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자외선 조사된 상피 줄기세포에 대한 붉나무 추출물의 보호 효과

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Protective Effect of *Rhus Semialata* M. extract on Epidermal Stem Cells against UV Irradiation

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요 약: 피부 기저막에 위치하고 있는 인간 상피 줄기 세포는 피부상피층의 항상성 유지에 중요한 역할을 담당하고 있다. 비록 상피 줄기 세포가 조직손상에 대한 반응으로 상처 회복에 필요한 새로운 세포들을 공급하고 있지만 일부 세포는 정지 상태로 남아 생존을 위해 분화와 노화로부터 보호된다. 이러한 관점에서 적소세포와 외부세포기질 단백질로 구성된 특정 미세환경인 줄기세포 적소는 줄기세포를 보호하기 위해 적절한 자극을 제공해 준다. 줄기세포 마커는 상피 줄기세포의 표면에 발현되며, 기저막의 세포외기질과 부착하여 장기간의 성장 잠재력을 가지며 외부 자극에 대한 세포 사멸의 저항성을 가진다. 본 연구에서는 외부자극의 주요 인자로써 자외선 조사가 인테그린 α2, β1 와 α6 의 발현을 저하시킴을 확인하였으며 붉나무 추출물이 자외선에 의해 유도되어지는 인테그린 발현 저하를 억제하는 것을 확인 할 수 있었다. 또한 붉나무 추출물은 콜라겐 IV와 라미닌과 같은 상피 줄기세포의 부착과 연관된 분자들의 발현을 상향조절 하였다. 이러한 결과는 붉나무 추출물이 줄기세포 표면의 인테그린의 발현을 증가시키고 적소에서의 세포외기질 성분의 발현을 증가시킴으로써 자외선 조사에 대한 보호효과가 있음을 확인하였다.

Abstract: Human epidermal stem cells(ESCs) residing in the basement membrane of the skin have an important role in maintenance of skin homeostasis of epidermal layer. Although, ESCs provide new cells to repair damaged tissue in response to tissue injury, subsets of stem cells remain in the quiescent state protected from differentiation and senescence for prolonged survivals. In this perspective, the stem cell niche, which is specific microenvironment composed of niche cells and an extracellular matrix(ECM), supplies the relevant signal to save stem cells from microenvironmental damages. The expression of stemness marker on the surface of ESCs contributes to the attachment on their ECM of the basement membrane, which lead to growth potential and apoptotic resistance against environmental stimuli. In this study, we observed that UV irradiation, a major factor of environmental stimuli, reduced the expression of α2, β1 and α6 integrin in ESCs. *Rhus Semialata* M extract(RSE) showed inhibitory effect on the UVB-induced reduction of integrin expression. Furthermore, RSE could upregulate the expression of Col-IV and Laminin, which contribute to the attachment of ESCs. These results indicated that RSE could be a potent ingredient for the protection of ESCs from UV irradiation by increasing the expression of integrin and substrate ECM components at their niche.

Keywords: *Epidermal stem cell, Rhus Semialata M, Extracellualr matrix, stem cell niche*

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1. Introduction

Epidermal keratinocyte stem cells adhered to the basement membrane can self-renew and differentiate into different lineage of cell type for the cell replacement after injury or aging. These cells express specific surface or intracellular markers, including keratin or integrins, to enhance attachment of the cells to extracellular matrix components that make up the stem cell niche[1,2]. Stem cell niches can regulate the proliferation of stem cells and inhibit the apoptosis or differentiation to maintain the numbers of stem cells by providing signals for stem cell renewal[3]. In this microenvironment, stem cells communicates with their niches component through the surface molecules and if they detach from the basement membrane they withdraw from the cell cycle and differentiate, move outward to skin surface[4]. Several studies revealed that restoration of extracellular matrix to basement membrane is important for stem cell maintenance in skin[5].

Stem cells are involved in the overall health of the skin, skin rejuvenation, restoration of skin damage, wound healing as well as hair coloring and regeneration. However the human body has a limited numbers of stem cells. As the aging progresses, the number of stem cells decreases, which leads to decrease of the renewal rate of epidermis[6,7]. Decreased renewal rate of epidermal cells is associated with a reduced expression of stem cell markers such as integrin β 1 and p63[8,9]. The decrease of the integrin β1 contributes to the reduced proliferation of keratinocytes that leads to disruption of epidermal regeneration[10]. p63, a p53 family member is reported to inhibit premature aging in clonogenic cells[11].

Stem cells are also decreased by daily threatens of ultraviolet rays, pollutants, internal aging factors inducing communication errors. Especially, todays the depletion of the ozone layer may result in the increasing the UV radiation reaching the ground. Although, epidermal stem cells are highly resistant to the DNA damage, repetitive exposure to UVB radiation could impair the epidermal stem cells as well as basement membrane components. Impared stem cell has been known to cause the skin premature aging and skin cancers[12]. High expression of stem cell marker such as integrin β1 and α6 in keratinocytes can attribute to exert anti-apoptotic effect against UV irradiation[13]. That indicate that increase of stem cell marker could protect epidermis from UV-induced damages.

Rhus Semialata Murray (*Rhus Semialata* M), which belongs to the genus *Rhus* and the family of *Anacardiaceae*, is widely distributed in northeastern Asian countries such as Korea, China and Japan. Recently, the bioactive ingredients from *Rhus Semialata* M have been studied for their pharmacological effect such as antiviral, antibacterial, anticancer, hepatoprotective and antioxidant activities[14]. Investigation for phytochemical ingredients on the components of *Rhus Semialata* M found to contain gallic acid, methyl gallate, β-sitosterol, morolic acid, (2S)-1-O-heptatriacontanoyl glycerol[15]. However, little is known about the anti-aging effect of *Rhus Semialata* M through the strength of the attachment between epidermal keratinocyte and ECM on the basement membrane.

In this study, we confirmed the characteristics of the early passage of epidermal keratinocyte by analyzing specific surface markers on keratinocyte stem cells. In addition, we have been investigated whether the UVB irradiation could alter the expression of surface marker on the epidermal keratinocytes and production of extracellular matrix from the dermal fibroblasts. In this regard, we find out that extract of *Rhus Semialata* M (RSE) have anti-aging effect with increase of collagen IV and laminin synthesis in the basement membrane and upregulation of the expression of surface markers of epidermal keratinocytes.

2. Experimental

2.1. Materials

ELISA kit for laminin and collagen IV were purchased from Cloud-Clone corp (USA). Anti-integrin β1 conjugated with FITC, anti-integrin α2 conjugated with PE, anti-integrin α6, anti-p63 and anti-cytokeratin 15 were purchased from Abcam (UK) and Life Technology (CA, USA). Primary antibodies for explant immunostaining of anti-collagen IV and anti-laminin V were purchase from Southern Biotechnology (USA) and Santa Cruz Biotechnology (USA) respectively.

EpiLife^{TM} Medium containing Human Keratinocyte Growth Supplement and Dulbecco's modified Eagle's medium (DMEM) were obtained from Thermo Fisher Scientific (USA) and Welgene (Korea).

2.2. Preparation of Extracts from *Rhus Semialata* M

The leaves and branches of *Rhus Semialata* M were cultivated in Jeollanamdo of South Korea. The dried and pulverized raw material (100 g) was extracted overnight with 70% (v/v) ethanol at room temperature, and the supernatant was collected by filtration. Ethanol was removed by rotary vacuum evaporation (Heidolph, Schwabach, Germany) from collected supernatant. The extract was lyophilized to yield RSE (2.1 g, yield 2.1 %). The lyophilized powder was dissolved in DMSO and used in this experiment.

2.3. Cell Culture

Human epidermal keratinocytes (HEKn) were obtained from Invitroge (CA, USA) and maintained in EpiLife (Invitrogen, USA), containing Human Keratinocyte Growth Supplement (HKGS, Invitrogen, USA) at 37 ℃, under 5% CO_2. Passage 2-3 cells were used in all experiments.

2.4. Measurement of Cell Viability and UVB Irradiation

Cell viability assay was determined by using the 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Duchefa, Haarlem, Netherlands) assay. Early stage of epidermal keratinocytes were treated with or without various concentration of RSE. After 6 h, the using cell were irradiated with 10 mJ/cm^2 of UVB by using LZC-UVB lamp (Luzchem, Ottawa, ONT, Canada), which had an emission spectrum of 280-370 nm and a peak at 312 nm. The UV dose was measured with a UV light meter UV-340 (Lutron, Coopersburg, PA, USA). After treatment, MTT reagent (1 mg/mL) was added to each well and incubated for an additional 3 h. After then the supernatant was removed and dimethylsulphoxide (DMSO) was added to dissolve the formazan crystal produced from MTT. Absorbance was measured at a wavelength of 570 nm using a spectrophotometer (Epoch^{TM} microplate spectrophotometer, BioTek, Winooski, Vermont, USA).

2.5. Measurement of Extracellular Matrix

Expression of collagen IV and laminin in culture supernatant from RSE treated early stage of epidermal keratinocytes were determined using a commercially available ELISA kit according to the manufacturer's instructions (Cloud-Clone Corp, USA). Epidermal keratinocytes were cultured in the presence of 1 and 10 ㎍/mL of RSE for 72 h. Cell culture supernatant was collected to determine the production of collagen IV and laminin. The absorbance was measured at 450 nm using an automatic Epoch microplate spectrophotometer (Biotek, USA) and all samples and standards were measured in duplicate.

2.6. *Ex-Vivo* Experiment

Human tissue samples collected as part of *Ex-vivo* study conducted by the Laboratoire BIO-EC were obtained in the frame of the contract signed between Laboratoire BIO-EC and Clinique du Mont-Louis. This contract was previously submitted to the Ethical Committee of the Clinique du Mont-Louis and approved in principles of the declaration of Helsinki. It includes information and patient consent signed for each of the samples used in this study. The samples used were surgical waste and were not collected as apart of a clinical study.

The 12 human skin explants of an average diameter of 11 mm (± 1 mm) were obtained from abdomen-plasty on the 50's Caucasian women after liposuction. The explants were maintained in culture at 37 ℃ in a humid, 5% CO_2 at mospheric condition in BEM culture medium in BIO-EC. 2 mg/cm^2 powder of RSE was topically applied on the skin explants, and were spread on day 0, 2, 5, 7. On day 9. The explants from each batch were collected and fixed in buffered formalin for 24 h. After the fixation, frozen samples were cut into 7 ㎛ thick sections and each sections were immunostaining with specific antibodies for collagen IV and Laminin V. To observe general morphological change, the paraffinized sections were stained with Masson's trichrome, Goldner variant. The microscopical observations were realized using an DMLB (Leica, Germany) or BX43 (OLYMPUS, Japan) microscope.

2.7. Flow Cytometric Analysis

Flow cytometry was performed to analyze the expression of integrin subunits α2 and β1 on the surface of epidermal stem cells. Early stage of epidermal stem cells were treated with or without various concentration of RSE. After 6 h, the cell were irradiated with 10 mJ/cm^2 of UVB or untreated as a control. After additional incubation for 24 h, the cells were harvested and washed with PBS containing 2% FBS and incubated with anti-integrin β1 labeled with FITC, anti-integrin α6 conguated with FITC, and anti-integrin α2 conjugated with PE for 1 h at 4 ℃ for analysis of surface markers. Also, intracellular markers such as anti-p63 and anti-cytokeratin 15 were stained after being permeabilized with 0.1% Triton X-100 . In the case of unlabeled primary antibodies, cells were treated with IgG isotype antibody. Fluorescence was measured by FACScalibur Flow Cytometer (Becton Dickinson, USA) and analyzed with CellQuest^{TM}Pro software.

2.8. Statistical Analysis

Statistical significance of data was determined by a Student's *t*-test. All results were expressed as the means ± standard deviation (N = 3). $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered to be significant.

3. Results and Discussion

3.1. Pattern of Integrin Expression on Early Stage of Epidermal Keratinocytes

To identify the characterization of the epidermal stem cells, we determined the expression of β1 and α2, α6, cytokeratin 15 and p63 which are typical surface marker proteins [3,7,10,16,17]. As shown in Figure 1, the levels of integrin β 1 and α2, α6, cytokeratin 15 and p63 was significantly upregulated in the early passage of growing human epidermal keratinocytes as compared to isotype IgG. These results suggested that early passage of epidermal keratinocytes could be used as stem cells in further experiments.

3.2. RSE Upregulates the Production of Laminin and Collagen IV in early stage of Epidemral Keratinocytes

In the skin, self-renewing epidermal stem cell reside in the basal layer, which make interactions with extracellular matrix of basement membrane. This regulatory signals from the niche could regulate the behavior of stem cells to differentiate or remains as stem cells. To examine whether the RSE involved in the upregulation of extracellular matrix formation, we evaluated the production of laminin and collagen IV after the treatment of RSE in epidermal keratinocyte. Laminin and collagen IV expression were increased in epidermal keratinocyte 72 h after the treatment of RSE (Figure 2A and B). These results demonstrated that RSE could enhance the expression of integrin subunits in the surface of epidermal keratinocytes as well as enhanced the expression of extracellular matrix in the basement membrane.

3.3. RSE Increase the Expression of ECM Molecules in *Ex-Vivo* Experiments

To confirm the RSE induced upregulation of extracellular matrix including laminin V and collagen IV as a marker of epidermal and dermal structure, we evaluate the expression of those markers in human living skin explants. RSE was topically applied to the surface of the explant for 7 days

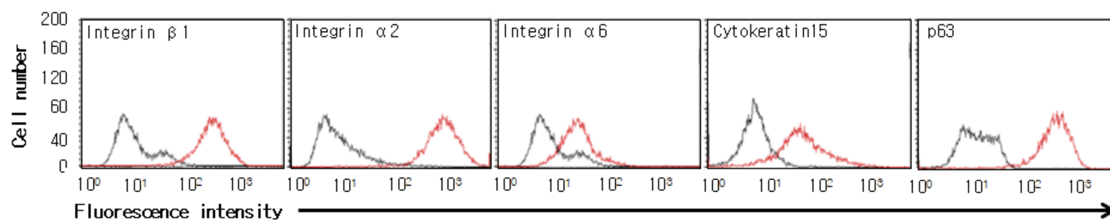

Figure 1. Detection of markers to characterize the epidermal keratinocytes as stem cells. Flow Cytometric analysis of integrin β1 and α2, α6, cytokeratin 15 and p63 (red) and Isotype IgG (black) in early stage of epidermal keratinocytes.

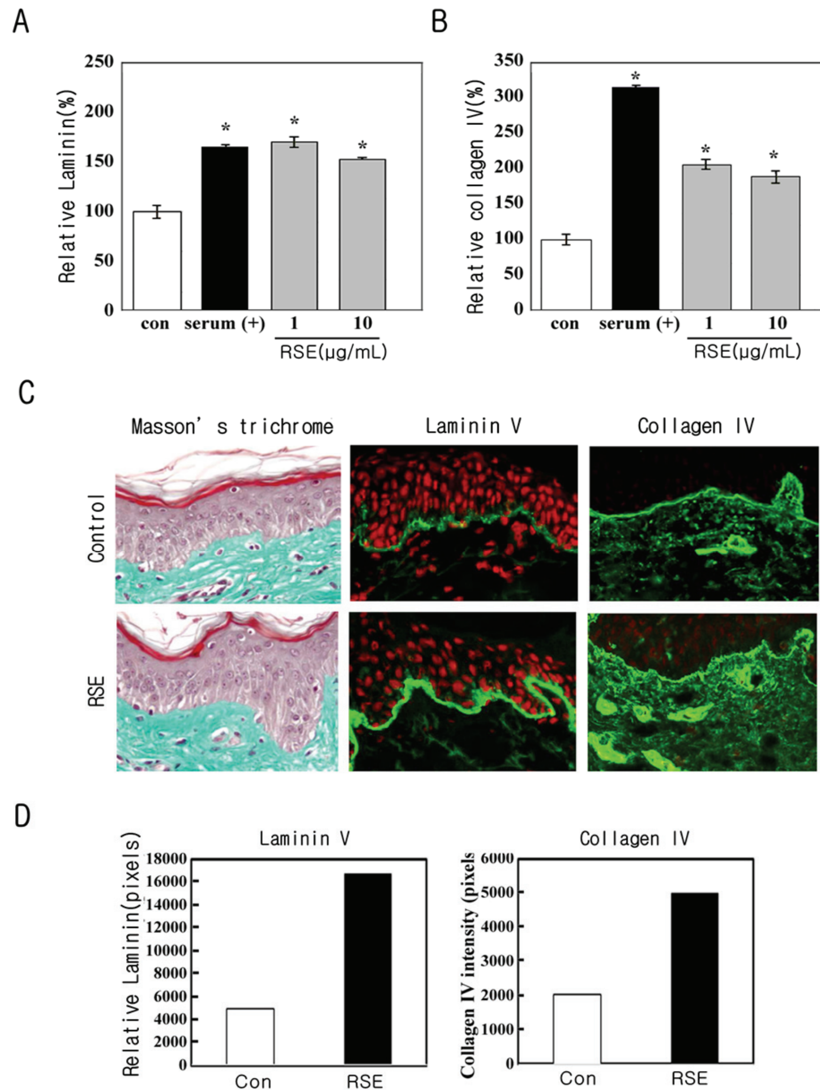

Figure 2. (A and B) Relative production of laminin V and collagen IV after the treatment of *Rhus Semialata* M in early stage of epidermal keratinocytes. (C) Immunostaining of Masson's trichrome, laminin V and collagen IV after the treatment of *Rhus Semialata* M in skin explants. (D) Quantification of immunnofluorescent intensity in *Rhus Semialata* M treated skin explant.

periodically. 2 days after the last treatment of RSE skin explant from the each batch were collected and the performed the staining for specific target proteins. As shown in Figure 2C, the p63 and integrin β1 which were critical for retardation of the production of laminin V and collagen IV component of basement membrane were increased with the treatment of RSE. These results means that RSE shows a quite good anti-aging activity by inducing a clear increase of laminin V and slight increase of collagen IV expression, an extracellular matrix molecules between basal layer and basement membrane separating dermis and epidermis.

3.4. RSE Protect Down-Regulation of UVB Induced Surface Marker Expression in Early Stage of Epidemral Keratinocytes

To investigate the effect of RSE on integrin expression,

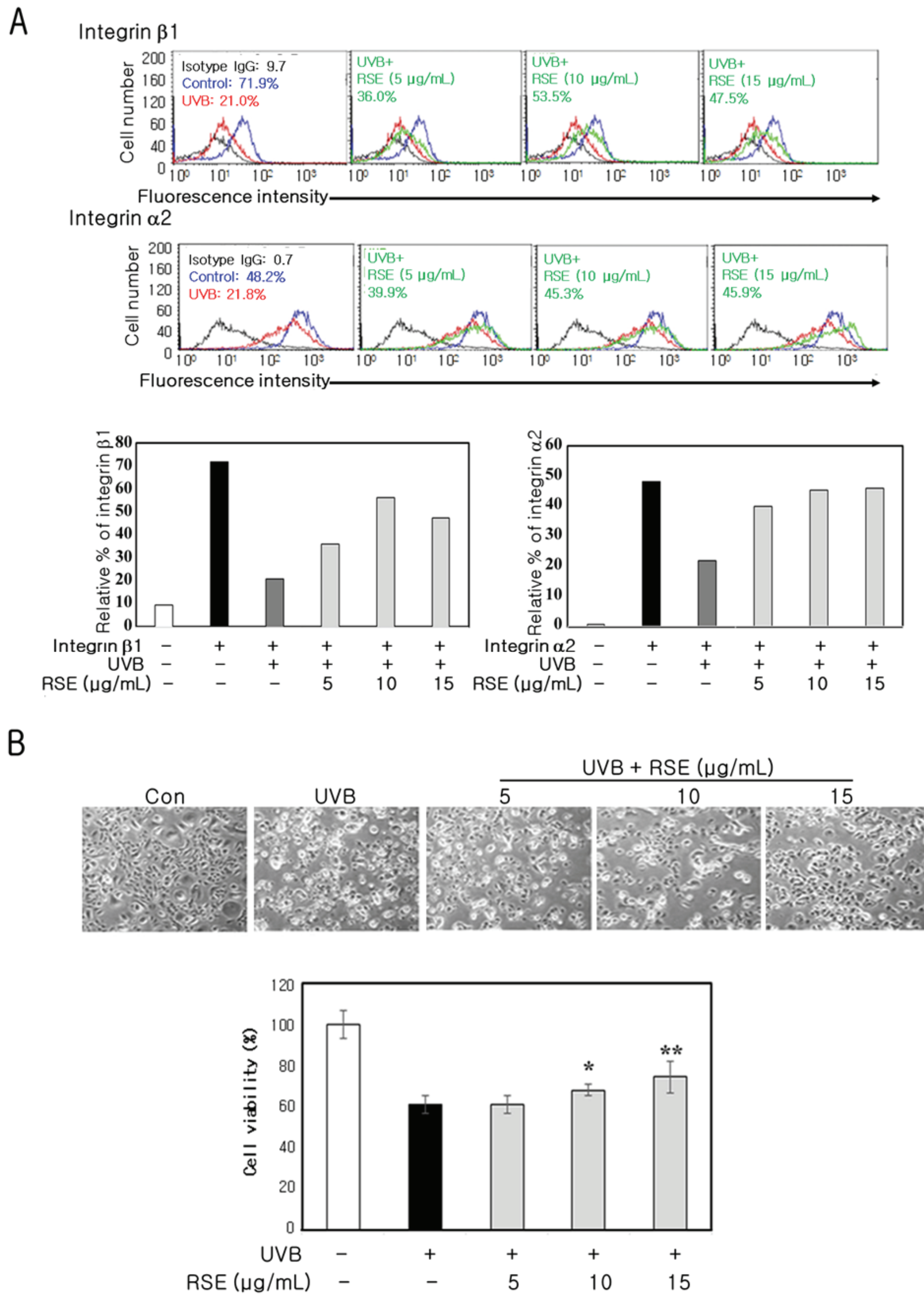

Figure 3. (A) The protective effect of *Rhus Semialata* M extract against UVB damaged expression of surface markers including integrinβ1 and integrin α2 on early stage of epidermal keratinocytes. (B) Cell morphology and viability of early stage of epidermal keratinocytes after exposure to UVB irradiation with pretreatment of *Rhus Semialata* M.

epidermal keratinocytes were pretreated with various concentration of RSE for 24 h before UVB irradiation. The cells were harvested and the expression patterns of integrin subunit β1 and α2 were determined by flow cytometry. RSE could recover the reduced expression of integrin subunits by UVB irradiation, in a dose dependent manner (Figure 3A). And the number of attached cells on the plate increased with pretreatment of RSE (Figure 3B). These results indicate that RSE protect the epidermal keratinocyte against UVB irradiation through the upregulate the expression of surface marker protein necessary for possessing epidermal stemness.

4. Conclusion

In this study, we evaluated the stem cell markers from early stage of epidermal keratinocytes. Furthermore, we demonstrated that RSE exhibited protective effects against UVB irradiated aging through the upregulation of surface markers and extracellular matrix molecules in epidermal keratinocytes in the basement membrane. As a result, RSE can be an effective natural anti-aging ingredient that protects stem cells from UVB damage.

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