# Mechanical Stretching *In Vitro* Regulates Signal Transduction Pathways and Cellular Proliferation in Human Epidermal Keratinocytes

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**Epidermal keratinocytes are continuously exposed to mechanical forces. The human skin surface can be thickened and enlarged by various stresses such as tissue expander or abrasive pressure. To investigate the mechanism of epidermal hyperproliferation by mechanical stress, keratinocytes were plated on flexible silicone dishes, which were continuously stretched by + 20%. Stretching of cells for 24 h caused upregulation of 5-bromo-2′-deoxyuridine (BrdU)-positive cells to 200%–220% and activation of extracellular signal-regulated kinases (ERK)1/2. Inhibition of mitogen and ERK with U0126 and phosphoinositide 3-OH kinase attenuated BrdU incorporation and ERK1/2 activation. The EGF receptor kinase inhibitor and the calcium channel inhibitor also inhibited BrdU incorporation and the activation of ERK1/2. Twenty-four hours of stretching stimulated reporter activity driven by activator protein 1 (AP-1), induction of K6, and suppression of K10, which were inhibited by U0126. Our results indicate that mechanical stretching induces proliferative signals on human keratinocytes via induction of calcium influx, phosphorylation of epidermal growth factor receptor (EGFR), and ERK1/2. These mechanisms may contribute to the hyperproliferative nature of the epidermis, which is mechanically stretched by various stimuli.**

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The skin, especially the epidermal keratinocytes, which protects the human body from the external environment, is continuously exposed to various stimuli. The effects of chemical and UV light stimulation of the skin and the epidermal keratinocytes have been investigated in detail (Hruza and Pentland 1990; Nickoloff *et al*, 1993), and phototherapy has been applied in dermatology as psoralen and ultraviolet A (PUVA) therapy. Little, however, is known about the consequences and mechanisms of action of mechanical stimuli to the skin, especially to the epidermis. The human skin surface can be stretched and enlarged by tissue expander apparatus for skin grafts. The abdominal skin of pregnant women is stretched and extended, which allows the skin to cover a markedly increased area. During wound healing, retracting adjacent tissue may stimulate marginal epidermal cells to cause epithelial spreading into the wound bed. Patients with acanthosis nigricans show skin lesions in intertriginous skin, which is assumed to be induced by continuous mechanical stretching or abrasion. Psoriasis vulgaris tends to affect the skin of rubbed areas such as the elbows and knees. Many dermatological diseases present the Köebner phenomenon, in which mechanically injured skin promotes new eruption. The mechanisms of these phenomena, however, remain to be fully elucidated. Recently, Kippenberger *et al* (2000) reported that $^3$H-thymidine uptake was induced and extracellular signal-related kinase (ERK)1/2 and c-Jun amino terminal kinase (JNK) were activated in response to keratinocyte stretching. As for fibroblast stretching, fibroblast–collagen matrix contraction causes isometric tension to fibroblasts, ERK and p38 MAP kinases are activated (Lee *et al*, 2000), and the level of c-fos mRNA increases (Rosenfeldt *et al*, 1998), which indicates that these events play a unique role for wound contraction and wound healing (Grinnell, 2000).

Mechanical forces are related to many biological phenomena. Isometric strength training and flexibility training of the human muscles thicken muscle fibers and increase the amount of these muscles (Goldspink *et al*, 1991; Timson, 1991; Booth *et al*, 1998). It is widely accepted that mechanical loading is necessary to construct the architecture of bone and to maintain bone mass (Salter *et al*, 2000; Kurata *et al*, 2001). These observations support the theories of athletic training and rehabilitation. In the cardiovascular field, mechanical forces applied to rat vascular smooth muscle cells (VSMC) were shown to induce the phosphorylation of epidermal growth factor receptor (EGFR) (Iwasaki *et al*, 2000) and mitogen-activated protein kinase (MAPK) (Komuro *et al*, 1996; MacKenna *et al*, 1998; Seko *et al*, 1999; Kushida *et al*, 2001), which then

promoted cell proliferation and hypertrophy of VSMC (Komuro *et al*, 1991; Sadoshima *et al*, 1992; Hishikawa *et al*, 1994). Other groups reported that exposure of human endothelial cells to fluid shear stress stimulated MAPK (Tseng *et al*, 1995). Shear stress was also reported to stimulate the migration and proliferation of endothelial cells (Ando *et al*, 1987) and enhance endothelial cell DNA synthesis during the repair of mechanical denudation (Ando *et al*, 1990). These results may support the mechanism of the alterations in cardiovascular and endothelial morphology and function induced by hypertension.

These phenomena surrounding human skin and other organs prompted us to further investigate the nature of stretched epidermal cells, including the signal transduction pathways induced by mechanical stretching. We demonstrated that mechanical stretching promoted the proliferation of cultured human keratinocytes by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation and that mechanical stretching also inhibited differentiation by the upregulation of keratin K6 and the downregulation of keratin K10.

# Results

**BrdU incorporation was induced by mechanical stretching** To verify observations on the nature of the epidermis surrounding intraepidermal tumors, we examined whether the stretching signal really induced proliferation of cultured keratinocytes on flexible silicone chambers (see Fig 1).

We showed that continuous mechanical stretching of keratinocytes by $+20\%$ for 24 h significantly induced BrdU incorporation by $2.2 \pm 0.4$-fold in NHK (Fig 2) and $2.0 \pm 0.5$-fold in HaCaT (not shown) compared to non-stretched controls ($p < 0.05$). U0126 (10 μM), wortmannin (1 μM), AG1478 (200 nM), and gadolinium (150 μM), inhibitors of MEK1/2, PI 3-K, EGF receptor, and calcium influx, respectively, strongly inhibited BrdU incorporation induced by mechanical stretching (Fig 2). This suggested that
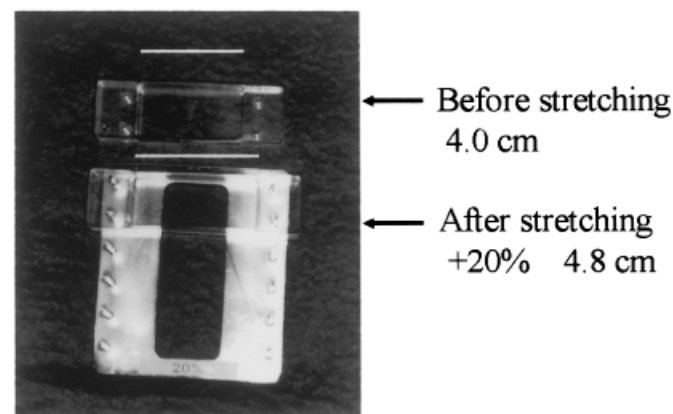
mechanical stretching induced keratinocyte proliferation, i.e., S-phase entry, and that keratinocyte proliferation induced by mechanical stretching was MEK1/2 pathway-, PI 3-K pathway-, EGFR-, and calcium channel-dependent.

**Mechanical stretching activates ERK1/2** The above results indicated that mechanical stretching induces cell proliferation through ERK1/2 and PI 3-K pathways, which are also activated by various growth factors and cytokines such as EGF. We examined ERK1/2 phosphorylation by mechanical stretching by harvesting keratinocytes after 1–60 min of stretching and performed western blotting using an antibody to phosphorylated ERK1/2.

Western blotting revealed that phosphorylation of ERK1/2 arose from 2 min of stretching, which reached the maximal level at 5 and 15 min, and then ceased after 30 and 60 min (Fig 3*A*). On the same membrane re-probed with anti-ERK2 antibody, the density of ERK2-positive bands was almost the same among all lanes (Fig 3*B*). The phosphorylation of ERK1/2 by EGF (50 ng per mL) was strongly detected from 1 min and lasted up to 60 min (Fig 3*C*). These results suggested that mechanical stretching activated the ERK1/2 signaling pathway in epidermal keratinocytes *in vitro*.

**ERK1/2 activation by mechanical stretching occurs through both MEK1/2 and PI 3-K pathways, while activation by EGF occurs only through MEK1/2** MEK1/2 and PI 3-K are the two major kinases activated by growth stimulatory signals such as EGF. We next investigated whether MEK1/2 and PI 3-K pathways are involved in ERK1/2 activation by mechanical stretching. ERK1/2 activation by mechanical stretching was abolished by pre-treatment with both MEK1/2 inhibitor U0126 (30 min) and PI 3-K inhibitor wortmannin (60 min) in a concentration-dependent manner, while ERK1/2 activation by EGF (50 ng per mL) was affected only by U0126 (10 μM) but not by wortmannin in all doses (Fig 4*A*, *B*). These results indicated that both EGF and
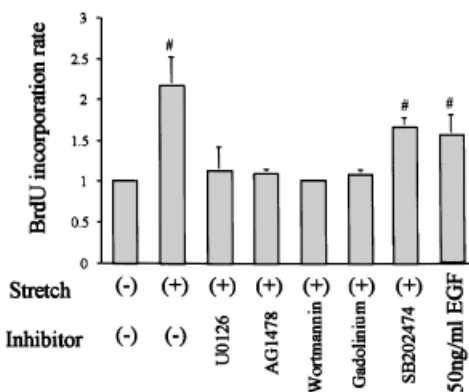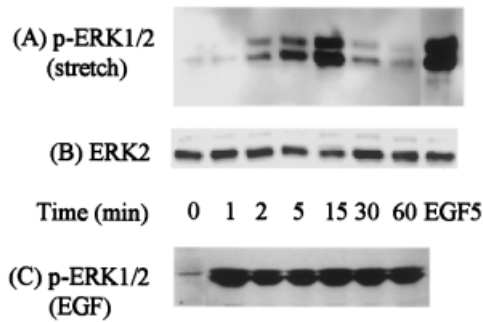


**Figure 1**
**Stretching apparatus.** Cells were seeded and grown in transparent and cubic silicone chambers, the bottom of which was covered with collagen type I as described in *Materials and Methods*. The overall view of the flexible silicone chamber before stretching and after stretching to $+20\%$ continuously and longitudinally using a stainless-steel stretching device was shown.



**Figure 2**
**BrdU incorporation was induced by mechanical stretching.** Continuous stretching of NHK for 24 h and EGF (50 ng per mL) stimulation caused upregulation of BrdU-positive cells to 200%–220% as detected using a commercial BrdU ELISA kit. This upregulation was inhibited by MEK1/2 inhibitor U0126, PI 3-K inhibitor wortmannin, EGFR phosphorylation inhibitor AG1478, and calcium channel blocker gadolinium ($Gd^{3+}$). SB202474 was used as a negative control and did not inhibit BrdU upregulation by mechanical stretching (#: $p < 0.01$).

**Figure 3**
**Mechanical stretching causes ERK1/2 phosphorylation.** Time courses of ERK1/2 phosphorylation induced by mechanical stretching and EGF treatment of NHK. Equal amounts of protein extracts were immunoblotted with antibodies specific for (*A*) phosphorylated ERK1/2 (p-ERK1/2) and (*B*) ERK2 (after stripping the same membrane). p-ERK1/2-positive bands showing phosphorylation were seen after 5–15 min of stretching and disappeared after 30–60 min of stretching. (*C*) On the other hand, strong phosphorylation of ERK1/2 induced by EGF (50 ng per mL) was maintained for longer periods than that induced by stretching.

mechanical stretching activated ERK1/2. EGF utilized only the MEK1/2 pathway however, while stretching utilized both MEK1/2 and PI 3-K pathways.

**Calcium influx is indispensable for activation of ERK1/2 by mechanical stretching, but not for its activation by EGF** Calcium metabolism is an important factor in the activation of ERK1/2 in several lines of cells. To investigate the effects of calcium metabolism in the activation of ERK by mechanical stretching, we utilized the calcium influx inhibitor gadolinium ($Gd^{3+}$). ERK1/2 phosphorylation by mechanical stretching as determined by western blotting was almost completely inhibited by $Gd^{3+}$ pre-treatment (for 60 min, 150 μM), and the inhibition of ERK1/2 phosphorylation by mechanical stretching was concentration-dependent, i.e., 50 μM $Gd^{3+}$ showed moderate inhibition, and 15 μM $Gd^{3+}$ showed only a slight inhibitory effect. On the other hand, activation of ERK1/2 by EGF stimulation (50 ng per mL) was not inhibited by all concentrations of $Gd^{3+}$ (Fig 5). These phenomena, in addition to the experiment with MEK1/2 and PI 3-K inhibitor, delineated the EGF signal transduction pathway and the mechanical stretching-induced signal transduction pathway.

**ERK1/2 activation by mechanical stretching requires EGF receptor phosphorylation** We inhibited EGF receptor phosphorylation to determine whether it affects the activation of ERK1/2 by mechanical stretching. The EGF receptor

kinase inhibitor AG1478, which specifically inhibits EGF receptor autophosphorylation, inhibited the activation of ERK1/2 in NHK by mechanical stretching as well as the activation of ERK1/2 by EGF in a concentration-dependent manner. AG1478 at 200 nM showed almost perfect inhibition, 20 nM AG1478 showed moderate inhibition, and 2 nM AG1478 showed only a slight inhibitory effect on ERK1/2 activation both by mechanical stretching and by EGF (Fig 6). These results suggested that mechanical stretching induced ERK1/2 activation through the activation of EGF receptors probably without stimulation by its ligand.

**Mechanical stretching also induced the phosphorylation of EGFR** As EGFR autophosphorylation is responsible for the activation of ERK1/2 by mechanical stretching as described above, we next directly investigated EGFR phosphorylation using anti-phospho-EGFR (Tyr845, Tyr992, Tyr1045, Tyr1068) antibodies. Western blotting analysis showed that only Tyr845-EGFR phosphorylation was augmented at 2 and 5 min, as compared to the control (0 min), but decreased thereafter. Tyr992, Tyr1045, or Tyr1068-EGFR phosphorylation was not detected by mechanical stretching, although EGF stimulation strongly phosphorylated all phospho-EGFR antibodies (Fig 7*A*–*E*). Immunoprecipitation analysis showed that EGFR phosphorylation was augmented at 5–10 min, as compared to the control (0 min), but decreased thereafter (Fig 7*F*). These results suggested that mechanical stretching induced phosphorylation of a part of EGFR prior to ERK1/2 phosphorylation.

**AP-1-driven reporter activity is induced by mechanical stretching** AP-1, which is a complex of Jun, Fos, or activating transcription factor (ATF), is one of the transcription factors activated by EGF and other cytokines such as TNF and IL-1. We investigated whether the mechanical stretching signal induces AP-1 activation. We transfected keratinocytes with an AP-1 site-driven firefly luciferase construct together with TK promoter-driven renilla luciferase vector, and performed dual luciferase assay. AP-1 luciferase activities were normalized with renilla luciferase activity. Six to forty-eight hours of stretching signal significantly stimulated AP-1 consensus sequence-driven reporter activity in both NHK and HaCaT (not shown) as compared to non-stretched keratinocytes, similar to the effect of EGF (50 ng per mL) (Table I). The activation of AP-1-driven reporter activity by mechanical stretching and EGF stimulation was strongly suppressed by MEK1/2 inhibitor U0126 pre-treatment (30 min, 10 μM) to almost the same level as in

**Figure 4**
**Mechanisms of ERK1/2 activation were different between mechanical stretching and EGF stimulation.** Both (*A*) U0126 and (*B*) wortmannin suppressed p-ERK1/2 by mechanical stretching in a concentration-dependent manner in NHK. Only U0126 (10 μM) suppressed p-ERK1/2 in cells stimulated with EGF. The densities of the ERK2 bands in each lane were almost equivalent (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).
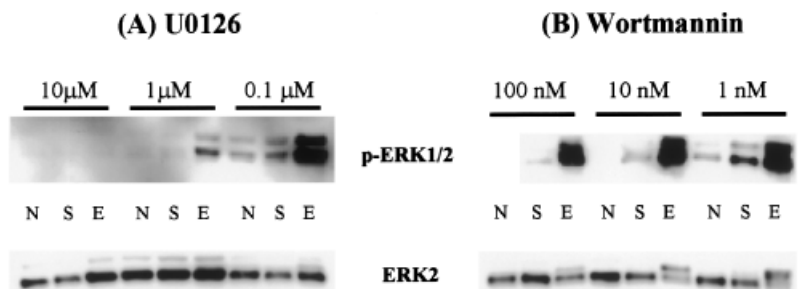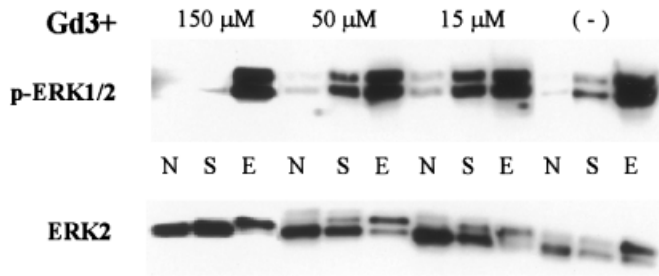
**Figure 5**
**ERK1/2 phosphorylations by mechanical stretching require cal-cium influx.** $Gd^{3+}$ inhibited ERK1/2 phosphorylation by mechanical stretching in a concentration-dependent manner in NHK (15, 50, 150 μM concentration), while it did not inhibit phosphorylation of ERK1/2 induced by EGF stimulation at these concentrations (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).
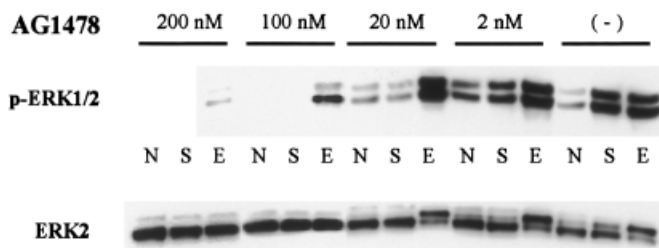


**Figure 6**
**ERK1/2 activation by mechanical stretching requires EGF receptor phosphorylation.** AG1478 inhibited ERK1/2 phosphorylation induced by mechanical stretching and EGF stimulation in a concentration-dependent manner in NHK (N, non-stretched NHK lysate; S, NHK stretched for 15 min; E, NHK stimulated with 50 ng per mL EGF for 15 min).

non-stretched controls (Table I). Our results indicated that mechanical stretching induces AP-1 activity via MEK1/2 activation in human keratinocytes.

**Mechanical stretching induces epidermal keratinocyte activation with K6 induction and K10 suppression *in vitro*** Keratins are convenient markers to investigate keratinocyte differentiation. We examined two of the keratins, keratin K6 and keratin K10, which represent activated and differentiated keratinocytes, respectively, in cultured human keratinocytes. Western blotting analysis indicated that 24 h stretched and 50 ng per mL EGF-treated keratinocytes showed strong K6 expression compared to sparse K6 expression in non-stretched controls. Pre-treatment with the MEK1/2 inhibitor U0126 (30 min, 10 μM) abolished these effects (Fig 8*A*, *B*). On the other hand, K10 was not observed in stretched and 50 ng/ml EGF-treated keratinocytes, while non-stretched keratinocytes showed strong K10 expression. U0126 pretreatment (30 min, 10 μM) decreased K10 suppression caused by mechanical stretching and EGF (50 ng per mL) (Fig 8*C*, *D*). These results suggested that mechanical stretching makes quiescent keratinocytes activated rather than differentiated via MEK1/2 activation.

## Discussion

We could demonstrate several effects of mechanical stretching *in vitro*. As described in *Materials and Methods*,

**Table I. AP-1-driven reporter activity is induced by mechanical stretching**

| Stretching time (h) | AP-1 activation ratio (against no stretch) |
|---|---|
| +20% strech | |
| 6 | $1.52 \pm 0.37$ times[*] |
| 12 | $2.05 \pm 0.55$ times[*] |
| 24 | $1.87 \pm 0.24$ times[*] |
| 48 | $1.41 \pm 0.23$ times[**] |
| EGF 24 | $2.08 \pm 0.71$ times[*] |
| U0126 pre-treatment | |
| 12 | $1.04 \pm 0.08$ times |
| 24 | $1.08 \pm 0.08$ times |
| EGF 24 | $1.15 \pm 0.32$ times |

Luciferase activity in NHK cell lysates was measured using a luminometer. About 6–24 h of stretching signal stimulated AP-1 consensus sequence-driven luciferase reporter activity, similar to the effect of EGF. The activation of AP-1-driven luciferase reporter activity by mechanical stretching and EGF was suppressed by U0126 pretreatment.
[*]$p < 0.01$,
[**]$p < 0.05$ by using Student's *t* test.

we utilized a unique culturing and stretching system. In this system, basal collagen film itself does not contract or peel off, and it stretches synchronously with a silicon chamber, which enabled attached keratinocytes to be stretched exactly by +20%. First, stretching of keratinocytes seeded on silicone wells for 24 h caused the upregulation of BrdU-positive cells to 200%–220%, and this upregulation was inhibited by the MEK1/2 inhibitor U0126, PI 3-K inhibitor wortmannin, EGFR phosphorylation inhibitor AG1478, and calcium channel blocker $Gd^{3+}$. These observations indi-cated that mechanical stretching induces the S-phase entry of keratinocytes via MEK1/2, PI 3-K, EGFR, and calcium channel-dependent pathways. Although there is also a possibility that the effects of stretching are mediated by releasing soluble factors from stretched keratinocytes themselves, this is the first study illustrating the signaling molecules that induce BrdU incorporation by mechanical stretching in human keratinocytes.

ERK1/2 activation in various types of cells is indispen-sable in cell proliferation and survival (Boulton *et al*, 1991; Guyton *et al*, 1996; Whelchel *et al*, 1997). It is reported that during fibroblast contraction of stressed collagen matrices under isometric tension, both ERK and p38 MAP kinases were activated (Lee *et al*, 2000). We demonstrated that mechanical stretching induced ERK1/2 phosphorylation in a time-dependent manner via a calcium channel and EGFR activation-dependent pathway, thereby leading to the stimulation of AP-1 activity in NHK and HaCaT cells. Moreover, Akt phosphorylation was also induced by mechanical stretching in a time-dependent manner via EGFR activation and calcium channel activation, leading to an anti-apoptotic effect (Yano *et al*, unpublished data). Interestingly, wortmannin and U0126 both inhibited ERK1/2 phosphorylation induced by mechanical stretching. On the other hand, ERK1/2 phosphorylation due to EGF stimulation was not inhibited by wortmannin but was inhibited only by

**(A) p-EGFR (Tyr845)**

**(B) p-EGFR (Tyr992)**

EGF5   0   1   2   5   15  30  60        EGF5   0   1   2   5   15  30  60

**(C) p-EGFR (Tyr1045)**

**(D) p-EGFR (Tyr1068)**

EGF5   0   1   2   5   15  30  60        EGF5   0   1   2   5   15  30  60

**(E) EGFR**

**(F) IP: EGFR, IB: 4G10**

EGF5   0   1   2   5   15  30  60        EGF5   0   1   2   5   10  15  30  60

**Figure 7**
**Mechanical stretching induces phosphorylation of EGFR.** Time course (0–60 min) of EGFR phosphorylation after stretching NHK. (*A*) EGFR phosphorylation (Tyr845) was seen only after 2–5 min of stretching and disappeared after 15–60 min of stretching. (*B–D*) EGFR phosphorylation (Tyr992, Tyr1045, Tyr1068) was not detected. (*E*) EGFR bands detected on the same membrane were also shown. The densities of bands in all lanes were almost equivalent. (*F*) After immunoprecipitation (IP) with EGFR antibody, EGFR phosphorylation (4G10) was seen only after 5–10 min of stretching and disappeared after 15–60 min of stretching (IP, immunoprecipitation, IB, immunoblot).
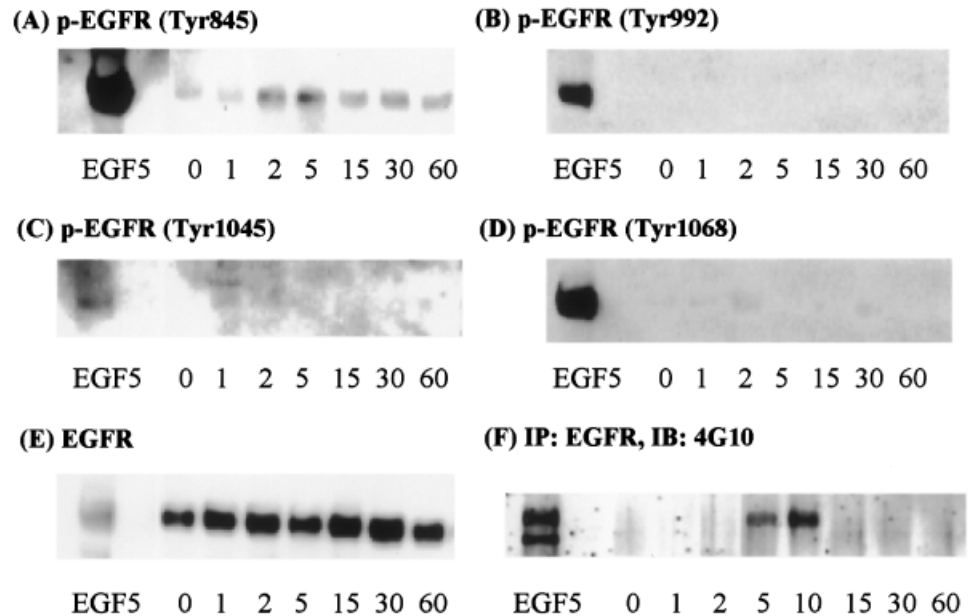
U0126. These results suggested that PI 3-K and MEK1/2 pathways may function as activators of ERK in mechanical stretching in these cells, and that both EGF and mechanical stretching activate ERK1/2 through EGFR phosphorylation, while their downstream signal transduction pathways are not exactly the same. The results of the present study represent the first evidence that PI 3-K is responsible for ERK1/2 activation by mechanical stretching in human keratinocytes.

Calcium channel activation on the cell membrane and calcium influx play crucial roles in controlling cell growth (Taylor and Simpson, 1992; Dascalu *et al*, 2000; Wang *et al*, 2000). We showed in this study that the calcium channel blocker gadolinium inhibited the phosphorylation of ERK1/2 induced by mechanical stretching. This result indicated that calcium channels receive mechanical stimuli, and then transmit the signals to downstream pathways. The observation that the calcium channel blocker gadolinium did not affect the phosphorylation of ERK1/2 due to EGF stimulation supports the idea that there are differences in signal transduction between mechanical stretching and EGF stimulation, although both cause EGFR phosphorylation. EGFR exists on the cell membrane, and receives stimulation from EGF and other members of the EGF family. The binding of integrin $\alpha 2\beta 1$ and EGFR is required for EGFR phosphorylation in A431 cells (Yu *et al*, 2000). Therefore, we hypothesized that EGFR received mechanical stimuli and was phosphorylated, and then downstream signals of EGFR such as MEK1/2 and PI 3-K were activated. We showed that EGFR phosphorylation (Tyr845) occurred after 2–5 min of keratinocyte stretching, and that AG1478, an inhibitor of EGFR phosphorylation, blocked the phosphorylation of ERK1/2 induced by mechanical stretching. We also showed EGFR phosphorylation by immunoprecipitation. These results suggested that the mechanical stimulation by stretching of human keratinocytes is received by EGFR, which results in MAPK activation. Similar phenomena were reported in VSMC, which showed the phosphorylation of EGFR (Iwasaki *et al*, 2000), followed by activation of the Ras and Raf pathways (Yamazaki *et al*,

1995; Li *et al*, 1999) upon mechanical stretching. We showed that mechanical stretching phosphorylated only the Tyr845 site compared to EGF stimulation which phosphorylated many sites (Tyr845, Tyr992, Tyr1045, Tyr1068) and that the density of positive bands by mechanical stretching was weaker than that by EGF stimulation (Fig 8*A*, *B*). These suggested that the effect on EGFR by mechanical stretching is neither more various nor stronger than that by EGF stimulation and that their downstream signal transduction pathways are consequently different from each other.

Keratins constitute a large family of cytoskeletal proteins, differentially expressed in various epithelial cells (Schweizer, 1993). K6 is a marker of activated keratinocytes (Komine *et al*, 2000) and is expressed in psoriasis and carcinomas, but not in the healthy interfollicular epidermis. EGF and TNF$\alpha$ induce K6 in keratinocytes (Jiang *et al*, 1993; Komine *et al*, 2000) and there is an AP-1 binding site in the K6 gene promoter (Bernerd *et al*, 1993; Ma *et al*, 1997). We showed that mechanical stretching of keratinocytes for 24 h induced K6 upregulation, which was strongly inhibited by MEK1/2 inhibitor U0126. This result suggests that mechanical stretching induced the signals resulting in keratinocyte activation and proliferation, which were probably mediated via the MEK1/2 activation and AP-1 activation pathway. Although there is a discrepancy between the strong activation of K6 expression and the relatively small AP-1 activation induced by stretching, we suggest that the expression of K6 gene requires not only AP-1 activation but also other signaling or stimulating factors. Unexpectedly, the expression of K10 was clearly abolished when keratinocytes were stretched for 24 h, and K10 downregulation was simultaneously suppressed by U0126. K10 is a marker of differentiated keratinocytes (Ivanyi *et al*, 1989) and is expressed in the differentiating suprabasal layers. These phenomena suggested that mechanical stretching suppresses keratinocyte differentiation probably via the MEK1/2 activation pathway. In the epidermis of normal human palm and sole skin, keratin K9, K6, K16, and K17 were shown to be expressed at the bottom of the deep

primary epidermal ridges possibly due to the greater stress that ridged skin has to withstand (Swensson *et al*, 1998). This study, however, is the first to demonstrate that mechanical forces can alter the expression patterns of keratin, the constituent of intermediate filaments expressed specifically in keratinocytes.

Our investigations elucidated that early activation of signaling pathways such as EGFR, ERK1/2, and PI 3-K in response to mechanical stretching is involved in epidermal cell proliferation, and keratinocyte activation including keratin K6 expression and keratin K10 suppression. Although it shares many features with EGF signaling, substantial parts of the response to mechanical stretching are probably different from those of the response to EGF stimulation. Moreover, we can hypothesize that mechanical stretching on the epidermis or keratinocytes enables epidermal regeneration and establishes new methods of keratinocyte proliferation. Our data and model are useful for explaining cutaneous changes caused by mechanical stress in cases of tissue expander, pregnant women, wound healing, and the Köebner phenomenon, and present additional insights for signaling pathways in human keratinocytes. We are now investigating further functions and clinical application using mechanical stretching systems.

## Materials and Methods

**Antibodies and materials** Anti-phospho-ERK1/2, anti-phospho-EGFR (Tyr845, Tyr992, Tyr1045, Tyr1068), and anti-EGFR were purchased from Cell Signaling (Beverly, Massachusetts). Anti-ERK2, anti-mouse IgG horseradish peroxidase (HRP) conjugate, and anti-rabbit IgG HRP conjugate were from Santa Cruz (Santa Cruz, California). HRP-conjugated anti-phosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, New York). Anti-cytokeratin 6 (K6) was from Progen (Heidelberg, Delaware). Anti-keratin 10 (K10) was from Sanbio (San Diego, California). The mitogen and extracellular signal-regulated kinase (MEK1/2) inhibitor U0126 was from Promega (Madison, Wisconsin). The phosphoinositide 3-OH kinase (PI 3-K) inhibitor wortmannin, the EGFR kinase inhibitor AG1478, and negative control SB202474 were from Calbiochem (San Diego, California). The calcium channel blocker gadolinium ($Gd^{3+}$) was from Sigma (St Louis, Missouri).

**Cell culture** Normal human keratinocytes (NHK) were purchased from Clonetics (San Diego, California) and were cultured in keratinocyte-SFM (KBM) supplemented with epidermal growth factor and bovine pituitary extract (KGM, Life Technologies, Rockville, Maryland), and used at passage 3 to 5. Spontaneously immortalized human keratinocyte cells (HaCaT) were a generous gift from Dr. Kuroki (Showa University, Japan) with permission from Dr Fusenig (Institute Fur Zell- und Tumourbiologie, Deutsches Kresforschungszentrum, Heidelberg, Germany) and were maintained in minimal essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum, and used at passage 41 to 50. Both types of cells were plated at $1 \times 10^5$ cells per $cm^2$ into flexible and transparent silicone chambers (Taiyo Kogyo, Tokyo, Japan) (Fig 1). Prior to plating, we covered the bottom of chambers with collagen type I (Sigma, 2.5 μg per $cm^2$) solution, and then dried them overnight. Subsequently, a thin collagen film was formed on the surface of the chamber, which enabled keratinocytes to attach the chambers. Chambers were cultured at 37°C and in a 5% $CO_2$ incubator. After reaching confluence, the cells were incubated in KBM or MEM for 24 h and then continuously stretched longitudinally by +20% on a stainless-steel stretching device (Towa Kagaku, Tokyo, Japan) (Fig 1). The stretching chambers was

achieved by hand carefully, and then we laid them over the stretching device. It took 30 s to stretch and lay them. Finally, chambers and devices were cultured at 37°C and in a 5% $CO_2$ incubator.

**Proliferation assay** Confluent NHK or HaCaT cells were stretched continuously for 24 h as described above. For the last 3 h of the 24 h period of stretching, cells were pulsed with 100 μM BrdU. Subsequently, cells were scraped into PBS, and then transferred to 96-well plates and centrifuged. After drying the plates, the incorporation rate of BrdU was determined by Cell Proliferation ELISA with a BrdU colorimetric system (Roche, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells were fixed with ethanol for 20 min and then incubated with peroxidase-conjugated anti-BrdU antibody (1:100) for 60 min. After washing the cells three times, the reaction with tetramethylbenzidine as a substrate was performed for 15 min. The reaction was stopped by adding 1 N $H_2SO_4$. Absorbance at 450 nm was measured immediately using a microplate reader (Model 550, BIO-RAD, Hercules, California).

**Immunoblotting and immunoprecipitation** Cells were stretched for the indicated periods, scraped into lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 1 μg per mL leupeptin), sonicated, and microcentrifuged for 10 min at 4°C. Supernatants containing equal amounts of protein were boiled for 5 min at 95°C with 2 × SDS sample buffer, separated by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with anti-phospho-EGFR (1:1000), anti-phospho-ERK1/2 (1:1000), anti-ERK2 (1:500) or anti-EGFR (1:1000) antibody overnight at 4°C. The bound primary antibodies were detected using appropriate secondary antibodies conjugated with HRP (1:1000) for 60 min at room temperature and visualized using an ECL detection kit (Cell Signaling). The membranes were exposed to X-ray film, which was developed and visualized.

Immunoprecipitations were performed as described (Fujimoto *et al*, 2000). Briefly, cell lysates extracted above were incubated with anti-EGFR (1:100) antibody and protein A/G agarose for 3 h at 4°C. After washing, the pellets were mixed with 2 × SDS sample buffer and boiled. After centrifuging, the supernatants were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted with HRP-conjugated anti-phosphotyrosine 4G10
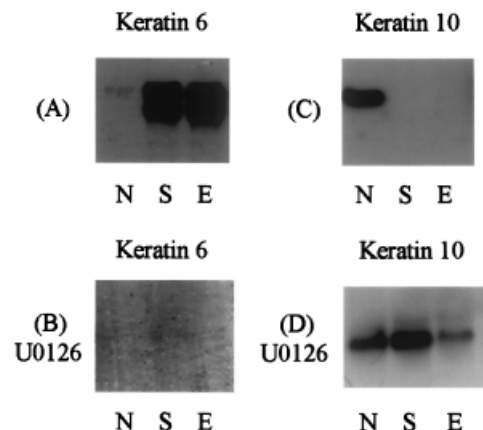


**Figure 8**
**Mechanical stretching induces epidermal keratinocyte activation with keratin K6 induction and K10 suppression *in vitro*.** NHK were stretched for 24 h, and then collected and assayed as described in *Materials and Methods*. (*A, C*) Keratin K6 induction and keratin K10 suppression by mechanical stretching and EGF stimulation were detected. (*B, D*) U0126 abolished these effects on keratin K6 induction and keratin K10 suppression (N, non-stretched NHK lysate; S, NHK stretched for 24 h; E, NHK stimulated with 50 ng per mL EGF for 24 h).

(1:10,000) antibody. All figures present representative results from three independent experiments, all of which yielded similar results.

**DNA transfection and luciferase reporter assay** The plasmid containing four activator protein 1 (AP-1) binding sites (TG-AGTCAGTGAGTCACTGACTCACTGACTCATGAGTCAGCTGACTC) and the firefly luciferase reporter gene (Clonetech, Palo Alto, California), and a control plasmid containing the herpes simplex virus thymidine kinase (TK) promoter region and renilla luciferase reporter were co-transfected into confluent cells using the polybrene-DMSO shock method (Jiang *et al*, 1991). After transfection, NHK or HaCaT were incubated in medium with KGM or MEM with 10% FCS, respectively, for 24 h and then incubated without EGF or FCS for another 24 h, respectively. Then, cells were stretched for the indicated time. Using a Dual Luciferase Reporter Assay system (Promega), cells were collected and lysed, and then the dual intracellular luciferase activity was measured with a luminescencer-PSN (Atto, Tokyo, Japan).

**Keratin extraction and immunoblotting** After cells reached confluence, they were continuously incubated in KGM and then stretched for 24 h. Cells were collected into PBS with a cell scraper and centrifuged. The pellets were lysed using buffer A (25 mM Tris, 1.5 M KCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM EGTA, 2 mM PMSF), and the keratin was extracted from insoluble pellets using buffer B (25 mM Tris, 9.5 M urea). These extracts were separated by SDS-PAGE, transferred onto PVDF membranes, and immuno-blotted with anti-keratin K6 (1:200) and K10 (1:200) antibodies. The bound primary antibodies were detected using anti-mouse-IgG HRP conjugate (1:1000). The membranes were exposed to X-ray films, which were developed and visualized. All figures present representative results from three independent experiments, all of which yielded similar results.

**Statistics** Proliferation assays and luciferase reporter assays were performed in four independent experiments, which yielded highly comparable results, respectively. Data are presented as mean values $\pm$ SD as indicated in the results and Table I. Differences between mean values were analyzed with Student's *t* test, and $p < 0.05$ was considered to be statistically significant.

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