

## Short Communication

# Mechanical stretching modulates growth direction and MMP-9 release in human keratinocyte monolayer

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Cells within human skin are exposed to mechanical stretching that is considered a trigger stimulus for keratinocyte proliferation, while its effect on keratinocyte migration has been poorly investigated. In order to explore the effect of stretching on keratinocyte migration spontaneously immortalized human keratinocyte (HaCaT) monolayers seeded onto collagen I-coated silicon sheets were stimulated three times for 1 hour every 24 hours (total time = 72 hours) by mechanical stretching increasing substrate deformations (10%) applied both as static (0 Hz) and cyclic (0.17 Hz) uniaxial stretching. At the end of stimulations monolayer areas measured in both static and cyclic samples appeared reduced and strongly oriented in a direction perpendicular to the stress direction compared to unstimulated ones. Moreover during the mechanical stimulation period HaCaT monolayers strongly increased the release in the medium of matrix metalloproteinase 9 (MMP-9), a proteolytic enzyme necessary for keratinocyte migration.

## Introduction

The influence of mechanical forces on skin structure and remodeling has been studied extensively to understand phenomena such as wound healing and hypertrophic scarring reduction<sup>1,2</sup> and balloon expanders induced skin surface increasing.<sup>3</sup> As all these phenomena involve keratinocyte proliferation and migration, a large number of investigations have been focused on the effect of mechanical stimulations on keratinocyte cellular behavior. Skin can undergo to both compression and stretching and these two types of mechanical force applied from the external can be transduced in opposite way in the skin.<sup>4</sup> In fact, mechanical pressure induce in vitro overexpression of differentiation markers in a keratinocyte cell line<sup>5</sup> reducing cell proliferation,<sup>6</sup> while the application of stretch seems to trigger keratinocyte growth and proliferation.<sup>7,8</sup>

In particular, considering the uniaxial stretching, it has been pointed out that in human keratinocyte cyclic strain affects protein kinase C (PKC) activity,<sup>9</sup> increasing cell proliferation, DNA and protein synthesis<sup>8,10</sup> compared to stationary or constantly loaded cells, where mechanical stretching induces activation of MAPK extracellular signal regulated kinase 1/2 (ERK 1/2) via  $\beta$ 1 integrin activation.<sup>11</sup>

In this paper activation of cell migration in human keratinocyte monolayers by mechanical stretching has been evaluated measuring the monolayers area and orientation along with the production of MMP-9 and MMP-2, two proteolytic enzymes involved in the keratinocyte migration.<sup>11</sup>

## Results and Discussion

Figure 1A shows representative images of HaCaT monolayers before (CT0) and after 72 hours in control condition (CT) and 10% substrate deformation stretching both in static (Stat) or cyclic (Cycl) condition. After 72 hours the strip area covered by keratinocyte was increased in every condition compared to the CT0 samples (mean area  $\pm$  standard deviation (S.E.) =  $136 \pm 5$  mm<sup>2</sup>, Fig. 1A and B), nevertheless after stretching monolayers area was reduced compared to CT samples (Fig. 1A and B,  $213 \pm 7$  mm<sup>2</sup>) both for static ( $178 \pm 3$  mm<sup>2</sup>,  $p < 0.001$ ) and cyclic ( $197 \pm 5$  mm<sup>2</sup>,  $p < 0.05$ ) stimulation. The light reduction observed in monolayers area in stimulated samples seemed in contrast with the observed cell proliferation induced by 24 hours continued mechanical stretching both in static<sup>9,11</sup> and cyclic mode.<sup>8</sup>

Moreover, it was possible to observe that HaCaT monolayers were oriented toward a direction perpendicular to stress direction (Fig. 1A).

However, in our model, the mechanical stimulation applied was smaller and shorter than the one indicated in other reports.<sup>8</sup> Nevertheless the same authors observed single cell alignment perpendicular to force vector,<sup>8</sup> even if our results seemed more dramatic.

In order to explore the effect of mechanical stretching on cell growth/migration the presence and activity of two matrix metalloproteinases, the MMP-2 (Gelatinase A, 72 KDa) and MMP-9 (Gelatinase B, 92 KDa) have been monitored by substrate zymography as described in the experimental design. As shown in the

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representative zymogram (Fig. 2A), the expression of MMP-2, already present in the serum (Sr), did not change significantly both in unstimulated and stimulated samples during the experimental period. On the contrary, the production of MMP-9 enzyme in medium conditioned by CT samples increased during all the experimental period (Fig. 2A and B) as shown by the increase in the optical density of pro-MMP-9 and MMP-9 bands (Fig. 2B). The MMP-9 was not present in the serum and its production was strongly increased 24 hours after the first and second stimulation both in static and cyclic samples (Fig. 2B). Static stimulation induced also MMP-9 intense activation (Fig. 2A). However after the third stimulation (72 hours) the production of MMP-9 was reduced compared to the CT samples ( $86 \pm 1\%$  and  $77 \pm 3\%$  of CT respectively for Stat e Cycl samples) (Fig. 2A and B) as probably the growth/migration front reached the silicon strip limit (Fig. 1A), therefore cells could not migrate/growth anymore and downregulated their MMP-9 production. The MMP-9 is an important proteolytic enzyme which expression is induced in keratinocyte by growth factors<sup>12,13</sup> and integrin stimulation<sup>14</sup> via PKC and stress activated kinases<sup>15</sup> and it is essential for keratinocyte migration during wound healing.<sup>16,17</sup> So far the modulation of a matrix metalloproteinase by mechanical stimulation in keratinocyte is a novel report. Even if there are no reports about MMP-9 or other MMPs modulation induced in keratinocyte by mechanical stretching, it has been observed in vitro that fibroblast not aligned with the force direction during stretching show a several fold increase in matrix metalloproteinases activity (MMP-1, MMP-2, MMP-3) suggesting that cells that are unable to align with the direction of the applied load, remodel their matrix more rapidly than oriented cells.<sup>18</sup> A similar phenomenon could occur in keratinocyte stretched in our study, even if it has been reported that normal human keratinocyte downregulated the expression of urokinase-type plasminogen activator (uPA), an activator of MMP-9 in the skin,<sup>19</sup> when a strong mechanical stretch (30% elongation) was applied.<sup>20</sup> Nevertheless our results indicate a correlation between the morphological effect of mechanical stretching on human keratinocyte and the production of MMP-9 involved in cell migration.

A possible mechanism for MMP-9 overexpression induced by stretching could be linked to the integrin-ERK system as it has been demonstrated that static stretching induces in keratinocyte a

Figure 2. (A) Representative zymogram of MMP-9 and MMP-2 activity present in media conditioned by HaCaT cells after 24, 48 and 72 hours according to the stimulation protocol described in the Materials and Methods section. Std = MMP-2 and MMP-9 standard, pro-MMP-9 = inactive form, MMP-9 = active form, Sr = 10% serum. (B) densitometric analysis of the MMP-9 (both active and inactive) bands observed in zymograms obtained from three different experiments. \*\* $p < 0.001$ , \* $p < 0.05$  compared to the CT samples at the same time point.

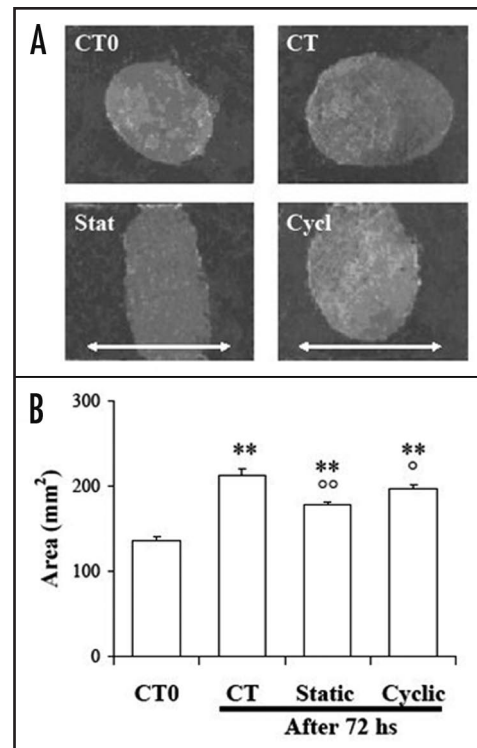
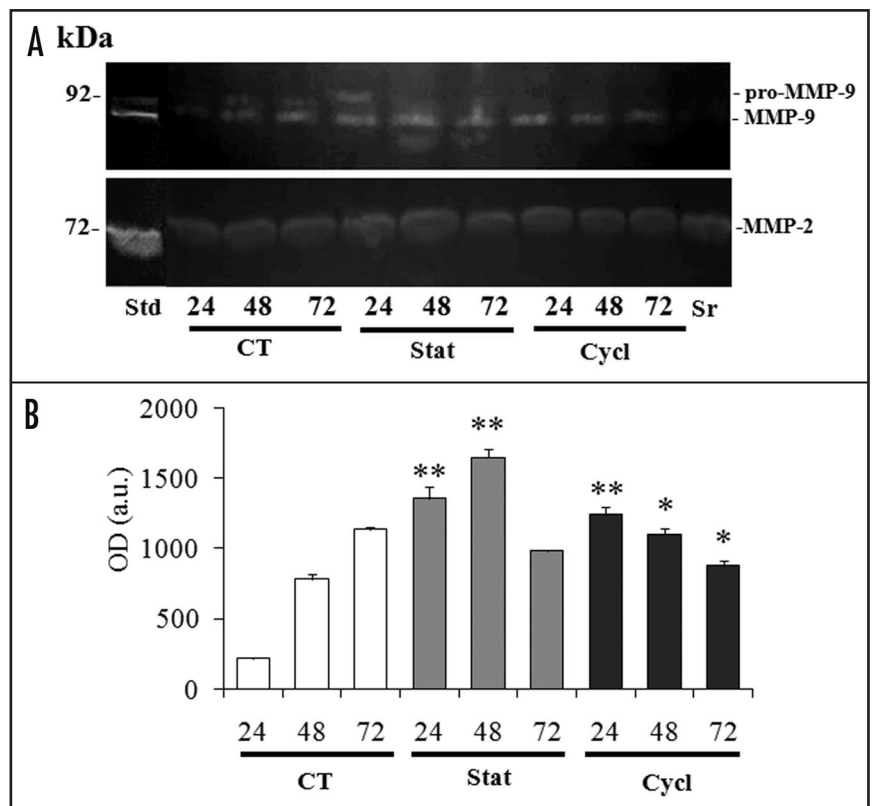


Figure 1. (A) Representative images of HaCaT monolayers at time 0 (CT0) and after 72 hours in control conditions (unstimulated, CT), after mechanical stretching in static and cyclic conditions (arrows indicate the stress direction); (B) quantification of the area of keratinocytes monolayers (n = 3) expressed in mm<sup>2</sup> ± Standard Error (S.E.). \*\* $p < 0.001$  compared to CT0, ° $p > 0.05$  and °° $p < 0.001$  compared to CT.



rapid activation of mitogen-activated protein kinases (MAPKs),<sup>11</sup> regulated via  $\beta$ 1-integrin, a “mechano-transducer” linked to the focal adhesion kinase activity.<sup>21</sup> A similar mechanism could act in our model as  $\beta$ 1-integrin regulates MMP-9 mRNA stability<sup>14</sup> and MAPK activation induces MMP-9 expression in keratinocyte.<sup>13,15</sup> Moreover the mechanical stretching decreases cAMP and protein kinase A (PKA) activity in keratinocyte,<sup>7</sup> two biochemical signals that inhibit MMP-9 induction and cell migration.<sup>22</sup> Finally, in this paper, we evaluated only two MMPs, but keratinocyte migration is a composite mechanism that involves often different cell populations (e.g., dermal fibroblast) and different MMPs working in cooperation such as MT-MMP-1 and MMP-13 as clearly demonstrated in skin tumors.<sup>23</sup> Future studies could be aimed to verify similar complex interaction also in mechanical stimulated migration. In conclusion, in our study keratinocyte monolayers grown onto collagen I-coated silicon strips have been stimulated by 10% substrate stretching both with static (0 Hz) and cyclic (0.17 Hz) mode. The results of these stimulations can be summarized in two main points: (1) mechanical stretching lightly reduced monolayer area and dramatically oriented them, (2) mechanical stimulation quickly increased the production and activation of MMP-9.

Our *in vitro* findings indicate that a keratinocyte monolayer, similar to a normal basal layer in the skin, can be stimulated by mechanical stretching to produce MMP-9 allowing cell growth and migration in a direction perpendicular to stretching force, suggesting a possible mechanism for some strain-dependent responses observed in the skin.

## Materials and Methods

Immortalized human keratinocytes (HaCaT),<sup>24</sup> were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (Euroclone, Milan, Italy) in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C. HaCaT cell suspension (300  $\mu$ l, 8.3 x 10<sup>5</sup> cell/ml) in serum-free DMEM was added to silicone sheet (3 x 2 cm) (SMI Specialty Manufacturing Inc., Saginaw, MI, USA) sterilized by autoclaving and coated with 1  $\mu$ g/ml sterile Collagen Type I (Calbiochem, Darmstadt, Germany). After 4 h incubation, unattached cells were removed by washing with fresh DMEM and adherent cells were incubated for further 24 hours at 37°C before stretching. Samples were connected with tweezers to an Instron 5564 testing Instrument (Instron Corporation, Canton, MA, USA) equipped with a loading frame with a load capacity of 2.5 N in tension. Samples were stretched for one hour every 24 hours (total experimental time = 72 hours) in a culture vertical chamber (Ugo Basile, Milan, Italy) filled with serum free DMEM (30 ml) at 37°C in a 5% CO<sub>2</sub> atmosphere.<sup>25</sup> Substrate deformation applied was 10%<sup>11</sup> both in static (0 Hz) and cyclic (0.17 Hz)<sup>8</sup> uniaxial stretching. Control (CT) samples were mounted into the loading chamber for 1 hour every 24 hours, but not stimulated. Three samples of HaCaT monolayer grown for 24 hours onto collagen I-coated silicon stripes were fixed before stimulation (time 0 untreated samples, CT0) using formaldehyde 3.7% and sucrose 3% solution, while all the others samples were fixed at the end of

treatments. Monolayer images were taken using Gel Doc imaging system (BIORAD, Milan Italy) at 2x magnification and analyzed using Leica Qwin software. Monolayer areas were expressed in mm<sup>2</sup>  $\pm$  standard error (S.E.). Conditioned medium obtained from HaCaT monolayer 24 hours after each mechanical stimulation was analysed by gelatine substrate zymography to assess MMP-2 and MMP-9 presence and activity. Conditioned medium (15  $\mu$ l, protein content = 5  $\mu$ g protein) was electrophoresed without boiling or reduction in SDS-polyacrylamide gels copolymerized with 0.2% gelatin.<sup>12</sup> After SDS removal with Triton X-100, gels were incubated in 0.05 M Tris, pH 7.5, containing 5 mM CaCl<sub>2</sub> and 5 mM ZnCl<sub>2</sub> at 37°C overnight, stained with Coomassie blue and destained. Both proenzyme and active gelatinases were detected as clear bands against the blue background of the stained gelatine. Positive controls for gelatinase A and B (Std, Chemicon Europe, Chandlers Ford, UK) were used to identify the two enzymes and their activated forms. Clear bands densitometric analysis was performed using NIH Image 1.62 software and results were expressed as arbitrary units (a.u.) of optical density (O.D.).

Statistical analysis of data was performed using Graph Pad Prism 2.01 software. Anova Test followed by Bonferroni's post hoc test method was used taking  $p < 0.05$  as the minimum level of significance.

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