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개 지방 유래 줄기세포를 이용한
진피 유두 유사조직의 형성

Canine Adipose-Derived Stem Cell
Aggregates as a Viable Substitute to Actual
Canine Dermal Papillae

2015년 8월

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Canine Adipose-Derived Stem Cell Aggregates as a Viable Substitute to Actual Canine Dermal Papillae

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Abstract

Hair loss is a major dermatological disease in veterinary and human medicine.

Active studies on hair regeneration with mesenchymal stem cells have been

performed in an effort to solve the limitations of conservative treatments in human

medicine. Our understanding of the canine hair follicle (HF), considering a useful model for the study of the human alopecia, is limited. This study was designed to broaden our understanding of canine dermal papilla (DP), and to reconstruct dermal papilla-like tissue (DPLT) using canine adipose-derived mesenchymal stem cells (AD-MSCs), as an alternative to actual DP. We cultured canine DPs, observed their culture patterns and compared their expression level of DP-related genes and proteins with those of DPLTs by performing RT-PCR analysis and Western blotting. Canine dermal papilla cells (DPCs) showed multilayer culture patterns with pseudo-papillae. Reconstruction of DPLTs was performed successfully. They were morphologically similar to actual DPs and we observed similarities between DPC and DPLT in molecular characteristics. These findings suggested that DPLT was a viable substitute for DP. This study will not only be helpful for understanding the cultural, morphological, and molecular

characteristics of canine DPCs, but may also serve as a basis for understanding human hair follicle biology and potential therapeutic strategies for alopecia.

Key words: dog, hair regeneration, adipose-derived mesenchymal stem cells (AD-MSCs), dermal papilla, dermal papilla-like tissue (DPLT)

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I. INTRODUCTION

Alopecia is a common dermatological disease in veterinary and human medicine. It may be caused by inflammation, infection, traumatic damage as a result of chemotherapy or radiotherapy, nutritional deficiencies, hormonal or autoimmune disease, or stress. Even though there is more complex classification of alopecia in human, certain types of human alopecia can be found in canine hair loss. (1-3)

Especially in humans, the condition may be mentally stressful to patients going through it due to its effect on their appearance, which is why many different treatment methods have been tried; of these, auto hair transplantation has been the most effective. In addition, because of critical limitations on cost, amount of hair and number of operations, (4) studies on hair regeneration have been attempted using cultured hair follicle cells or mesenchymal stem cells. (4-8)

Hair growth is a result of active epidermal and dermal interactions. (6, 9-14) In general, dermal component is thought as inducers and epidermal component as responders in the process of hair growth. (15) Dermal papilla (DP) is a cluster of highly specialized fibroblasts located at the base of the hair follicle (HF). It is believed that DP not only determines the length, thickness and shape of the hair shaft (16) but also regulate hair follicle development and growth. For this reason, active research on DPs has been done.

Hair regeneration has been tried using tissue and cellular recombination of epidermal and dermal component. (15, 17) However, DP isolation is technically laborious and difficult, and cultured dermal papilla cells (DPCs) lose their hair inductive abilities after a few passages. To overcome these, studies on the reconstruction of dermal papilla-like tissues have been performed with human umbilical cord-derived mesenchymal stem cells. (4, 5, 7)

This study represents the first reconstruction of DPLTs done using canine adipose-derived mesenchymal stem cells (AD-MSCs); we compare them to actual canine DPCs to evaluate DPLTs as an alternative to actual DPs. We expect the findings of this study to broaden our understanding of the morphological and molecular characteristics of canine DPCs, and to represent a valuable source for the investigation of canine and human hair regeneration.

II. MATERIALS AND METHODS

1. Canine AD-MSC Cultures

We used canine AD-MSCs that were supplied from Seung Hoon Lee (Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Seoul National University); these AD-MSCs were previously characterized. (18) The AD-MSCs were cultured in low-glucose Dulbecco's modified eagle medium (DMEM, Gibco®, Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco®, Life Technologies, Grand Island, NY, USA), 100 units /mL penicillin and 100 mg/mL streptomycin at 37.0 °C, in a 5% CO₂ incubator.

2. Canine DP Isolation and Cell culturing

Full-thickness skin biopsies obtained from the flank region of healthy beagle dogs roughly 3 years old were immediately placed in phosphate-buffered saline (PBS). Under dissecting microscope, the hair follicles were isolated using a No. 15 blade, watchmaker's forceps, and 23G needles. Isolated hair follicles were then transferred to a fresh medium-containing dish for further isolation. Hair cycle stage was evaluated by the position of DP and tip of hair shaft and DPs in late anagen were isolated. (19) Five DPs were placed together in a single well of a 12-

well cell-culture cluster, which contained 1.2 mL of AmnioMAXTM-C100 complete medium (AmnioMAXTM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% inactivated and steroid-free canine serum at 37°C, in a 5% CO₂ incubator. (6, 8, 20-22) We observed culture patterns of canine DPCs every day and the culture medium was replaced every third day. Primary cultured DPCs were harvested when confluent and then passaged on every tenth day. All procedures were approved by Seoul National University's Institutional Animal Care and Use Committee (SNU-140729-5).

3. Reconstruction of DPLTs

Canine AD-MSCs were subjected to a monolayer culture in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 mg/mL streptomycin, until the cells occupied approximately 80% of the culture dish. Next, the culture medium was replaced with dermal papilla-forming medium (DPFM) (23) that contained 10 ng/mL hydrocortisone, 5 mL Insulin-Transferrin-Selenium liquid media (ITS, Gibco®, Life Technologies, Grand Island, NY, USA), and 100 units/mL penicillin, 100 mg/mL streptomycin, and 20 ng/mL HGF (recombinant human HGF, Gibco®, Life Technologies, NY, USA). The medium was changed every third day for three weeks, after which the culture was treated with the

StemPro® Accutase® Cell Dissociation Reagent (Accutase®, Gibco®, Life Technologies, Grand Island, NY, USA) at concentrations ranging from 20 to 40 $\mu\text{L}/\text{cm}^2$ to detach the cells from the culture dish (detach-attach step). Cell distribution was changed and cell aggregation was observed 24 h after treatment with Accutase; the aggregated cell mass became suspended in the medium five to seven days after the treatment. The suspended cell aggregates were then isolated from the culture by centrifugation at 500 rpm for 3 min. We morphologically compared DPLTs to actual canine DPs. All procedures were performed based on those of previous studies. (4, 23)

4. RT-PCR analysis

Total RNA from all three cell types (canine AD-MSCs, DPCs and DPLTs) was isolated using Hybrid-R™ with RiboEx® (GeneAll®, Seoul, Korea) according to the manufacturer's instructions. cDNA was then synthesized, using 1 microgram of the total RNA, with a PrimeScript™ RT-PCR Kit (Takara Bio Inc., Shiga, Japan) for RT-PCR processing according to the manufacturer's instructions. Cycling conditions included initial denaturation for 10 min at 94 °C, followed by thirty-five 15-s cycles at 95 °C and 60-s cycles at 60 °C. The products were then analyzed by electrophoresis on a 1.6% agarose gel and subsequently visualized by

the RedsafeTM Nucleic Acid Staining Solution (iNtRON Biotechnology, Gyeonggi-do, Korea). We tested well-known hair-inductive markers of canine DPCs, such as alkaline phosphatase detection (ALP), Wnt inhibitory factor 1(WIF1), laminin, vimentin, versican, and prominin-1. (1, 8, 13, 15, 24) Glyceraldehyde 3-phosphate de hydrogenase was used as the reference gene. The primer used as follows (forward).

Marker	Primer sequence
ALP	5' – GGC GTC CAC GAG CAG AAC TA – 3'
WIF1	5' – GGC ACG GAA GAC ACT GCA AT – 3'
Laminin	5'- AGA CCA CCG GAT ACT GAT CG - 3'
Vimentin	5' – CTG CCA AGA ACC TTC AGG AG – 3'
Versican	5' – GTA GAA AAT GCC AAG ACC TT – 3'
Prominin-1	5' – TTG TGA TTC TGA TGC CGC TG – 3'
GAPDH	5' – CAA GGC TGT GGG CAA GGT – 3'

5. Western Blot

The cell and tissue extracts were prepared using the PRO-PREPTM protein extraction solution (iNtRON Biotechnology, Gyeonggi-do, Korea). Protein samples were run on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto a nitrocellulose membrane, and incubated with an anti-human versican (VCAN) antibody (R&D systems, Minneapolis, MN, USA), anti-laminin

antibody (ab11575, Abcam, Cambridge, MA, USA), and anti-vimentin antibody (ab8069, Abcam, Cambridge, MA, USA). We used an anti-beta actin antibody (sc-47778, Santa Cruz Biotechnology, Paso Robles, CA, USA) as an internal control. Blots were then incubated with a peroxidase-conjugated secondary antibody and visualized using a Novex® ECL Chemiluminescent Substrate Reagent Kit (ECL, Novex®, Grand Island, Life Technologies, NY, USA).

6. Statistical Analysis

A student's *t*-test was used for comparing the size between the canine DPCs and DPLTs. A value of $p < 0.05$ was considered to be statistically significant. The statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA).

III. RESULTS

1. Morphological and Cultural Characteristics of Actual Canine DPs and DPCs

The DPs were teardrop-shaped (Fig. 1a). Their approximate sizes were measured and compared with those of DPLTs (Fig. 1b). The mean actual canine DP maximal width was $265.8 \pm 11.50 \mu\text{m}$. The first outgrowth of dermal papilla cells presented after five days, and the cells had a flattened, polygonal morphology (Fig. 1c) with multiple cytoplasmic processes. We also observed the aggregative growth pattern in the multi-layer culture with the formation of the pseudo-papillae (Fig. 1d) and cells formed multilayered conglomerates on day 8.

2. Reconstruction of the DPLTs

After the cell detach-attach process, cell aggregates were formed within five days. The mean DPLT size was $296 \pm 12.43 \mu\text{m}$, and there was no statistical difference when compared to actual DP size ($p = 0.1465$) (Fig. 1e). Various factors affected DPLT formation; of these, cell inoculation density was one of the most prominent factors. We tried DPLT formation with different cell inoculation densities, from $2.0 \times 10^4 \text{ cells/cm}^2$ to $8.0 \times 10^4 \text{ cells/cm}^2$. It was found that the

optimal inoculation cell density was from 6.0×10^4 cells/cm² to 8.0×10^4 cells/cm² (data not shown). Cell migration only occurred in densities lower than 2.0×10^4 cells/cm². Even though significant aggregation was observed from a density of 4.0×10^4 cells/cm², in the optimal density, the number of DPLTs was higher and the sizes were more regular than those of DPLTs in a density of 4.0×10^4 cells/cm².

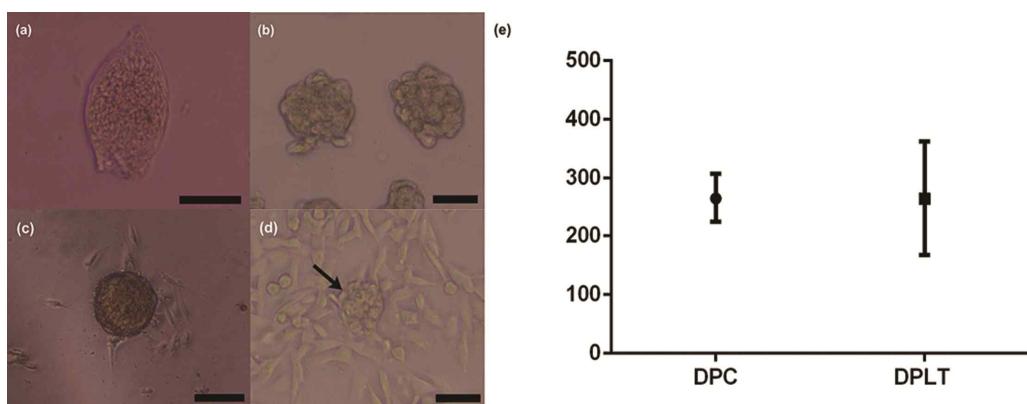


Fig. 1 Dermal papilla (DP), cultured dermal papilla cells (DPCs) and dermal papilla-like tissues (DPLTs). (a) Isolated DP (200X). (b) Reconstructed DPLTs from canine AD-MSCs (100X). (c) Primary DPCs culture. The first outgrowth cell was seen on day 5 (100X). (d) Typical multilayered, aggregative DPC growth pattern and the formation of pseudopapillae (arrow) (100X). (e) Comparison of DPs and DPLTs. There was no statistically significant difference in the maximal width between two groups.

3. Evaluating DPLTs as an Alternative to DPs

We performed RT-PCR and Western blotting to evaluate molecular similarities between DPLTs and DPs. Well-known hair-inductive markers, as previously mentioned, were assessed for evaluating the hair-inductive abilities of three groups on a genetic level. In addition, we assessed the quantitative protein expression level with laminin, vimentin and versican.

According to previous studies, ALP was used as an indicator for hair inductivity (6, 15), and versican was expressed in anagen hair follicles but absent in telogen hair follicles. This might support the idea that DP played an important role in anagen induction and maintenance. (15, 24) WIF1, vimentin and laminin were related to signal transduction, cellular junction and cytoskeleton, and extracellular matrix and cell adhesion of DPs, respectively. (12) In previous studies, WIF1 was considered to positively relate to *in vivo* murine hair-inductive capacity and was significantly over-represented in freshly isolated canine DPs. (1, 6, 11, 13) Vimentin is known for the functions of promoting the proliferation of DPCs and increasing cell migration and growth factor expression, and laminin plays an important role in promoting DP development and function during early hair morphogenesis. (25)

RT-PCR analysis revealed that AD-MSCs, DPCs, and DPLTs expressed all

markers as mentioned above (Fig. 2). In western blotting, all three groups showed similar versican expression level, which was reflected by their hair-inducing ability. However, we also observed that vimentin and laminin had a higher level of expression in DPCs and DPLTs compared to AD-MSCs (Fig. 3), which might mean that DPLTs have higher hair inductivity than AD-MSCs and the potential to be a viable substitute for actual DPs.

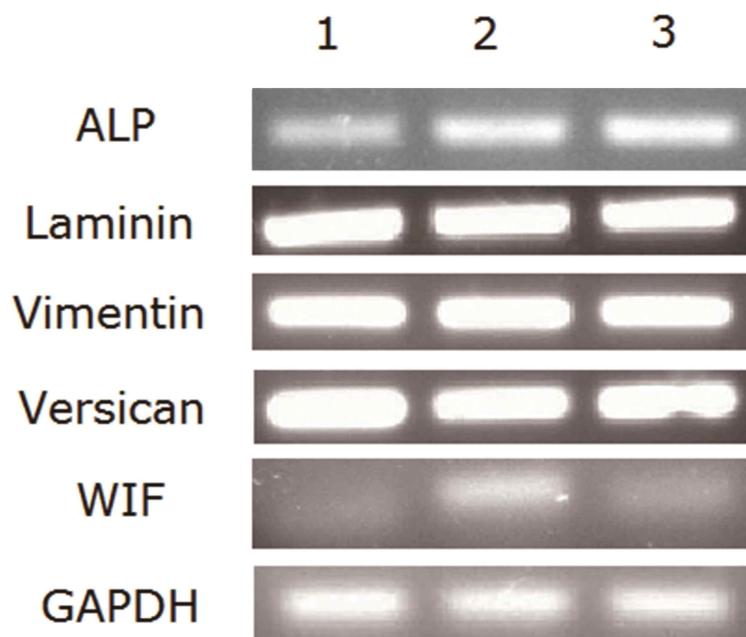


Fig. 2 RT-PCR of reconstructed DPLTs. Lane 1, monolayer cultured canine AD-MSCs (third passage). Lane 2, third passage of cultured canine DPCs. Lane 3,

DPLTs reconstructed from the third-passage canine AD-MSCs. Hair-inductive markers, ALP, WIF1, laminin, vimentin, versican, and prominin-1, were similarly expressed in all three groups.

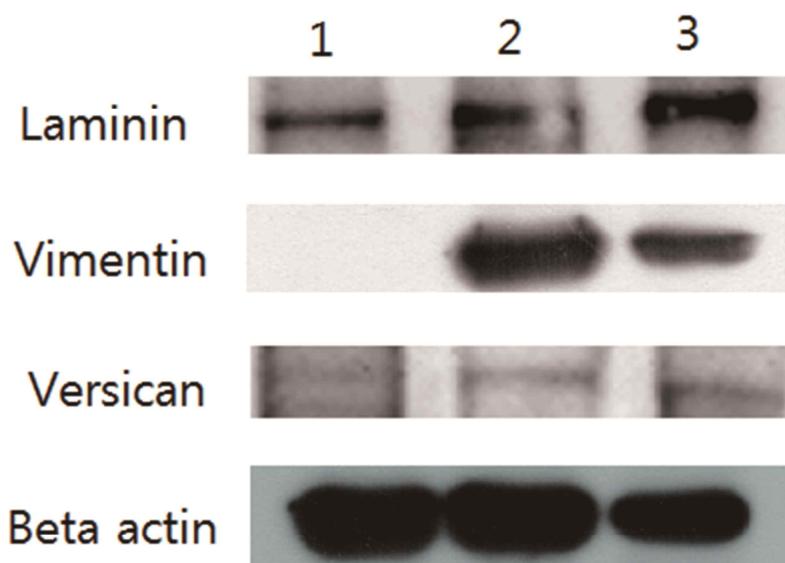


Fig. 3 Western blot of reconstructed DPLTs. Lane 1, monolayer cultured canine AD-MSCs (third passage). Lane 2, third passage of cultured canine DPCs. Lane 3, DPLTs reconstructed from third passage canine AD-MSCs. Vimentin, and laminin were expressed more in DPC and DPLT than in ASCs.

IV. DISCUSSION

Limited research has been done on canine hair follicles, and our understanding of hair follicles in veterinary and human medicine relies heavily on murine studies. (14, 15, 26) Not only are canine primary anagen hair follicles morphologically analogous to human anagen hair follicles in terms of size and structure but dogs also develop most major human alopecia. (2, 27) Therefore, canine hair follicles could be a helpful model for the investigation of human hair follicle biology.

There have been many trials and successes of regeneration of hair with epidermal and dermal components, both *in vitro* and *in vivo*. (15) Yoo *et al.* (23) also observed hair regeneration on the scalps of mice through transplantation of epidermal and mesenchymal compartments. The difference was that they applied aggregates from human umbilical cord-derived mesenchymal stem cells as an alternative to the dermal components comparing to former studies.

DP plays an important role not only in regulating hair follicle development and growth, but also as a reservoir of mesenchymal stem cells. Driskell *et al.* (27) noted that DPC expansion and generation might be one way of strategically treating alopecia. However, cultured DPCs lose their trichogenic properties *in vitro* after few passages. (15, 27, 28) Reconstructing DPLTs is one of the ways to overcome that limitation.

In this study, we found that the expression level of hair-inductive markers and proteins between DPCs and DPLTs showed similar patterns, which suggested that cell aggregates reconstructed from AD-MSCs might be excellent substitutes for actual DPs. DPLTs may have the potential to be a key structure in a future treatment method of alopecia that does not response to medical treatments, and in auto-transplantations. We may also consider a new trial to treat alopecia in veterinary medicine.

In previous study, it was demonstrated that AD-MSC, which had various cytokine-secreting properties and paracrine functions, showed an effect on hair growth by promoting the survival and proliferation of DPCs. (29) In addition, Osada *et al.* (30) demonstrated that aggregates made from later passage DPCs (more than 10 passages) did induce new hair follicles unlike dissociated DPCs that lost their hair follicle-inducing ability at later passage (over passage 4). Considering the results of previous and present studies, we cautiously speculate that DPLTs would have a direct effect on hair regrowth as an alternative structure of DPs and show analogous behavior patterns *in vivo* or under epidermal-mesenchymal interactions (EMIs) compared to dissociated AD-MSCs.

In the culture of canine DPCs, they showed typical growth pattern and morphology, as in previous studies. (1, 21, 30) We hypothesized that early-passage canine DPCs cultured in AminioMAX had the same capacity for hair

induction as canine DPs, based on previous studies. (1, 21) We used third-passage DPCs and observed their aggregative culture patterns. In the DP microdissection procedures, we selected late-anagen-phase hair follicles based on morphological criteria; to make the procedures simple, we chose large hair follicles, which could explain why DP sizes were measured larger than expected.

In conclusion, in this study, we reconstructed canine DPLTs with AD-MSCs, which are a more easily accessible source than other mesenchymal stem cells and DPCs, and evaluated them as alternatives to canine DPs. We believe that DPLTs may form the basis of potential therapeutic applications. However, further investigations on DPLTs under active EMIs or *in vivo* should be performed.

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VI. 국문초록

개 지방 유래 줄기세포를 이용한

진피 유두 유사조직의 형성

배 소희

서울대학교 대학원

수의학과

임상수의학 (수의외과학) 전공

(지도교수: 김완희)

탈모는 수의와 인의에서 중요한 피부질환이다. 특히 인의에서는 기존의 처치의 한계점을 극복하기 위해 중간엽 줄기세포를 이용한 모발 재생에 대한 연구가 활발하게 이뤄지고 있다. 개의 모낭은 사람 탈모 연구의 유용한 모델로 평가되나, 정작 개의 모낭에 대한 이해는 제한적이다. 이 연구는 개의 모낭, 그 중 진피유두에 대한 이해를 넓히고 이러한 진피유두의 대체제로서의 진피유

두 유사조직을 개 지방유래 중간엽 줄기세포를 이용하여 형성시키는 것에 그 목적이 있다. 본 연구자들은 개 진피유두를 1차배양하고, 배양과정을 관찰하였으며 RT-PCR과 western blot법을 이용하여, 진피유두와 관련된 유전자와 단백질 발현정도를 진피유두 유사조직과 비교, 평가하였다. 개 진피유두 세포는 여러층으로 겹쳐자라는 배양 양상을 보였으며, 가 진피 유두의 형성 또한 관찰할 수 있었다. 진피유두 유사조직은 성공적으로 형성되었다. 그들은 실제 개 진피유두와 유사한 형태학적 특성을 보였으며 유전자적, 단백질 발현양상 또한 유사하였다. 이러한 결과는 진피유두 유사조직이 실제 진피유두의 대체제로서의 사용될 수 있는 가능성 을 제시한다. 본 연구는 개 진피유두의 세포 수준의 배양적, 형태학적 그리고 유전자, 단백질 수준의 특성을 이해하는데 도움이 될 뿐 아니라 사람 모낭의 생물학적 이해와 잠재적 탈모치료 전략의 초석이 될 수 있다고 생각한다.

주요어: 개, 모발재생, 지방유래 간엽 줄기세포, 진피유두, 진피유두 유사조직

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