Differential involvement of estrogen receptor α and estrogen receptor β in the healing promoting effect of estrogen in human keratinocytes

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Abstract

Estrogen affects proliferation and migration of different skin components, thus influencing wound healing processes. The human keratinocyte cell line NCTC 2544 has been used to examine the effects of estrogen, dissect its mechanism of action and characterize receptor subtypes involved. Western blot and immunocytochemical analyses confirmed the expression of estrogen receptors (ERs) α and β , with prevalence in the nuclear and extranuclear compartment, for ER α and ER β respectively. Treatment with 10 nM 17 β -estradiol (17 β -E₂) and the ER α and ER β selective agonists, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT; 100 nM), and diarylpropionitrile (DPN; 1 nM) produced a slight but significant increase in cell proliferation, as by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and bromodeoxyuridine incorporation assays, only after a long-term treatment (96 h). Analysis of cell migration by a scratch wound assay showed that 17 β -E₂ (10 nM) accelerated

migration between 5 and 24 h after scratching, an effect confirmed by the transwell migration assay. PPT and DPN elicited similar effects. Pre-treatment with the mitogen-activated protein kinase inhibitor, U0126 (1 μ M), abolished the ability of 17 β -E₂ and DPN, but not of PPT, to accelerate wound closure. TGF- β 1 (10 ng/ml) produced a similar positive effect on wound closure and the TGF- β 1 receptor antagonist, SB431542 (10 μ M), reduced the ability of 17 β -E₂ and PPT to accelerate cell migration, but did not modify DPN effect. It is suggested that estrogen positively affects *in vitro* wound healing by stimulating cell proliferation after long-term exposure but mainly by accelerating cell migration within a few hours from treatment. Selective activation of ER β may result in favorable stimulation of wound healing without any increase of transforming growth factor- β 1 production.

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Introduction

It is largely known that the regulatory roles of estrogen extend well beyond the reproductive system. In all tissues and cell types, estrogen exerts its actions by interacting with two specific receptor subtypes, α and β (ERs). These are transcription factors, which dimerize following ligand binding, recruit a number of different co-regulators, and finally bind to target sequences on gene promoters. However, extremely rapid responses triggered by estrogen, not ascribable to the ‘classical’ genomic action, have also been described. They are likely linked to membrane events, coupled to intracellular second messengers that in turn activate kinase signaling cascades. Among these are the mitogen-activated protein kinase (MAPK), the phosphatidylinositol 3-kinase, the protein kinase A, and protein kinase C pathways (Cheskis *et al.* 2007). The actual identity of putative membrane receptors has not been determined. While evidence points to a membrane localization of ER α and/or ER β , several studies on different cell types have suggested the existence of a specific membrane receptor (Verdier-Sevrain

et al. 2006). Rapid signaling has been shown to impact genomic activity, resulting in the integration of membrane and nuclear actions of estrogen, thus granting plasticity to the cellular response to the hormone (Levin 2005).

Skin represents one of estrogen’s targets outside the reproductive system. Different skin cell types are directly affected by the hormone in both physiological and pathological conditions (Thornton 2005). Particularly relevant is the ability of the hormonal environment to affect wound healing that is a complex, multi-stage process involving inflammatory response, angiogenesis, and extracellular membrane deposition and remodeling. *In vivo* studies have shown that healing is worsened in ovariectomized rats, but improves following estrogen administration (Ashcroft *et al.* 1997, 1999). Accordingly, hormone replacement therapy improved post-wounding epithelialization in post-menopausal women, restoring a healing rate similar to that of premenopausal women (Ashcroft *et al.* 1997). In addition, topical and systemic estrogen treatments ameliorated acute wound healing in the elderly of both sexes (Ashcroft *et al.* 1997, 1999).

Estrogen impacts different stages of the healing process; 17 β -estradiol (17 β -E₂) has been shown to have a proliferative effect on epidermal keratinocytes in aged human skin *in vivo* (Son *et al.* 2005) and on primary human keratinocytes *in vitro*, where it induces cyclin D2 and increases cell distribution in the S phase of cell cycle (Kanda & Watanabe 2004a). 17 β -E₂ has also been demonstrated to enhance the production of granulocyte-macrophage colony-stimulating factor, which is involved in improving wound repair ability (Kanda & Watanabe 2004b).

Although the positive effects of estrogens on skin wound healing have been widely demonstrated both *in vivo* and *in vitro*, very little is known about the mechanisms underlying such action and the relative contribution of ER α and ER β . In the present study, we used the human keratinocyte cell line NCTC 2544 as an *in vitro* model to address these issues. We show that ER α and ER β differentially affect keratinocyte cell migration and proliferation, key events during wound repair and that both membrane and genomic actions are involved in these effects.

Materials and Methods

Cell culture and reagents

NCTC 2544 human keratinocyte cells (Interlab Cell Line Collection, Genoa, Italy) were maintained at 5% CO₂ and 37 °C in phenol red-free DMEM supplemented with 10% FCS and penicillin/streptomycin (all from Invitrogen). All experiments were carried out in phenol red-free and serum-free DMEM.

17 β -E₂ (Sigma–Aldrich Co), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole, and diarylpropionitrile (PPT and DPN; Tocris Cookson Ltd, North Point, UK) were dissolved in ethanol; BSA-conjugated 17 β -E₂ (Sigma) was dissolved in 50% ethanol; U0126 and SB431542 (Tocris Cookson) were dissolved in dimethyl sulfoxide (Sigma). Stock solutions were usually at a concentration of 10 mM. Subsequent dilutions for treatments were made in aqueous solution so that all controls received an amount of vehicle that was always less than 0.01%.

Transforming growth factor- β 1 (TGF- β 1; Peprotech, Rocky Hill, NJ, USA) was dissolved in an acidic 0.1% BSA sterile solution.

Western blot analysis

NCTC 2544 cells were harvested in 10 mM Tris lysis buffer containing 5 mM EDTA, 1% Triton X-100, and a protease- and phosphatase-inhibitor cocktail mix (Sigma). Full lysis was obtained by sonication of the samples. Fractionation of nuclear and membrane/cytosolic protein extracts was carried out as described by Dai *et al.* (2007). Fifty micrograms of protein extract were separated by SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL, Amersham

Biosciences Europe GmbH) using a Transblot semidry transfer cell. Membranes were then blocked in 3% milk in PBS containing 0.1% Tween-20, and processed for immunodetection with the following antibodies: rabbit anti-ER α (1:200), rabbit anti-ER β (1:500), rabbit anti-TGF- β 1 (1:400; all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA), rabbit anti-ERK and anti-phospho-ERK (1:1000, Cell Signaling Technology, Danvers, MA, USA), mouse anti-actin (1:1000, Sigma), and HRP-conjugated secondary antibodies (all from Santa Cruz). Detection of specific bands was carried out using the Immobilon detection system (Millipore, Billerica, MA, USA).

Immunocytochemistry

NCTC 2544 cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 when necessary, and saturated with 3% BSA. Cells were then incubated with mouse anti-ER α (1:50) or rabbit anti ER β (1:200) primary antibodies (both from Santa Cruz). For diaminobenzidine-based detection, cells were incubated for 2 h with biotinylated anti-mouse or anti-rabbit secondary antibodies followed by the ABC detection system (all from Vector Laboratories, Burlingame, CA, USA). For double-labeling experiments, FITC-conjugated anti-mouse (1:100, cat. n. AP192F, Chemicon, Temecula, CA, USA) and Texas Red-conjugated anti-rabbit (1:200, Santa Cruz) secondary antibodies were used.

Analysis of the prediploid cell population

Cells were collected and fixed in ice-cold 70% ethanol, treated for 1 h at 37 °C with 100 μ g/ml ribonuclease and incubated with 50 μ g/ml propidium iodide (both from Sigma) just prior to analysis of DNA content and ploidy by flow cytometry, carried out with a Coulter-Elite Flow Cytometer.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay

NCTC 2544 cell proliferation was evaluated by a colorimetric assay based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into blue formazan (absorbance = 560 nm) by mitochondrial activity of viable cells. After incubation with the MTT substrate (Sigma) for 2 h at 37 °C, cells were disrupted for 30 min at 37 °C with an isopropanol/acetic acid solution containing 1% SDS, and absorbance was measured in a plate reader.

Bromodeoxyuridine incorporation assay

In parallel, NCTC 2544 cell proliferation was analyzed by evaluation of bromodeoxyuridine (BrdU) incorporation. Three micromolar BrdU (Sigma) was supplemented to the cells in the last 24 h of a 96-h treatment. Cells were fixed with 95% EtOH, subjected to acid treatment (2 N HCl) for DNA

denaturation, followed by neutralization with 0.1 M Tris–borate and finally processed for immunostaining with mouse anti-BrdU (MAB3510, 1:2000, Chemicon) for 2 h and biotinylated anti-mouse (1:200, Vector) for 1 h. Cells were stained with the ABC detection system (Vector) using diaminobenzidine (Sigma) as a substrate. Immunopositive cells were counted in three randomly selected fields/wells from at least six wells per experiment.

Scratch wound migration assay

Subconfluent monolayers of NCTC 2544 cells were scratched with a sterile P200 pipette tip. After removal of the resulting debris by repeated washes, cells were subjected to treatment and scratch wound closure was monitored by phase microscopy capturing images of the same field with a 10× objective at 0, 5, 10, and 24 h. The cell free area was determined with the aid of the image processing software 'Image J' developed by NIH and in the public domain.

Transwell migration assay

NCTC 2544 cells were plated in the upper chamber of 8 µm pore size transwell insert (Corning, Lowell, MA, USA). Cells were immediately exposed to treatment and allowed to migrate for 24 h toward chemoattracting 10% FCS-supplemented medium present in the lower chamber. Non-migrating cells in the upper chamber were removed with the aid of a cotton swab, while migrated cells adhering to the lower surface of the membrane were stained with eosin and hematoxylin for cell counting (five randomly selected fields/transwell from at least three transwells per experiment).

Statistical analysis

All experiments were repeated three to six times and were analyzed by one-way ANOVA followed by Newman–Keuls test for significance, as indicated in figure legends. Where appropriate, a two-way ANOVA followed by a Bonferroni test for significance was applied. $P < 0.05$ was taken as the criterion for statistical significance.

Results

The expression of estrogen receptor subtypes α and β in NCTC 2544 human keratinocytes was analyzed by immunocytochemical analysis. When cells were permeabilized with Triton X-100 (0.1%), allowing antibodies to reach both the cytoplasm and the cell nucleus, the two receptors appeared to be located in different compartments. ER α showed very strong nuclear labeling, while ER β was expressed at high levels in the extranuclear region, yet showing a remarkable staining also inside the nucleus (Fig. 1a). Double immunocytochemistry with both anti-ER α and anti-ER β antibodies confirmed such complementary expression. Most cells showed in fact intense nuclear ER α staining (Fig. 1b) and a more diffuse ER β immunopositivity involving both nuclear and membrane compartments (Fig. 1b). Omission of the permeabilization step, a previously reported method for analysis of expression of cell surface antigens (Verdier-Sevrain *et al.* 2004), supported the nuclear localization of ER α , which was not anymore detectable, and the extranuclear expression of ER β , whose labeling was still evident (Fig. 1a). To further confirm these results, western blot analysis of nuclear or

Figure 1 (a) Expression of ER α and ER β in human NCTC 2544 keratinocytes. Immunocytochemical analysis of ER α and ER β in the presence (a; left panels) or absence (a, right panels) of the permeabilization agent Triton X-100 (0.1%). Arrow and arrowhead indicate nuclear and extranuclear expression of ER β respectively. On the right panels (b), double immunocytochemical analysis of ER α (green staining), ER β (red staining) and co-localization of the two receptors in the nuclei (yellow staining after merge). (c) Western blot analysis of ERs expression in cytoplasm/membrane (C/M) and nuclear (N) protein fraction. Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-08-0050>.

cytosolic/membrane (C/M) fractions was carried out. Results showed that ER α was strongly enriched in nuclear fractions, while only a faint band was detectable in the C/M compartment. By contrast, ER β yielded a fairly intense band in both fractions (Fig. 1c).

17 β -E₂ effects on NCTC 2544 cell proliferation were examined. Cells were plated at a low density and treated with 10 nM 17 β -E₂ in phenol red-free and serum-free medium for 24, 48, or 96 h, and proliferation assessed by the MTT assay. At earlier time points, there was no significant effect on cell proliferation (not shown), but after 96 h of exposure to the hormone a small but significant induction of cell proliferation became evident (Fig. 2a). This effect was clearly mediated by ERs as shown by its prevention in the presence of 1 μ M of the ER antagonist ICI 182 780 (137 ± 4.3 and $112 \pm 6.2\%$ of control in 17 β E₂- and 17 β E₂±ICI 182 780-treated conditions respectively). Interestingly, treatment with 100 nM PPT and 1 nM DPN, selective agonists of ER α and ER β respectively, fully mimicked the effect of 17 β -E₂ (Fig. 2a). Pre-treatment with 1 μ M of the MAPK pathway antagonist U0126 for 30 min, prevented the stimulatory effect on keratinocyte proliferation induced by exposure for 96 h to

17 β -E₂ or the selective ER β agonist, DPN for 96 h, but had only a partial, not significant effect, against 100 nM PPT (Fig. 2b). To rule out the possibility that the observed effects were due to an increased viability of cells during treatment rather than increased proliferation, BrdU uptake assay was performed. Cells were treated with 10 nM 17 β -E₂, 100 nM PPT, and 1 nM DPN for 96 h, and exposed to 3 μ M of the nucleotide analog BrdU during the last 24 h of treatment. Immunocytochemical analysis followed by cell count showed that 17 β -E₂, PPT, and DPN, all significantly increased the number of cells that had incorporated BrdU compared with control (Fig. 2c). No significant effect was observed after shorter time incubation with the ER agonists (data not shown). In addition, cytofluorometric analysis of the prediploid, apoptotic population by propidium iodide staining was carried out, showing that the percentage of cell death was negligible (data not shown).

The ability of 17 β -E₂ and of the ER α and ER β agonists, PPT and DPN, to modify the migration rate of NCTC 2544 cells was tested by the scratch wound closure assay. After scratching a subconfluent monolayer of cells with a P200 pipette tip and completely removing resulting debris by

Figure 2 Effect of 17 β -E₂ and selective ER agonists on NCTC 2544 cell proliferation. (a) Cells were treated with 17 β -E₂ (E; 10 nM), the selective ER α agonist PPT (100 nM) or the selective ER β agonist DPN (1 nM) for 96-h prior to incubation with tetrazolium salts and evaluation of cell proliferation by the MTT reduction assay. In b, cells were pre-treated with 1 μ M U0126 for 30 min prior to addition of 17 β -E₂, PPT or DPN for 96 h and proliferation analysis by the MTT assay. In data reported in c, 3 μ M BrdU was added during the last 24 h of a 96-h incubation. Cells were then processed for immunocytochemical analysis and counted. Data are mean $\pm$ s.e.m. of five to six independent experiments each run in quadruplicates. * P < 0.05 versus control and ** P < 0.05 vs 17 β -E₂ and DPN alone by one-way ANOVA followed by Newman-Keuls *post hoc* test for significance.

Figure 3 Time-course analysis of the effect of 17β -E₂ and selective ER agonists on NCTC 2544 cell migration. Subconfluent cell monolayers were scratched with a P200 pipette tip and treated with 17β -E₂ (E; 10 nM), PPT (100 nM), or DPN (1 nM). Migration rates were determined by measuring the free area outlined by the edge of the migratory front at 0, 5, 10, and 24 h after scratching (a). Representative images from vehicle- and 17β -E₂-treated cultures at different time points are shown in a. In b, the percentage of cells migrated through the 8 μ m pore size membrane of a transwell insert is reported (* $P < 0.05$ versus control by Student's *t*-test). Bars represent mean $\pm$ S.E.M. of five to eight independent experiments each run in triplicates. * $P < 0.05$ versus control by two-way ANOVA followed by Bonferroni *post hoc* test for significance.

repeated washing, cells were treated with either 10 nM 17β -E₂, 100 nM PPT or 1 nM DPN in phenol red-free and serum-free medium. Images of the same field were captured at time 0 and after 5, 10, and 24 h, allowing monitoring of cell migration into the scratched area. Analysis at earlier time points did not allow detection of any change in migration rate in control and ER agonist-treated cells versus time 0. As shown in Fig. 3a, 17β -E₂ (E), PPT, and DPN were able to significantly and comparably accelerate cell migration towards the free area. This effect was significant already at 5 h after scratching and was maintained at all time points examined, up to 24 h (Fig. 3a). Similar results were obtained when migration was analyzed in NCTC 2544 keratinocytes plated on a laminin-coated substrate (data not shown).

In parallel, cell migration was evaluated by the transwell migration assay, which confirmed the ability of 10 nM 17β -E₂ to increase cell migration, as shown by the greater number of cells migrated toward a serum-rich medium used as chemoattractant (Fig. 3b).

Due to the early appearance of the observed effect on keratinocyte migration, the involvement of rapid, non-genomic pathways of estrogen action was hypothesized. Accordingly, 17β -E₂, PPT, and DPN all comparably increased the levels of phospho-ERK protein after a 15 min pulse, as shown by western blot analysis (Fig. 4a). However, blockade of the mitogen activated protein kinase (MAPK) pathway by pre-treatment of cells for 30 min with 1 μ M U0126, prevented the acceleration of cell migration induced

by a 24-h treatment with 17β -E₂ and DPN, but did not modify the effect induced by PPT (Fig. 4b). Consistent with the involvement of a rapid membrane-activated pathway, treatment for 24 h with 100 nM of the membrane impermeable BSA-conjugated 17β -E₂ (E-BSA), that is able to trigger only rapid, membrane-related events, mimicked 17β -E₂ effect, producing a significant acceleration of wound healing, sensitive to U0126 pre-treatment (Fig. 4c).

In an attempt to identify a mediator of the observed effects of estrogen on NCTC 2544 keratinocytes, TGF- β 1 was tested as a likely candidate. In contrast to what was expected, the stimulatory effect of 17β -E₂ on NCTC2544 cell proliferation did not involve TGF- β 1 as it was not modified by pre-treatment of cells with the TGF- β 1 antagonist SB431542 (Fig. 5a).

TGF- β 1 was instead likely involved in keratinocyte migration as treatment of a scratched monolayer of NCTC 2544 cells with 10 ng/ml rhTGF- β 1 for 24-h mimicked 17β -E₂ action by significantly increasing the rate of cell migration (Fig. 5b). More importantly, pre-treatment of the cells with 10 μ M of the TGF- β 1 receptor antagonist SB431542 for 30 min, prevented the stimulatory effect on cell migration induced by subsequent exposure to 17β -E₂ for 24 h (Fig. 5c). However, treatment with the TGF- β 1 receptor antagonist SB431542 (10 μ M for 30 min) inhibited PPT-induced wound closure, but did not modify the effect induced by DPN (Fig. 5c). Accordingly, evaluation of NCTC 2544 cellular content of TGF- β 1 protein by western blot analysis

Figure 4 Involvement of MAPK pathway in estrogen-induced NCTC 2544 cell migration. (a) The effect of a 15 min pulse with 17β-E₂ (10 nM), PPT (100 nM), or DPN (1 nM) on phospho-ERK 1/2, as by western blot analysis is shown. A representative immunoblot is shown; densitometric analyses, reported in the histogram, have been normalized over constitutive ERK 1/2 expression and represent mean ± S.E.M. of three independent experiments. In b, the effect of a pre-treatment of a scratched cell monolayer with 1 μM U0126 for 30 min, prior to addition of 17β-E₂ (E; 10 nM), PPT (100 nM), or DPN (1 nM) for 24 h. Treatment of the scratched monolayer with 100 nM membrane-impermeable BSA-conjugated 17β-E₂ (E-BSA) for 24 h is reported in c. Bars represent mean ± S.E.M. of three to five independent experiments each run in triplicates. *P < 0.05 versus respective controls by one-way ANOVA followed by Newman–Keuls *post hoc* test for significance. **P < 0.05 vs 17β-E₂ and DPN alone by one-way ANOVA followed by Newman–Keuls *post hoc* test for significance.

revealed that TGF-β1 levels were, unexpectedly, significantly increased by exposure for 24 h to 10 nM 17β-E₂ (E) and 100 nM PPT, but were not modified by 1 nM DPN, as shown by changes in a specific band at 25 kDa (Fig. 5d). A visible proof of increased TGF-β1 was provided by immunocytochemical analysis showing that cells treated for 24 h with

10 nM 17β-E₂ (E) had a much stronger TGF-β1 staining compared with vehicle-treated cultures (Fig. 5d).

Discussion

A role for estrogen in skin function, especially under conditions of cutaneous wound healing has long been analyzed (Gilliver *et al.* 2007). This may appear particularly relevant with aging when skin undergoes significant structural and functional changes, such as decrease in proliferative and migratory properties of cells, flattening of the dermo-epidermal junction and disorganization of microcirculation. As a whole, these events lead to thinning, wrinkling and impairment of the re-epithelialization ability after a lesion (Ashcroft *et al.* 2002). In addition, the reduced levels of circulating estrogens in postmenopausal women have been related to an exacerbation of the detrimental effects of aging on skin. Estrogen are known to affect wound healing by influencing different skin cellular components, including fibroblasts, vascular endothelial cells, and keratinocytes as well as infiltrating inflammatory cells (Gilliver *et al.* 2007).

Keratinocytes play a key role in wound healing and our results show that estrogen affects not only proliferation but also migration of human keratinocytes. These effects seem to occur independently, they both involve ERα and ERβ, but differential mechanisms in modifying proliferation and migration for the two receptor subtypes are suggested.

The expression of ERα and ERβ in human keratinocytes has been previously demonstrated although some controversies have emerged (Kanda & Watanabe 2003, Thornton *et al.* 2003, Verdier-Sevrain *et al.* 2004) and a role for cell surface GPR30 in mediating estrogen effects on keratinocytes has also been suggested (Kanda & Watanabe 2004a). We now confirm that ERα is expressed in human keratinocytes and that its expression predominates in the nuclear compartment, whereas ERβ is equally present in the nuclear as well as in the non-nuclear fraction. Both receptor subtypes contribute to a stimulatory action on keratinocyte proliferation, an effect that, as already shown (Urano *et al.* 1995), appears only after a long-term exposure to 17β-E₂ (Kanda & Watanabe 2004a, Verdier-Sevrain *et al.* 2004) or to the selective ERα and ERβ agonists, PPT, and DPN. This is also consistent with the recent report on the involvement of both ERα and ERβ in the positive effect of estradiol in keratinocytes following trauma-hemorrhage (Moeinpour *et al.* 2008). While the effect of 17β-E₂ and DPN was prevented by pre-treatment with the MAPK inhibitor U0126, under the same conditions, the stimulatory action of PPT was only reduced, suggesting that at least part of the stimulatory effect of estrogen on keratinocyte proliferation involves ERβ that is expressed on the cell membrane and is responsible for rapid, non-genomic signaling. In addition, activation of keratinocyte proliferation by 17β-E₂ does not imply increase in TGF-β1 as it was not modified by treatment with the TGF-β1 receptor antagonist SB431542.

Figure 5 Involvement of TGF-β1 in estrogen effects on NCTC 2544 cell proliferation and migration. The lack of effect of TGF-β1 receptor blockade with SB431542 (SB; 10 μM) on NCTC 2544 cell proliferation (measured by MTT reduction) is shown in d. A scratched cell monolayer was treated with 10 ng/ml hrTGF-β1 and cell migration was assessed at 5, 10, and 24 h as described in Materials and Methods section (b). In c the effect of a 30 min pre-treatment with 10 μM SB431542 prior to addition of 17β-E₂ (E; 10 nM), PPT (100 nM), or DPN (1 nM) for 24 h, in a scratch wound assay is shown. In d a representative immunoblot (top panel) and immunocytochemical analysis (bottom panel) of TGF-β1 expression following treatment with 17β-E₂ (E), PPT, and DPN; data in the accompanying histogram are normalized over β-actin expression and represent mean ± S.E.M. of three independent determinations. Bars represent mean ± S.E.M. of three to five independent experiments each run in triplicates. *P < 0.05 versus other groups by one-way ANOVA followed by Newman-Keuls *post hoc* test for significance. §*P* < 0.05 versus respective control by two-way ANOVA followed by Bonferroni *post hoc* test for significance.

TGF-β1 has a well-established regulatory role in wound healing influencing all stages of wound repair, mainly through induction of extracellular matrix deposition and down-regulation of matrix degradation (Son *et al.* 2005, Faler *et al.* 2006, Lamar *et al.* 2008). In aged skin, topical application as well as systemic administration of TGF-β1 affects tissue repair rates (Beck *et al.* 1993), accelerating wound closure and restoring normal healing (Pierce *et al.* 1989, Son *et al.* 2005). Estrogen positive effects on skin repair have been linked to TGF-β1, although a direct correlation has been reported in dermal fibroblasts (Ashcroft *et al.* 1997) but no evidence exists, to date, in keratinocytes. In our experiments, however, that TGF-β1 *per se*, was able to accelerate wound closure, seemed to play a role in the reparative effects of estrogen being involved in 17β-E₂ property to stimulate keratinocyte migration. This effect was in fact prevented by the TGF-β1 receptor antagonist SB431542. Interestingly, enough, a different role for ERα

and ERβ emerged, as revealed by the distinct behavior of the ERα and ERβ selective agonists. Thus, in contrast to ERα, ERβ seems to mediate estrogen's effect on keratinocyte migration independently of TGF-β1, as the effect of the selective ERβ agonist DPN was not modified by pre-treatment with SB431542 and DPN was not able to mimic 17β-E₂ in inducing TGF-β1 expression. This suggests that selective stimulation of ERβ may result in enhanced keratinocyte proliferation and migration without any increase in the production of TGF-β1. Such an effect may be particularly beneficial, bearing in mind the favorable outcomes that a limited production of TGF-β1 may have on the quality of scarring that follows wound repair (Ashcroft *et al.* 1997, Philipp *et al.* 2004, Riedel *et al.* 2007). In support of the possibility to target selectively ERβ is the preferential cell surface localization of this receptor subtype that is predominantly expressed in the membrane compartment and whose activation generates rapid, non-genomic signaling.

Accordingly, treatment with the MAP kinase inhibitor U0126 prevented the ability of the ER β selective agonist DPN to increase keratinocyte migration while not modifying the effect induced by the ER α agonist, PPT. Furthermore, the effect of 17 β -E₂ on keratinocyte migration was fully mimicked by E₂-BSA, a non-cell permeable estrogen agonist. All these data point to cell surface ER β as a potential selective target to stimulate keratinocyte migration and proliferation thus contributing to wound healing.

Although preferentially mediating genomic actions, we cannot exclude that ER α is also expressed on the cell surface of keratinocytes, as from our results on assessment of receptor expression (immunocytochemistry and western blot), and activation of rapid, membrane-generated signaling (phospho-ERK induction). However, the possibility exists that activation of ER β is sufficient to obtain the stimulatory effect on wound repair through a mechanism that is unknown at present, but may be advantageous as it does not involve excessive production of TGF- β 1. Several other factors are known to be involved in wound healing, among which granulocyte/macrophage colony stimulating factor (GM-CSF) that is produced by epidermal keratinocytes may play a central role (Mann *et al.* 2006, Fang *et al.* 2007). Specifically, GM-CSF that is increased in response to estrogen stimulation of a membrane receptor (Mann *et al.* 2001, Kanda & Watanabe 2004b) promotes migration of keratinocytes and endothelial cells (Mann *et al.* 2001).

In summary, the present data indicate a common function for ER α and ER β in mediating changes in keratinocyte proliferation and migration. However, a unique role for ER β that does not involve enhanced production of TGF- β 1 has been highlighted, opening a promising field for the development of ligands selective for a membrane-localized ER β . This may appear particularly valuable in view of the increasing interest toward innovative and alternative approaches, including TGF- β 1 targeting, for regenerative wound healing (Rhett *et al.* 2008).

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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