, either an ANOVA (one-way ANOVA) corrected by Tukey’s multiple comparison test or a Kruskal–Wallis test corrected by Dunn’s multiple comparison test were applied. The values with 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

Table 1 Sequences of primers used in qRT-PCRs

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers 5’–3’</th>
<th>Reverse primers 5’–3’</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
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<td>Ar</td>
<td>GTCTCCGGAAATGTTATGAA</td>
<td>AAGCTGCCCTCTGCAAG</td>
<td>58</td>
</tr>
<tr>
<td>Erα</td>
<td>CGGTGCGAATGACTATGCC</td>
<td>GTGCTTCAAGATTTTCCCTCT</td>
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<tr>
<td>Il17a</td>
<td>TCATCCGCAATGACTATGCC</td>
<td>TTCATTTGCGGAGGAGTCC</td>
<td>58</td>
</tr>
<tr>
<td>Il6</td>
<td>CCGGAGAGGACCTCAAG</td>
<td>CAGAAATTGCGACCACCAAC</td>
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</tr>
<tr>
<td>Muc1</td>
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<td>TCGGCCAACCTCCTCATGGGG</td>
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<tr>
<td>Muc2</td>
<td>GCGAAGTCCGCAAAACCAC</td>
<td>TGGAGGAGTCTGGGCAGTCA</td>
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<tr>
<td>Tgfβ1</td>
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<td>GAAAGGCTATGGCTGTTACGTA</td>
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<tr>
<td>Tnf</td>
<td>CCGCATGGGATGGATGGA</td>
<td>CACTTGTGTTTGCTCAAG</td>
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Increased frequency of mucinous metaplasia in the prostates of FGF8b-TG-BERKO<sub>FVB</sub> mice

Histological evaluation revealed mild changes in the VP and DLP of 1-year-old BERKO<sub>FVB</sub> mice. Compared with WT mice, the prostates of the BERKO<sub>FVB</sub> 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, B, C, and D); the prostates were otherwise normal (Fig. 1A).

Consistent with results described in our previous report (Elo et al. 2010), the VP and DLP of the 1-year-old FGF8b-Tg mice contained a high frequency of epithelial and stromal abnormalities (Fig. 1A) such as epithelial and stromal hypercellularities with atypical 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, and F). However, mucinous metaplasia foci, defined by Goblet-like cells in the prostate epithelium in the HE-stained sections, were significantly more frequent (χ²-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 (Fig. 1B). PAS-positive cells and others with a decreased frequency of p63-positive cells were positive for AR in all mouse groups (Fig. 3A, B, C, and D). A similarly high percentage of AR-positive cells was also present in the hypercellular areas of the prostate epithelium of a BERKO<sub>FVB</sub> mouse (Fig. 1A). In addition, both epithelial and stromal hypercellularities containing atypical cells were slightly increased in the prostates of FGF8b-Tg mouse compared with FGF8b-Tg-BERKO<sub>FVB</sub> 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<sub>FVB</sub> prostates (adenocarcinomas 5 vs 0%; sarcomas or carcinosarcomas, 10 vs 0%) (Fig. 1A).

Alterations in the prostate stroma of FGF8b-TG-BERKO<sub>FVB</sub> mice

To examine 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 reported previously (Imamov et al. 2004). Our results indicated that in particular the hypercellular foci of the prostate epithelium in BERKO<sub>FVB</sub> 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 those areas with an increased 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 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 described in FGF8b-Tg mice (Elo et al. 2010). A total of 90–100% of the normal prostate epithelial cells were positive for AR in all mouse groups (Fig. 3A, B, C, and D). A similarly high percentage of AR-positive cells was present in the hypercellular areas of the prostate epithelium of a BERKO<sub>FVB</sub> mouse (Fig. 3B). Consistent with results described in 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 hypercellularities 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). Hypercellular stroma seemed to be more extensive in the prostates of FGF8b-Tg mice when compared with the FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fig. 1E, F, I, and J). In addition, alterations in the prostate stroma of FGF8b-Tg-BERKO<sub>FVB</sub> mice have previously been described in FGF8b-Tg mice (Elo et al. 2010, 2012).
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 χ<sup>2</sup>-test. Exact P values were used. *P < 0.05. (B) The scores for inflammation in the mouse prostates evaluated on a scale of 0–3 as defined in the Materials and methods section. The mean values and s.d.s are shown. (C) The normal histology of a 12.5-month-old WT mouse in the 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-BERKO<sub>FVB</sub> mouse showing PAS positivity in the epithelial cells. The images were obtained using 20× and 40× objectives. The scale bars represent 100 μm.
FGF8b-Tg and FGF8b-Tg-BERKOFVB mice (Fig. 3C). In the normal prostate stroma, the percentage of AR-positive cells varied between 35 and 50% in all mouse groups. These results were also consistent with findings described in our previous report (Elo et al. 2010), the percentage of AR-positive cells was reduced in the hypercellular and atypical 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-BERKOFVB mouse prostates (Fig. 3D).

The composition of prostate stroma was studied by IHC staining for SMA (Fig. 3E, F, G, and H) and by Masson’s trichrome staining (Fig. 3 I and J). The hypercellular stroma in both FGF8b-Tg and FGF8b-Tg-BERKOFVB prostates contained wider areas of blue–green-staining (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 with the FGF8b-Tg-BERKOFVB mice.

**Gene expression patterns in VPs of FGF8b-Tg-BERKOFVB and FGF8b-Tg mice**

To examine whether there were changes in the expression of genes that have been 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, BERKOFVB, FGF8b-Tg, and FGF8b-Tg-BERKOFVB mice (Fig. 4). The expression of FGF8b mRNA, which is an indicator of the function of the transgene construct, was increased by several hundred-fold in the prostates of FGF8b-Tg and FGF8b-Tg-BERKOFVB mice compared with WT and BERKOFVB mice (Fig. 4). Consistent with results described in our previous report (Elo et al. 2010), the expressions of Spp1 and Ctgf mRNAs were significantly upregulated in the prostates of FGF8b-Tg and FGF8b-Tg-BERKOFVB mice compared with WT mice.
The level of Spp1 or Ctgf mRNA did not differ significantly between the VPs of BERKO<sub>FVB</sub> and WT mice. As we have previously demonstrated that there are changes in the expression levels of the c-isoforms of Fgfr1 and Fgfr3 in the epididymes 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 with the VPs of WT mice, the expression of Fgfr1c was significantly upregulated and that of Fgfr2c was downregulated in the VPs of FGF8b-Tg and FGF8b-Tg-BERKO<sub>FVB</sub> mice (Fgfr1c, Kruskal–Wallis test, 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> and WT mice.

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the prostate was slightly upregulated in BERKO FVB 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 FVB and FGF8b-Tg mice was statistically significant (ANOVA, P<0.0032). No significant differences were detected in the mRNA levels of Erα between any of the mouse groups.

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. β-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 at 1. The mean values and s.d.s are shown. The differences between groups were tested by one-way ANOVA corrected with Tukey’s multiple comparison test or by a Kruskal–Wallis test corrected with Dunn’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001.
As prostatic inflammation was frequent among the mouse groups, the mRNA levels of several pro-inflammatory cytokines, such as tumor necrosis factor α (Tnfα) and interleukins 6 (Il6) and 17 (Il17a), were analyzed by qRT-PCR (Fig. 5). Compared with WT mice, the level of Tnfα mRNA was significantly upregulated in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO FVB mice (Kruskal–Wallis test, \( P \approx 0.0347 \) and \( P \approx 0.044 \) respectively). The expression of IL17a mRNA was undetectable in most of the samples (19 out of 26 samples, including all the WT prostates) but was upregulated in the prostate of some of the BERKO FVB and FGF8b-Tg-BERKO FVB mice and in one FGF8b-Tg mouse. However, no statistically significant differences between the groups were found for the Il6 or Il17 mRNA levels.

Finally, the expression of several markers associated with a mucinous phenotype and/or prostate cancer progression, such as mucin 1 (Muc1) and mucin 2 (Muc2) and transforming growth factor β 1 (Tgfb1), was analyzed using qRT-PCR (Fig. 5). The prostates of FGF8b-Tg-BERKO FVB mice showed a tendency toward higher levels of Tgfb1 compared with other groups, but due to large inter-individual variation, the difference was not statistically significant (Kruskal–Wallis test, \( P = 0.12 \)). The mean level of both Muc1 and Muc2 mRNAs was highest in the prostate of FGF8b-Tg mice, but there were no significant differences in the level of Muc1 or Muc2 mRNAs between the mouse groups.

**Discussion**

Several studies have provided evidence for anti-proliferative and differentiation-promoting effects of ERβ and tumorigenic effects of ERα 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. (In Press)). Recently, the anti-tumorigenic effect of ERβ was supported by results from a study, which compared the incidence of prostate tumorigenesis in genistein- and casein-fed transgenic adenocarcinoma of the mouse prostate (TRAMP), BERKO-TRAMP, and ERKO-TRAMP mice (Slusarz et al. 2012). In the current study, we examined how the deficiency of ERβ affects prostate tumorigenesis that had been previously observed in FGF8b-Tg mice (Elo et al. 2010) by crossing FGF8b-Tg mice with BERKO FVB mice. We examined the hypothesis that two genomic, putative tumor-promoting modifications might generate a model with accelerated or more advanced prostate tumorigenesis, as has been reported previously for several
mouse models bearing more than one genomic modification (Jeet et al. 2010, Ittmann et al. 2013).

Consistent with results described in our previous report, a high frequency of the 1-year-old FGF8b-Tg mouse prostates contained epithelial and stromal hypercellularities, inflammation, and mPIN lesions. In addition, some FGF8b-Tg mouse prostates contained cancers (adenocarcinoma, sarcoma, or carcinosarcoma) (Elo et al. 2010). The prostate of a 1-year-old BERKOFVB mouse contained relatively mild changes, including epithelial hypercellularity and inflammation. This is in contrast to the results from a study by Weihua et al. (2001), which reported a higher frequency of more pronounced hyperplasia in the prostates of their BERKO mice. This result is interesting because the BERKOFVB mice used in this 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 results regarding the expression pattern of p63 differ from those described by Imamov et al. (2004) because even though we noticed a tendency toward a higher frequency of p63-positive cells in the hypercellular foci of BERKOFVB 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 BERKOFVB mice. According to our results, including the IHC staining and qRT-PCR analysis of the AR, the level of AR mRNA or protein was not significantly increased in the prostates of BERKOFVB mice compared with those of WT mice, but interestingly, the level of AR mRNA was significantly higher in the prostates of BERKOFVB mice compared with those of FGF8b-Tg mice; this may be due to a previously reported decreased level of AR in the PIN lesions and in the hypercellular stroma of the FGF8b-Tg mice (Elo et al. 2010).

In contrast to the results expected according to the original hypothesis, the FGF8b-Tg-BERKOFVB mice did not contain more mPIN lesions or more advanced neoplastic changes than the FGF8b-Tg mice. The epithelial and stromal hypercellularities were, in fact, slightly more frequent in the prostates of FGF8b-Tg-BERKOFVB mice when compared with FGF8b-Tg mice, while the hypercellular changes, especially in the stroma, were less extensive in the FGF8b-Tg-BERKOFVB 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-BERKOFVB mice. Taken together, these data indicate that ERβ 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 ERβ, which have shown that ERβ 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-BERKOFVB mice than in any of the other mouse groups studied. This indicates that even if the structural changes in the prostate histology of BERKOFVB mice were mild, the lack of ERβ combined with overexpression of FGF8b influences the differentiation of the epithelial cells. An increased frequency of mucinous metaplasia has been reported previously in the prostates of old FGF8b-Tg mice (Elo et al. 2010), but a lack of ERβ 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 for several genetically modified mouse models (Ittmann et al. 2013).

We have reported previously 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 indicated a tendency towards an increased frequency of inflammation in the prostates of FGF8b-Tg-BERKOFVB mice than in those of the FGF8b-Tg mice, indicating that a deficiency of ERβ could facilitate the formation of inflammatory lesions in the FGF8b-Tg mice. This is consistent with results from
previous animal studies; it has been reported that ER\(\alpha\) mediates neonatal estrogen-treatment-induced prostatic inflammation (Prins et al. 2001), and the anti-inflammatory effects of ER\(\beta\) 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. This study did not find any statistically significant differences in the mRNA levels of proinflammatory cytokines (Il6, Il17a and Tnf) between the prostates of WT and BERKO\_FVB mice or between FGF8b-Tg and FGF8b-Tg-BERKO\_FVB mice. However, a trend toward higher Il17a levels in BERKO\_FVB and Tg-BERKO\_FVB mice was observed. In addition, Tnf was upregulated in both FGF8b-Tg and FGF8b-Tg-BERKO\_FVB prostates. This upregulation is most probably associated with the ongoing inflammatory process. TNF 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 and Ctgf) previously found to be upregulated in the prostate and epididymis of FGF8b-Tg mice (Elo et al. 2010, 2012) was increased in the prostate of FGF8b-Tg-BERKO\_FVB mice. Interestingly, upregulation of the mRNA for Fgfr1c and downregulation of the mRNA for Fgfr2c were found in the prostates of FGF8b-Tg and FGF8b-Tg-BERKO\_FVB mice. This observation is consistent 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. Results described in a previous report have demonstrated 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-isofoms, but not the b-isofoms, 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 FGF8b-Tg-BERKO\_FVB 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 results from 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, Prins et al. 2001, Weihua et al. 2001, Antal et al. 2008). Our BERKO\_FVB model is the first one, to our knowledge, 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 this study is not a complete knockout model because it was generated by insertion of NEO-cassette into exon 3 of the Er\(\beta\) gene (Krege et al. 1998). The presence of shorter forms of Er\(\beta\) mRNA in BERKO mice, which resulted from alternative splicing of Er\(\beta\) mRNA, has been described previously with respect to the ovaries of BERKO mice (Krege et al. 1998, Dupont et al. 2000). Our experiments demonstrated similar shorter forms of Er\(\beta\) mRNA and IHC-based evidence for a protein product (truncated ER\(\beta\)) for the first time, to our knowledge, in the prostates of BERKO mice (Supplementary Figure 2, see section on supplementary data given at the end of this article). According to Krege et al. (1998) and our results, two of these Er\(\beta\)KO mRNAs (Er\(\beta\)KO1 and Er\(\beta\)KO2) lack the disrupted exon 3 and contain early stop codons in the exon 4. They would therefore be translated into protein products lacking the DNA-binding domain (DBD) and the ligand-binding domain (LBD) (Supplementary Figure 2). The third Er\(\beta\)KO mRNA (Er\(\beta\)KO3, Krege et al. 1998), which seemed to be present only occasionally at low levels in the prostate of BERKO\_FVB mice, lacks only exons 3 and 4. It would thus be translated into a protein product devoid of the DBD but containing the AF1 and AF2 (and the LDB) domains. These shortened Er\(\beta\)KO protein products are not capable of exerting the classical functions of the ER\(\beta\), but they may, especially the Er\(\beta\)KO2 product, possess some of the non-classical functions of ER\(\beta\), such as those mediated by the activation of AF1 sites (Price et al. 2001).

One feature that complicates our understanding of the function of ER\(\beta\) is the presence of functionally different isoforms of ER\(\beta\) in the prostate. In a pattern consistent with the results described in previous studies of ER\(\beta\) expression in the rat prostate (Petersen et al. 1998, Hanstein et al. 1999), we observed the expression of Er\(\beta\) isoforms 1 (‘mEr\(\beta\)2, wt isoform’) and 2 (mEr\(\beta\)2) in the prostates of WT mice (Supplementary Figure 2a).
The structure and nomenclature of ERβ isoforms differ between mice and humans, and for example, hERβ2 and mERβ2 are not homologs (Lu et al. 1998, Moore et al. 1998). In fact, normal human tissues do not express an ERβ isofrom homologous to the mERβ2 isofrom (Lu et al. 1998), but a corresponding isofrom (different from hERβ1, hERβ2, hERβ3, hERβ4, and hERβ5) is expressed in some human cancer cell lines (Hanstein et al. 1999). The mERβ2 has ligand- and coactivator-binding properties that are different from mERβ1 (Zhao et al. 2005), but its physiological function is unclear. It has been suggested that mERβ2 acts as a negative regulator of ERα (Zhao et al. 2005) and/or ERβ1 (Lu et al. 2000). In humans, the different isoforms of ERβ are functionally different and have differential, even opposite roles in prostate tumorigenesis (Leung et al. 2010, Dey et al. 2012, Kawashima & Nakatani 2012), which may partly explain conflicting results regarding the role of ERβ in the prostate.

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

On the whole, ERβ may have different roles in the different phases of prostate development and the different phases of prostate tumorigenesis (Nelson et al. (In Press)). Therefore, a conditional inducible knockout model of ERβ would be required to examine the effect of ERβ abrogation in prostate tumorigenesis completely and to eliminate the potential consequences of the lack of a 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 is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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