Totipotent vegetal stem cells activate Smad2 signalling pathway and stimulate epidermal and dermal physiology

Betty Nusgens¹, Amélie Thépot², Charlotte Lequeux², Alain Colige¹, Christiane Montastier³, Odile Damour²

¹ Laboratory of Connective Tissues Biology, University of Liège, Belgium, ² Laboratoire des Substituts Cutanés, Hôpital Edouard Herriot, Lyon, France, ³ Helena Rubinstein, Levallois-Perret, France

Introduction

Skin, like all organs, experiences progressive morphological and physiological deteriorations with time due to intrinsic aging further worsened by cumulative environmental aggressions such as solar radiation, smoking, among the most well recognized. Besides its protective role towards deleterious environment, skin is a crucial organ for the maintenance of temperature and the barrier function against fluids and electrolyte diffusion. It has also an endocrine function by synthesizing a variety of hormones, growth factors, sex steroids and essential vitamins such as vitamin D. With aging, not only the structural and morphological aspects of skin deteriorate but also its physiological functions.

Aged skin is characterized by thinning of the epidermis, impaired proliferation and differentiation of keratinocytes and flattening of the dermo-epidermal junction among other. A marked atrophy and loss of elasticity of the dermal connective support is caused by a reduction and disorganization of the major extracellular matrix components such as collagen and elastic fibers and the highly hydrated extracellular gel made of hyaluronic acid and proteoglycans. This degenerative process compromises the interactions of dermal fibroblasts with their microenvironment, which are no longer mechanically solicited. As demonstrated in vitro, such a loss of mechanical regulation induces an alteration of fibroblastic phenotype characterized by a downregulation of extracellular matrix components synthesis [1]. It leads also to an increased production of matrix metalloproteinases [2] which contributes to the perpetuation of the atrophying process. Several studies suggest that the progressive loss of dermal connective tissue in aging is associated with a diminished expression of the matricellular connective tissue growth factor (CTGF/CCN2) [3,4], a gene target of the TGFβ signaling dependent on transcription factors of the Smad family. The TGFβ/Smad/CTGF axis is influenced notably by mechanical forces and is pivotal to fibroblasts function by activating the transcription of a repertoire of genes participating in the extracellular matrix homeostasis [4].

Crithmum maritimum, also called samphire or rock samphire, is one among these unique plants able to adapt to hostile environments. This plant can indeed grow in contact with high salt concentration such as seawater and in arid soil and climate and synthesizes large amounts of antioxidants. Totipotent dedifferentiated cells from C. maritimum (dCMC), have a large
proliferative potential, can be amplified in culture and display a high capacity for regeneration and defense. These properties could have beneficial effects by counteracting degenerative alterations in skin aging. We used a three-dimensional Skin Equivalent (SE) made of a collagen-glycosaminoglycans-chitosan porous substrate populated by human dermal fibroblasts and epithelialized with human keratinocytes to reconstruct a full thickness skin equivalent. This experimental model closely reproduces the in vivo dermal architecture and allows the complex dermo-epidermal interactions [5-8].

The effect of a long-term systemic administration of an extract of dCMC was evaluated

- **on the epidermal compartment:** by evaluating the proliferative capacity of keratinocytes (Ki67) and the expression of terminal differentiation markers (fillagrin, claudins),

- **on the dermal compartment:** by evaluating the stimulation of fibroblasts by measuring the activated form of Smad2 (p-Smad2), a TGFβ1/CTGF down-stream intracellular signaling pathway. Furthermore, we evaluated by immunochemistry and image analysis the proteoglycans deposited within the neosynthetized dermal matrix and fibrillin-1, the main microfibrillar component governing the formation of elastic fibers and known to control the release of TGFβ1.

**Materials and methods**

**Skin equivalents:** they were prepared by seeding fibroblasts from a 57 year-old donor on a collagen-glycosaminoglycans-chitosan dermal substrate cultured 3 weeks before epidermalization by young keratinocytes to produce a Skin Equivalent (SE). After 7 days of submerged culture, the SE was raised to the air-liquid interface. The systemic administration of an extract of dCMC (Biotech Marine, Ponttrieux - France) at 0.1% was added to the culture medium 2 days after seeding fibroblasts until the end of the study. Non-treated SE controls were handled in parallel.

**Sample collection:** the SE samples were harvested on day 35 of culture. For all conditions, three samples were fixed in 4% paraformaldehyde and embedded in paraffin and three in Tissue-Tek®. 5 µm sections of 3 treated and 3 untreated SE were performed for each of the following analyses.

**Histology:** paraffin embedded sections were stained with hematoxylin phloxin and saffron for a global morphological analysis.

**Immunofluorescence:** frozen sections were fixed 10 min in acetone or acetone-methanol and labelled with anti-fibrillin-1 at 1/100 (Novocastra) or anti-claudin-1 at 1/100 (Invitrogen) followed by a secondary antibody Alexa 488 at 1/1000. Nuclei were stained with Hoechst reagent. For each condition, image analysis (ImageJ software) was realized at least on 2 areas of 3 different pictures (n=6).

**Immunostaining:** paraffin embedded sections were labelled with anti-p-Smad2 (Cell Signaling), anti-Ki67 (Dako) or anti-fillagrin (Novocastra) followed by a secondary peroxydase-coupled antibody revealed using AEC and counterstained with hematoxylin. The
number of positive cells per unit surface was determined in 5 zones in 3 treated and 3 untreated SE by image analysis (ImageJ software).

**Staining of proteoglycans:** sections were labelled with Blue-Alcian and staining was quantified in pixels per unit surface (ImageJ software) on 6 zones in 3 treated and 3 untreated SE.

**Statistical analyses:** were performed with Bonferroni test, Mann and Withney or t-test of Student.

**Results**

**Epidermal compartment**
The long term administration of dCMC accelerated the complete regeneration and differentiation of the epidermis as compared to the untreated SE. Histology showed a multi-layered, thick and differentiated epithelium after 35 days of culture (Figure 1). The number of basal and supra-basal cell layers was increased by a factor of 4.9 ± 0.4 as compared to the untreated control SE versus control (Mann and Withney, p<0.001).

![Control SE](image1)

![dCMC treated SE](image2)

Hematoxylin/phloxin/saffron staining

**Figure 1**

This increase was attributed to the greater proliferation of basal cells, the number of cells expressing the proliferation marker Ki67 being significantly higher in the dCMC treated SE (12.9±1.3, per unit surface) than in the control samples (5.0±0.5, Mann and Withney, p<0.001). No filaggrin expression was found in the control SE while a positive staining appeared in the dCMC supplemented SE (not shown) demonstrating the influence of dCMC on terminal differentiation.
The expression of claudin, a marker of keratinocytes junctions, was also significantly increased (x3) by dCMC treatment (Figure 2 A & B) demonstrating a well structured epidermis.

A  Control SE         dCMC treated SE

B

**Figure 2**

A Expression of claudin-1 in SE control (C) or treated with dCMC ; B Quantification by image analysis (ImageJ). *** p<0.0001, test de Bonferroni.

The extract of *Crithmum Maritimum* seems therefore to induce an improved epidermal homeostasis through a beneficial balance between proliferation and differentiation.
**Dermo-Epidermal Junction (DEJ)**

Fibrillin is a main microfibrillar component that dictates elastogenesis by inducing elastin deposition to form elastic fibers. It also plays a crucial role in the regulation of TGFβ availability by binding the LBTP/LLC/active TGFβ complex.

The treatment by dCMC induced a 2 fold higher expression of fibrillin 1 (p<0.05) versus control. (Figure 3 A & B).

![Figure 3 A & B](image_url)

**Figure 3**

A Expression of fibrillin in control SE and treated with dCMC ; B Quantification by image analysis (ImageJ). * p<0.05, test de Bonferroni.
A better dermo-epidermal junction organization was observed upon *Chrichtum maritimum* administration, with fibrillin microfibrils appearing perpendicular to DEJ, similarly to oxytalan fibers in normal human skin.

*Dermal compartment*

The administration of dCMC had also a strong effect in the reconstructed dermis, by stimulating the synthesis of a more abundant extracellular matrix filling the full thickness of the dermal substrate. We investigated the activation level of the phosphorylated activated form of Smad2 (p-Smad) which induces its translocation into the nucleus to act as a transcriptional regulator of the expression of a series of molecules involved in the extracellular matrix homeostasis and remodelling.

Phospho-Smad was exclusively observed as intranuclear staining as shown in the high magnification of a resident cell of the SE in figure 4.

![Figure 4](image.png)

The number of positive cells per unit surface was significantly increased (p=0.05) in the SE treated with dCMC as compared to the untreated SE.

| Control | Positive cells per unit surface : 212±28 |
The deposition of proteoglycans in the SE was visualized by an Alcian blue staining. As illustrated in figure 6 for a representative section, and quantified by image analysis (ImageJ), proteoglycans were much more abundant in the SE treated by dCMC by comparison to controls (C).

**Figure 5**

Phospho-Smad immunostaining of (A) control and (B) dCMC treated SE

**Figure 6**

<table>
<thead>
<tr>
<th></th>
<th>Alcian blue</th>
<th>Deconvolution</th>
<th>Binarization</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C</strong></td>
<td></td>
<td></td>
<td><strong>8.2±0.5</strong></td>
</tr>
<tr>
<td><strong>dCMC</strong></td>
<td></td>
<td></td>
<td><strong>14.8±1.2</strong>*</td>
</tr>
</tbody>
</table>

p=0.0004
CONCLUSIONS

Our data clearly show that dedifferentiated cells from C. maritimum induce the epidermis regeneration by stimulating both proliferation of basal cells and keratinocytes differentiation as evidenced by the increased expression of Ki67, filaggrin and claudins [4]. The extract of C. Maritimum seems therefore to induce an improved epidermal homeostasis through a beneficial balance between proliferation and differentiation.

They also stimulate the production and deposition of fibrillin1, a TGFβ1 binding protein, appearing as organized structures perpendicular to the DEJ, similarly to oxytalan fibers in human skin. A significantly increased deposition of proteoglycans in the dermal extracellular matrix indicates that these vegetal cells can also activate the phenotype of dermal fibroblasts. The higher number of cells showing an activation of Smad2 suggests that the dedifferentiated cells from C. maritimum may trigger the TGFβ1/CTGF signalling pathway and stimulate the extracellular matrix metabolism.

REFERENCES