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The influence of extracellular matrix and growth factors on early hair follicle development using hair patch assay

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Abstract

Background : The hair follicle (HF) undergoes cycles of growth, regression and quiescence throughout the lifetime of the individual. The organization of the HF requires the interaction of epithelial and mesenchymal cells. To understand the interaction of these cells in HF development, a variety of methods were examined to generate the HF structures in the laboratory.

Objective : In order to clarify the influence of extracellular matrix (ECM) molecules, the growth factors (GFs) affecting early HF development, we modified the method recently invented for generating HFs *in vivo* (patch assay method), and analyzed the effect on HF development of type IV collagen and laminin-5 as the ECMs, and keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF)-BB, hepatocyte growth factor (HGF), insulin-like growth factor (IGF)-1 and -2 as the GFs.

Methods : The dorsal skin avulsed from donor C57BL/6J mice (from embryonic day 14 to postnatal day 2) was dissociated into individual cells. Then cell suspensions with various ECM molecules and GFs were intracutaneously injected into the dorsal region of recipient nude (nu/nu) mice. The recipients were sacrificed 10 days after the injection, and the size of the generated patches and the number of HFs were analyzed.

Results : After the addition of type IV collagen, the generated patch areas with HF significantly increased in size compared with the control group. The addition of laminin-5 showed no significant influence in the size of the patches. A significant increase in the number of HF structures was observed after the addition of KGF (at a concentration of 50 ng/ml), PDGF-BB (5 ng/ml), HGF (100 ng/ml), IGF-1 (5, 10 ng/ml) and IGF-2 (10, 30, 100 ng/ml).

Conclusions : We confirmed the efficacy of ECM molecules and GFs on the acceleration of HF development by means of the patch assay method, which appeared to be a useful method for assessing the effect on hair growth of materials recently developed for the treatment of hair loss disorders.

Introduction

The hair follicle (HF) drastically alters its morphology throughout its life-cycle[1)2)]. The HF undergoes the cycles of growth, regression and quiescence, known as the 'anagen', 'catagen' and 'telogen' stages, respectively. In each stage of HF development, various follicular microenvironments are regulated by the extracellular matrix (ECM) and growth factors (GFs). The ECM is composed of collagen, laminin, fibronectin, elastin, and so on. Laminin, a kind of cell adhesion molecule[3)] and its receptor integrin[4)] have frequently been cited as factors regulating the development and growth of various kinds of cell.

The effect of GFs on the early HF morphogenesis has also been widely studied. Recently various methods have been developed to generate HF structures using an epithelial-mesenchymal interaction closely mimicking the

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natural process. For example, the silicon chamber method[5], its modified version[6], hanging drop cultures[7], granulation tissue beds[8], collagenous shells[9] and kidney capsule cultures[10][11] have been introduced. Ying Zheng et al.[12] created a novel method of artificially generating HF structures, their so-called 'hair patch assay system', involving the infusion of dissociated epithelial and mesenchymal cells from donor mice into the dorsal skin of recipient nude mice with various ratios of epithelial and mesenchymal cells. Eight to twelve days after infusion, artificial hair follicles were generated in the intracutaneous tissue of the recipient. This method provided narrow spaces in which epithelial mesenchymal cells could interact with each other to form HFs.

In the present study, we modified the method of Ying Zheng et al. by shortening the time required to dissociate the epithelial and mesenchymal cells by using three kinds of enzymes, and analyzed the effect on HF development of type IV collagen and laminin-5 as ECM molecules, and of KGF, PDGF-BB, HGF, IGF-1, IGF-2 as the GFs.

Materials and Method

Animals

Progenitor cells were obtained from C57BL/6J mice (donor mice) (Charles River Laboratories, Wilmington MA, USA) from embryonic day 14 (E14) to post natal day 2 (P2). Regeneration assays were performed with male nude (nu/nu) mice (recipient) (Charles River Laboratories) at 7-9 wks of age. These animals were fed a standard diet and water ad libitum and were housed at room temperature (24±2°C) on a normal day-night cycle (lightning from 6 am to 6 pm) in the Tokyo Medical University animal facility. All studies were approved by the Animal Care Committee of the Tokyo Medical University animal facility.

Reagents

The reagents of the ECM molecules were bovine type IV collagen (R&D systems Inc., Minneapolis, MN, USA) and mouse laminin-5 (R&D systems Inc.), used in concentrations of 0.3 mg/ml and 1.0 mg/ml, respectively. GFs used were recombinant human keratinocyte growth factor (KGF, FGF-7, PeproTech EC Ltd. London, UK), recombinant murine platelet derived growth factor-BB (PDGF-BB, PeproTech EC Ltd.), recombinant human hepatocyte growth factor (HGF, PeproTech EC Ltd.), recombinant murine insulin-like growth factor-1 (IGF-1, PeproTech EC Ltd.) and recombinant murine insulin-like growth factor-2 (IGF-2, R&D systems Inc.), deployed in concentrations of 10 to 100 ng/ml, 1 to 10 ng/ml, 30 to 500 ng/ml, 1 to 10 ng/ml, and 10 to 100 ng/ml, respectively.

Preparation of cells

The dorsal skin (1×1 cm in size) was avulsed from donor mice at the level of fat tissue. This avulsed skin was defatted and segmentalized into small pieces using micro forceps and scissors under a stereomicroscope, and rinsed in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS). These segmentalized pieces were dissociated into individual cells in the PBS solution with collagenase type I (1.0 mg/mL, Invitrogen Corp., New York, NY), Dispase (1.0 mg/mL, Invitrogen Corp.) and DNAase I (0.1 mg/mL, Worthington Biochemical Corp., Lakewood, NJ) at 37°C for 90 min with a shaker. At each 30 min interval, pipetting was performed to accelerate the dissociation of the cells. After 90 min incubation, the enzyme reaction was stopped with the addition of serum free-DMEM-F12 medium (Invitrogen Corp.). Then the cell suspension was prepared after filtering this solution with mesh-nylon (42 μm). The cells were counted and the dissociated cells were re-suspended in the serum free-DMEM-F12 medium for the adjustment of the medium volume up to 50 μl.

Application of prepared cells and analysis of hair follicle development

The prepared cell suspension was injected into the dorsal region of recipient mice intracutaneously using microsyringes (Hamilton Company, Reno, NV, USA). The mixed cells at 5.0×10^5 containing mesenchymal cells and epidermal cells from P2 mice in 50 μl serum free-DMEM-F12 medium were injected into 6 sites per mouse together with the ECM molecules. The mixed cells at 3.0×10^5 in 50 μl serum free-DMEM-F12 medium were injected into 6 sites per mouse together with the GFs. The recipient was anesthetized by diethylether (Wako Pure Chemical Industries Ltd., Osaka, Japan) before injection. The recipients were housed and sacrificed 10 days after the injection. Then photographs of the newly generated hair follicle formations (hair patch) were taken using a microscopic camera. The effects of the ECM and the GFs were evaluated in terms of the area of the resulting hair patch and the number of hair shafts generated, respectively. The area of the hair patch was calculated by multiplying the diameter of the long and short axes of the patch, each diameter length having been macroscopically measured to the nearest 100^{th} of a millimeter. The generated hair shafts were counted using a stereomicroscope. These data were presented as the mean ±standard deviation (SD) and data plot. Statistical analyses were performed using the Student's un-paired *t* -test.

Results

Determination of the minimal number of injected cells required to generate hair follicle structures

Hair patches generated using the modified method of

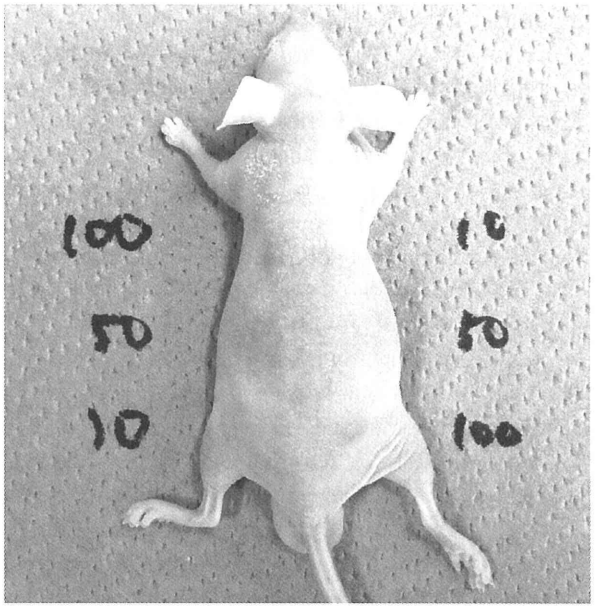

Fig. 1A

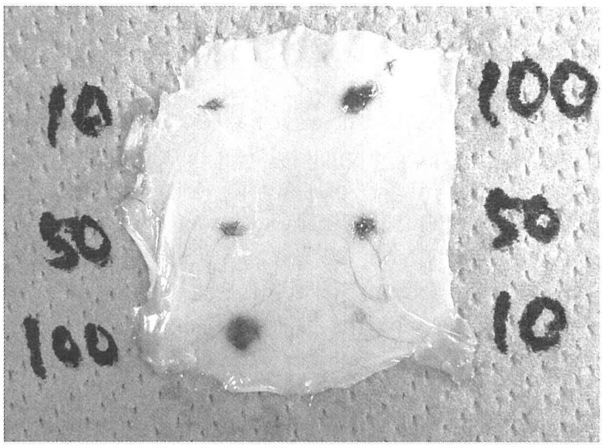

Fig. 1B

Zheng et al[12] were observed as black macules from the dorsal side and black plaques from the ventral side (Fig. 1A, B). The black plaque consisted of hair shafts arranged in a radial manner, as described in the paper of Zheng et al[12]. Repetition of these procedures confirmed that cells from mice older than over E14 generated HF structures whereas the mixed cells collected from P2 and pre-cultured in DMEM-F12 medium with bovine serum for two days failed to show any HF structures (data not shown). Our analysis of the influence of injected cell numbers on generating the HF structures indicated that the minimal number of injected cells required for the generation of HF structures had to be more than 1.0×10^5 cells per injection site ; after the injection of 1.0×10^5 cells into 6 sites, only 2 sites showed visible HF patches (Fig. 1C).

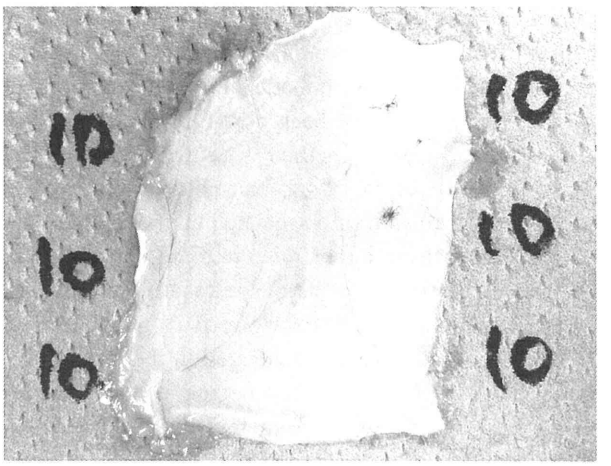

Fig. 1C

Fig. 1 Generated hair patches and plaques
Generated hair patch was observed macroscopically (**A**). The recipients were sacrificed 10 days after the injection. View from the ventral side of the avulsed skin (**B**). Result of the injection of 1×10^5 cells (**C**).

Effect of the ECM on HF development

In the presence of type IV collagen in the infusion medium at a concentration of 0.3 mg/ml, the numbers of generated HF structures significantly increased the area of follicular growth compared with the control group. Fig. 2A shows the area of the hair patches generated (mm^2) in the presence of type IV collagen, and indicates the significant difference between these groups (p-value=0.008, n=11). The addition of type IV collagen enhanced the number of HF generated, leading to an increase in the area of the hair patch. The same experiment using laminin-5 as another ECM molecule also led to the generation of larger areas of HF structures than in the control group, although no significant difference between the type IV collagen and laminin-5 groups was observed (Fig. 2B, p-value=0.07, n=7).

Effect of GFs on the system

In order to increase the concentration of GFs in the injected media, we reduced the cell numbers and counted the hair shafts instead of measuring the area of the HF patches in order to assess the generation of the HF.

The effect of KGF was assessed by adding concentrations of 10 ng/ml, 50 ng/ml, and 100 ng/ml to the infusion medium. The result was a significant (p=0.01) increase in the number of HF structures (Fig. 3A). PDGF-BB was tested at concentrations of 1 ng/ml, 5 ng/ml, and 10 ng/ml, with the result that 5 ng/ml of PDGF-BB was found to significantly increase (p=0.03) the number of HF in comparison with the control (Fig. 3B). HGF at a concentration of 100 ng/ml similarly increased the number of follicles (p=0.02) (Fig. 3C). The addition of

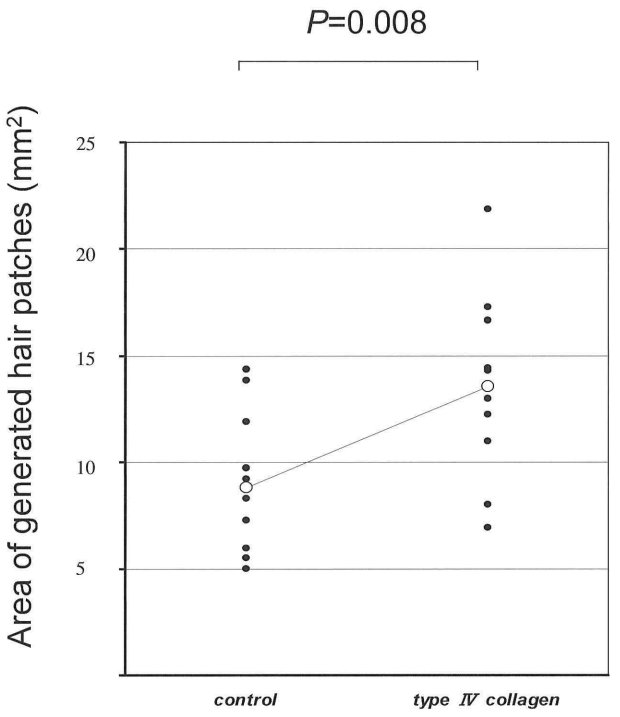

Fig. 2A

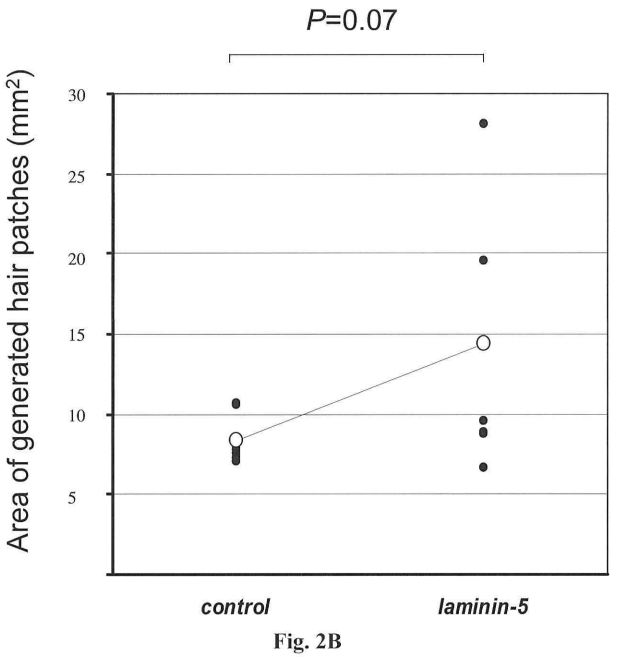

Fig. 2B

Fig. 2 Generation of hair follicle structures after the addition of type IV collage or laminin-5

(**A**) Mean area of generated hair patches (mm^2) of control and type IV collagen group was 8.8±3.4 and 13.6±4.2 (the mean±SD), respectively (n=11).

(**B**) Mean area of hair patches (mm^2) generated in the control and laminin-5 group was 8.4±1.6 and 14.4±8.0 (the mean±SD), respectively (n=7).

IGF-1 produced similar, significant effects at the concentrations of 5 ng/ml and 10 ng/ml (p=0.03 and 0.05, respectively) (Fig. 3D). IGF-2 produced significant enhancement of HF development at all of the tested concentrations (10 ng/ml, 30 ng/ml, 100 ng/ml ; CP=0.02, 0.01, and 0.03, respectively (Fig. 3E)). The optimal concentration for promoting HF morphogenesis was found to be 50 ng/ml for KGF, 5 ng/ml for PDGF-BB, 100 ng/ml for HGF, 5 ng/ml for IGF-1, and 30 ng/ml for IGF-2.

Discussion

The pre-conditions required for a successful hair patch assay system[12] are first, a large quantity of living HF cells and mesenchymal cells ; and second, a relatively compact space in which the injected cells will have ample opportunities to interact. In order to shorten the duration of enzyme treatment and decrease the concentration of Dispase, we modeled our procedure on the method adopted by Ying Zheng et al. of employing collagenase and dispase synergistically[12]. We also used DNase I to degrade the DNA from enzyme-treated cells and to avoid the aggregation of dissociated cells. In the previous study[12], injected cell suspensions were sometimes refluxed over the skin surface. However, our method, using Hamilton® syringes and needles, precluded reflux, and the cells were injected with precision into the target sites in the mouse skin.

Our results, indicating that injected cells older than E14 could form new HF structures, were consistent with the widely accepted view that murine HF morphogenesis generally begins at E13. Our results also indicated that cells older than E14 have already partially differentiated into HF structures, and that HF structures are generated only with difficulty if dissociated cells have been incubated in the media for several days (data not shown). This suggests that epithelial-mesenchymal interaction is always a pre-requisite for HF development, and that epithelial and mesenchymal cells consistently transmit various signals to each other during HF development. In the hair patch assay system, epithelial-mesenchymal interactions and the generation of new HFs do not occur if the number of injected cells is less than 1×10^5[5][12]. In the present study, at least 1×10^5 cells were needed to generate new HF, while 3×10^6 cells produced an abundance of HF structures.

We applied type IV collagen and laminin-5 to this assay in order to examine the relationship between HF morphogenesis and the extracellular matrix. Because the basement membrane is composed of type IV collagen, it serves as a robust scaffold for close communication between epithelial and mesenchymal cells. Alternatively, the collagen itself may directly impinge on cell development and differentiation. On the other hand, the

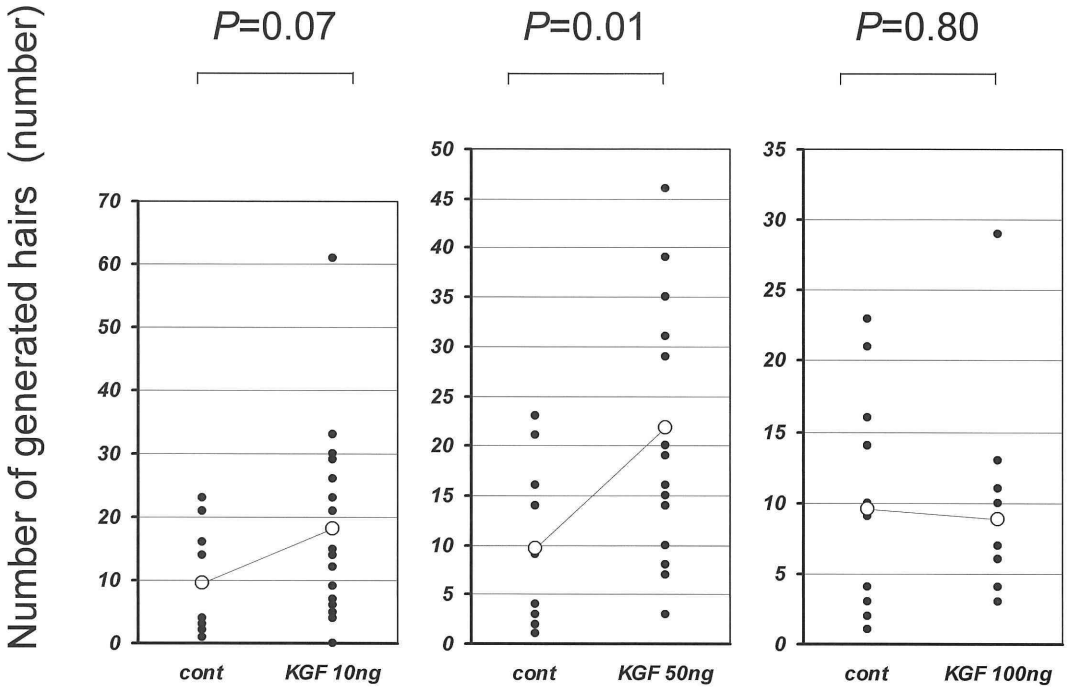

Fig. 3A

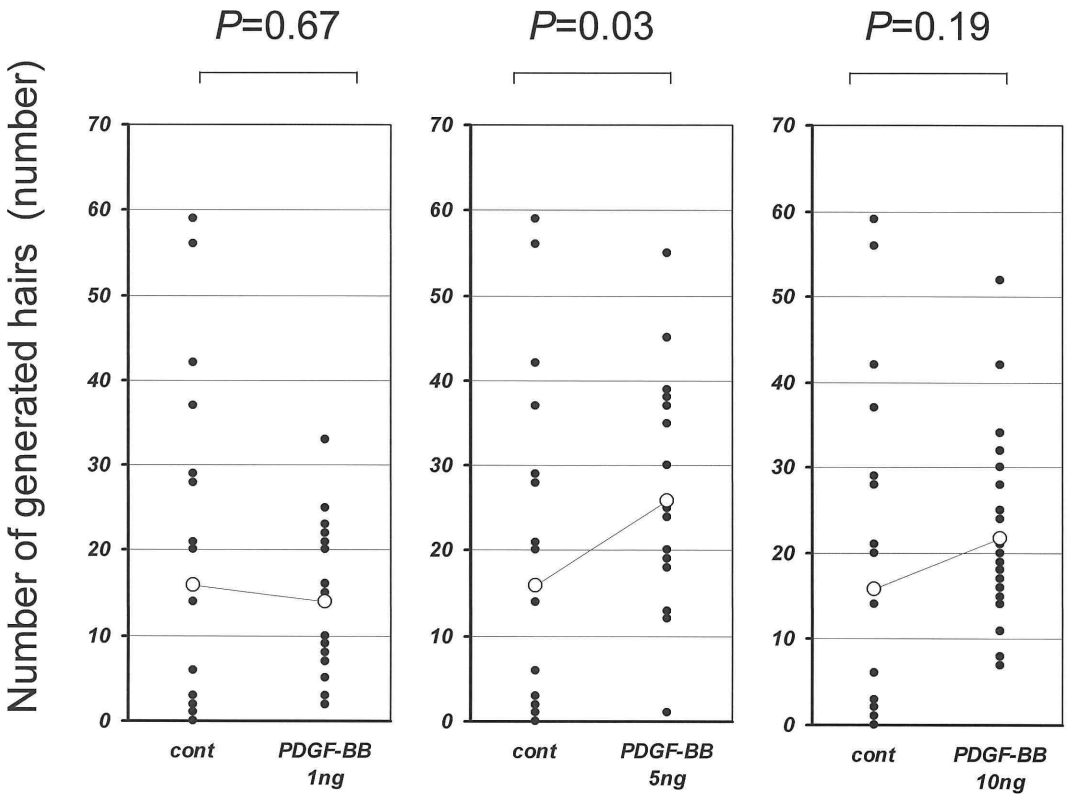

Fig. 3B

dysfunction of laminin-5 leads to Herlitz type junctional epidermolysis bullosa[4]. Further, it has already been reported that laminin-10 is essential for hair development[13]; Sugawara et al[14]. have reported that inclusion of laminin-10 in culture media enhances hair elongation compared with controls. Although both laminin-5 and -10 are found in the basement membrane, laminin-5 has a gamma 2 subunit which is known to interact with type IV collagen[15]. However, in the present study, laminin-5 did not increase the hair patch size significantly. It is also reported that laminin-5 suppresses hair growth driven by laminin-10 in *in vitro* cultures[16]. Further-

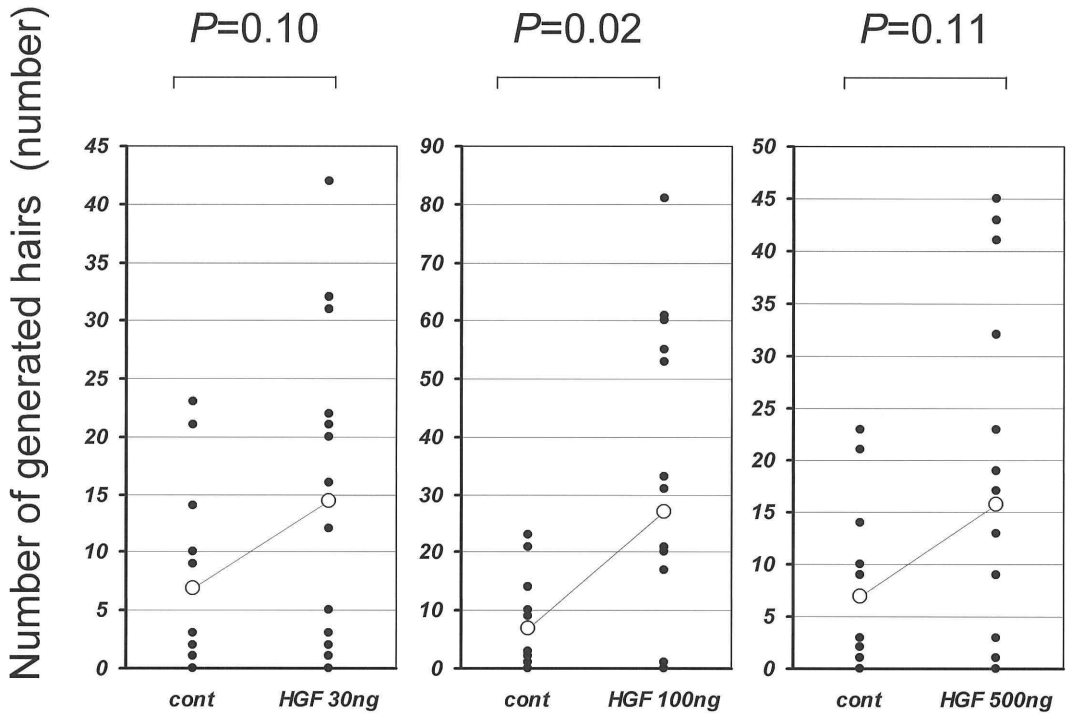

Fig. 3C

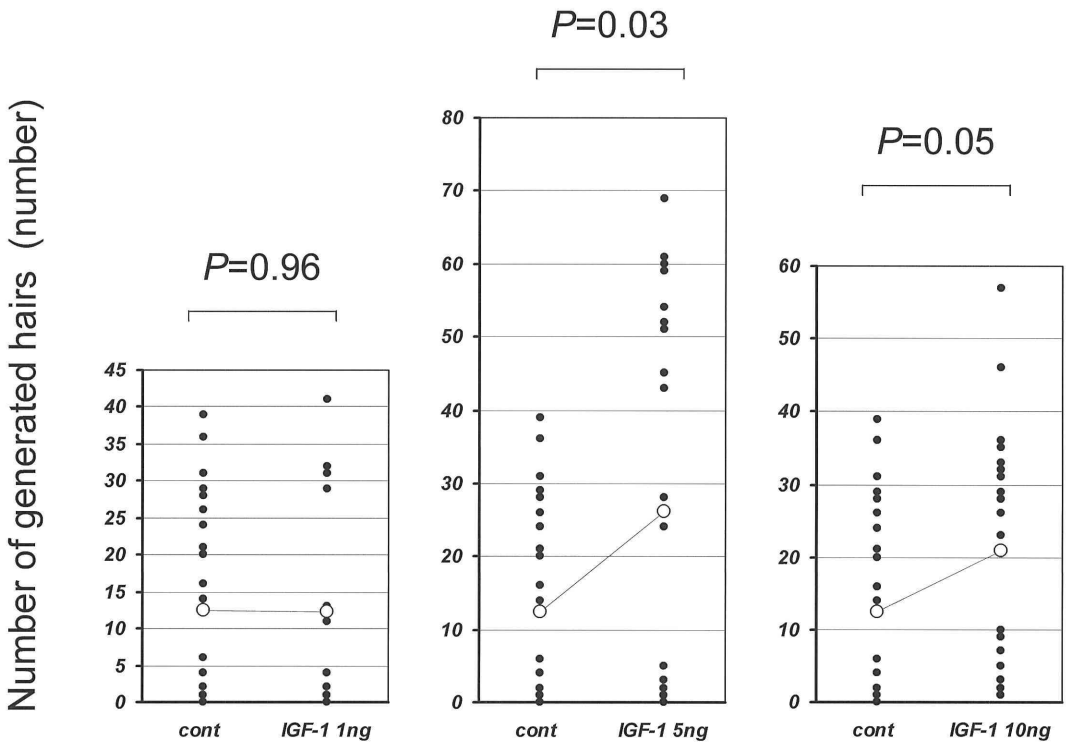

Fig. 3D

more, Sugawara et al[17]. suggested that fine-tuning of the relative expression of laminin-5 and laminin-10 is essential to the precise regulation of HF growth.

The function of growth factors as secretory signals in HF morphogenesis is also integral to the development of HF structures. The function of KGF, also known as FGF-7, is to mediate the migration and cell growth of keratinocytes[18]. Adenosine upregulates KGF gene expression, resulting in increased extracellular KGF protein levels and the promotion of hair growth[19]. Platelet-derived growth factor (PDGF) stimulates the migration and proliferation of mesenchymal cells mainly. Both PDGF-AA and BB injected into mouse back skin induced the anagen phase of the hair cycle in mature

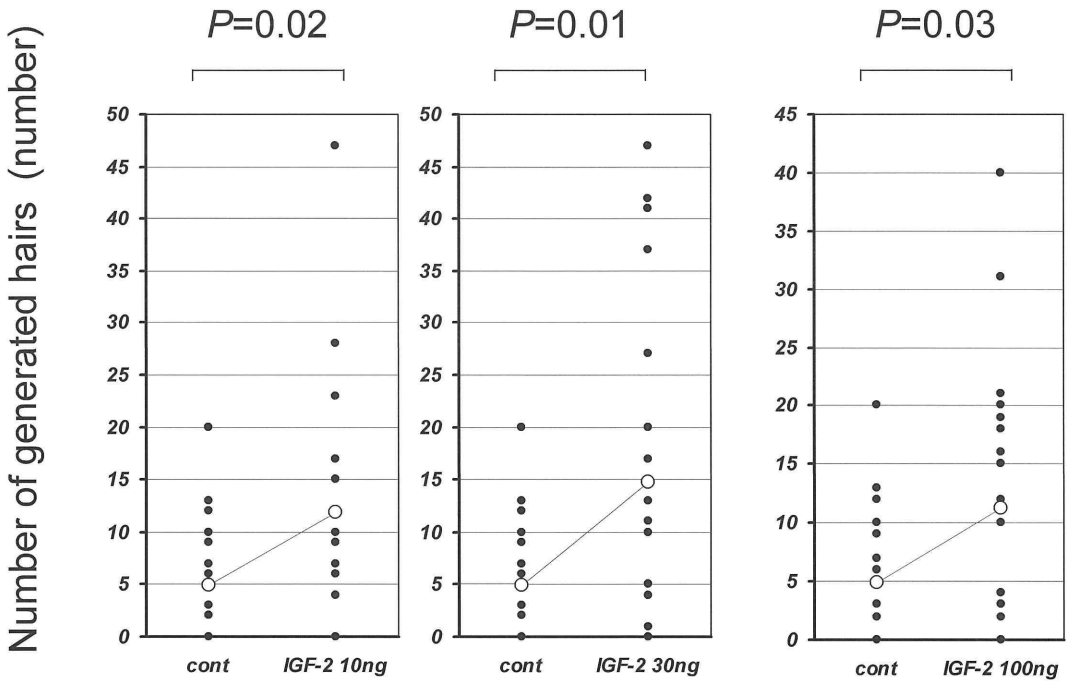

Fig. 3E

Fig. 3 Generation of hair follicle structures after the addition of growth factors

(**A**) Mean number of hairs generated in control, KGF (10 ng/ml), KGF (50 ng/ml) and KGF (100 ng/ml) was 9.6±7.6, 18.2±14.9, 21.8±13.6 and 8.8±7.0 (the mean±SD), *n*= 12, 17, 18 and 12, respectively.

(**B**) Mean number of hairs generated in the control, PDGF-BB (1 ng/ml), PDGF-BB (5 ng/ml), and PDGF-BB (10 ng/ml) was 15.8±18.3, 13.6±8.6, 25.8±12.7 and 21.7±10.9 (the mean±SD), *n*= 24, 21, 24 and 23, respectively.

(**C**) Mean number of hairs generated in the control, HGF (30 ng/ml), HGF (100 ng/ml) and HGF (500 ng/ml) was 6.9±8.5, 14.4±13.8, 27.1±27.3 and 15.7±16.5 (the mean±SD), *n*=12, 18, 16 and 16, respectively.

(**D**) Mean number of hairs generated in the control, IGF-1 (1 ng/ml), IGF-1 (5 ng/ml) and IGF-1 (10 ng/ml) was 12.5±13.6, 12.2±14.1, 26.1±26.5 and 21.0±15.5 (the mean±SD), *n*=24, 18, 24 and 24, respectively.

(**E**) Mean number of hairs generated in the control, IGF-2 (10 ng/ml), IGF-2 (30 ng/ml) and IGF-2 (100 ng/ml) was 5.0±5.5, 11.9±11.8, 14.8±15.7 and 11.2±11.3 (the mean±SD), *n*= 21, 18, 20 and 21, respectively.

individuals[20]. HGF promoted HF growth in mice[21] and human scalp hair in an organ culture[22]. IGF also plays an important role in HF regulation. Increased vibrissal growth was seen in transgenic mice overexpressing IGF-1[23]. In human beard hairs, androgen promotes the proliferation of outer root sheath cells via IGF-1[24]. In the present study, a significant increase in the number of HFs was observed after the addition of KGF (at a concentration of 50 ng/ml), PDGF-BB (5 ng/ml), HGF (100 ng/ml), IGF-1 (5, 10 ng/ml) or IGF-2 (10, 30, 100 ng/ml). The increased generation of HFs with GFs as well as type IV collagen indicates that our assay system is useful for examining the effects on hair growth of materials recently developed for the treatment of hair loss disorders.

Further studies can be performed to assess the topical application of the reagents such as minoxidil in ointment or lotion vehicles.

Abbreviations : HF, hair follicle ; ECM, extracellular matrix ; GF, growth factor ; EGF, epidermal growth factor ; TGF, tissue growth factor ; VEGF, vascular endothelial growth factor ; KGF, keratinocyte growth factor ; HGF, hepatocyte growth factor ; IGF, insulin-like growth factor ; FGF, fibroblast growth factor ; PBS, phosphate buffered saline ; DNase I, deoxyribonuclease I

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毛包パッチアッセイ法の、早期毛包発生に及ぼす細胞外基質と増殖因子の影響

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【背景】毛包は、個々の毛包の寿命の中で、成長、退行、休止の周期を繰り返す。毛包の構築には上皮細胞と間質細胞の相互作用が必要である。毛包発生におけるこれらの細胞の相互作用を理解するため、*in vivo* で毛包構造を作製する多くの実験手技が試みられてきた。

【目的】早期毛包発生における細胞外基質分子と増殖因子の影響を明らかにするため、我々は *in vivo* で毛包を構築する最近開発された手技（パッチアッセイ法）を改良し、細胞外基質としてIV型コラーゲンとラミニン5、増殖因子としてKGF、PDGF-BB、HGF、IGF-1、IGF-2の毛包発生における影響を解析した。

【方法】胎生14日から生後2日のC57BL/6Jマウスから背部皮膚を採取し、個細胞浮遊液とした。そして細胞浮遊液に種々の細胞外基質分子や増殖因子を添加し、ヌードマウスの背部に経皮的に注入した。注入10日後にヌードマウスを屠殺し、生じたパッチの面積と毛包数を解析した。

【結果】IV型コラーゲンを添加したところ、毛包組織を含むパッチの面積はコントロール群と比較して有意に増加した。しかし、ラミニン5では有意な影響が無かった。増殖因子としてKGF (濃度50 ng/ml)、PDGF-BB (5 ng/ml)、HGF (100 ng/ml)、IGF-1 (5, 10 ng/ml)、IGF-2 (10, 30, 100 ng/ml) を添加したところ、毛包構造の数が有意に増加した。

【結論】パッチアッセイ法により、細胞外基質分子や増殖因子の毛包発生促進作用が確認され、この方法が脱毛疾患の治療のために最近開発された薬剤の毛成長促進作用のアッセイ方法として有用であることが示された。

〈キーワード〉 毛包、発生、コラーゲン、ラミニン、増殖因子