Pharmacological activation of estrogen receptors-α and -β differentially modulates keratinocyte differentiation with functional impact on wound healing

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Abstract. Estrogen deprivation is considered responsible for many age-related processes, including poor wound healing. Guided by previous observations that estradiol accelerates re-epithelialization through estrogen receptor (ER)-β, in the present study, we examined whether selective ER agonists [4,4',4''-(4-propyl [1H] pyrazole-1,3,5-triyl)-trisphenol (PPT), ER-α agonist; 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), ER-β agonist] affect the expression of basic proliferation and differentiation markers (Ki-67, keratin-10, -14 and -19, galectin-1 and Sox-2) of keratinocytes using HaCaT cells. In parallel, ovariectomized rats were treated daily with an ER modulator, and wound tissue was removed 21 days after wounding and routinely processed for basic histological analysis. Our results revealed that the HaCaT keratinocytes expressed both ER-α and -β, and thus are well-suited for studying the effects of ER agonists on epidermal regeneration. The activation of ER-α produced a protein expression pattern similar to that observed in the control culture, with a moderate expression of Ki-67 being observed. However, the activation of ER-β led to an increase in cell proliferation and keratin-19 expression, as well as a decrease in galectin-1 expression. Fittingly, in rat wounds treated with the ER-β agonist (DPN), epidermal regeneration was accelerated. In the present study, we provide information on the mechanisms through which estrogens affect the expression patterns of selected markers, thus modulating keratinocyte proliferation and differentiation; in addition, we demonstrate that the pharmacological activation of ER-α and -β has a direct impact on wound healing.

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Introduction

It is well known that the efficiency of the wound healing process is reduced with aging, and the skin becomes more fragile and susceptible to trauma (1). Estrogen deprivation in post-menopausal women is considered responsible for a number of issues associated with aging, including poor wound healing (2-4). Notably, in females, estrogen replacement therapy (ERT) has been proven to reverse delayed wound healing which is related to aging, and this effect is mediated by at least two basic mechanisms: i) the downregulation of the expression of macrophage migration inhibitory factor (5), a key player in skin biology and wound healing (6); and ii) the increase of transforming growth factor-β1 (TGF-β1) production by dermal fibroblasts (7).

Cells with low-level differentiation potential have the ability to stimulate tissue renewal (8), which is of great significance in tumor growth and/or tissue repair (9,10). Keratinocytes proliferate and migrate over the wound to create a barrier between the outer and inner environments (11), through re-epithelialization. The level of keratinocyte differentiation can change during the process of epithelialization, determined by assessing the presence of distinct keratins (12). For example, the expression of keratin-10 is restricted to differentiated keratinocytes located in the suprabasal epidermal layer (13,14). By contrast, keratin-14 positivity is considered a marker of proliferating, non-terminally differentiated keratinocytes located in the basal layer of the epidermis (13,15). In addition, the expression of keratin-19 is confined to cells of hair follicles (16), a characteristic which exemplifies the stem cell-like character of keratinocytes (16,17).

As regards routes of biological information transfer, increasing attention has been paid to glycans attached to proteins and lipids. Notably, sugar-encoded information of glyco-conjugates is translated into cellular responses by endogenous lectins (18-20). Members of the family of adhesion/growth-regulatory galectins are known to be involved in these responses, and their expression is stringently controlled, e.g., during differentiation (21-23). Since galectins play an important regulatory role in cell proliferation, migration and extracellular matrix formation (24-26) and are expressed in tumors (cell lines and clinical specimens) as detected by hemagglutination and purification by affinity chromatography (27,28), it has been postulated that they are biorelevant modulators of wound/tumor microenvironments (29). For example, the extracellular matrix (ECM), which is rich in fibronectin and galectin-1, serves as an active substratum when feeder cells are substituted for keratinocytes (26). Galectin-1, a multifunctional effector in various compartments (30,31), is upregulated during the early phases of healing (25,32), and is known to have anti-inflammatory properties (33).

As keratinocytes are known to express estrogen receptors (ERs) (34), we can posit that the regeneration of the epidermis may be modulated through this route. In this context, it has been previously demonstrated that the administration of exogenous estrogen to ovariectomized ER-β knockout mice delays wound healing and that the beneficial effects of ERT are mediated through epidermal ER-β (35,36). However, little is known about the underlying mechanisms of estrogen regeneration, in particular the cell type-specific role of the two nuclear ERs, ER-α and ER-β. Therefore, in the present study, we aimed to assess the effects of two ER agonists on the expression of certain protein markers (Ki-67, keratins-10, -14 and -19, and galectin-1) in HaCaT keratinocytes in an attempt to better understand the mechanisms of the ER-β-mediated acceleration of re-epithelialization, which has been previously identified (36). In addition, the *in vivo* effects of the selective ER agonists were investigated using an open wound healing model with ovariectomized Sprague-Dawley rats.

Materials and methods

Drug preparation. 4,4',4''-(4-propyl [1H] pyrazole-1,3,5-triyl)-trisphenol (PPT), a selective ER-α agonist and 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), a selective ER-β agonist, were purchased from Tocris Bioscience (Bristol, UK) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

Human keratinocyte cell line, HaCaT. Cells of the HaCaT line (37) were obtained from CLS Cell Lines Service (Eppelheim, Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (streptomycin and penicillin) (all from Biochrom, Berlin, Germany). The cells were seeded on coverslips at a density of 5,000 cells/cm^2 and cultured for 24 h. The ER agonists, PPT and DPN, were added to the medium to reach a final concentration of 10 nM, as previously described (38), and the cells were then cultured for 4 days.

HaCaT immunocytochemical analysis. The HaCaT cells were washed with phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde (pH 7.2). Non-specific binding of the secondary antibody was blocked by pre-incubation with normal swine serum (Dako, Glostrup, Denmark) diluted in PBS for 30 min. Details of the commercial antibodies used in the present study are presented in Table I; the anti-galectin-1 antibody was made in our laboratory, and we tested it to ensure that it was free of cross-reactivity against human galectins-2, -3, -4, -7, -8 and -9 by western blot analysis and enzyme-linked immunosorbent assays (ELISAs), as previously described (39). We controlled antigen-dependent specificity was by replacing the first-step antibody with an antibody of the same isotype directed against an antigen not present in the cells, or omitting the incubation stage with the antibody. The nuclei of the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), which specifically recognizes DNA.

RNA isolation, cDNA preparation by reverse transcription and ER-specific mRNA amplification by real-time (quantitative) PCR. Total RNA was extracted from the HaCaT cells using TRIzol reagent (Life Technologies, Woburn, MA, USA), according to the manufacturer's instructions. RT-PCR was carried out according to the instructions provided by Qiagen with the One-Step RT-PCR kit (Qiagen, Hilden, Germany). Briefly, for each sample, 150 ng of total RNA was added to a solution with RT-PCR buffer, deoxynucleotide triphosphate (dNTP) mix (10 mM of each dNTP), primers (10 μM each) and enzyme mix. The following primers were used: for ER-α detection forward, 5'-GGA GGG CAG GGG TGA A-3' and reverse, 5'-GGC CAG GCT GTT CTT CTT CTT AG-3'; for ER-β detection forward, 5'-AGA GTC CCT GGT GTG AAG CAA-3' and reverse, 5'-GAC AGC GCA GAA GTG AGC ATC-3'; and for β-actin detection forward, 5'-ACC AAC TGG GAC GAC ATG GAG AA-3' and reverse, 5'-GTA GCC GCG CTC GGT GAG GAT CT-3'. SYBR-Green Supermix (Bio-Rad iQ™; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used with the following thermal cycling steps: 30 min at 50˚C for reverse transcription, and 5 min at 95˚C for initial PCR activation using the LightCycler Carousel-Based system (Roche, Basel, Switzerland). PCR cycles were run as follows: 60 cycles of 15 sec at 95˚C, 30 sec at 55˚C, and 15 sec at 72˚C (cooling to 37˚C).

Table I. Commercial reagents used in immunocytochemical analysis.

Primary antibody	Abbreviation	Host	Produced by	Secondary antibody	Produced by	Channel
Ki-67	Ki-67	Mouse monoclonal	DakoCytomation	Goat anti-mouse	Sigma-Aldrich	TRITC-red
High-molecular-weight keratin	HMWK	Mouse monoclonal	DakoCytomation	Goat anti-mouse	Sigma-Aldrich	TRITC-red
Keratin-10	K10	Mouse monoclonal	DakoCytomation	Goat anti-mouse	Sigma-Aldrich	TRITC-red
Keratin-14	K14	Mouse monoclonal	Sigma-Aldrich	Goat anti-mouse	Sigma-Aldrich	TRITC-red
Keratin-19	K19	Mouse monoclonal	Dakopatts	Goat anti-mouse	Sigma-Aldrich	TRITC-red
Wide-spectrum keratin	WSK	Rabbit polyclonal	Abcam	Swine anti-rabbit	Santa Cruz Biotechnology	FITC-green
Sox-2	SOX-2	Rabbit polyclonal	Abcam	Swine anti-rabbit	Santa Cruz Biotechnology	FITC-green
Estrogen receptor-α	ER-α	Rabbit polyclonal	Sigma-Aldrich	Swine anti-rabbit	Santa Cruz Biotechnology	FITC-green
Estrogen receptor-β	ER-β	Rabbit polyclonal	Sigma-Aldrich	Swine anti-rabbit	Santa Cruz Biotechnology	FITC-green

Sigma-Aldrich (St. Louis, MO, USA); Santa Cruz Biotechnology (Santa Cruz, CA, USA); Abcam (Cambridge, UK); DakoCytomation (Glostrup, Denmark); Dakopatts (Glostrup, Denmark).

Figure 1. Location and dimensions of the excisional wound on the back of each rat.

Acquisition of microphotographs and processing of data. Microphotographs of processed samples labeled with fluorochromes were recorded with identical settings using an Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan) equipped with filterblocks for fluorescein isothiocyanate (FITC), tetramethylrhodamine (TRITC) and DAPI, and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany). Data were processed and analyzed with a LUCIA 5.1 computer-assisted image analysis system (Laboratory Imaging, Prague, Czech Republic).

Animal model. The experimental conditions complied with European rules on animal treatment and welfare. Our study was approved by the Ethics Committee of the Faculty of Medicine of Pavol Jozef Šafárik University in Košice and by the State Veterinary and Food Administration of the Slovak Republic.

Female Sprague-Dawley rats (n=20) at 4 months of age, were used in the present study. The rats were randomly divided into 4 groups with 5 animals in each group: i) the control group: sham-operated rats, treated with the vehicle (NOV-C group); ii) ovariectomized rats treated with the vehicle (OVX-C group); iii) ovariectomized rats treated with the selective ER-α agonist, PPT (OVX-PPT group); and iv) ovariectomized rats treated with the selective ER-β agonist, DPN (OVX-DPN group).

All surgical interventions were performed under general anesthetic induced by the administration of 33 mg/kg of ketamine (Narkamon a.u.v.; Spofa a.s., Prague, Czech Republic), 11 mg/kg xylazine (Rometar a.u.v.; Spofa a.s.) and 5 mg/kg tramadol (Tramadol-K; Krka, Novo Mesto, Slovenia).

Twelve weeks prior to beginning the wound-healing experiment, as previously described (40), rats from all the OVX groups underwent ovariectomies, whereas the rats from the control group were sham-operated.

One round full-thickness skin wound, 1 cm in diameter, was inflicted under aseptic conditions on the back of each rat (Fig. 1). After wounding, rats from the OVX-PPT and OVX-DPN groups were treated daily (during the first 7 days after surgery) with 1 mg/kg of PPT and DPN subcutaneously, while the other rats received the vehicle (1% DMSO), as previously described (41,42). On day 21 post-surgery, 5 animals from each group were sacrificed by ether inhalation, and the wound tissues were removed for further processing.

Histological analysis of skin wounds and semi-quantitative analysis of histological sections. Tissue specimens were processed routinely for light microscopy [fixed in 4% buffered formaldehyde, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E)]. The stained

Table II. Definition of scale in the semi-quantitative evaluation of the histological sections.

Scale	Epithelization	PMNL	Fibroblasts	Luminized vessels
0	Thickness of cut edges	Absent	Absent	Absent
1	Migration of cells (<50%)	Mild ST	Mild ST	Mild SCT
2	Migration of cells (≥50%)	Mild DL/GT	Mild GT	Mild GT
3	Bridging the excision	Moderate DL/GT	Moderate GT	Moderate GT
4	Keratinization	Marked DL/GT	Marked GT	Marked GT

ST, surrounding tissue; DL, demarcation line; SCT, subcutaneous tissue; GT, granulation tissue. PMNL, polymorphonuclear leukocytes.

sections were evaluated in a blinded manner (without knowing which section belonged with which rat group) using an Olympus BX51 microscope equipped with a DP50 CCD camera (Olympus, Tokyo, Japan).

A semi-quantitative method, which has been previously described (43), was used to monitor the re-epithelialization of the epidermis and the presence of inflammatory cells [polymorphonuclear leukocytes (PMNLs), fibroblasts, vessels and new collagen]. The sections were evaluated in a blinded manner according on a scale of 0 to 4 (Table II).

Statistical analysis. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test was used to compare the differences in the number (percentages) of Ki-67-, keratin-10-, keratin-14-, keratin-19- and galectin-1-positive cells (data are presented as the means ± standard deviation). Data from the semi-quantitative analysis are presented as median and were compared using the Kruskal-Wallis non-parametric test. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

ER detection in HaCaT cells. We noted that the HaCaT keratinocytes expressed both ER-α and -β, mainly in the cell nuclei (Fig. 2a). ERs were detected by RT-qPCR, and the gene transcription of the ER-β receptor was slightly higher when compared to that of the ER-α receptor (Fig. 3). Cytochemically, in comparison to the control and PPT-treated cells, treatment with the ER-β agonist (DPN) increased the percentage of Ki-67-positive cells (Fig. 2b, panels A1-A3). It should be noted that targeting ER-β abolished galectin-1 expression, whereas the control and ER-α agonist-treated cells were positive for galectin-1 (Fig. 2b, panesl E1-E3).

The majority (81±13%) of the HaCaT control cells expressed keratin-14 (Fig. 2b, panels C1-C3). Only a small percentage of the control cells expressed keratin-10 (5±4%; Fig. 2b, panels D1-D3) and keratin-19 (7±5%; Fig. 2b, panels B1-B3). The cells stimulated with the ER-α agonist (PPT) had similar percentages of keratin-based phenotypes (K10, 8±3%; K14, 71±15%; K19, 3±3%; Fig. 2b, panels B2, C2 and D2) compared to the untreated controls (Fig. 2b, panels B1, C1 and D1). The cells stimulated with the ER-β agonist (DPN) had less differentiated phenotypes, with a marked positivity of keratin-19 (64±19%) and the absence of keratin-10 (0±0%) (Fig. 2b, panels B3, C3 and D3). Of note, the level of keratin-14 following treatment with DPN remained relatively consistent (K14, 89±11%). In all the groups, no effects on the expression level of Sox-2 (0±0%) were observed (Fig. 2b, panels B1-B3).

Table III. Semi-quantitative analysis of histological structures/changes 21 days post-surgery (data are presented as the median).

Group	Epithelialization	PMNLs	Fibroblasts	Luminized vessels
NOV-C	4	0	3	2
OVX-C	4	0	3	2
OVX-PPT	4	0	3	3
OVX-DPN	4	0	3	3

NOV-C, control [sham-operated rats treated with the vehicle (1% DMSO)]; OVX-C, ovariectomized rats treated with the vehicle; OVX-PPT, ovariectomized rats treated with PPT; OVX-DPN, ovariectomized rats treated with DPN; PMNLs, polymorphonuclear leukocytes.

Skin wounds. During the post-surgical period, all animals remained healthy and did not exhibit any clinical symptoms of infection. Of note, the inflammatory phase passed in all groups with no presence of PMNLs noted and only very minor occurrences of tissue macrophages at the sites of injury. The results of semi-quantitative analysis of the histological sections are summarized in Table III.

On day 21 after wounding, a thin keratin layer was present in all wounds (Fig. 4), demonstrating that a normal keratinocyte differentiation had occurred. However, differences were noted in the process of epidermal regeneration. In the rats from the OVX-C and OVX-PPT groups, we noted that hair follicle regeneration and epidermal thickening were both delayed (Fig. 4). Treatment with the ER-β agonist (Fig. 4; OVX-DPN) resulted in a normalized process of epidermal regeneration, comparable to that of the sham-operated animals (NOV-C). In comparison to both control groups (OVX-C and NOV-C), the number of luminized vessels slightly increased upon treatment with the estrogen agonists treatment (OVX-PPT and OVX-DPN). A moderate number of fibroblasts in the granulation tissues of all wounds was observed, reflecting the progression of in tissue fibrosis. Of note, no significant differences were observed

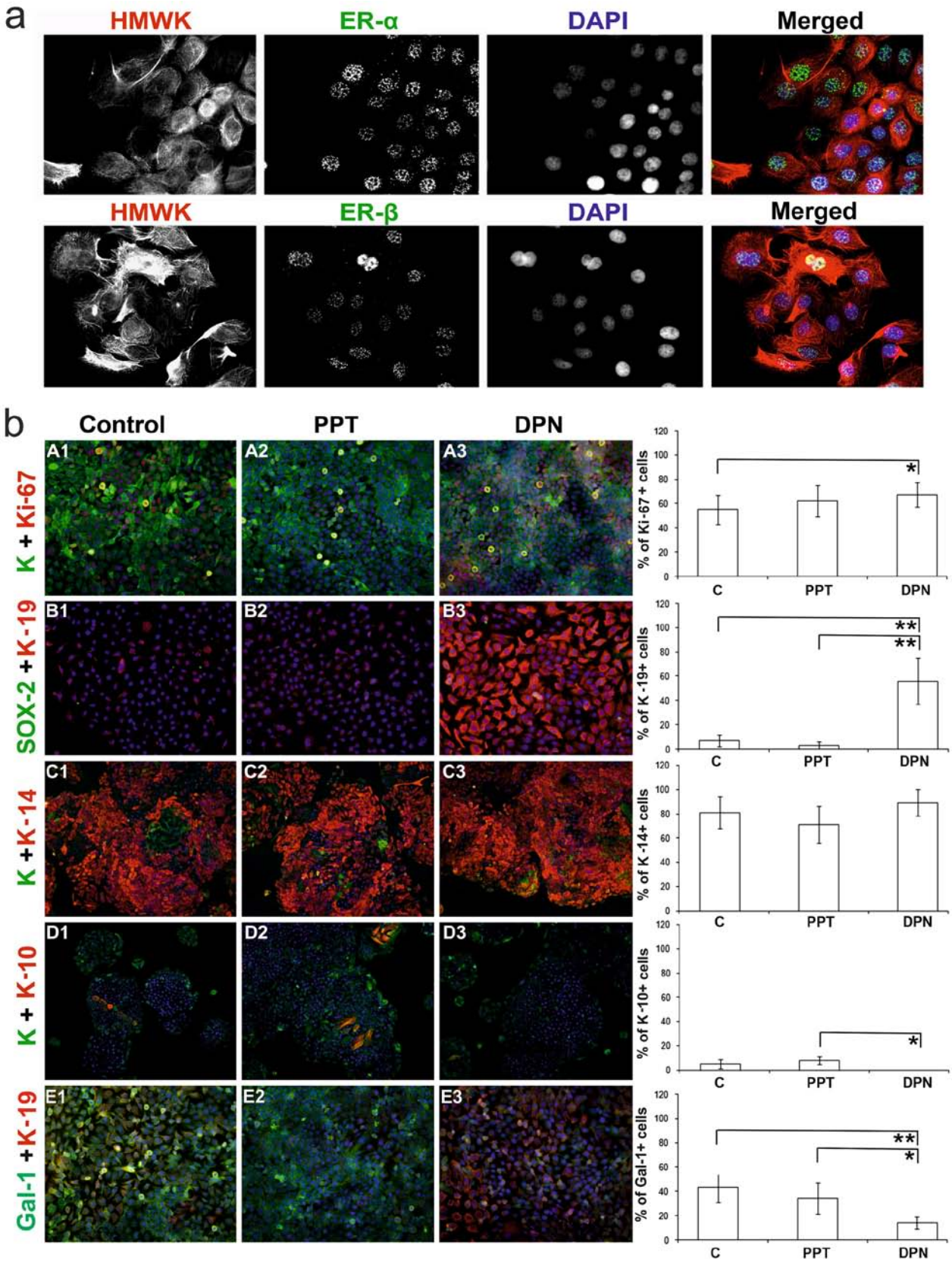

Figure 2. (a) Culture of HaCaT keratinocytes (magnification, x600), which express both estrogen receptor (ER)-α and -β mainly in the cell nuclei; cytoskeleton is stained with high-molecular-weight keratins (HMWK), cell nuclei are visualized by DAPI. (b) HaCaT keratinocytes cultured under the influence of selective ER agonists; first line of horizontal panels (A1-A3, magnification, x200): detection of the proliferation marker Ki-67 and wide-spectrum keratin; second horizontal panel (B1-B3, magnification, x200): presence of keratin-19 (marker of poorly differentiated keratinocytes with stem-like phenotype) and Sox-2 (stem cels marker); third horizontal panel (C1-C3, magnification, x100): positivity for keratin-14 (marker of poorly differentiated keratinocytes) and wide-spectrum keratin; fourth horizontal panel (D1-D3, magnification, x100): expression of keratin-10 (marker of differentiated keratinocytes) and wide-spectrum keratin; fifth horizontal panel (E1-E3, magnification, x100): presence of keratin-19 and galectin-1 (Gal-1). PPT-, 4,4',4''-(4-propyl [1H] pyrazole-1,3,5-triyl)-trisphenol, a selective ER-α agonist; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile, a selective ER-β agonist; *P<0.05 and **P<0.01. C, control; K, wide-spectrum keratin.

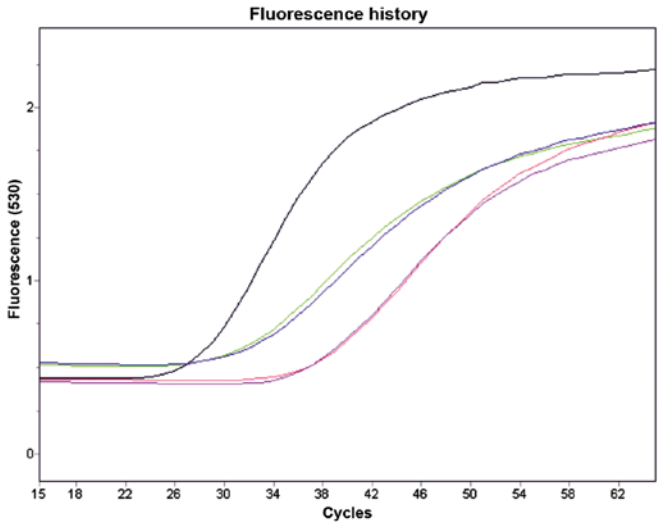

Figure 3. Estrogen receptor (ER) expression in HaCaT cells evaluated by quantitative RT-qPCR. Black line, house keeping gene; green and blue lines, ER-β; red and purple, ER-α).

between the groups in terms of the presence of luminized vessels or of fibroblasts.

Discussion

Monitoring markers for differentiation revealed that the ER-β agonist, DPN, decreases the expression of galectin-1 in HaCaT cells. The expression of this lectin is known to be sterol-sensitive (please see below), and this observation provides direction to further assessments of its impact on other members of the galectin network and also on glycosylation, making cells susceptible to galectins. These lectins can have site-specific additive or antagonistic effects, and when co-expressed they form a network, as in tumors (44-46). Therefore, their regulation may alter the clinical course of a tumor, and in this context, the phytoestrogen, genistein, and its potential chemopreventive effects on breast cancer also deserve attention (47,48). Following initial immunohistochemical detection of this class of tumors, galectin-1 has been shown to be upregulated in invasive breast carcinoma with a positive correlation with the TNM staging system (49,50). Fittingly, as previously demonstrated, the silencing of galectin-1 in a breast carcinoma model overcame breast cancer-associated immunosuppression, inhibited tumor growth and prevented metastatic disease (51). Of note, in our previous studies, we found that galectin-1 was upregulated during the early phases of wound healing (25,32). Since long-term estradiol deprivation enhances estrogen sensitivity (52), by upregulating ER-α expression, further studies focusing on this receptor are warranted in order to reduce tumor cell proliferation (53). Antagonizing ER-α, together with agonizing ER-β, may ameliorate galectin-1-induced immunosuppression in breast cancer; however, further consideration based on detailed network and glycosylation studies is necessary.

Although estrogen is considered a key regulator of wound healing, an incomplete understanding of the molecular mechanisms of action of estrogen, as well as the well-documented adverse effects of estrogens during menopause in clinical trials, preclude the common clinical use of ERT as a wound-healing treatment. One example of the negative effects of estrogens is that the activation of ER-α leads to a decrease in the tensile strength

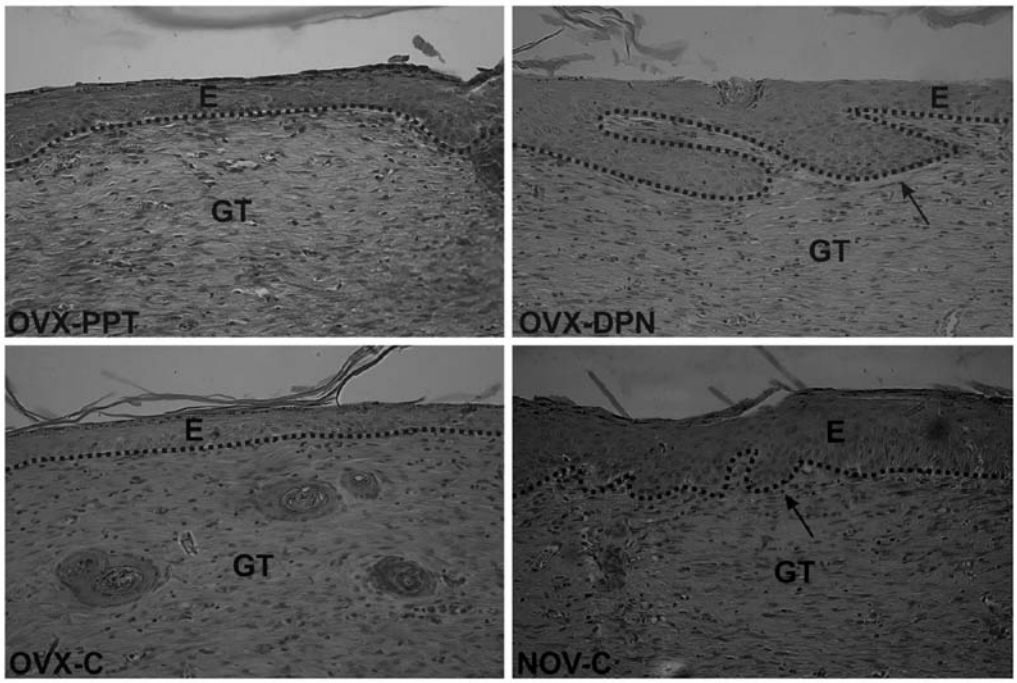

Figure 4. Healing of skin wounds 21 days post-surgery (magnification, x400). The maturation phase of healing was noted in all groups, and differences were observed in epidermal regeneration, which was impaired in the ovariectomized rats treated with the vehicle (1% DMSO; OVX-C) and ovariectomized rats treated with the ER-α agonist, PPT (OVX-PPT. Black arrows indicate growing hair follicles in sham-operated vehicle-treated rats (NOV-C) and ovariectomized rats treated with the ER-β agonist, DPN (OVX-DPN). The dotted line distinguishes the epidermis from the granulation tissue; staining was done with H&E. E, epidermis; GT, granulation tissue; PPT-, 4,4',4"-(4-propyl [1H] pyrazole-1,3,5-triyl)-trisphenol, a selective ER-α agonist; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile, a selective ER-β agonist.

of wounds during the proliferation phase of healing (41,54), whereas the activation of ER-α and/or -β significantly increases this parameter during the early maturation phase (54). The ER-α agonist, TGF-β1-dependently increases fibroblast migration and keratinocyte proliferation. By contrast, the ER-β agonist does not affect cell migration (55,56) and increases keratinocyte proliferation in a TGF-β1-independent manner (57). Accordingly, *in vivo* experiments have shown that targeting ER-β, but not ER-α leads to accelerated re-epithelialization in mice (36) and rats (54). Furthermore, ER-α has been proven to be responsible for impaired wound healing in male mice (58). In the present study, we demonstrated that the pharmacological activation of ER-β, but not that of ER-α, led to a significant alteration in the pattern of differentiation and the proliferation activity of keratinocytes. In relation to markers, previous research has demonstrated that the ER-β agonist does not induce Sox-2 expression, a characteristic of stem-like properties (59), in keratin-19-positive cells.

In conclusion, our data suggest that marker-based cytochemical monitoring provides new information on ER-modulated keratinocyte differentiation and proliferation. The stimulation of epidermal regeneration may ensue after treating wounds with an ER-β agonist. In order to activate the TGF-β1 pathway to this end, ER-α should be targeted (57). However, the nature of the animal model and restrictions on extrapolations must be taken into consideration, as we did in our study, by combining a human *in vitro* model with *in vivo* data on rats.

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