Journal of Dermatological Science 96 (2019) 66–72

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

Invited Review Article

Human keratinocyte stem cells: From cell biology to cell therapy

Daisuke Nanba

Department of Stem Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan

ARTICLE INFO

Article history:
Received 10 October 2019
Received in revised form 15 October 2019
Accepted 15 October 2019

Keywords:
Human keratinocyte culture
Keratinocyte stem cells
Stem cell dynamics
Regenerative medicine
Cultured epidermal autografts

ABSTRACT

Human keratinocyte cultures contain keratinocyte stem cells, and have been involved in significant progress regarding stem cell biology as well as keratinocyte biology. Such cultures have also been applied in cell therapy for extensive severe burns for more than three decades, and in genetic disorders of the skin recently. Human keratinocyte stem cells were firstly characterized as holoclones by *ex post* clonal analysis, but *in situ* identification of keratinocyte stem cells is required for clinical applications. Recently, it was demonstrated that human keratinocyte stem cells display a unique rotational motion at early stages of culture, with subsequent dynamic collective motion at later stages. This finding enables image-based identification of keratinocyte stem cells, and noninvasive evaluation of their proliferative capacity, which can be applied for the quality assurance of human keratinocyte cultures. This review summarizes the historical development of human keratinocyte cultures and its applications for cell biology and cell therapy. This article also introduces recent advances in keratinocyte stem cell research with medical relevance and discusses the next-generation of regenerative medicine using human keratinocyte stem cells.

1. Introduction

Human cells can be isolated from tissues and maintained and expanded *ex vivo* under appropriate cell culture conditions. Cultured human cells still possess some properties when placed within the original tissue, which has advanced human biology and medical research. In particular, cultures of patient-derived cells have contributed to improved understanding of the pathogenic mechanisms of a number of diseases, and also to develop therapeutic treatments. Obviously, cultured cells are not identical to cells *in vivo*, and a comprehensive approach is required for a better understanding of human physiology and pathology. Also, conventional cell cultures cannot reconstitute structural and systemic environments. However, organoid technology and "organs-on-a-chip" or "body-on-a-chip" devices are now intend to overcome these issues [1–3]. Hence, human cell culture is still a promising technology for advancing human biology and medical research.

Cell cultures are definitely indispensable for stem cell-based regenerative medicine which reconstitutes human tissues *ex vivo* by using pluripotent and tissue-specific stem cells. Keratinocytes mainly compose the epidermis, and keratinocyte stem cells produce their differentiating progeny and maintain epidermal homeostasis for a lifetime. Human keratinocytes, including keratinocyte stem cells, can be isolated from the skin and massively expanded in culture, generating squamous sheets, which has allowed the transplantation of regenerated epidermal sheets in burn patients [4,5], currently known as the first successful application of cultured human cells for regenerative medicine [6]. This technology has been adapted to cell therapy for ocular surface reconstruction [7,8] and conceptually followed by other stem cell-based regenerative medicine including autologous transplantation of sheets of retinal pigment epithelial cells generated from iPS cells derived from patients [9]. Thus, further investigation and technological development surrounding human keratinocyte culture will contribute to advance stem cell-based regenerative medicine by using pluripotent and other tissue-specific stem cells.

2. Development of human keratinocyte culture

Several groups have described their attempts at culture of human epidermal keratinocytes since the early 1960s, but they

E-mail address: nanbscm@tmd.ac.jp (D. Nanba).

https://doi.org/10.1016/j.jdermsci.2019.10.002
0923-1811/

could not expand and subculture isolated keratinocytes *in vitro*. In 1975, Rheinwald and Green reported that murine teratoma cells gave rise to epithelial colonies against a background of fibroblasts [10]. These epithelial cells grew very poorly when isolated, but grew very well and were similar to keratinocytes when co-cultured with murine fibroblast 3T3 cells [10] that were also previously established in the Green lab [11]. To prevent 3T3 cells from overgrowth of the keratinocyte population, this fibroblast cell line was irradiated in order to lose mitotic ability but remain metabolically active. Rheinwald and Green then applied this protocol to human epidermal keratinocytes, and generated progressively growing colonies of human keratinocytes from single cells on feeder layers of irradiated 3T3 cells [12]. Such growing colonies consisted of a basal layer of proliferative cells and upper layers of differentiating squamous cells. Co-culture with mitotically inactivated feeder cells also enabled serial expansion of human keratinocytes. Initially, this culture was maintained in a 3:1 mixture of the Dulbecco-Vogt modification of Eagle's medium (DMEM) and Ham's F12 medium supplemented with fetal bovine serum (FBS) and hydrocortisone, which was then improved by the addition of adenine, cholera toxin, insulin, triiodothyronine, and epidermal growth factor [13]. The feeder cells were also optimized for human keratinocyte culture. 3T3-J2 cells, a subclone of the original 3T3 line, are now commonly used as a feeder layer for growth of human keratinocytes, and mitomycin-C treatment is an alternative to irradiation of feeder cells. This keratinocyte culture system is currently known as the Rheinwald and Green method and allowed for the massive expansion of human keratinocytes for the first time [13].

3. Application of human keratinocyte cultures for regenerative medicine

Green and his colleagues confirmed that cells in the upper layer of stratified human keratinocyte colonies start to differentiate and develop a cornified envelope [14]. They subsequently developed the technologies such that a functional keratinocyte sheet resulting from the fusion of a number of stratified growing colonies can be detached from cell cultures dish by using dispase and then transplanted onto a graft bed prepared in athymic mice [15,16]. Transplanted grafts regenerated the epidermis with the cornified layer and remained intact for as long as 108 days after transplantation [16]. Logistics of large-scale production of human keratinocytes from a small skin biopsy were also estimated [15]. By the early 1980s, Green and colleagues performed pre-clinical work for cell therapy with cultured human keratinocytes.

3.1. Burns

The first therapy using cultured human keratinocytes was performed on two patients with burns in 1980. Small skin biopsies were taken from the patients, and the isolated keratinocytes were expanded to produce epidermal grafts in the Green lab. Dispase-detached grafts were transplanted to prepared wound surfaces and regenerated the epidermis in both patients [4]. The next advance involving cell therapy for burns was the large-scale production of epidermal grafts from autologous keratinocyte cultures in 1983. Two patients with third-degree burns to more than 80% of their body surface received the transplantation of numerous cultured grafts made from their own skin [5]. The grafts were successfully engrafted, and the two patients survived for over 20 years [6].

Compton et al. investigated the regeneration of skin from cultured epidermal autografts by analyzing transplanted grafts in pediatric patients from 6 days to 5 years after grafting [17]. Six days after transplantation, fully stratified epidermis with the cornified layer regenerated, and the dermal-epidermal junction, including hemidesmosomes, basal lamina, and anchoring fibrils, started to be reformed along the attachment surface of the grafts. Within 3 to 4 weeks, the dermal-epidermal junction was complete, and rete ridges regenerated from 6 weeks to 1 year after grafting. Furthermore, the sub-epidermal connective tissue initially healed with normal scarring, but was remodeled to produce papillary and reticular dermis, similar to intact dermis within 4 to 5 years.

3.2. Epidermolysis bullosa

Cultured epidermal grafts produced using the Rheinwald and Green method have been also applied to *ex vivo* gene therapy for an inherent skin disease. Junctional epidemolysis bullosa (JEB) is a severe blistering disorder of the skin in which epithelia are poorly anchored to the basement membrane due to mutations in genes for laminin-332 (*LAMA3*, *LAMB3*, and *LAMC2*), type XVII collagen, α6 and β4 integrin. De Luca and colleagues expanded epidermal keratinocytes isolated from a JEB patient with LAMB3 deficiency, and retrovirally transduced the full-length *LAMB3* gene into cultured keratinocytes [18]. Genetically-modified autologous cultured epidermal grafts expressing LAMB3 were then produced and transplanted onto surgically prepared wound beds. Successfully engrafted keratinocytes stably expressed *LAMB3* mRNA in the transplanted area and maintained themselves for at least 6.5 years [19]. Recently, they restored approximately 80% of the total body surface area of a 7-year-old child suffering from a life-threatening form of JEB with *LAMB3* mutation by transplantation of genetically modified cultured epidermal grafts [20]. These studies provide a therapeutic framework for a number of inherent diseases by combining *ex vivo* cell and gene therapies.

Gene transduction with retroviral vectors has a risk of viral integration into the host genome. Droz-Georget Lathion et al. have demonstrated safer epidermal gene therapy for recessive dystrophic epidermolysis bullosa (RDEB) [21], characterized by an extremely severe skin blistering due to poor adherence of epidermis to the dermis caused by mutations in the type VII collagen gene (*COL7A1*), the major component of anchoring fibrils. They clonally expanded genetically-corrected keratinocytes isolated from a RDEB patient, and each clone was characterized using a number of criteria including long-term proliferative potential, viral integration, karyotype, and tumorigenicity. Selected safer clones were then expanded again and used for the generation of cultured epidermal grafts. This strategy provides a safe and homogeneous genetically-modified cultured epidermal graft, but is not yet in clinical use.

4. Identification and isolation of human keratinocyte stem cells

Tissue-specific stem cells can produce unaltered daughters and also give rise to another type of daughter cells that have more differentiated properties. These cells sustain turnover and repair in tissues throughout life. Keratinocyte stem cells reside in the basal layer of the epidermis, and the terminally differentiating progeny leaves this layer to move toward the skin surface and function as the structural barrier of the skin.

4.1. Identification

Long-term expansion of human keratinocyte populations *ex vivo*, and permanent coverage of epidermal defects by transplantation of cultured epidermal grafts, indicated that human keratinocyte cultures contain keratinocyte stem cells. Barrandon and Green demonstrated the existence of human keratinocyte stem cells by clonal analysis [22]. Single keratinocytes were isolated under a microscope and individually cultured. After 7 days of cultivation, the resultant growing colonies were individually

trypsinized and subcultured into two indicator dishes for 12 days until the cultures were fixed and stained.

From the appearance of the colonies produced in indicator dishes, they assigned each single keratinocyte to one of three clonal types. The holoclone has the greatest proliferative capacity and gives rise to fewer than 5% of terminally differentiated colonies (terminal colony) in indicator dishes. The paraclone has the least proliferative capacity and generates only terminal colonies. The meroclone exhibits an intermediate phenotype and has a broad range of proliferative capacity. Holoclones, currently known as keratinocyte stem cells, can undergo at least 180 doublings, and a single holoclone can theoretically produce enough cultured epidermal grafts to cover the entire body of an adult human. Meroclones can regenerate an epidermis in the short term, whereas paraclones cannot regenerate an epidermis at all.

4.2. Clonal conversion

Holoclones are eventually converted into meroclones or paraclones during serial cultivation, which results in termination of a culture of human keratinocytes. This phenomenon, termed clonal conversion, results in progressive and irreversible restriction of the growth potential of human keratinocyte stem cells [23,24]. Clonal conversion is accelerated by stress, suboptimal culture conditions, serial cultivation, and age of donor. Although mechanisms underlying clonal conversion remain unclear, it has been reported that several proteins including p63 [25,26], 14-3-3 [27], Rac1 [24], and YAP [28] are involved in clonal conversion.

4.3. Isolation and enrichment

Human keratinocyte stem cells can be also identified by the expression of cell surface proteins including α1, α2, α4, α6, β1, and β4 integrin subunits [29–31], transferrin receptor [31], Delta1 [32], melanoma chondroitin sulfate proteoglycan [33], leucine-rich repeats and immunoglobulin-like domains protein [34], CD46 [35], and ATP-binding cassette subfamily G member 2 [36]. These proteins, however, are not stem cell-specific, and the ratio of clonogenic keratinocytes in populations enriched by cell sorting employing these markers is always lower than that of cells simply isolated based on size ($\leq$11 μm in diameter) with a Pasteur pipette [37]. Although there might be other reliable cell surface markers for human keratinocyte stem cells, the cell sorting procedure might also negatively impact such stem cells.

These cell surface proteins are used for isolation and enrichment of keratinocyte stem cells when directly isolated from human skin. In addition, we have demonstrated that the expression level of α6 integrin subunit is correlated with proliferative capacity of human keratinocytes, even though cells were isolated from culture [38]. When keratinocytes in progressively growing colonies were fractionated into α6 integrin high- and low-expressing populations ($\alpha6^{high}$ and $\alpha6^{low}$), only $\alpha6^{high}$ keratinocytes maintained proliferative capacity through serial cultivation. Flow cytometric analysis also revealed that the $\alpha6^{high}$ keratinocyte population contains the smallest cells, which possess higher clonogenic ability [37]. Furthermore, we have recently reported that expression of collagen XVII (COL17A1), which is a transmembrane component of hemidesmosomes and interacts with α6 integrin subunits, is also associated with proliferative capacity of human keratinocytes [39]. COL17A1 negative and low ($COL17A1^{low/-}$) and COL17A1 positive ($COL17A1^{+}$) cells were collected by cell sorting, and $COL17A1^{+}$ but not $COL17A1^{low/-}$ keratinocytes sustained a high clonogenic potential after serial cultivation. Importantly, in these experiments, small keratinocytes comprising progressively growing colonies were sorted by α6 integrin or COL17A1 expression. The culture of human keratinocytes on a feeder layer of 3T3 cells also contains terminal colonies composed of large and flattened basal cells with differentiated phenotypes, and these cells strongly express hemidesmosomal components. Therefore, keratinocyte populations with the highest expression of both α6 integrin and COL17A1 were derived from terminal colonies and must be excluded from the analysis [24,38].

5. Human keratinocyte stem cell dynamics

Human keratinocytes exhibit dynamic behavior in culture. In particular, improved understanding of human keratinocyte stem cell dynamics would provide significant insights into epidermal homeostasis and wound healing in humans. The dynamic behavior of such stem cells is also closely relevant to *ex vivo* maintenance and expansion of human keratinocyte stem cells and the production of cultured epidermal autografts.

5.1. Rotational motion in the two-cell stage colony

The dynamic behavior of single keratinocytes has been well investigated; however, it still remains unknown how the collective dynamics of keratinocytes observed in keratinocyte cultures and living skin emerge from the properties of individual keratinocytes. In culture, seeded single keratinocytes adhere to the surface of culture dishes and subsequently start to proliferate. The first mitosis generates two daughter cells that remain adhered to each other and give rise to two-cell stage colonies. This is the first time that individual keratinocytes have a neighbor, hence the behavior of two-cell colonies is fundamental for understanding the collective dynamics of keratinocytes. Intriguingly, two-cell colonies of human keratinocytes exhibit rotational motion, and this motion is related to the proliferative capacity of human keratinocytes.

Hata et al. reported that the number of rotating two-cell colonies of human keratinocytes decreases along with passage numbers [40]. We subsequently clearly demonstrated that the rotational speed of two-cell colonies is positively associated with the clonal growth of normal human keratinocytes [38] and HaCaT keratinocytes [41] (Fig. 1). We also found that the rotational speed was associated with the expression of involucrin (IVL), a differentiation marker of keratinocytes [38]. The rotational speed of IVL-negative two-cell colonies was significantly greater than that of IVL-positive colonies. This result is consistent with the level of IVL expression being negatively related to the proliferative capacity of cultured human keratinocytes [29,42]. These results indicate that rotating two-cell colonies maintain their proliferative capacity and generate large colonies by clonal expansion.

5.2. Collective motion in growing colonies

The collective motions of human keratinocytes in growing colonies was first described in an early study by Sun and Green [14], and are currently observed by conventional time-lapse imaging with a phase–contrast microscope [38] (Fig. 2). We supposed that rotational motion generates collective motion of keratinocytes in progressively growing colonies, since human keratinocytes with significant proliferative capacity exhibit rotational motion when they form two-cell colonies. To prove this hypothesis, we performed a simulation experiment to analyze whether collective motion could emerge if a hundred cells with rotational ability accumulated as a colony [38]. This simulation experiment clearly demonstrated that rotational motion of individual cells gives rise to the collective motion observed in growing colonies. Furthermore, the locomotion speed of cells in the colony was dependent on the rotational speed of individual cells. These results theoretically revealed that keratinocyte colonies derived from a holoclone can be identified by measuring

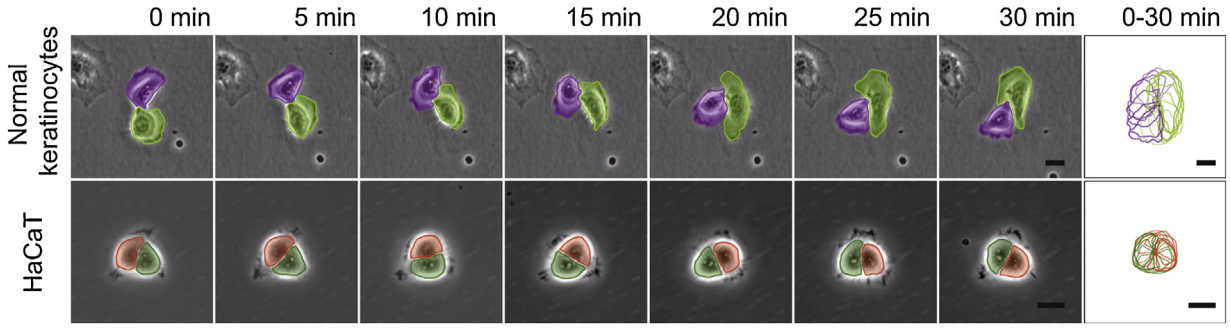

Fig. 1. Rotational motion of human keratinocytes.
Two-cell stage colonies were derived from normal human epidermal keratinocytes (upper) and HaCaT cells (lower). Images were obtained at 5 min intervals. The panels at the right end are merged images of contours of rotating keratinocytes. Bars, 20 μm.

cell locomotion speed, since rotational speed of keratinocytes is associated with their proliferative capacity.

Combined experiments with cell motion analysis and clonal analysis of human keratinocytes were then performed to prove this prediction, and clearly demonstrated that colonies consisting of greater number of locomotive cells contain keratinocytes with higher growth potential [38]. The results of combination analysis also indicated that a keratinocyte colony with long-term proliferative capacity, which is derived from a holoclone, shows the highest locomotive capacity among growing colonies of keratinocytes, as predicted from the simulation. These findings indicated that human keratinocyte stem cells (holoclones) can be identified by only analyzing cell locomotion speed by time-lapse observation using a conventional phase-contrast microscope.

5.3. Application for regenerative medicine

Ex vivo maintenance and expansion of keratinocyte stem cells are definitely associated with successful transplantation for epidermal regeneration. For cell therapy using cultured keratinocytes, determination of the ratio of holoclones in keratinocyte populations is the best criteria to assess the quality of the cultures [23]. A holoclone assay, however, takes 19 days to perform [22], which has limited its usefulness for regenerative medicine. Furthermore, clonal analysis can only discriminate holoclones retrospectively from meroclones and paraclones. Image-based noninvasive identification of cultured human keratinocyte stem cells enables us to monitor and validate their successful culture *in situ*. This technology can be also applied for selection of normal and genetically modified keratinocyte stem cells among cultured keratinocyte populations derived from patient skin [21], or differentiated from patient-specific induced pluripotent stem cells for gene therapy [43]. Therefore, cell motion analysis is the basis of a noninvasive method that estimates the growth capacity of keratinocytes in culture, and monitors and validates the quality of medical cultures of human keratinocytes (Fig. 3).

6. Improvement of culture conditions

6.1. Xenobiotic free cultures

The Rheinwald and Green culture method involves murine fibroblasts and FBS, which has potential risk of transmission of microbes from animals. Since the early 1980s, Ham and colleagues

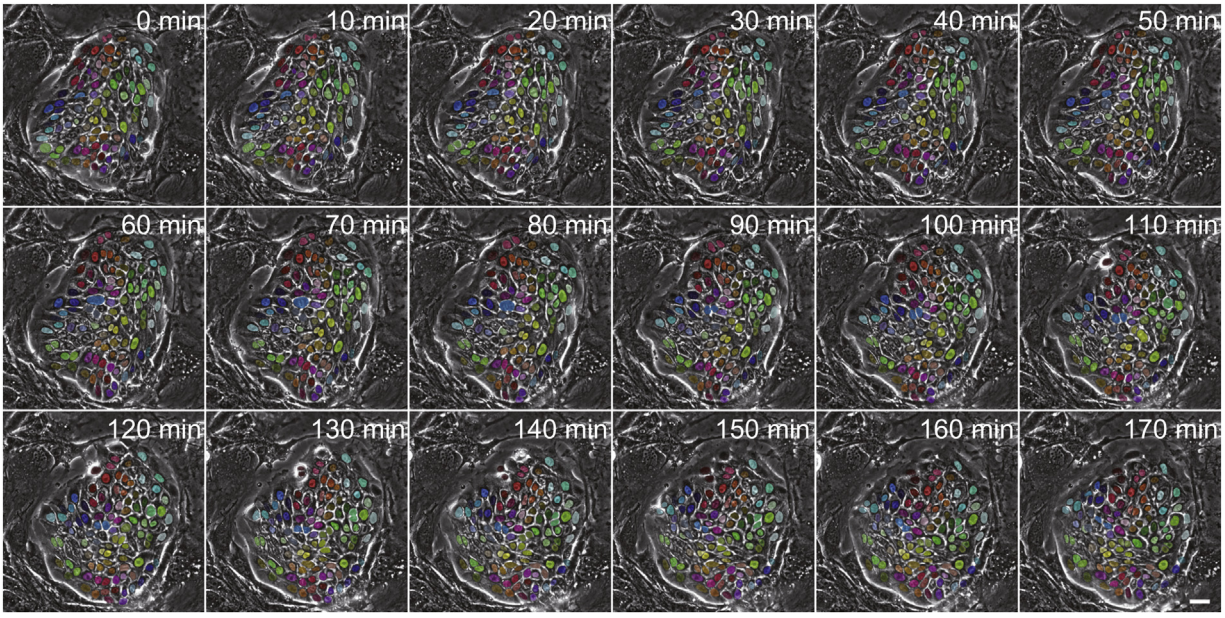

Fig. 2. Collective motion dynamics of human keratinocytes.
Tracing of cell locomotion in a progressively growing colony derived from a single normal human epidermal keratinocyte. Images were obtained at 10 min intervals. Time moves from left to right and then from top to bottom. Bar, 20 μm.

have developed a chemically defined culture medium that enables clonal growth and serial culture of normal human keratinocytes without murine feeder cells and bovine serum [44]. However, this medium still requires a small amount of whole bovine pituitary extract for primary cultures and for massive expansion. Currently, several types of chemically defined media that are serum-free and do not require feeder cells are commercially available for human keratinocyte culture. Under culture conditions using these media, the dynamics of human keratinocytes are quite different from those of keratinocytes cultured with the Rheinwald and Green method [38]. Furthermore, it is still unclear whether these serum- and feeder-free culture systems can maintain keratinocytes stem cells for long periods *ex vivo*, and be available for the production of cultured epidermal grafts that enable long-term maintenance of regenerated epidermis after transplantation. Therefore, the Rheinwald and Green method is still the gold standard for human keratinocyte stem cell research and epidermal regenerative medicine [13].

Recently, Tjin et al. reported that recombinant laminins LN-511 and LN-421, components of the basement membrane, can be replaced with murine 3T3 feeder cells [45]. The culture system using recombinant LN-511 and LN-421 also did not require bovine serum, and normal human keratinocytes could be expanded with a chemically defined keratinocyte culture medium, even if in initial expansion. Tjin and co-workers have also demonstrated that this laminin system is comparable to 3T3 co-culture systems in terms of gene expression profile, colony-forming efficiency, the ability to produce epidermis *ex vivo*, and long-term maintenance of regenerated epidermis after transplantation in animal models. These results strongly suggest that this system provides a new platform for human keratinocyte stem cell research and safer cell therapy involving cultured human keratinocytes.

6.2. Small molecules

Enhanced expansion of human keratinocytes *ex vivo* with small chemical molecules that can inhibit intracellular signaling has also been investigated. Currently, a Rho-kinase inhibitor (Y-27632) [46,47], a γ-secretase inhibitor (DAPT) [48], SMAD inhibitors (DMH-1 and A-83-01) [49], and TGFβRI/ALK5 inhibitor (RepSox) [50] have been reported as signal inhibitors that can increase human keratinocyte proliferation in the feeder-dependent or independent culture. Although it remains to investigate whether these small molecules can also maintain and expand keratinocytes stem cells for long periods *ex vivo*, the combination of feeder- and serum-free media with small molecules promises safer and less labor intensive cell therapy for epidermal regeneration.

7. Conclusions and perspectives

Human keratinocyte cultures were developed more than 40 years ago, and are currently used in cell biology, medical research, clinics, and industry. Nevertheless, further investigations and technological

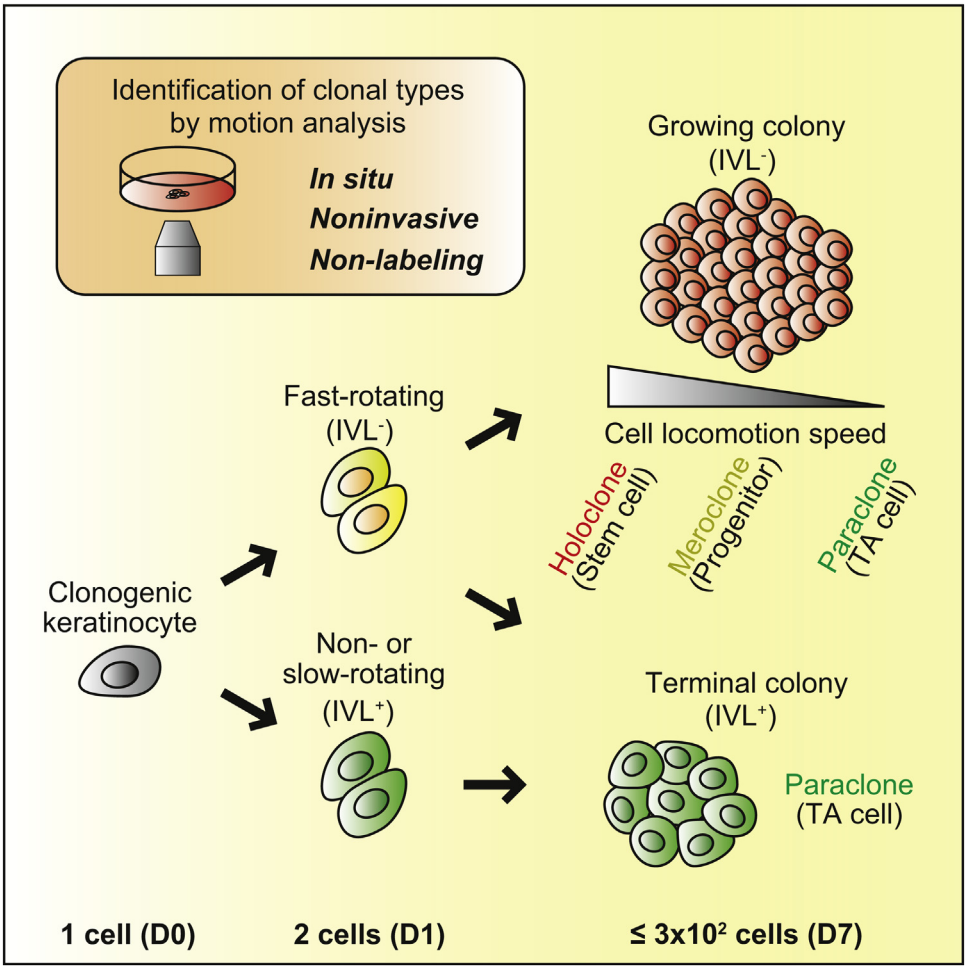

Fig. 3. Identification of human keratinocyte stem cells (holoclones) by motion analysis.
Rotational speed of two-cell colonies can predict their clonal growth and the expression of involucrin (IVL). Locomotion speed of cells in growing colonies was also associated with long-term proliferative capacity of keratinocytes.

development are still ongoing, worldwide. The addressing of the following issues brings the next-generation of regenerative medicine of the skin. *Ex vivo* expansion and long-term maintenance of keratinocyte stem cells with xeno-free culture systems realize safer cell therapy using human keratinocytes. Noninvasive, *in situ* identification, and label-free isolation of cultured keratinocyte stem cells increase successful treatment using cultured epidermal grafts and decrease the cost of therapeutic cell culture. Understanding the mechanisms of keratinocyte stem cell dynamics will innovate new approaches for epidermal regeneration.

Funding

This work was supported by the Japan Society for the promotion of Science KAKENHI grants (17K10231).

Declaration of Competing Interest

The author has no conflicts of interest to declare.

Acknowledgments

The author thanks Prof. Yann Barrandon (Institute of Medical Biology, A*STAR, Singapore), Prof. Koji Hashimoto (Ehime Prefectural University of Health Science, Japan), Prof. Shigeki Higashiyama (Ehime University), and Prof. Emi K. Nishimura (Tokyo Medical and Dental University, Japan) for their continuous encouragement and support. The author apologizes to the contributors in this field, whose work is not cited due to space constraints. This work was supported by the Japan Society for the promotion of Science KAKENHI grants (17K10231).

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Daisuke Nanba received his Ph.D. from Osaka University, where he studied skin appendage development including hair follicles and mammary glands. He then worked on EGFR-ligands/EGFR signaling in keratinocytes at Ehime University, and *ex vivo* maintenance and expansion of human keratinocyte stem cells at École Polytechnique Fédérale de Lausanne (EPFL) in Switzerland as a Postdoctoral Fellow. He moved back to Ehime University as a Senior Research Fellow and studied human keratinocyte stem cell dynamics and dermal fibroblast heterogeneity, and was then appointed as an Associate Professor. He is currently an Associate Professor of the Department of Stem Cell Biology, Medical Research Institute at Tokyo Medical and Dental University.