Deficiency of ER β 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 (ER α (ESR1)) and β (ER β (ESR2)). Hyperplasia and decreased differentiation of epithelial cells in the prostate have been reported in ER β knockout (BERKO) mice. Herein, we studied the effect of ER β deficiency on prostate tumorigenesis by crossing BERKO_{FVB} 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_{FVB} mice exhibited mild epithelial hypercellularity and inflammation. The prostate phenotypes of FGF8b-Tg-BERKO_{FVB} 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_{FVB} 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_{FVB} 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_{FVB} mice. Our results indicate that ER β 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 ER β in the prostate of FGF8b-Tg mice.

Key Words

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

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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 & Risbridger 2009, Hartman *et al.* 2012).

In target tissues, estrogens act via estrogen receptor α (ER α) (ESR1) and ER β (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, 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 *Era*-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, Torlakovic *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 (Bonkhoff *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-BERKO_{FVB}) 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-BERKO_{FVB}, *FGF8b*-Tg, BERKO_{FVB}, 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)SQC, 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). For 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 χ^2 -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

Genes	Forward primers 5'–3'	Reverse primers 5'–3'	Annealing temperature (°C)
<i>Ar</i>	GTCTCCGGAAATGTTATGAA	AAGCTGCCTCTCTCCAAG	58
<i>Erα</i>	CCGTGTGCAATGACTATGCC	GTGCTTCAACATTCTCCCTCCTC	58
<i>Il17a</i>	TCATCCCTCAAAGCTCAGCG	TTCATTGCGGTGGAGAGTCC	58
<i>Il6</i>	CCGGAGAGGAGACTTCACAG	CAGAATTGCCATTGCACAAC	60
<i>Muc1</i>	GTGCCAGTGCCGCCGAAAGA	TGCCGAAACCTCCTCATAGGGGC	60
<i>Muc2</i>	GCCAGATCCCGAAACCAC	TGTAGGAGTCTCGGCAGTCA	60
<i>Tgfb1</i>	CAACAATTCTGGCGTTACCTTGG	GAAAGCCCTGTATTCCGTCTCCTT	60
<i>Tnf</i>	CCCCAAAGGGATGAGAAGTT	CACTTGGTGGTTTGCTACGA	60

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 described previously 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 1-year-old BERKO_{FVB} mice. Compared with 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, 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_{FVB} 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 (χ^2 -test, $P=0.028$) in the *FGF8b*-Tg-BERKO_{FVB} 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 epithelium of the majority of *FGF8b*-Tg (71%) and *FGF8b*-Tg-BERKO_{FVB} (89%) prostates that were defined as goblet-cell-positive in the initial analysis (Fig. 1I and J). In contrast, the majority of BERKO_{FVB} (100%) and WT (67%) prostates that were defined as goblet-like-cell-positive did not contain PAS-positive cells in their epithelia (Fig. 1G and H). In addition, inflammation was slightly, but not to a statistically significant extent, more frequent in the prostates of *FGF8b*-Tg-BERKO_{FVB} 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_{FVB} mice (0.9 vs 1.0) (Fig. 1B). Epithelial and stromal

hypercellularities were present at similar frequencies in the prostates of *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} 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 when compared with the *FGF8b*-Tg-BERKO_{FVB} 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 mice compared with *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 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_{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 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 also present in the hypercellular areas of the prostate epithelium of a BERKO_{FVB} 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

Figure 1

(A) Frequency of histological changes in the prostates of 10–14-month-old WT, BERKO_{FVB}, *FGF8b*-Tg, and *FGF8b*-Tg-BERKO_{FVB} mice. Differences in frequencies between *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} mice were tested by χ^2 -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_{FVB} 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_{FVB} 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_{FVB} 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 an mPIN lesion. (J) PAS staining of VP of a 12.5-month-old *FGF8b*-Tg-BERKO_{FVB} mouse showing PAS positivity in the epithelial cells. The images were obtained using 20 $\times$ and 40 $\times$ objectives. The scale bars represent 100 μ m.

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_{FVB} mouse prostate with a focally increased frequency of p63-positive cells (arrow). (C and D) A decreased frequency of p63-positive cells in the mPIN lesions (*) of *FGF8b*-Tg (C) and *FGF8b*-Tg-BERKO_{FVB} mice (D). The images were obtained using a 40 $\times$ objective, and the insets show

2 $\times$ magnification of the images. The scale bars represent 100 μ m. (E) The percentages of p63-positive cells in the VP epithelia of WT, BERKO_{FVB}, *FGF8b*-Tg, and *FGF8b*-Tg-BERKO_{FVB} mice. A minimum of one thousand epithelial cells from multiple prostatic acini from sections stained for p63 were analyzed by IHC ($n=5-7$ /mouse group). The mean values $\pm$ s.d.s are shown. There were no statistically significant differences between the mouse groups (one-way ANOVA and Tukey's multiple comparison test).

FGF8b-Tg and *FGF8b*-Tg-BERKO_{FVB} 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 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-BERKO_{FVB} 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. 3I and J). The normal prostate stroma of the WT and BERKO_{FVB} mice contained SMA-positive cells in a ring-like structure surrounding the prostatic acini (Fig. 3E and F). The hypercellular stroma of both *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} 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_{FVB} mice (Fig. 3G and H). The staining patterns were similar in the prostates of WT and BERKO_{FVB} mice for Masson's trichrome staining, which stains collagen fibers blue-green and smooth muscle red

(Fig. 3I and J). The hypercellular stroma in both *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} prostates contained wider areas of blue-green-staining (Fig. 3K 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-BERKO_{FVB} mice.

Gene expression patterns in VPs of *FGF8b*-Tg-BERKO_{FVB} 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, BERKO_{FVB}, *FGF8b*-Tg, and *FGF8b*-Tg-BERKO_{FVB} 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-BERKO_{FVB} mice compared with WT and BERKO_{FVB} 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-BERKO_{FVB} mice compared with WT mice

Figure 3

Immunohistochemical (IHC) staining of AR (A, B, C, and D) and SMA (E, F, G, and H) and Masson's trichrome staining (I, J, K, and L) in the VPs of 10–15-month-old WT, BERKO_{FVB}, *FGF8b*-Tg, and *FGF8b*-Tg-BERKO_{FVB} mice. (A) A representative image 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_{FVB} mouse in which epithelium and stroma showed similar staining patterns for AR to those of the WT mice. (C) The prostate of an *FGF8b*-Tg mouse in which the PIN lesion (arrow) and hypercellular stroma (*) contained a decreased frequency of AR-positive cells compared with WT mice. (D) An *FGF8b*-Tg-BERKO_{FVB} mouse VP with hypercellular epithelium and stroma (*) with ~100 and 50% 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

an *FGF8b*-Tg mouse with wide areas of SMA-negative hypercellular and atypical stroma surrounding the acini (*). (H) The VP of an *FGF8b*-Tg-BERKO_{FVB} 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_{FVB} mouse showing a similar staining pattern to that in the WT mouse. (K) The VP of an *FGF8b*-Tg mouse with hypercellular and atypical stroma displaying wide areas of blue–green staining and collagen-rich stroma. (L) The VP of an *FGF8b*-Tg-BERKO_{FVB} mouse, showing an increased amount of blue–green staining and collagen-rich stroma around the prostatic acini. These images were obtained using a 20 $\times$ objective, and the insets show a 2 $\times$ magnification of these images. The scale bars represent 100 μ m.

(Kruskal–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_{FVB} 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 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 with the VPs of WT mice, the expression of *Fgfr1c* was significantly upregulated and that of *Fgfr2c* was down-regulated in the VPs of *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} mice (*Fgfr1c*, Kruskal–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 the BERKO_{FVB} mice, the expressions of *Fgfr1c* and *Fgfr2c* mRNAs were similar to those of WT mice. There were no significant differences in the expressions of *Fgfr1b* and *Fgfr2b* mRNAs in the VPs of different mouse groups. The expressions of *Fgfr3b* and *Fgfr3c* mRNAs could not be quantified because the levels of these mRNAs were below the detection limit of qRT-PCR.

To examine whether the deficiency of ER β expression in BERKO_{FVB} and *FGF8b*-TG-BERKO_{FVB} mice affected the expression of other steroid hormone receptors in the prostate, the expression of ER α and AR was analyzed by qRT-PCR (Fig. 4). The upregulation of the AR in BERKOs has been reported previously by Imamov et al. (2004). Compared with WT mice, the expression of *Ar* mRNA in

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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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 *Era* between any of the mouse groups.

Figure 5

The expression of genes for cytokines (*Tnf*, *Il6*, and *Il17a*), prostate cancer-promoting factors (*Tgfb1*), and proteins related to the mucin-secreting phenotype (*Muc1* and *Muc2*) as analyzed by qRT-PCR. β -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 at 1. In case of *Il17* qRT-PCR, the average of all the CT value data was used as a

reference artificially set at 1 because no signal could be detected in any of the WT prostates. 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. * $P < 0.05$.

As prostatic inflammation was frequent among the mouse groups, the mRNA levels of several pro-inflammatory cytokines, such as tumor necrosis factor α (*Tnf*) and interleukins 6 (*Il6*) and 17 (*Il17a*), were analyzed by qRT-PCR (Fig. 5). Compared with WT mice, the level of *Tnf* mRNA was significantly upregulated in the prostates of *FGF8b*-Tg and *FGF8b*-Tg-BERKO_{FVB} mice (Kruskal–Wallis test, $P = 0.0347$ and $P = 0.044$ 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 ER β and tumorigenic effects of ER α 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 ER β 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 ER β 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 BERKO_{FVB} 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 BERKO_{FVB} 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 BERKO_{FVB} 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_{FVB} 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 BERKO_{FVB} mice compared with those of WT mice, but interestingly, the level of *Ar* mRNA was significantly higher in the prostates of BERKO_{FVB} 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-BERKO_{FVB} 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-BERKO_{FVB} mice when compared with *FGF8b*-Tg mice, while the hypercellular changes, especially in the stroma, were less extensive in the *FGF8b*-Tg-BERKO_{FVB} 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 indicate that ER β 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 ER β , which have shown that ER β 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 of the other mouse groups studied. This indicates that even if the structural changes in the prostate histology of BERKO_{FVB} mice were mild, the lack of ER β 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 ER β 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-BERKO_{FVB} mice than in those of the *FGF8b*-Tg mice, indicating that a deficiency of ER β 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 α mediates neonatal estrogen-treatment-induced prostatic inflammation (Prins *et al.* 2001), and the anti-inflammatory effects of ER β 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-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 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 β* gene (Krege *et al.* 1998). The presence of shorter forms of *Er β* mRNA in BERKO mice, which resulted from alternative splicing of *Er β* 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 β* mRNA and IHC-based evidence for a protein product (truncated ER β) 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 β* KO mRNAs (*Er β* KO₁ and *Er β* 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 β* KO mRNA (*Er β* 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 β* KO protein products are not capable of exerting the classical functions of the ER β , but they may, especially the *Er β* KO₃ product, possess some of the non-classical functions of ER β , such as those mediated by the activation of AP1 sites (Price *et al.* 2001).

One feature that complicates our understanding of the function of ER β is the presence of functionally different isoforms of ER β in the prostate. In a pattern consistent with the results described in previous studies of ER β expression in the rat prostate (Petersen *et al.* 1998, Hanstein *et al.* 1999), we observed the expression of *Er β* isoforms 1 ('*mEr β 2*, wt isoform') and 2 (*mEr β 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 isoform homologous to the mERβ2 isoform (Lu *et al.* 1998), but a corresponding isoform (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 forms 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-13-0480>.

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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