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Regulation of Cell Signaling and Function via Changes in Growth Factor Presentation

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Abstract— **The ability to control cell migration has significance in virtually all areas of wound repair and regeneration, and the creation of biomaterials that actively control the migration rate and direction of cells has implications for both the clinic and basic science. In this work, we describe the covalent modification of materials with immobilized growth factor in order to: 1) explore how growth factor presentation impacts cellular response to materials, and 2) pursue the development of bioactive, growth factor-modified materials for dermal wound healing. Specifically, we have cultured human keratinocytes in the presence of epidermal growth factor (EGF) in a soluble form, tethered in a homogeneous pattern, or tethered in a gradient pattern, followed by analysis of cellular signaling, proliferation, and migration in response to these EGF cues. Keratinocyte migration was strongly dependent upon EGF presentation, with soluble EGF eliciting a strong proliferative response and tethered EGF eliciting a strong migratory response. The greatest acceleration of keratinocyte migration was achieved using gradients of immobilized EGF. Such acceleration of cell migration may have a significant impact on the development of treatments to enable faster, more cost-effective wound repair in many different types of tissues. Moreover, better understanding the interaction of cells with soluble vs. immobilized growth factors can help us to elucidate native healing events and achieve greater control over cell function.**

I. Introduction

GROWTH factors and other mitogens often provide the molecular cues that induce cell migration [1]. One of the most important factors in epidermal cell growth and migration is epidermal growth factor (EGF) [2, 3]. EGF is released in abundance by platelets at the wound site and is one of several growth factors that are deficient in chronic wounds. This growth factor has been credited with playing a prominent role in wound closure through stimulation of epithelial cell migration and proliferation; EGF also reduces scarring by preventing excessive wound contraction [4].

Biomolecule Immobilization. Despite the past decade's surge in biomolecule immobilization, little is known regarding differences in cell response to immobilized vs. soluble biomolecules. Immobilized biomolecules have been incorporated in applications ranging from simple patterning and cell adhesion studies to more advanced tissue engineering and biochemistry applications, such as immobilization of platelet derived growth factor to induce differentiation in neural stem cells [5], and nano-scale protein encapsulation to study protein folding [6]. Notably, immobilization of growth factors has emerged as a popular method to create "smarter" biomaterials for tissue engineering applications [5, 7-10]. Growth factors degrade rapidly in the body, leading to increased costs and decreased efficacy of many tissue-engineered materials that deliver growth factors in a soluble form. Biomolecule immobilization prolongs growth factor availability and allows spatial control, reducing cost and increasing effectiveness. Moreover, covalent immobilization also mimics the naturally-occurring biomolecule tethering or sequestration often mediated by the extracellular matrix.

Growth Factors in Wound Healing. In dermal tissue repair, the open wound is closed by the migration of keratinocytes from the wound edges, with re-epithelialization viewed as a hallmark of successful wound care. Unfortunately, individuals with chronic wounds are frequently deficient in the biological cues that stimulate migration (i.e., growth factors), thus contributing to their inability to heal [11]. Treatment strategies for these wounds range from basic wound care techniques (debridement, passive dressing) to use of more advanced materials which incorporate soluble growth factors. However, in addition to being expensive, these growth factor-containing dressings have been met with only limited clinical success, largely due to the inadequate delivery and persistence of the growth factor at the wound site (the soluble growth factor concentration can decrease by 50% within 4 hours of wound dressing application [12]). Covalent tethering of growth factors to biomaterials has the potential to ameliorate many of these problems, and possibly result in increased availability of active growth factor. Moreover, our group has demonstrated that tethering these growth factors in a spatially-defined manner (i.e., in a concentration gradient) can enable precise control over cell migration direction and speed [13]. Ultimately, the clinical goal is to apply these methods to create a multi-functional 3-D patterned wound dressing designed to work with the patient's body to accelerate wound healing, decrease the duration and cost of treatment, and significantly minimize scarring, infection, and serious complications associated with delayed healing.

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II. Materials and Methods

A. Growth Factor Modification and Patterning

Recombinant human EGF (Peprotech, Inc., Rocky Hill, NJ) was rendered photoactive via conjugation to Sulfo-SANPAH (sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino] hexanoate; Pierce Biotechnology, Inc., Rockford, IL) using methods previously described by our group [13, 14]. In an adaptation of the methods used in Ito et al. [15], EGF was photo-immobilized onto polystyrene plates via the phenyl azide functionality of the coupled Sulfo-SANPAH (SS). To create surfaces patterned with gradients of growth factor, standard photo-immobilization techniques were used in combination with a gradient-patterned photomask film created in Adobe Illustrator 10 (Imagesetter, Madison, WI). The slope of the gradient was controlled via alterations in photomask pattern design. The SS-EGF solution was pipetted into tissue culture polystyrene (TCPS) dishes, allowed to dry in an oven at 40°C, covered with film photomasks, and then exposed to ultraviolet light at 365nm wavelength and 90 mW/cm^2 for 120 seconds (Novacure 2001, EXFO UV Curing, Mississauga, Ontario). Upon UV exposure, the phenyl azide group enables immobilization of the SS-EGF to the dish as described previously [14].

B. EGFR and Erk 1/2 Detection

EGF was homogeneously immobilized on culture substrates at two concentrations (1 or 10 ng/cm^2) as previously described [14]. Treatment with soluble EGF was performed by adding EGF in solution (10 or 100 ng/ml) to the culture medium upon cell seeding. The negative control consisted of keratinocytes seeded onto unmodified TCPS surfaces and treated with serum-free DMEM alone. Keratinocytes were seeded in a 96-well plate at 62,500 cells/cm^2, with 6 wells per condition. Quantitative ELISAs for total and phosphorylated EGF receptor (EGFR; Dual Detect CELISA EGFR Assay Kit, Millipore, Danvers, MA), and Erk 1/2 (Dual Detect CELISA Erk Assay Kit, Millipore) were used according to manufacturer's instructions to assay cultured cells at 30 minutes, 1 hour, and 24 hours following exposure to either soluble or immobilized EGF. EGFR or Erk activation was expressed as the amount of phosphorylated EGFR or Erk relative to total EGFR or Erk.

C. Keratinocyte Proliferation

After three days of culture on immobilized EGF (10 ng/cm^2), soluble EGF (10 ng/ml), or the TCPS control, keratinocytes were fixed in 10% formalin for 5 min and rinsed twice with PBS. Proliferating cells were detected via standard immuncytochemical detection of Ki67 (Abcam, Cambridge, MA; 100 µg/ml), using goat anti-rabbit Alexa Fluor 488 as the secondary antibody (Invitrogen, Eugene, OR; 5 µg/ml) and DAPI (1 µg/ml) as a nuclear counterstain. Images of three randomly-selected fields of viewed (100x magnification) were captured in each well of each condition (n = 9). Positive staining for proliferation was indicated by the appearance of green fluorescence, and total cell count was obtained from the number of cells stained with DAPI in each photomicrograph. Proliferation index was then calculated as the number of proliferating cells as percent of the total cell count in each field of view.

D. Keratinocyte Migration in Immobilized vs. Soluble EGF Environments

A modified fence method [16] was used to examine radial migration of keratinocytes. To construct this system, 1 cm diameter silicone elastomer cylinders were placed in the center of each well of a 6 well tissue culture polystyrene plate; these removable cylinders were used to temporarily cover the area containing the radial gradient pattern of immobilized EGF, the homogeneous (non-gradient) immobilized EGF, or an unpatterned area of the same dimensions for the control and soluble EGF. Keratinocytes were seeded at 5 x 10^5 cells/ml in each well surrounding the cylinders in reduced-serum medium (5% FBS). After allowing 24 hours for cell attachment, the reduced-serum medium was replaced with serum-free medium and the cylinders were removed, thus allowing the cells to migrate in the uncovered circular area. For the soluble EGF condition, 10 ng/ml EGF was added daily to the cultures. Cells were fixed in 10% formalin, rinsed with PBS, stained with hematoxylin, and photographed at days 0, 3, and 7.

E. Keratinocyte Alignment

After three days of migration on immobilized radial gradient patterns of EGF, immobilized homogeneous EGF, or unpatterned TCPS in the presence or absence of soluble EGF, keratinocytes were fixed in 10% formalin and stained with phalloidin (5 units/ml) to visualize cytoskeletal f-actin, and DAPI (1 µg/ml), a nuclear counterstain. Photomicrographs (100x) were taken of the cells that were located one field of view behind the leading edge of migration in three random locations on each of three wells for each condition. Photomicrographs were taken such that the center of the well was always at a 90 degree orientation.

A minimum of one hundred cells in each photomicrograph (n = 9) was measured, using DAPI images and ImageJ software. Individual DAPI-stained nuclei were manually circled, and then, using ImageJ software, the nuclei were fit to an ellipse, and the orientation angle measured. Orientation angles were grouped as +/- 15 degrees at 0, 30, 60, 90, 120, and 150 degrees relative to the center of the well. An orientation angle of 90 degrees indicates that the long axis of the cell is aligned with the radii of the circular area the cells migrated into, and thus oriented to be "facing" toward the center of the well.

III. Results

A. Impact of GF Presentation on Cellular Signaling

Keratinocyte intracellular signaling was examined upon culture in environments that presented EGF in either a tethered or soluble form. As shown in Figure 1, significant differences were found in the keratinocyte response to immobilized vs. soluble EGF. At thirty minutes post-seeding

(Figure 1), culture of keratinocytes on immobilized EGF stimulated significantly greater levels of EGFR activation than treatment with soluble EGF ($p<0.0001$) or culture on the TCPS control ($p<0.02$). EGFR activation demonstrated a dose-dependent response to both the immobilized and soluble EGF conditions, as the higher concentrations of immobilized and soluble EGF induced higher phospho-EGFR levels than their lower concentration counterparts ($p<0.005$). Similar trends continued at one hour (not shown), with both concentrations of immobilized EGF eliciting significantly greater EGFR activation than the TCPS control ($p<0.02$) and both soluble EGF treatments ($p<0.0001$). By twenty-four hours post-seeding (not shown), levels of EGFR activation on immobilized EGF conditions were lower in comparison to previous time points, although they still retained their dose-dependent trends ($p<0.005$).

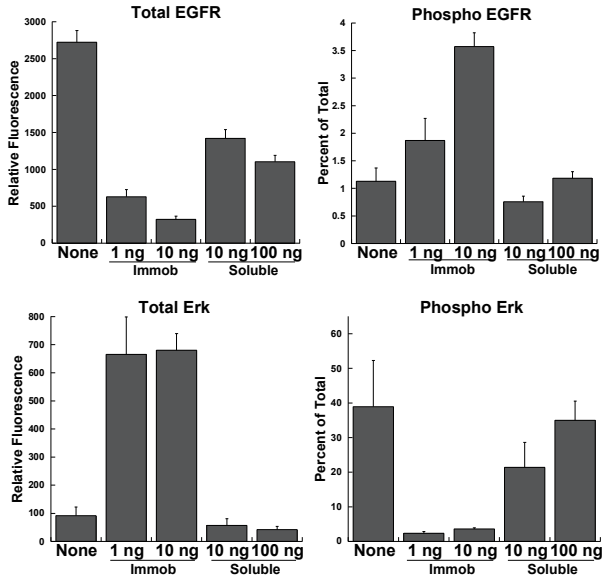

Figure 1. EGFR and Erk activation in keratinocyte cultures 30 minutes post-seeding. Top: Total EGFR (left) and phosphorylated (right) EGFR as percent of total. Bottom: Total Erk 1/2 (left) and phosphorylated (right) Erk 1/2 as percent of total.

To further explore differences in the response of keratinocytes to immobilized versus soluble EGF, cell signaling downstream of EGFR was examined via quantification of Erk 1/2 activation under conditions that were identical to those used for the EGFR experiments. As seen in Figure 1, the hyperstimulation of EGFR by immobilized EGF was accompanied by a large downregulation in Erk phosphorylation ($p<0.002$ vs. soluble EGF). Similar trends continued at one hour (not shown), with both concentrations of immobilized EGF eliciting lower levels of Erk 1/2 activation than the TCPS control ($p<0.01$) and both soluble EGF conditions ($p<0.005$). By twenty-four hours (not shown), Erk 1/2 activation had increased in all conditions except the TCPS control, although Erk 1/2 activation in keratinocytes cultured on immobilized EGF still remained significantly lower than that found in keratinocytes treated with soluble EGF ($p<0.005$).

B. Impact of GF Presentation on Proliferation

Proliferation of keratinocytes cultured for three days on immobilized EGF (10 ng/cm^2), unpatterned surfaces treated with soluble EGF (10 ng/ml), or the TCPS control was examined via staining for Ki67. No proliferation was detected on the TCPS controls, which is not unusual given the serum-free and EGF-free nature of this condition. Meanwhile, keratinocytes treated with soluble EGF demonstrated significantly increased proliferation in comparison to those cultured on immobilized EGF (17% vs. 2.8% proliferating cells, $p<0.00002$).

C. Impact of GF Presentation on Keratinocyte Migration and Alignment

In a modified fence assay, keratinocytes were allowed to migrate on circular areas that had been patterned with EGF (homogeneous pattern or gradient pattern), on unpatterned areas that were treated with EGF in solution ("soluble" EGF), or on unpatterned, untreated TCPS. As seen in Figure 2, the trend in cell migration was: immobilized EGF gradient > homogeneously immobilized EGF > soluble EGF treatment > unpatterned, untreated TCPS, where only keratinocytes on immobilized gradient patterns of EGF migrated to fully close the circular "wound" within 7 days of culture. In all other conditions (TCPS without growth factor, homogeneously immobilized EGF, and soluble EGF), the circular "wound" was not closed within 7 days.

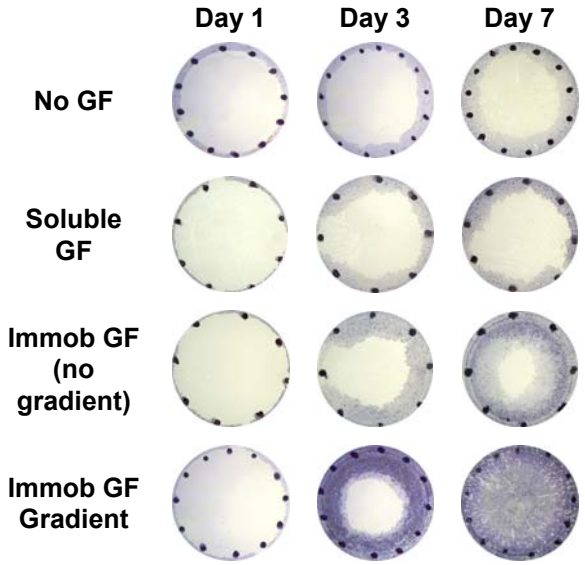

Figure 2. Stereoscope photomicrographs of radial migration of keratinocytes on the untreated and unpatterned TCPS control, unpatterned TCPS treated with soluble EGF, homogeneous patterns of immobilized EGF, and gradient patterns of immobilized EGF at time points of 0, 3, and 7 days following removal of the migration fence. The small black markings were used to indicate the initial boundary of the fence at day 0, and keratinocytes were stained (blue) with hematoxylin at each specified time point.

A quantitative analysis of the trends evident in these images demonstrated that the general manner of EGF presentation – as either an immobilized or soluble molecule

– had the greatest impact on keratinocyte migration, with migration on both immobilized EGF conditions exceeding that achieved with either soluble EGF treatment or the control. By Day 7, wounds receiving no EGF or soluble EGF were 32.9% and 47.7% closed, respectively, while exposure to either a homogeneous or gradient pattern of EGF resulted in 86.2% and 100% wound closure, respectively. Thus, while the effect of changing the type of EGF immobilization – homogeneous vs. gradient – was still significant ($p<0.05$), it was much more modest than the differences observed in comparing immobilized vs. soluble conditions ($p<0.0001$).

Qualitative observations of keratinocyte morphology during radial migration experiments revealed coordinated alignment of keratinocytes cultured on both immobilized presentations of EGF (homogeneous pattern and gradient), but not upon delivery of soluble EGF or culture on the TCPS control. To quantify keratinocyte alignment, DAPI-stained nuclei were measured for orientation relative to the center of the radial migration area, with a 90-degree angle of orientation indicating that the long axis of the cell was oriented along the radial "spokes" of the circle. As seen in Figure 3, keratinocytes on the immobilized EGF condition exhibited a Gaussian distribution trend in orientation in which the majority of cells were at 90 ± 30 degrees. On both the homogeneous pattern of immobilized EGF (Figure 3) and the gradient of immobilized EGF (not shown), significantly more keratinocytes were oriented at 90 degrees than any other orientation angle ($p<0.005$). No trends in cell alignment were evident in either keratinocytes treated with soluble EGF or those cultured on the TCPS control.

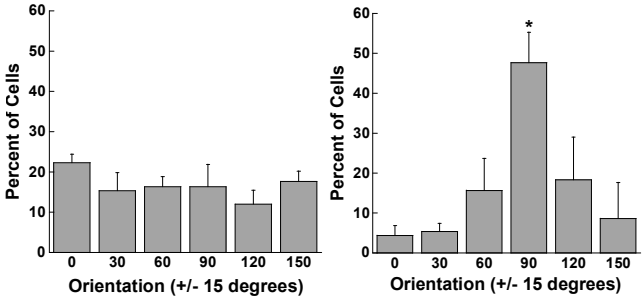

Figure 3. Keratinocyte alignment toward center of circular migration area on day 3 of radial migration experiments. Each bar represents percent of cells oriented in a particular direction, with 90 degrees being oriented toward the center. *Left*: Unpatterned TCPS treated with soluble EGF; *Right*: Homogeneously immobilized EGF. *$p<0.005$ compared to all other orientations.

IV. Conclusions

The goals of engineering controlled environments that regulate cell function or attempting to translate our extensive body of knowledge on soluble growth factors to create immobilized GF systems necessitates characterization of the mechanisms by which cells interact with tethered GFs. Our results suggest that cells interact quite differently with immobilized GFs than they do soluble GFs, which may result in different functional outcomes for the cells. Namely, our findings demonstrate that keratinocytes respond to soluble EGF by engaging in highly proliferative behavior, while their response to tethered EGF is dominated by migration, with very little proliferation. Revealing such differences between immobilized vs. soluble GF recognition and signaling may be useful in both designing bioactive materials and understanding natural physiological phenomena.

With respect to biomaterial applications, many types of tissue repair involve the need to implant materials into a wound site and coerce surrounding cells to infiltrate and populate the material (e.g., polymeric nerve conduits, vascular grafts). With respect to basic science, these materials not only provide a means to control a specific cell function (which has many implications in itself), but they also enable us to characterize how cells recognize and interact with tethered biomolecules, which is both a natural phenomenon that is highly understudied and a synthetic technique that is gaining much use in tissue engineering. The work described herein provides a launching point for the development of more complex wound healing systems that include the incorporation and synergism of multiple immobilized and patterned growth factors, and a better understanding of cellular response to immobilized vs. soluble biomolecules.

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