Accepted Preprint first posted on 25 January 2018 as Manuscript ERC-17-0476

HSD17B1 EXPRESSION INDUCES INFLAMMATION-AIDED RUPTURE OF MAMMARY GLAND MYOEPITHELIUM

Päivi Järvensivu[1,3], Taija Heinosalo[1,3], Janne Hakkarainen[1], Pauliina Kronqvist[2], Niina Saarinen[1,4], Matti Poutanen[1,4]

[1]Institute of Biomedicine, Research Centre for Integrative Physiology and Pharmacology and Turku Center for Disease Modeling, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland

[2]Institute of Biomedicine, Research Center for Cancer, Infections and Immunity, University of Turku and Department of Pathology, Turku University Hospital, Kiinamyllynkatu 10, 20520 Turku, Finland

[3]equal contribution

[4]these authors jointly supervised this work

Correspondence should be sent to

Matti Poutanen (matti.poutanen@utu.fi) or Niina Saarinen (niina.saarinen@utu.fi)

Word count: 4972

Short title: HSD17B1-driven mammary myoepithelium breakage

Key words: Hydroxysteroid (17-beta) dehydrogenase type 1, HSD17B1, mammary gland, myopithelial cells, periductal inflammation

ABSTRACT

Hydroxysteroid (17-beta) dehydrogenase type 1 (HSD17B1) converts low active estrogen estrone to highly active estradiol. Estradiol is necessary for normal postpubertal mammary gland development; however, elevated estradiol levels increase mammary tumorigenesis. To investigate the significance of the human HSD17B1 enzyme in the mammary gland, transgenic mice universally overexpressing human HSD17B1 were used (HSD17B1TG mice). Mammary glands obtained from HSD17B1TG females at different ages were investigated for morphology and histology, and HSD17B1 activity and estrogen receptor activation in mammary gland tissue were assessed. To study the significance of HSD17B1 enzyme expression locally in mammary gland tissue, HSD17B1-expressing mammary epithelium was transplanted into cleared mammary fat pads of wild-type females, and the effects on mammary gland estradiol production, epithelial cells and the myoepithelium were investigated. HSD17B1TG females showed increased estrone to estradiol conversion and estrogen-response element-driven estrogen receptor signaling in mammary gland tissue, and they showed extensive lobuloalveolar development that was further enhanced by age along with an increase in serum prolactin concentrations. At old age, HSD17B1TG females developed mammary cancers. Mammary-restricted HSD17B1 expression induced lesions at the sites of ducts and alveoli, accompanied by peri- and intraductal inflammation and disruption of the myoepithelial cell layer. The lesions were shown to be estrogen dependent, as treatment with an antiestrogen, ICI 182,780, starting when lesions were already established reversed the phenotype. These data elucidate the ability of human HSD17B1 to enhance estrogen action in the mammary gland *in vivo* and indicate that HSD17B1 is a factor inducing phenotypic alterations associated with mammary tumorigenesis.

INTRODUCTION

Postpubertal mammary gland development is extensively hormonally regulated, and 17-beta-estradiol (E2) is a hormone essential for normal postpubertal mammary gland development. In hormone-deprived mice, additive and sequential administration of E2 in concert with progesterone and prolactin in combination with cortisol and growth hormone can recapitulate mammary gland development (Nandi 1958, Brisken & O'Malley 2010). Estrogen receptor alpha (ESR1) is expressed in both stromal and epithelial compartments of the mammary gland. E2 exerts its mammotropic effect mainly through epithelial ESR1, as demonstrated by mammary gland transplantation experiments. There was a complete lack of postpubertal ductal development in the ESR1-deficient mammary epithelium transplanted into mammary fat pads of wild-type (WT) female mice, whereas normal mammary gland development was observed for WT epithelium transplanted into mammary fat pads devoid of ESR1 (Mallepell *et al*. 2006).

The importance of estrogens in the etiology of human breast cancer is widely recognized. Prolonged lifetime estrogen exposure due to early menarche, late menopause, nulliparity, prolonged oral contraceptive use and hormone replacement therapy increases the risk of breast cancer, whereas pregnancy at younger age and breastfeeding are protective against breast cancer (Dall & Britt 2017). Furthermore, an increased free E2 concentration in the plasma of postmenopausal women is associated with increased breast cancer risk (Yue *et al*. 2013), demonstrating a direct association between circulating estrogen levels and breast cancer risk. In experimental settings, E2 enhances mammary cancer development by triggering the neoplastic transformation of breast epithelial cells (Russo & Russo 2006) and by inducing the proliferation and survival of ESR1-positive breast cancer cells (Frasor *et al*. 2003).

Hydroxysteroid (17-beta) dehydrogenase type 1 (HSD17B1) is an enzyme catalyzing the last steps

of E2 biosynthesis together with CYP19A1 (Cytochrome P450 family 19 subfamily A member 1). CYP19A1, also known as aromatase, converts androstenedione and testosterone to estrone (E1) and E2, respectively, and HSD17B1 efficiently catalyzes the conversion of biologically low-active E1 to highly active E2 (Poutanen *et al*. 1993, Miettinen *et al*. 1996, Puranen *et al*. 1997, Day *et al*. 2008). Human HSD17B1 is mainly expressed in estrogen-producing tissues, the ovary (Tremblay *et al*. 1989, Ghersevich *et al*. 1994) and placenta (Fournet-Dulguerov *et al*. 1987) but is also expressed at lower levels in peripheral estrogen target tissues, such as the breast (Poutanen *et al*. 1992, Miettinen *et al*. 1999) and endometrium (Mäentausta *et al*. 1991, Dassen *et al.* 2007). In women, increased HSD17B1 expression has been reported in breast premalignant lesions, ductal carcinoma *in situ,* and breast cancer (Poutanen *et al*. 1992, Sasano *et al*. 1996, Ariga *et al*. 2000, Oduwole *et al*. 2004, Sasaki *et al*. 2010). HSD17B2 is a counteracting enzyme for HSD17B1 that has been shown to convert highly potent E2 to less potent E1 and its expression is often lost in breast cancer cells (Luu-The *et al*. 1995, Miettinen *et al*. 1996). In epidemiological studies, increased HSD17B1 expression is linked to increased breast cancer risk. In women with ESR-positive breast cancer, high HSD17B1 expression is associated with late recurrence (Gunnarsson *et al*. 2001, 2005), and amplification of *HSD17B1* decreases survival rates (Gunnarsson *et al*. 2003, 2008). Furthermore, patients with HSD17B1 expressing tumors have significantly shorter overall and disease-free survival (Oduwole *et al*. 2004, Salhab *et al*. 2006). In addition to HSD17B1 expression alone, alterations in the HSD17B1 to HSD17B2 ratio modify breast cancer risk. In women with ESR-positive breast cancer, high HSD17B1 to HSD17B2 ratios are associated with worse prognosis and increases the risk for recurrence (Gunnarsson *et al*. 2001, 2005). Respectively, high intratumoral HSD17B2 or high HSD17B2 to HSD17B1 expression ratios are linked to improved prognosis and reduced risk of recurrence (Gunnarsson *et al*. 2001, 2005).

In healthy breast tissue of post- and premenopausal women, the serum E2 concentration is the major

97 determinant of the intratissue E2 concentration (Depypere *et al.* 2015). Interestingly, in breast
98 cancer patients, higher intratumoral E2 concentrations than those in the plasma are often reported
99 (Geisler 2003, Stanczyk *et al*. 2015). The higher intratumoral E2 concentration is considered to be
100 due to local intratumoral estrogen synthesis from circulating androgen precursors (Sasano *et al.*
101 2008). Together with aromatase and a few other reductive HSD17B enzymes, HSD17B1 is
102 suggested to contribute to the maintenance of high intratumoral E2 levels (Miyoshi *et al.* 2001). In
103 xenograft tumors derived from human ESR-positive breast cancer cells, HSD17B1 expression
104 increases intratumoral E2 synthesis, and thus, increases the potency of E1 to stimulate tumor growth
105 (Day *et al*. 2008, Husen *et al*. 2006). Similarly, increased HSD17B1 expression in tumor tissue is
106 linked to increased intratumoral E2 levels in postmenopausal women (Sasano *et al.* 2006, Miki *et*
107 *al*. 2009). These findings suggest that increased HSD17B1 expression may contribute to the
108 development of breast lesions and estrogen-responsive breast cancer.
109
110 In this study, we investigated the impact of HSD17B1 expression on mammary gland tissue by
111 using transgenic mice with universal overexpression of human HSD17B1 (HSD17B1TG mice)
112 (Saloniemi *et al.* 2007). We previously reported that HSD17B1TG mice have increased *in vivo*
113 production of E2 from E1 (Saloniemi *et al.* 2010, Järvensivu *et al.* 2015) and that HSD17B1TG
114 female mice develop a classical estrogen-induced endometrial hyperplasia phenotype, indicating
115 enhanced estrogen action in estrogen target tissues. In the present study, we report HSD17B1-
116 induced effects on the mammary gland both in HSD17B1TG female mice and in wild-type (WT)
117 mice with local human HSD17B1 overexpression in the mammary gland induced by transplanting
118 human HSD17B1-expressing mammary epithelium in to WT mammary gland. Our findings
119 elucidate the role of increased HSD17B1 expression in the process of mammary gland
120 tumorigenesis.
121

ANIMALS, MATERIALS AND METHODS

Experimental animals and timeline of studies

Animal care and use were conducted in accordance with the Finnish Act on Animal Experimentation and with EU laws, guidelines, and recommendations. The studies were approved by the National Animal Experiment Board OF Finland (2007-01367, 2010-04888, 257/04.10.07/2013, 10605/04.10.07/2016).

The mice were housed under a 12h light/dark cycle at 21 ± 1°C and they had free access to soy-free RM3 chow (Special Diet Service, Whitman Essex, UK) and tap water. The generation and maintenance of FVB/N mice expressing human HSD17B1 under the chicken beta-actin promoter (HSD17B1TG mice) have been previously described (Saloniemi *et al*. 2010, Järvensivu *et al*. 2015). Briefly, HSD17B1TG males from line 013 and WT females were bred together, and heterozygous HSD17B1TG female offspring were used in the studies. In addition, bi-transgenic ERELuc-HSD17B1TG mice carrying an estrogen-response element (ERE)-driven luciferase reporter gene (Lemmen *et al*. 2004) in the HSD17B1 genetic background were used for mammary gland estrogen activity assays *ex vivo*. The maintenance and genotyping of these mice have been previously described (Järvensivu *et al*. 2015). A summary timeline diagram of the mouse experiments and analyses performed is presented in Supplementary Fig. 1.

Histological and morphological analyses of mammary gland tissues

For histological analysis, tissues were fixed in 10% (v/v) formalin at room temperature for at least 24 h. The tissues were dehydrated, embedded in paraffin, cut into 4-µm thick sections and stained with hematoxylin and eosin. Slides were then scanned with a Pannoramic 250 Flash II digital slide scanner (3DHISTECH, Budapest, Hungary). For morphological analysis, the fourth inguinal mammary gland was removed, spread on a glass slide, and fixed in Carnoy's fixative (acetic acid-

147 ethanol) at +4°C. These mammary gland whole-mount slides were rinsed with ethanol and tap water
148 and were stained with carmine-alum for 3-4 days. The stained slides were then dehydrated in a
149 series of ethanol washes (70-100% ethanol), cleared in xylene, and finally mounted onto glass slides
150 in Pertex (Histolab Products AB, Gothenburg, Sweden).
151
152 **Serum prolactin measurements**
153 At sacrifice, blood was collected by heart puncture under tribromoethanol (Avertin, Sigma-Aldrich,
154 St. Louis, MO, USA or Alfa Aesar, Karlsruhe, Germany) terminal anesthesia (600-1000 µl of 2.5%
155 (v/v) solution *i.p.*) for serum measurements. Blood samples, stored at +4°C for 24h, were separated
156 for serum collection by centrifugation and further stored at -20°C. The serum prolactin
157 concentration was measured by radio-immuno assay as previously described (Rulli *et al*. 2002).
158
159 **Determination of HSD17B1 activity in mammary gland *in vivo***
160 HSD17B1 activity *in vivo* was determined in 4-month-old HSD17B1TG and WT females.
161 Radioactive [^{3}H]-E1 (Perkin Elmer) dissolved in ethanol:saline (20:80 by vol.) was slowly injected
162 *i.v.* (2.5 µl/g, 1.6 Mbq/mouse). Mice were terminally anesthetized with tribromoethanol (Avertin,
163 600-1000 µl of 2.5% (v/v) solution *i.p.*, Sigma-Aldrich, St. Louis, MO or Alfa Aesar, Karlsruhe,
164 Germany), and 15 minutes after [^{3}H]-E1 substrate injection, blood was withdrawn from the heart.
165 After cervical dislocation of the mice, the mammary glands were dissected, snap-frozen in liquid
166 nitrogen and stored at −80°C. The frozen tissues were homogenized by Ultra-Turrax in ice-cold 50
167 mM Tris-HCl buffer (pH 7.4) and were extracted twice with isopropyl ether. The ether was then
168 evaporated to dryness at +37°C under nitrogen flow. Then, the extracted steroids were redissolved
169 in acetonitrile-water (48:52 by vol.) and separated with an HPLC apparatus (Waters™ 2695, Waters
170 Corporation, Milford, MA) equipped with a Nova-Pak C18 column (3.9 x 150 mm; Waters Co.) and
171 Nova-Pak C18 guard column (Waters Co.) with acetonitrile (Rathburn, Walkerburn, Scotland) –

water (48/52 v/v) as a mobile phase using a flow rate of 1.2 ml/min. The eluent was mixed on-line with scintillant (Ecoscint A, National Diagnostics, Atlanta, GA), and the beta emission of separated steroids was counted on-line with a scintillation analyzer (Packard model 150TR; Perking Elmer Co.). HSD17B1 activity was based on the percentage of [^{3}H]-E1 converted to [^{3}H]-E2.

Estrogen receptor activation in mammary gland tissue of HSD17B1TG mice

Frozen tissues from ERELuc and ERELuc-HSD17B1TG mice were homogenized by Ultra Turrax in 500 µl of lysis buffer [25 mM Tris acetate (pH 7.8), 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM dithiothreitol, and Complete Mini Proteinase Inhibitor (Roche Diagnostics, Penzberg, Germany)]. The homogenate was centrifuged for 30 min at 1000g, and luciferase activity in the supernatant was determined by a Luciferase assay kit (BioThema, Handen, Sweden) according to the manufacturer's instructions. Luminescence was measured with a Victor Multilabel Counter (PerkinElmer) and the obtained luminescence values were normalized against the samples' protein concentrations, which were determined with a Pierce BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA).

Mammary gland transplantations

In transplantation experiments, 18-20-day-old WT female mice were used as hosts, and 18-20-day-old or 4-6-month-old HSD17B1TG and WT females were used as donors. The mice were preoperatively administered 0.15 mg/kg buprenorphine *i.p.* (Temgesic, Shering-Plough, Kenilworth, NJ) and 5 mg/kg carprofen *s.c.* (Rimadyl, Pfizer, NY) was administered as a postoperative analgesic. The developing mammary parenchyma of the host was removed under isoflurane anesthesia by clearing the fat pad between nipples 4-5 and the inguino-abdominal lymph node from both right and left side mammary glands. A piece of mammary epithelium from an HSD17B1TG donor was transplanted into the right side, and WT epithelium was transplanted into the left side of

197 the remaining host mammary fat pads. Postoperative analgesia was continued for 3 days with daily
198 buprenorphine (0.15 mg/kg *i.p.*) and carprofen (5 mg/kg, *s.c.*) injections. The transplanted hosts
199 were sacrificed 4 months (n=21) or 17-18 months (n=8) later with CO_2 asphyxiation. After cervical
200 dislocation of the mice, the transplanted mammary glands were collected, divided into three parts
201 and processed for histological and morphological analyses and RNA expression analyses as
202 described below.
203
204 **HSD17B1 activity measurement in tissues *ex vivo*, and analysis of human HSD17B1**
205 **expression**
206 HSD17B1 activity in HSD17B1TG and WT transplanted mammary glands was analyzed by
207 measuring the conversion of [^{3}H]-E1 to [^{3}H]-E2. Tissues were homogenized in 10 mM KH_2PO_4
208 (pH 7.5) containing 1 mM EDTA supplemented with protease inhibitor cocktail (Complete Mini,
209 Roche Diagnostics GmbH, Mannheim, Germany), 0.01% (v/v) BSA and 10% (v/v) glycerol.
210 Protein concentrations of the homogenates were determined by using a Pierce BCA Protein Assay
211 Kit (Thermo Scientific) according to the manufacturer's instructions. Five micrograms of protein
212 was mixed with [^{3}H]-E1 (Perkin Elmer, Waltham, MA, USA; final [^{3}H]-E1 concentration in the
213 reaction 6.3 nM, and 1.4 mM NADPH, and the reaction was incubated at 37°C for 40 minutes.
214 Steroids were extracted from samples twice with diethyl ether (Merck) and redissolved into 48%
215 (v/v) acetonitrile in water, and [^{3}H]-E1 conversion to [^{3}H]-E2 was analyzed with an HPLC
216 equipped with a scintillation counter as described above.
217 Analysis of human HSD17B1 expression in transplanted mammary glands was measured by
218 quantitative RT-qPCR as previously described (Saloniemi et al. 2007) in triplicate reactions.
219
220 **Immunohistochemistry**

Paraffin-embedded mammary glands were cut into 4 μm sections. The sections were deparaffinized and rehydrated in xylene and ethanol series. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) in a pressure cooker or in 10 mM TRIS-EDTA-buffer, except that cytokeratin 19 (KRT19) and calponin (CNN1) antibodies (pH 9.0) were used for antigen retrieval. The endogenous peroxidase was then blocked by incubating the sections in 3% (v/v) H_2O_2 for 20 min, and then, the slides were incubated overnight at +4°C with one of the following primary antibodies: 1) rat anti-mouse Ki-67 (clone TEC-3, 1:500 dilution, Dako), 2) mouse anti-human ESR1 (1D5, 1:100, Dako), 3) rabbit anti-human progesterone receptor (PGR, A0098, 1:100, Dako), 4) rabbit anti-human keratin 5 (KRT5, RM-2106, 1:100, Thermo Scientific), 5) mouse anti-human alpha actin (ACTB, sc-32251, 1:1000, Santa Cruz Biotechnology), 6) rabbit monoclonal anti-human cytokeratin 19 (KRT19, 1 h incubation at RT, Clone EPR1579Y, 1:500, Epitomics); 7) Rabbit monoclonal anti-human calponin (CNN1, Clone EP798Y, 1:2000, Epitomics), or 8) mouse anti-human-HSD17B1 (1 μg/ml, developed in our group) antibody. The primary antibodies were detected by using an anti-mouse or anti-rabbit Dako Envision+ system (Dako). Visualization was made with DAB+ substrate (Dako). All sections were counterstained with Mayer's hematoxylin and scanned with a Pannoramic 250 Flash II digital slide scanner (3DHISTECH, Budapest, Hungary). For Ki-67 quantitation, over 200 mammary epithelial cells from different fields were counted, and the labeling index was calculated as the percentage of positive cells over the total number of cells counted. The epithelial expression of ESR1 and PGR was assessed according to the Allred scoring method (Allred *et al.* 1998).

ESR antagonist and HSD17B1 inhibitor treatment

Five-month-old host female mice with contralateral mammary tissues grafted with WT or HSD17B1TG epithelium at the age of 3 weeks were treated *s.c.* with 17 mg/kg the ESR antagonist ICI 182,780 (Fulvestrant, Selleckhem, Houston, TX), hereafter referred to as ICI, twice a week or

246 with 10 mg/kg the HSD17B1 inhibitor EC-15 (compound 21 in Messinger *et al*. 2009 provided by
247 Forendo Pharma Ltd., Turku, Finland) once a day for 4 consecutive weeks. The selected dose of ICI
248 was effective in our earlier studies with estrogen-responsive mouse mammary cancer (Wärri et al.,
249 unpublished results). Then the mice were sacrificed and mammary gland tissues were collected for
250 morphological analysis and for analysis of the mammary KRT19-positive epithelial cell Ki-67
251 proliferation index. A subgroup of mice in the ICI treatment group were also treated with 5 mg/kg
252 carprofen (Rimadyl, Pfizer, NY), which did not affect the lesion phenotype. Thus, for the final data
253 analysis, data obtained from ICI and ICI + carprofen treated mice were combined.
254
255 **Statistical analyses**
256 All statistical analyses were performed with GraphPad Prism version 7.02 for Windows. The
257 statistical difference between the two groups was determined by Student's *t*-test or by Mann-
258 Whitney U test for normally and not normally distributed data, respectively. For multiple
259 comparisons, one-way ANOVA followed by Tukey's post hoc test or Kruskal-Wallis one-way
260 ANOVA of ranks followed by Dunn's multiple comparisons test was performed for normally and
261 not normally distributed data, respectively. Differences were considered statistically significant at
262 $P<0.05$. Data are expressed as the mean ± standard error of the mean (SEM).

RESULTS

HSD17B1TG females show a mammary gland phenotype resembling that in pregnancy and present with increased incidence of mammary cancer

The effect of life-long HSD17B1 overexpression on the adult mammary gland phenotype was initially investigated in female HSD17B1TG mice and age-matched WT mice as controls. At the age of 4 months, the HSD17B1TG mammary duct lumens were enlarged and filled with secretion, in contrast to wild-type (WT) female mice presenting ducts with small lumens devoid of secretion (Fig. 1A). At the age of 10 months, HSD17B1TG females showed enhanced alveolar development accompanied by ducts filled with a milk-like secretion and lipid-filled droplets resembling the mammary phenotype observed at mid-pregnancy (Fig. 1B). The lactating mammary phenotype was further enhanced in 18-month-old female HSD17B1TG mice (Fig. 1C). Furthermore, by the age of 18 months, three of six HSD17B1TG females had developed mammary tumors, while no tumors were observed in WT mice of a similar age. Some of the tumors developed histopathological features resembling those of grade I or II human ductal carcinoma (Fig. 1C). Inflammatory cells were observed in the tumor tissues in parallel with the lactating phenotype (Fig. 1C). In HSD17B1TG mice, unlike in WT mice, serum prolactin concentrations were increased with aging, and the highest concentrations were measured in 12-month-old-mice (Fig. 1D). The HSD17B1TG mice developed pituitary adenomas (data not shown) that likely explain the increased circulating prolactin concentrations.

Estrogenicity is increased in HSD17B1TG female mammary gland tissue

To investigate the impact of *HSD17B1* expression on HSD17B1 activity in mammary gland tissue *in vivo*, E1 was injected into the tail veins of HSD17B1TG and WT female mice, and the formed E2 was measured in the mammary gland tissue. In HSD17B1TG female mammary tissue, a significant

proportion (57-85%) of the administered E1 was present as E2, while in WT mammary tissue, only 6-11% of the E1 provided was converted to E2 (Fig. 2A). The impact of HSD17B1 expression on mammary tissue estrogenicity *in vivo* was further investigated in ERELuc reporter mice and in ERELuc-HSD17B1TG mice. HSD17B1 expression significantly increased the ERELuc reporter activity in mammary gland tissue (Fig. 2B), indicating increased ESR activity in the tissue *in vivo*.

Mammary gland-restricted HSD17B1 expression induces the formation of mammary lesions, epithelial cell proliferation and intratissue estradiol production

The impact of mammary-specific HSD17B1 expression on mammary gland morphology was investigated in WT female mice with contralateral inguino-abdominal mammary fat pads bearing mammary ductal epithelium transplanted from WT or HSD17B1TG donors. The analysis of whole mounts collected 4 months after mammary epithelial engraftment revealed lesions at the sites of ducts and alveoli in mammary glands originating from the HSD17B1TG mice, while no lesion formation was observed in WT-transplanted glands (Fig. 3A). Histopathological analyses of the mammary glands with lesions indicated massive intra- and periductal inflammation surrounding the ducts at the sites of lesions, while such inflammatory cell infiltration around parenchyma developed from WT epithelium was not observed (Fig. 3A). Furthermore, no lactation phenotype in WT or HSD17B1TG mammary gland was observed (Fig. 3A). As expected, marked *HSD17B1* mRNA expression was measured in the mammary glands with HSD17B1TG epithelium, which resulted in a strongly increased capacity of the tissue to produce E2 from E1 *ex vivo* (Fig. 3B and C). However, a local increase in E2 production capacity in HSD17B1-expressing mammary gland did not alter the uterine weight compared to uterine weight of WT mice, indicating no significant change in systemic E2 levels (Fig. 3D). Along with the increased capacity for E2 production, a significantly increased proportion of cells positive for a proliferation marker, Ki-67, was observed in HSD17B1-expressing mammary epithelial cells compared to WT epithelial cells (Fig. 4A and B). However, ESR1 and

PGR expression levels were similar in the transplanted HSD17B1TG and WT mammary epithelium (Fig. 4A, C and D). Despite the increased epithelial cell proliferation, mammary gland tumors did not form after mammary gland transplantation within the 18-month follow-up period (data not shown), suggesting that HSD17B1 alone is not sufficient for tumor formation.

HSD17B1-driven periductal mastitis is associated with disruption of the luminal epithelial cell layer and with the breakdown of continuous myoepithelium

The structures of the HSD17B1-induced lesions were further studied with immunohistochemical analysis of epithelial and basal/myoepithelial cell markers. The expression pattern of the luminal epithelial cell marker KRT19 and basal/myoepithelial cell markers KRT5, CNN1 and ACTB revealed an association between HSD17B1-induced periductal inflammation and focal disruption of epithelial luminal and myoepithelial cell layers at the site of inflammatory cell infiltration (Fig. 5). At the sites of a broken or absent myoepithelial cell layer (Fig. 5B-D), the luminal epithelial cells were disorganized and devoid of polarity (Fig. 5A).

Downregulation of ESR signaling rescues the mammary gland lesion phenotype

The role of estrogen signaling in HSD17B1-induced inflammatory lesions was investigated by treating the mammary gland-transplanted mice with the anti-estrogen ICI or with the HSD17B1 inhibitor EC-15 starting 4 months after transplantation, *i.e.*, starting at the time point when mammary gland lesions were already established (Fig. 3A). Interestingly, the ICI treatment reversed the established lesions, which was observed as a significant reduction in lesion number accompanied by significant rescue of the histological phenotype, while HSD17B1 inhibitor treatment tended to rescue the phenotype, but the effect did not reach statistical significance (Fig. 6A). Accordingly, the ICI treatment significantly reduced mammary epithelial cell proliferation, while epithelial cell proliferation in vehicle- and HSD17B1 inhibitor-treated mice was similar (Fig.

338 6B). These data indicate that HSD17B1-induced inflammation-associated mammary gland lesions
339 are dependent on ESR signaling and thus are reversible with ESR antagonist treatment.
340
341 **DISCUSSION**
342 In this study, we show that *HSD17B1* overexpression induces mammary cancer in transgenic mice.
343 Furthermore, we demonstrate that in WT mice with mammary gland-specific HSD17B1 expression,
344 there are responses that are considered relevant for breast carcinogenesis. More specifically,
345 HSD17B1 increases epithelial cell proliferation and ESR signaling in the mammary gland *in vivo*,
346 which is due to increased intratissue production of E2 from its circulating precursor E1. Similar
347 factors, *i.e.*, increased breast epithelial proliferation (Huh *et al*. 2016), altered ESR signaling
348 (Huang *et al*. 2015) and increased E2 concentrations (Yue *et al*. 2013), are linked to increased
349 breast cancer risk in women. We also demonstrate that in mammary gland tissue, HSD17B1
350 expression leads to infiltration of inflammatory cells into the ductal stroma, where destruction of the
351 myoepithelial cell layer at the sites of inflammation was observed. These types of tissue responses
352 have not yet been described in the context of HSD17B1 and the mammary gland. However, in
353 women, both periductal inflammation and coincident loss of myoepithelium have been described
354 (Man & Sang 2004) and are considered carcinogenesis-promoting responses for breast
355 tumorigenesis (Polyak & Kalluri 2010, Yeong *et al*. 2017). Thus, our current findings support the
356 role of HSD17B1 as a factor contributing to mammary tumorigenesis.
357
358 In healthy pre- and postmenopausal women, the serum and breast tissue estrogen profiles are
359 similar (Depypere *et al*. 2015). Interestingly, in breast cancer, the tissue estrogen profile differs
360 from the serum profile (Geisler 2003, Stanczyk *et al*. 2015), and the enzymes expressed locally in
361 the cancer tissue are thought to be responsible for steroid accumulation in cancer tissue. The
362 steroid-metabolizing enzymes aromatase, estrone sulfatase and certain HSD17Bs, such as

363 HSD17B1, HSD17B7 and HSD17B12, are suggested to control local estrogen production in breast
364 cancer tissues by converting circulating androgen and estrogen precursors to active forms with high
365 affinity to steroid receptors (Poutanen *et al*. 1992, Laplante *et al*. 2009, Shehu *et al*. 2011).
366 However, the catalytic efficacy of HSD17B1 for the conversion of E1 to E2 is markedly higher
367 (Puranen *et al*. 1997) than that of HSD17B7 or HSD17B12 (Törn *et al*. 2003, Luu-The *et al*. 2006),
368 and even relatively low HSD17B1 expression levels in peripheral tissues are sufficient for E2
369 production (Delvoux *et al.* 2014). In the present study, we demonstrate that E1 administered into the
370 blood circulation of HSD17B1TG mice via *i.v.* injection or used as a substrate *ex vivo* was
371 efficiently converted to E2 in the mammary gland tissue, supporting HSD17B1 expression as a
372 significant determinant of the mammary gland intratissue E2 concentration.
373
374 Local expression of estrogen-producing enzymes in the mammary gland tissue is also linked to
375 mammary tumorigenesis. In a study conducted with MMTV-aromatase transgenic female mice,
376 local aromatase expression in the mammary gland increased mammary tissue E2 concentrations and
377 induced the development of mammary gland abnormalities, such as epithelial hyperplasia and
378 ductal dysplasia (Tekmal *et al*. 1999, Diáz-Cruz *et al*. 2011). Importantly, in our current study,
379 increased mammary gland -restricted HSD17B1 expression, along with increased E2 production
380 capacity, induced the development of preneoplastic lesions in the mammary gland. The lesions were
381 ameliorated by blocking ESR signaling with ICI, known to be an effective and full ESR antagonist
382 in mouse mammary gland (Silberstein et al. 1994). This indicated that the lesions were dependent
383 on ESR signaling. We previously reported that HSD17B1-induced imbalances in sex steroid
384 hormones were linked to the formation of preneoplastic changes in other estrogen target tissues,
385 such as the ovary and endometrium (Saloniemi *et al*. 2007, 2010). In HSD17B1TG females, we
386 showed the formation of benign ovarian serous cystadenomas and endometrial hyperplasia
387 (Saloniemi *et al*. 2007, 2010) that are known to predispose patients to ovarian and endometrial

388 cancer, respectively. However, no ovarian or endometrial cancers were detected within the 18-
389 month-long follow-up period (Saloniemi *et al*. 2007, 2010). Similarly, no mammary cancers were
390 established in this study in mice with mammary gland-restricted HSD17B1 expression. These
391 findings indicate that HSD17B1 expression and increased sex steroid exposure alone are not
392 sufficient to induce tumorigenesis in reproductive tissues.
393
394 Prolactin is an essential hormone required for alveologenesis and for the differentiation of
395 mammary epithelial cells into milk-producing cells (Nandi 1958, Brisken & O'Malley 2010).
396 Therefore, lactation phenotype of mammary gland is considered as a biological marker for systemic
397 prolactin exposure in ovary-intact female mice. HSD17B1TG mice with advanced age showed
398 increased lobuloalveolar development and lactating phenotype with a co-incident increase in
399 circulating prolactin levels. Thus, the increased circulating prolactin is a likely explanatory factor
400 for the lactating mammary gland phenotype. However WT female mice bearing mammary fat pads
401 with transplanted HSD17B1TG epithelium or WT mammary epithelium showed no lactation
402 phenotype indicating that the local or systemic prolactin levels were not affected in transplanted
403 mice. Increased circulating prolactin levels and sustained prolactin signaling are linked to increased
404 risk of breast cancer (Tworoger *et al*. 2008, Fernandez *et al*. 2010). Accordingly in experimental
405 rodent models, hyperprolactinemia induced in mice either by ectopic pituitary grafts or by
406 overexpression of human chorionic gonadotropin induce the development of cancer in mammary
407 glands (Huseby et al. 1985, Rulli et al. 2002). Mammary tumorigenesis induced by
408 hyperprolactinemia in combination with estrogen is considered more relevant for human breast
409 carcinogenesis than prolactin alone (Tworoger *et al*. 2008). In a previous study, the importance of
410 co-operation with prolactin and E2 in mammary tumorigenesis was demonstrated in transgenic mice
411 with mammary-targeted prolactin expression (Arendt et al. 2009). In those mice, the exposure to E2
412 together with endogenous prolactin decreased mammary tumor latency compared to mice exposed

413 to prolactin alone, indicating that E2 enhances the prolactin-induced mammary gland neoplastic
414 process. HSD17B1TG females with an increased circulating prolactin concentration and enhanced
415 capacity for intratissue E2 production developed mammary cancers with 50% penetrance during the
416 18-month-long follow-up period, while no cancers were observed in WT mice. Consequently,
417 increased prolactin and E2 production are supposed factors inducing mammary cancer in
418 HSD17B1TG female mice. In rodents, increase in systemic E2 is known to cause pituitary
419 adenomas and prolactiomas, shown by us (Rulli *et al*., 2002) and others (Elias *et al*. 1984). In
420 HSD17B1TG mice, HSD17B1 is expressed in pituitary gland (Saloniemi et al. 2007) and, thus,
421 local E2 synthesis from its precursor E1 in the pituitary gland is possible. Therefore, we cannot
422 distinguish the difference between paracrine and endocrine effect of E2 as a causal factor for
423 pituitary adenomas observed in HSD17B1TG female mice. Interestingly, the HSD17B1-induced
424 mammary tumors had histological similarities to human breast cancer, unlike prolactin-induced
425 mammary cancers in general. This finding suggests that overexpression of HSD17B1 may
426 contribute to the development of mammary cancers with a clinically relevant histotype.
427
428 In the normal mammary gland, myoepithelial cells positioned between the luminal cells and the
429 basement membrane maintain the basement membrane, mediate luminal epithelial cell polarity and
430 contribute to branching and differentiation processes (Man & Sang 2004, Brisken & O'Malley
431 2010, Polyak & Kalluri 2010). At early stages of mammary tumorigenesis, myoepithelial cells
432 provide a barrier preventing the invasion of cancerous epithelial cells into the surrounding stroma.
433 Therefore, loss of myoepithelial cell layer integrity and the consequent loss of basal membrane are
434 crucial events for carcinogenesis (Man & Sang 2004, Yeong *et al*. 2017). In clinical ductal
435 carcinoma *in situ* samples, most specimens with a focally disrupted myoepithelial cell layer are
436 reported to display increased leucocyte infiltration and increased proliferation of epithelial cells at
437 the site of the disrupted myoepithelium (Man & Sang 2004). Interestingly, similar myoepithelium-

related changes, *i.e.*, a focally disrupted myoepithelial cell layer co-incident with leucocyte infiltration and increased luminal epithelial cell proliferation, were observed in this study in mice with mammary gland-restricted HSD17B1 expression. Normal estrus cycle is likely an important factor driving peri- and intraductal inflammation and myoepithelial breakage as that phenotype was not observed in HSD17B1TG females that are devoid of estrus cycle (Saloniemi et al. 2009). Moreover, in a previous *in vitro* study, HSD17B1 expression was linked to increased tumorigenic potential of the human mammary-derived premalignant epithelial cell lines (Fu et al. 2010). These data suggest that HSD17B1 expression in mammary epithelium induces phenomena that are similar to those identified to be critical at early phases of mammary carcinogenesis. Whether a comparable association between the expression of HSD17B1 and mammary tumorigenesis occurs in clinical specimens remains to be explored.

In conclusion, this is the first *in vivo* evidence that increased human HSD17B1 expression in mammary gland epithelium 1) enhances the conversion of E1 to E2 in mammary gland tissue, 2) increases luminal epithelial cell proliferation and 3) induces the formation of mammary gland lesions. These lesions display focal disruption of the myoepithelial cell layer, a phenomenon co-existent with peri- and intraductal lymphocyte infiltration. Treatment with an estrogen receptor antagonist, ICI, ameliorates the ductal phenotype, indicating an estrogen receptor-dependent mechanism for lesion development. Furthermore, ubiquitous HSD17B1 expression present in HSD17B1TG mice induces mammary cancer with a histotype also found in human breast cancer. These data enlighten the impact of HSD17B1 expression in the mammary gland and indicate its importance in phenomena related to mammary tumorigenesis *in vivo*.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest for any of the authors that could be perceived

as prejudging the impartiality of the reported research.

FUNDING

This work was supported by the Academy of Finland (#277624), Sigrid Juselius Foundation, University of Turku Graduate School Drug Research Doctoral Programme – DRDP, Finnish Cultural Foundation, Varsinais-Suomi Regional Fund, and The Finnish Funding Agency for Innovation, TEKES (#598/31/2015).

ACKNOWLEDGEMENTS

Forendo Pharma Ltd. is acknowledged for providing the HSD17B1 inhibitor. Turku Center for Disease Modeling (TCDM) personnel are acknowledged for their skillful assistance.

REFERENCES

Allred DC, Harvey JM, Berardo M & Clark GM 1998 Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Modern Pathology* **11** 155-168.

Arendt LM, Evans LC, Rugowski DE, Garcia-Barchino MJ, Rui H & Schuler LA 2009 Ovarian hormones are not required for PRL-induced mammary tumorigenesis, but estrogen enhances neoplastic processes. *Journal of Endocrinology* **203** 99-110.

Ariga N, Moriya T, Suzuki T, Kimura M, Ohuchi N, Satomi S, Sasano H 2000 17 beta-Hydroxysteroid dehydrogenase type 1 and type 2 in ductal carcinoma in situ and intraductal proliferative lesions of the human breast. *Anticancer Research* **20** 1101-1108.

Brisken C & O'Malley B. Hormone action in the mammary gland 2010 *Cold Spring Harbor Perspectives in Biology* doi: 10.1101/cshperspect.a003178.

Dall GV & Britt KL 2017. Estrogen effects on the mammary gland in early and late life and breast cancer risk. *Frontiers in Oncology* doi:10.3389/fonc.2017.00110.

488 Dassen H, Punyadeera C, Kamps R, Delvoux B, Van Langendonckt A, Donnez J, et al. 2007
489 Estrogen metabolizing enzymes in endometrium and endometriosis. *Human Reproduction* **22** 3148-
490 3158.
491 Day JM, Foster PA, Tutill HJ, Parsons MF, Newman SP, Chander SK, et al. 2008 17beta-
492 hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of
493 hormone-dependent breast cancer. *International Journal of Cancer* **122** 1931-1940.
494 Delvoux B, D'Hooghe T, Kyama C, Koskimies P, Hermans RJ, Dunselman GA & Romano A 2014
495 Inhibition of type 1 17β-hydroxysteroid dehydrogenase impairs the synthesis of 17β-estradiol in
496 endometriosis lesions. *Journal of Clinical Endocrinology and Metabolism* **99** 276-284.
497 Depypere HT, Bolca S, Bracke M, Delanghe J, Comhaire F & Blondeel P 2015 The serum estradiol
498 concentration is the main determinant of the estradiol concentration in normal breast tissue.
499 *Maturitas* **81** 42-45.
500 Díaz-Cruz ES, Sugimoto Y, Gallicano GI, Brueggemeier RW & Furth PA 2011 Comparison of
501 increased aromatase versus ERα in the generation of mammary hyperplasia and cancer. *Cancer*
502 *Research* **71** 5477-5487.
503 Elias KA, Weiner RI 1984 Direct arterial vascularization of estrogen-induced prolactin-secreting
504 anterior pituitary tumors. *Proc Natl Acad Sci U S A* **81** 4549-4553.
505 Fernandez I, Touraine P & Goffin V 2010 Prolactin and human tumourogenesis. *Journal of*
506 *Neuroendocrinology* **22** 771-777.
507 Fournet-Dulguerov N, MacLusky NJ, Leranth CZ, Todd R, Mendelson CR, Simpson ER &
508 Naftolin F 1987 Immunohistochemical localization of aromatase cytochrome P-450 and estradiol
509 dehydrogenase in the syncytiotrophoblast of the human placenta. *Journal of Clinical Endocrinology*
510 *and Metabolism* **65** 757-764.
511 Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR & Katzenellenbogen BS 2003 Profiling of
512 estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene

networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* **144** 4562-4574.

Fu J, Weise AM, Falany JL, Falany CN, Thibodeau BJ, Miller FR, et al. 2010 Expression of estrogenicity genes in a lineage cell culture model of human breast cancer progression. *Breast Cancer Research and Treatment* **120** 35-45.

Geisler J 2003 Breast cancer tissue estrogens and their manipulation with aromatase inhibitors and inactivators. *Journal of Steroid Biochemistry and Molecular Biology* **86** 245-253.

Ghersevich SA, Poutanen MH, Martikainen HK & Vihko RK 1994 Expression of 17 beta-hydroxysteroid dehydrogenase in human granulosa cells: correlation with follicular size, cytochrome P450 aromatase activity and oestradiol production. *Journal of Endocrinology* **143** 139-150.

Gunnarsson C, Olsson BM, Stål O & Southeast Sweden Breast Cancer Group 2001 Abnormal expression of 17beta-hydroxysteroid dehydrogenases in breast cancer predicts late recurrence. *Cancer Research* **61** 8448-8451.

Gunnarsson C, Ahnström M, Kirschner K, Olsson B, Nordenskjöld B, Rutqvist LE, Skoog L & Stål O 2003 Amplification of HSD17B1 and ERBB2 in primary breast cancer. *Oncogene* **22** 34-40.

Gunnarsson C, Hellqvist E & Stål O 2005 17beta-Hydroxysteroid dehydrogenases involved in local oestrogen synthesis have prognostic significance in breast cancer. *British Journal of Cancer* **92** 547-552.

Gunnarsson C, Jerevall PL, Hammar K, Olsson B, Nordenskjöld B, Jansson A & Stål O 2008 Amplification of HSD17B1 has prognostic significance in postmenopausal breast cancer. *Breast Cancer Research and Treatment* **108** 35-41.

Huang B, Warner M & Gustafsson JÅ 2015 Estrogen receptors in breast carcinogenesis and endocrine therapy. *Molecular and Cellular Endocrinology* **418** 240-244.

Huh SJ, Oh H, Peterson MA, Almendro V, Hu R, Bowden M, et al. 2016 The proliferative activity of mammary epithelial cells in normal tissue predicts breast cancer risk in premenopausal women. *Cancer Research* **76** 1926-1934.

Huseby RA, Soares MJ & Talamantes F 1985 Ectopic pituitary grafts in mice: hormone levels, effects on fertility, and the development of adenomyosis uteri, prolactinomas, and mammary carcinomas. *Endocrinology* **116** 1440-1448.

Husen B, Huhtinen K, Saloniemi T, Messinger J, Thole HH & Poutanen M 2006 Human hydroxysteroid (17-beta) dehydrogenase 1 expression enhances estrogen sensitivity of MCF-7 breast cancer cell xenografts. *Endocrinology* **147** 5333-5339.

Järvensivu P, Saloniemi-Heinonen T, Awosanya M, Koskimies P, Saarinen N & Poutanen M 2015 HSD17B1 expression enhances estrogen signaling stimulated by the low active estrone, evidenced by an estrogen responsive element-driven reporter gene in vivo. *Chemico Biological Interactions* **234** 126-134.

Laplante Y, Rancourt C & Poirier D 2009 Relative involvement of three 17beta-hydroxysteroid dehydrogenases (types 1, 7 and 12) in the formation of estradiol in various breast cancer cell lines using selective inhibitors. *Molecular and Cellular Endocrinology* **301** 146-153.

Lemmen JG, Arends RJ, van Boxtel AL, van der Saag PT & van der Burg B 2004 Tissue- and time-dependent estrogen receptor activation in estrogen reporter mice. *Journal of Molecular Endocrinology* **32** 689-701.

Luu-The V, Zhang Y, Poirier D & Labrie F 1995 Characteristics of human types 1, 2 and 3 17 beta-hydroxysteroid dehydrogenase activities: oxidation/reduction and inhibition. *Journal of Steroid Biochemistry and Molecular Biology* **55** 581-587.

Luu-The V, Tremblay P & Labrie F 2006 Characterization of type 12 17beta-hydroxysteroid dehydrogenase, an isoform of type 3 17beta-hydroxysteroid dehydrogenase responsible for estradiol formation in women. *Molecular Endocrinology* **20** 437-443.

562 Mallepell S, Krust A, Chambon P & Brisken C 2006 Paracrine signaling through the epithelial
563 estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland.
564 *Proceedings of the National Academy of Sciences of the United States of America* **103** 2196-2201.
565 Man YG & Sang QX 2004 The significance of focal myoepithelial cell layer disruptions in human
566 breast tumor invasion: a paradigm shift from the "protease-centered" hypothesis. *Experimental Cell*
567 *Research* **301** 103-118.
568 Messinger J, Husen B, Koskimies P, Hirvelä L, Kallio L, Saarenketo P & Thole H 2009 Estrone
569 C15 derivatives--a new class of 17beta-hydroxysteroid dehydrogenase type 1 inhibitors. *Molecular*
570 *and Cellular Endocrinology* **301** 216-224.
571 Miettinen MM, Mustonen MV, Poutanen MH, Isomaa VV & Vihko RK 1996 Human 17 beta-
572 hydroxysteroid dehydrogenase type 1 and type 2 isoenzymes have opposite activities in cultured
573 cells and characteristic cell and tissue-specific expression. *Biochemical Journal* **314** 839-845.
574 Miettinen M, Mustonen M, Poutanen M, Isomaa V, Wickman M, Söderqvist G, et al. 1999 17Beta-
575 hydroxysteroid dehydrogenases in normal human mammary epithelial cells and breast tissue. *Breast*
576 *Cancer Research and Treatment* **57** 175-182.
577 Miki Y, Suzuki T & Sasano H 2009 Intracrinology of sex steroids in ductal carcinoma in situ
578 (DCIS) of human breast: comparison to invasive ductal carcinoma (IDC) and non-neoplastic breast.
579 *Journal of Steroid Biochemistry and Molecular Biology* **114** 68-71.
580 Miyoshi Y, Ando A, Shiba E, Taguchi T, Tamaki Y & Noguchi S 2001 Involvement of up-
581 regulation of 17beta-hydroxysteroid dehydrogenase type 1 in maintenance of intratumoral high
582 estradiol levels in postmenopausal breast cancers. *International Journal of Cancer* **94** 685-689.
583 Mäentausta O, Sormunen R, Isomaa V, Lehto VP, Jouppila P & Vihko R 1991
584 Immunohistochemical localization of 17 beta-hydroxysteroid dehydrogenase in the human
585 endometrium during the menstrual cycle. *Laboratory Investigations* **65** 582-587.

586 Nandi S 1958 Endocrine control of mammary-gland development in the C3H/He Crgl mouse.
587 *Journal of National Cancer Institute* **21** 1039-1063.
588 Oduwole OO, Li Y, Isomaa VV, Mäntyniemi A, Pulkka AE, Soini Y & Vihko PT 2004 17beta-
589 hydroxysteroid dehydrogenase type 1 is an independent prognostic marker in breast cancer. *Cancer*
590 *Research* **64** 7604-7609.
591 Polyak K & Kalluri R 2010 The role of the microenvironment in mammary gland development and
592 cancer. Cold Spring Harbour Perspectives in Biology **2** a003244. doi:
593 10.1101/cshperspect.a003244.
594 Poutanen M, Isomaa V, Lehto VP & Vihko R 1992 Immunological analysis of 17 beta-
595 hydroxysteroid dehydrogenase in benign and malignant human breast tissue. *International Journal*
596 *of Cancer* **50** 386-390.
597 Poutanen M, Miettinen M & Vihko R 1993 Differential estrogen substrate specificities for
598 transiently expressed human placental 17 beta-hydroxysteroid dehydrogenase and an endogenous
599 enzyme expressed in cultured COS-m6 cells. *Endocrinology* **133** 2639-2644.
600 Puranen T, Poutanen M, Ghosh D, Vihko P & Vihko R 1997 Characterization of structural and
601 functional properties of human 17 beta-hydroxysteroid dehydrogenase type 1 using recombinant
602 enzymes and site-directed mutagenesis. *Molecular Endocrinology* **11** 77-86.
603 Rulli SB, Kuorelahti A, Karaer O, Pelliniemi LJ, Poutanen M & Huhtaniemi I 2002 Reproductive
604 disturbances, pituitary lactotrope adenomas, and mammary gland tumors in transgenic female mice
605 producing high levels of human chorionic gonadotropin. *Endocrinology* **143** 4084-4095.
606 Russo J & Russo IH 2006 The role of estrogen in the initiation of breast cancer. *Journal of Steroid*
607 *Biochemistry and Molecular Biology* **102** 89-96.
608 Salhab M, Reed MJ, Al Sarakbi W, Jiang WG & Mokbel K 2006 The role of aromatase and 17-
609 beta-hydroxysteroid dehydrogenase type 1 mRNA expression in predicting the clinical outcome of
610 human breast cancer. *Breast Cancer Research and Treatment* **99** 155-162.

611 Saloniemi T, Lamminen T, Huhtinen K, Welsh M, Saunders P, Kujari H & Poutanen M 2007
612 Activation of androgens by hydroxysteroid (17beta) dehydrogenase 1 in vivo as a cause of prenatal
613 masculinization and ovarian benign serous cystadenomas. Molecular Endocrinology **21** 2627-2636.
614 Saloniemi T, Järvensivu P, Koskimies P, Jokela H, Lamminen T, Ghaem-Maghami S, et al. 2010
615 Novel hydroxysteroid (17beta) dehydrogenase 1 inhibitors reverse estrogen-induced endometrial
616 hyperplasia in transgenic mice. *American Journal of Pathology* **176** 1443-1451.
617 Sasaki Y, Miki Y, Hirakawa H, Onodera Y, Takagi K, Akahira J, et al. 2010 Immunolocalization of
618 estrogen-producing and metabolizing enzymes in benign breast disease: comparison with normal
619 breast and breast carcinoma. *Cancer Science* **101** 2286-2292.
620 Sasano H, Frost AR, Saitoh R, Harada N, Poutanen M, Vihko R, Bulun SE, Silverberg SG &
621 Nagura H 1996 Aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 in human breast
622 carcinoma. *Journal of Clinical Endocrinology and Metabolism* **81** 4042-4046.
623 Sasano H, Suzuki T, Nakata T & Moriya T 2006 New development in intracrinology of breast
624 carcinoma. *Breast Cancer* **13** 129-136.
625 Sasano H, Suzuki T, Miki Y & Moriya T 2008 Intracrinology of estrogens and androgens in breast
626 carcinoma. *Journal of Steroid Biochemistry and Molecular Biology* **108** 181-185.
627 Stanczyk FZ, Mathews BW & Sherman ME 2015 Relationships of sex steroid hormone levels in
628 benign and cancerous breast tissue and blood: A critical appraisal of current science. *Steroids* **99** 91-
629 102.
630 Shehu A, Albarracin C, Devi YS, Luther K, Halperin J, Le J, Mao J, Duan RW, Frasor J & Gibori
631 G 2011 The stimulation of HSD17B7 expression by estradiol provides a powerful feed-forward
632 mechanism for estradiol biosynthesis in breast cancer cells. *Molecular Endocrinology* **25** 754-766.
633 Silberstein GB, Van Horn K, Shyamala G, Daniel CW 1994 Essential role of endogenous estrogen
634 in directly stimulating mammary growth demonstrated by implants containing pure antiestrogens.
635 *Endocrinology* **134** 84-90.

636 Tekmal RR, Kirma N, Gill K & Fowler K 1999 Aromatase overexpression and breast hyperplasia,
637 an in vivo model--continued overexpression of aromatase is sufficient to maintain hyperplasia
638 without circulating estrogens, and aromatase inhibitors abrogate these preneoplastic changes in
639 mammary glands. *Endocrine Related Cancer* **6** 307-314.
640 Tremblay Y, Ringler GE, Morel Y, Mohandas TK, Labrie F, Strauss JF 3rd & Miller WL 1989
641 Regulation of the gene for estrogenic 17-ketosteroid reductase lying on chromosome 17cen----q25.
642 *Journal of Biological Chemistry* **264** 20458-20462.
643 Tworoger SS & Hankinson SE 2008 Prolactin and breast cancer etiology: an epidemiologic
644 perspective. *Journal of Mammary Gland Biology and Neoplasia* **13** 41-53.
645 Törn S, Nokelainen P, Kurkela R, Pulkka A, Menjivar M, Ghosh S, et al. 2003 Production,
646 purification, and functional analysis of recombinant human and mouse 17beta-hydroxysteroid
647 dehydrogenase type 7. *Biochemical and Biophysical Research Communications* **305** 37-45.
648 Yeong J, Thike AA, Tan PH & Iqbal J 2017 Identifying progression predictors of breast ductal
649 carcinoma in situ. *Journal of Clinical Pathology* **70** 102-108.
650 Yue W, Yager JD, Wang JP, Jupe ER & Santen RJ 2013 Estrogen receptor-dependent and
651 independent mechanisms of breast cancer carcinogenesis. *Steroids* **78** 161-70.

FIGURE LEGENDS

Figure 1. Morphology of mammary glands of wild-type (WT) and transgenic female mice expressing human HSD17B1 (HSD17B1TG). Representative whole-mount (WM) and hematoxylin-eosin (HE) staining of WT and HSD17B1TG mammary glands at the age of A) 4 months and B) 10 months. C) Representative whole-mount (WM) and hematoxylin-eosin (HE) staining in WT mammary glands and mammary and mammary cancer (MCa) histology in HSD17B1TG female mice at the age of 18 months. Inflammatory cells in MCa are indicated with an arrow. D) Serum prolactin levels of transgenic HSD17B1-expressing (HSD17B1TG) female mice. Each time point represents data obtained from 4-8 mice. Statistical differences between groups were determined by Kruskal-Wallis one-way ANOVA of ranks followed by Dunn's multiple comparisons test. * $P<0.05$. *** $P<0.001$.

Figure 2. HSD17B1 expression increases the estrogenicity in mammary glands of HSD17B1TG female mice. A) Percentages of estrone (E1) and estradiol (E2) in mammary gland *in vivo* in wild-type (WT) and HSD17B1TG mice. B) Estrogen receptor reporter (ERELuc) activity in mammary tissue of ERELuc mice and ERELuc-HSD17B1TG mice at the age of 4 months.

Boxplots indicate the median and the lower and upper quartiles. Whiskers indicate the maximum and minimum. Blots represent data from 3 to 6 mice per group. Statistical differences between the two groups were determined by Student's *t* test. ** $P<0.01$. *** $P<0.001$. Data are expressed as the mean±SEM.

Figure 3. Local human HSD17B1 expression in mammary epithelium induces the formation of lymphocyte-associated lesions, increases the conversion of estrone (E1) to estradiol (E2) in the

mammary tissue but does not affect wild-type (WT) host uterine weight, a biomarker of estrogenicity.

A) Representative whole-mount (WM), hematoxylin-eosin (HE), and HSD17B1 immunohistochemical (IHC) staining of mammary glands transplanted with WT or HSD17B1-expressing (HSD17B1TG) mammary epithelium. Some lesions in WM of HSD17B1TG mammary gland are indicated with arrows. B) Human *HSD17B1* mRNA expression in mammary gland transplanted with WT or HSD17B1TG mammary epithelium (n=4 in both groups). C) HSD17B activity measured *ex vivo* as the conversion of E1 to E2 in mammary tissue with WT (n=5) or HSD17B1TG (n=6) mammary epithelium. D) The uterine weight of intact WT mice (n=5) and WT mice transplanted with HSD17B1TG mammary gland epithelium (n=21). All samples for analyses were collected 4 months after the transplantations. Boxplots indicate the median and the lower and upper quartiles. Whiskers indicate the maximum and minimum. Blots represent data from 4 to 6 mice per group. Statistical differences between the two groups were determined by Student's *t* test. **, $P<0.01$. Non-significant, ns.

Figure 4. Local human HSD17B1 expression in mammary gland induces epithelial proliferation.

A) Expression of Ki-67, estrogen receptor alpha (ESR1) and progesterone receptor (PGR) in mammary glands transplanted with wild-type (WT) or transgenic HSD17B1-expressing (HSD17B1TG) mammary epithelium. Samples were collected 4 months after the transplantation. B) Ki-67 index in mammary epithelial cells (positivity in WT-transplanted gland was set to one in each mouse). C) Allred score of ESR1 and D) PGR expression in mammary epithelium of WT and HSD17B1TG transplanted glands.

Boxplots indicate the median and the lower and upper quartiles. Whiskers indicate the maximum and minimum. Blots represent data from 3 to 7 mice per group. Statistical difference between WT and HSD17B1TG groups was determined by Student's *t* test. **, $P<0.01$.

Figure 5. Local HSD17B1 expression in mammary epithelium induces inflammation-assisted breakage of the myoepithelial layer. A) Keratin 19 (KRT19), B) keratin 5 (KRT5), C) calponin (CNN1), and D) actin (ACTB) immunohistochemistry in mammary glands transplanted with wild-type (WT) or transgenic HSD17B1 expressing (HSD17B1TG) mammary epithelium. Samples were collected 4 months after mammary epithelial transplantations.

Figure 6. Antiestrogen treatment reduces mammary lesion number and epithelial proliferation.

A) Number of lesions counted from the mammary whole mounts after the treatments. B) Mammary epithelial cell proliferation index after the treatments. The proliferation in HSD17B1-expressing mammary epithelium in vehicle-treated mice was set to 1. C) Representative figures of mammary gland whole mounts (WM) and Ki-67 proliferation marker staining after the treatments.

For the study, female WT host mice with one HSD17B1-expressing mammary parenchyma were treated with HSD17B1 inhibitor daily or with ICI 182,780 (ICI) twice a week for 4 consecutive weeks starting 4 months after the cleared mammary fat pads of 18-20-day-old WT female mice were transplanted with HSD17B1TG donor epithelium (*i.e.*, starting at the time point when mammary gland lesions were already established). The bars represent data obtained from 11-18 mice. Statistical differences between the groups were determined by one-way ANOVA followed by Tukey's post hoc test. *, $P < 0.05$. **, $P < 0.01$. Data are expressed as the mean±SEM.

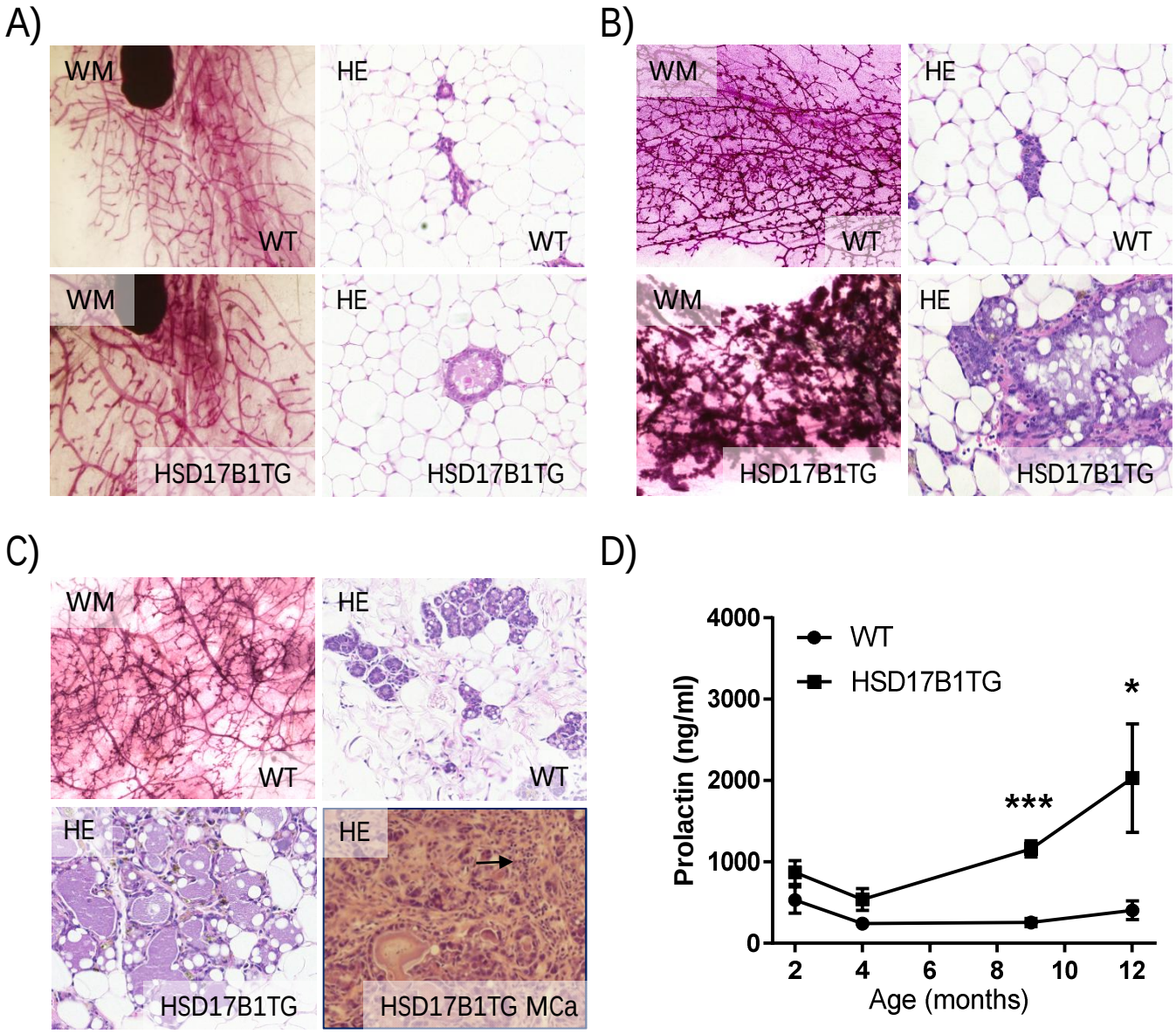
A)
WM
HE
WT
WT
WM
HE
HSD17B1TG
HSD17B1TG
B)
WM
HE
WT
WT
WM
HE
HSD17B1TG
HSD17B1TG
C)
WM
HE
WT
WT
HE
HE
HSD17B1TG
HSD17B1TG MCa
D)
Prolactin (ng/ml)
4000
3000
2000
1000
0
WT
HSD17B1TG
*
***
2
4
6
8
10
12
Age (months)

Figure 1.

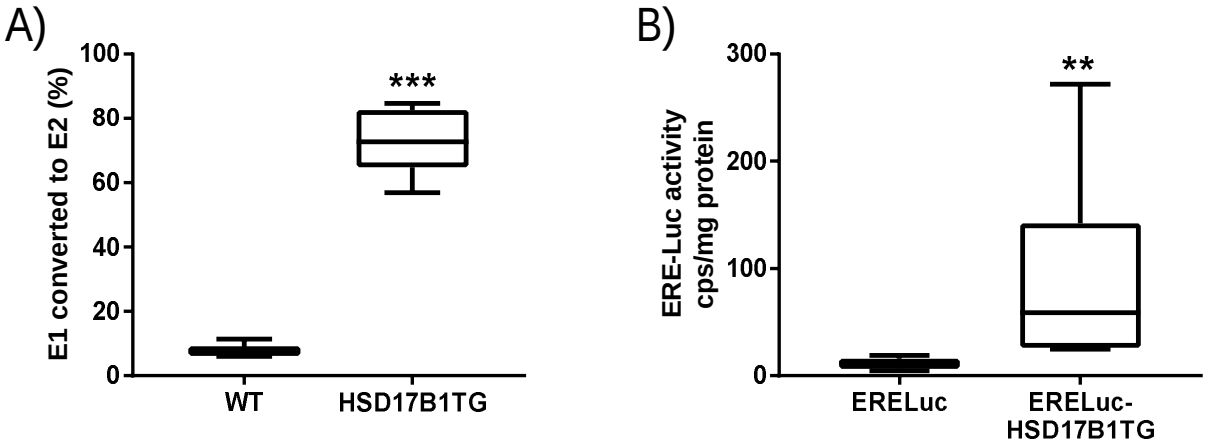
A)
E1 converted to E2 (%)
100
80
60
40
20
0
***
WT
HSD17B1TG
B)
ERE-Luc activity
cps/mg protein
300
200
100
0
**
ERELuc
ERELuc-
HSD17B1TG

Figure 2.

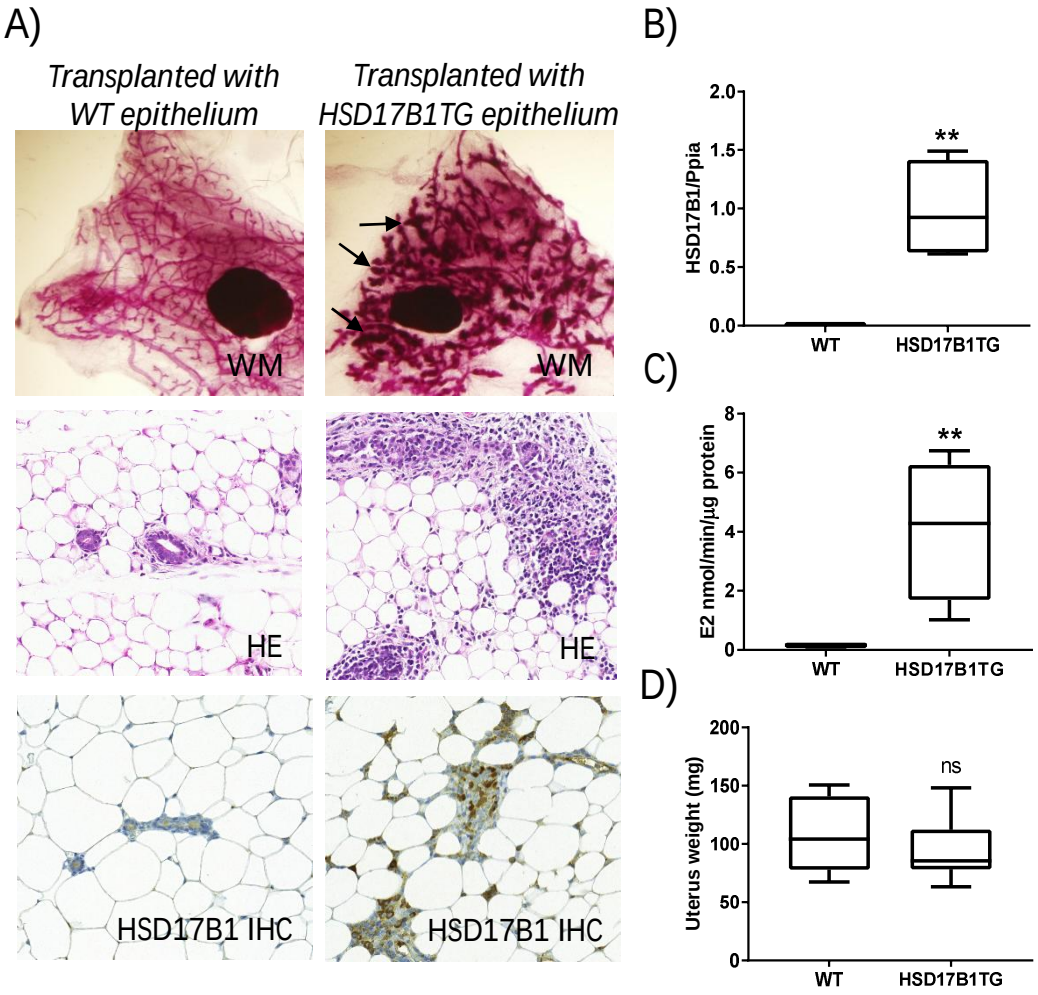
A)
Transplanted with WT epithelium
Transplanted with HSD17B1TG epithelium
WM
WM
HE
HE
HSD17B1 IHC
HSD17B1 IHC
B)
HSD17B1/Ppia
2.0
1.5
1.0
0.5
0.0
**
WT
HSD17B1TG
C)
E2 nmol/min/µg protein
8
6
4
2
0
**
WT
HSD17B1TG
D)
Uterus weight (mg)
200
150
100
50
0
ns
WT
HSD17B1TG

Figure 3.

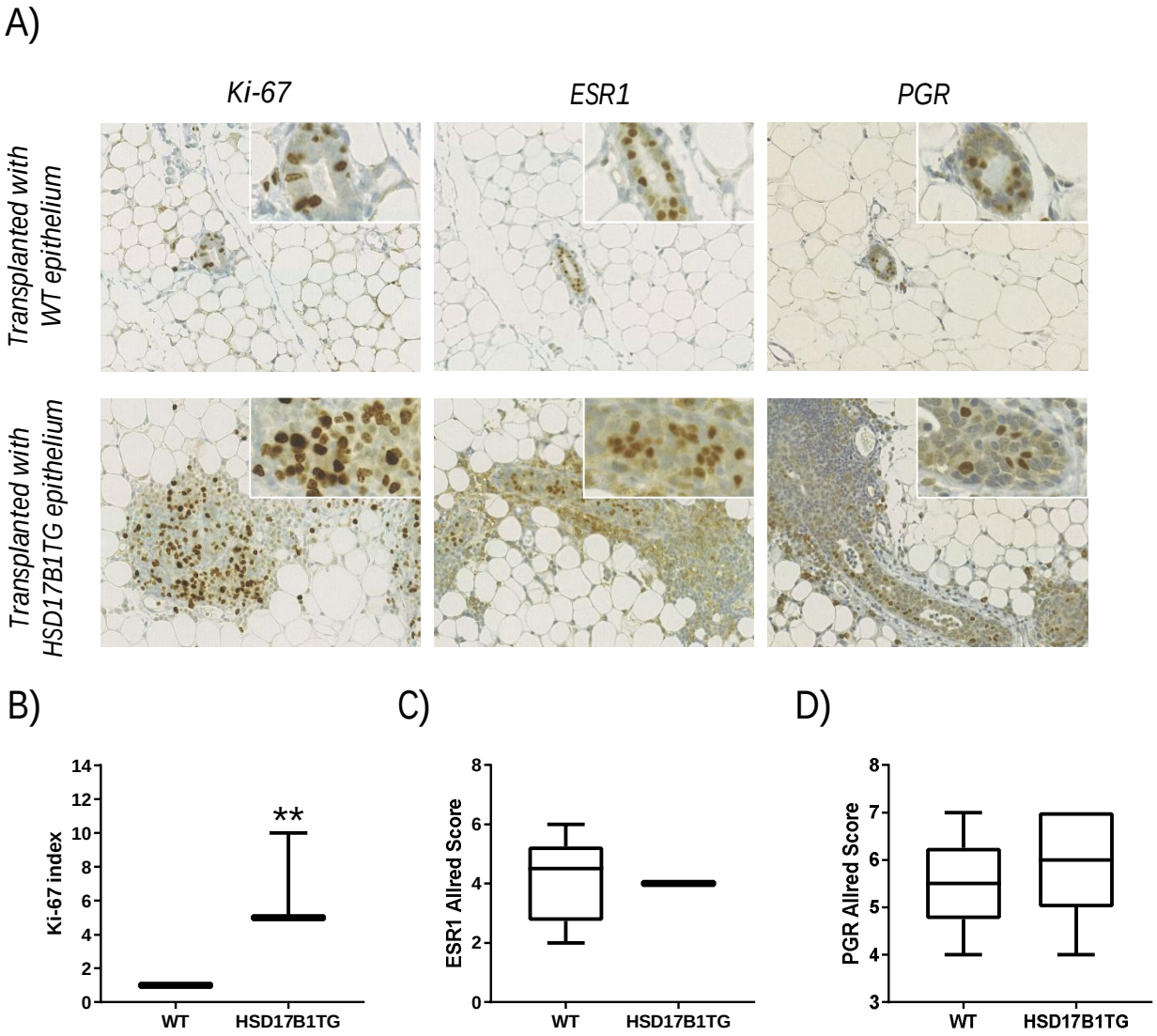
A)
Ki-67
ESR1
PGR
Transplanted with WT epithelium
Transplanted with HSD17B1TG epithelium
B)
Ki-67 index
**
WT
HSD17B1TG
C)
ESR1 Allred Score
WT
HSD17B1TG
D)
PGR Allred Score
WT
HSD17B1TG

Figure 4.

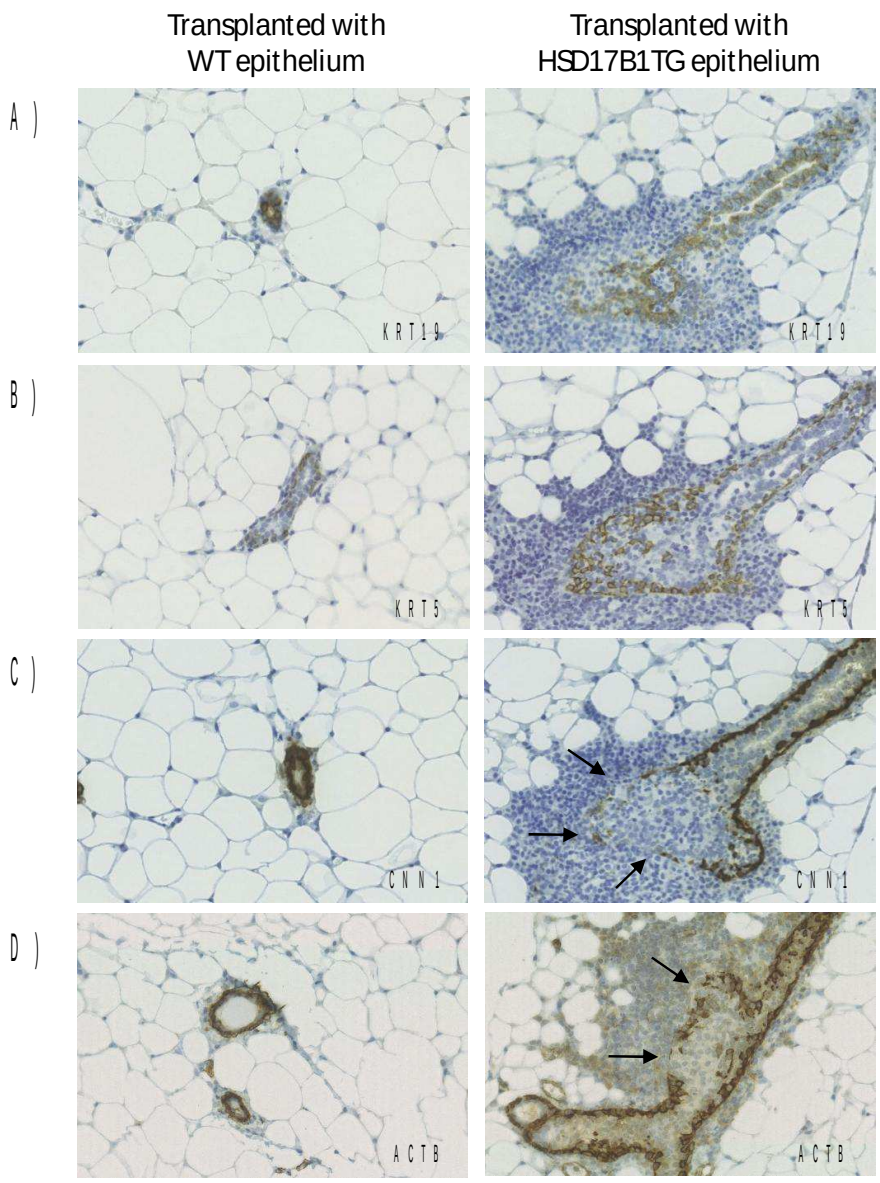
Transplanted with
WT epithelium
Transplanted with
HSD17B1TG epithelium
A)
B)
C)
D)
KRT19
KRT19
KRT5
KRT5
CNN1
CNN1
ACTB
ACTB

Figure 5.

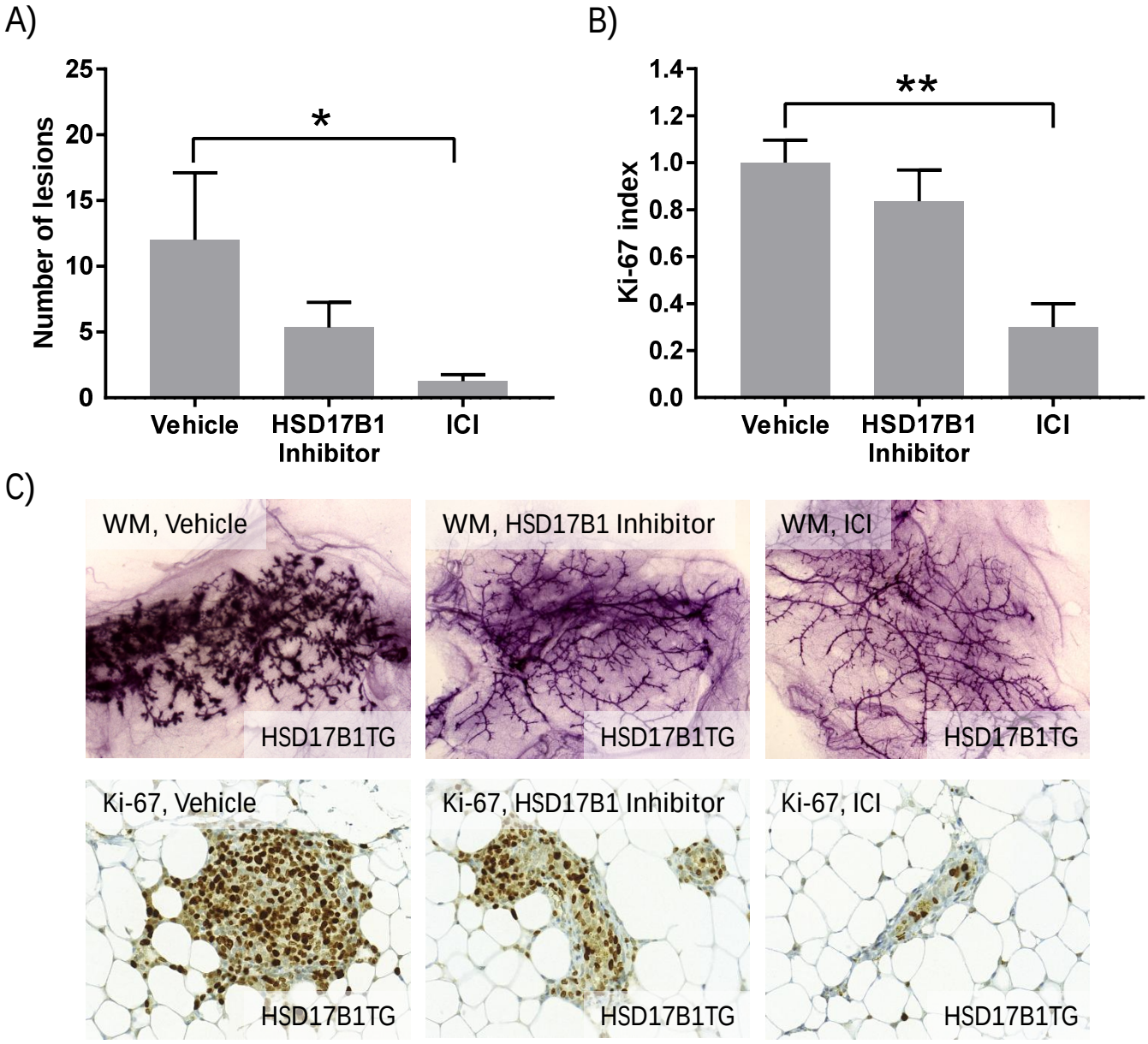
A)
Number of lesions
25
20
15
10
5
0
*
Vehicle
HSD17B1 Inhibitor
ICI
B)
Ki-67 index
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Vehicle
HSD17B1 Inhibitor
ICI
C)
WM, Vehicle
HSD17B1TG
WM, HSD17B1 Inhibitor
HSD17B1TG
WM, ICI
HSD17B1TG
Ki-67, Vehicle
HSD17B1TG
Ki-67, HSD17B1 Inhibitor
HSD17B1TG
Ki-67, ICI
HSD17B1TG

Figure 6.