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# Primary cell culture and morphological characterization of canine dermal papilla cells and dermal fibroblasts

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**Abstract** Skin biopsies were taken from female dogs, the primary hair follicles isolated and the dermal papilla dissected. After incubation in supplemented Amniomax complete C100 medium in 24-well culture plates, the dermal papilla cells (DPC) grew to confluence within 3 weeks. Thereafter, they were subcultivated every 7 days. Dermal fibroblast (DFB) cultures were established by explant culture of interfollicular dermis in serum-free medium, where they reached confluence in 10 days. They were subcultivated every 5 days. For immunohistochemistry, cells were grown on cover slips for 24 h, fixed and stained with antibodies against collagen IV and laminin. DPC showed an aggregative growth pattern and formation of pseudopapillae. Intensive staining for collagen IV and laminin could be observed until the sixth passage. DFB grew as branching, parallel lines and showed only weak staining for collagen IV and laminin.

*Keywords*: canine, cell culture, collagen IV, dermal fibroblasts, dermal papilla cells, dog, hair follicle, immunohistochemistry, laminin.

## INTRODUCTION

The dermal papilla, which is a mesenchyme-derived structure located at the base of the hair follicle, is believed to play a major role in regulating the hair growth cycle. Since the first report of successful culture of rat dermal papilla cells by Jahoda and Oliver in 1981, valuable insights into the hair cycle regulation of many species have been gained by studying the *in vitro* capabilities of these cells.[1]

We report here, for the first time, a method for culturing canine dermal papilla cells and canine dermal fibroblasts, providing the basis for further specific research of canine hair growth. As it is indispensable for further research in canine hair growth to have cells available *in vitro* with similar capabilities as the cells have *in vivo*, we investigated the expression of two components of basal membranes (collagen IV and laminin) in skin biopsies (for the *in vivo* expression), as well as in cultured cells from the first to the ninth passage.

## METHODS

### *Cell culture*

Full-depth skin samples obtained from the abdominal region of bitches at the time of surgical removal of mammary gland tumours were immediately placed in phosphate buffer (PBS/EBSS 1:1, pH 7.2, Life Technologies, Eggenstein, Germany). In the laboratory, the skin was cut into small strips (1 × 3 mm) and the epidermis was removed with a scalpel blade. Under a dissecting microscope the hair follicles were isolated using watchmakers forceps and 20 G no. 1 injection needles. In a second preparation step, primary hair follicles were isolated and placed in a Petri dish with Amniomax complete C100 medium (Life Technologies). Under 40-fold magnification the dermal papillae were microdissected by removing the hair bulb from the rest of the follicle. Whereas the distal part of the bulb was fixed with forceps, the fibrous sheath was excised by applying gentle pressure with the tip of a 20 G no. 1 needle. After opening the fibrous sheath, the dermal papilla were detached from the matrix cells and could be dissected from the capillary stalk using two 27 G no. 20 needles.

Three dermal papillae were placed together in a single well of a culture plate (24-well cell culture cluster, Costar, CN 3524, Cambridge, MA, USA) in 1 mL of Amniomax complete C100 medium supplemented with 10% inactivated (56 °C, 2 h) and steroid-free canine serum (Promocell GmbH, Heidelberg, Germany). The papillae were incubated at 37 °C in a humid atmosphere containing 5% $CO_2$. The culture medium was changed every third day.

When the papilla cells had grown to full confluence, they were rinsed with PBS, detached from the dishes with trypsin (Trypsin/EDTA, 1:250, Life Technologies) and

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subjected to the first subcultivation at a density of 10 000 cells $mL^{-1}$. Subsequently, they were serially subcultivated by passaging every 7 days in a similar manner.

Dermal fibroblast cultures were established by explant culture of small cubes (2 × 2 mm) of interfollicular dermis from the same biopsy pieces. Serum-free Amniomax complete C100 medium was used to obtain conditions similar to those for dermal papilla cells. After reaching confluence, they were serially subcultivated by passaging every 5 days in a similar manner as dermal papilla cells.

*Immunofluorescence*

Cells from the first to the ninth passage were grown on cover slips for 24 h, then rinsed with PBS and fixed in ice-cold methanol (100%) for 10 min. The cover slips were mounted with DPX on glass slides. The cells were permeabilized for 10 min with Triton X-100 (Fluka, Buchs, Switzerland) in PBS, rinsed with PBS and incubated with 1.5% goat serum for 30 min at room temperature in a moist chamber. Cells were incubated with the primary antibody for 2 h, then rinsed with PBS and incubated for an additional hour with the secondary antibody. Cell nuclei were counterstained with propidium iodide (2 μL 50 $mL^{-1}$ aqua dest.). After a final rinse with PBS the slides were mounted with Mowiol (Hoechst, Frankfurt, Germany) and Propylgallate (Sigma-Aldrich, Wien, Austria).

As a positive control, sections of canine skin were used. Skin samples were obtained from dogs which were presented for euthanasia to local veterinarians. Within half an hour after euthanasia, skin biopsies (2 × 0.5 cm) were taken, fixed in 4% buffered formaldehyde for 24 h and embedded in paraffin wax. From these tissue blocks 4-μm sections were cut and control sections were stained with haematoxylin and eosin in order to detect histopathological changes. Only healthy skin was included in this study.

After blocking endogenous peroxidase activity with methanol/$H_2O_2$, antigen retrieval was accomplished by digesting with 0.1% protease (Sigma-Aldrich) in 0.05 M Tris/HCl buffer (pH 7.2) for 5 min at 37 °C. Slides were rinsed twice for 5 min using cold Tris buffer and incubated with 1.5% goat serum (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with the primary antibodies overnight at 4 °C, rinsed with PBS and incubated with mouse (Collagen IV) or rabbit (Laminin) Dako EnVision system. Antibody binding was detected with the chromogen DAB (Sigma-Aldrich) in Tris buffer (pH 7.4), rinsed with tap water and counterstained with Mayer's haematoxylin.

As a negative control the primary antibody was replaced by PBS. Staining results were evaluated with a confocal laser scanning microscope (Leica TCS-NT, Germany) and a standard microscope (Labophot 2, Nikon, Badhovedrop, the Netherlands), respectively.

The following antibodies were used:

1 Collagen IV: mouse monoclonal antibody, clone CIV22 (Quartett Immunodiagnostika u. Biotechnologie GmbH, Berlin, Germany). Dilution 1:25 for cells, 1:100 for skin samples.
2 Laminin: rabbit anti-laminin (DAKO, Glostrup, Denmark). Dilution 1:200 for cells, 1:1000 for skin samples.
3 Secondary antibody: Alexa™ anti-mouse (Molecular Probes, Oregon, USA).
4 Fluorescein anti-rabbit IgG (H+L) (Vector Laboratories).

## RESULTS

*Dermal papilla cells*

The first outgrowth of dermal papilla cells was observed after 4–7 days and they reached confluence after 2–3 weeks. The cells displayed a flattened, polygonal morphology, exhibited multiple cytoplasmic processes and showed an aggregative growth pattern in monolayer culture from the very beginning. Two weeks after initiation of the culture, formation of the so-called pseudopapillae was obvious. This characteristic growth pattern was retained until the end of the life-span of the cells, which was reached after nine subcultures. Until the sixth subculture, the passaging interval was 7 days, after which the proliferative activity of the dermal papilla cells slowed and the passaging interval had to be extended to 14 days (Fig. 1).

*Dermal fibroblasts*

The first outgrowth of dermal fibroblasts was observed after 2–5 days and they reached confluence after 10 days. From the very beginning of cell migration they showed the typical growth pattern of fibroblasts: they had a bipolar, spindle-shaped appearance and grew as branching, parallel lines, which remained as monolayers. Ten days after initiation of the culture they could be passaged for the first time. DFB could be subcultured 16 times, the passaging interval of 5 days could be retained till the tenth subculture and had then to be extended to 10 days. When the dermal fibroblasts were rinsed with PBS prior to detaching them with trypsin, the monolayer detached easily. This was in contrast to the dermal papilla cells, which adhered more strongly to the culture dish.

*Immunofluorescence*

*DPC.* Cell lines from four dogs were stained for collagen IV from the first to the ninth passage. During the first two passages an intense fine-grained staining at the cell border could be observed (Fig. 2a). In the third passage, collagen IV expression was very intense at the cell border (Fig. 2c). Beyond the fifth passage, collagen IV could be detected as a broad cloudy halo around the cells (Fig. 2e). In higher passages, collagen IV expression decreased gradually.

Cell lines from five dogs were stained for laminin expression from the first to the ninth passage. In the first and second passage a small, but intensely stained,

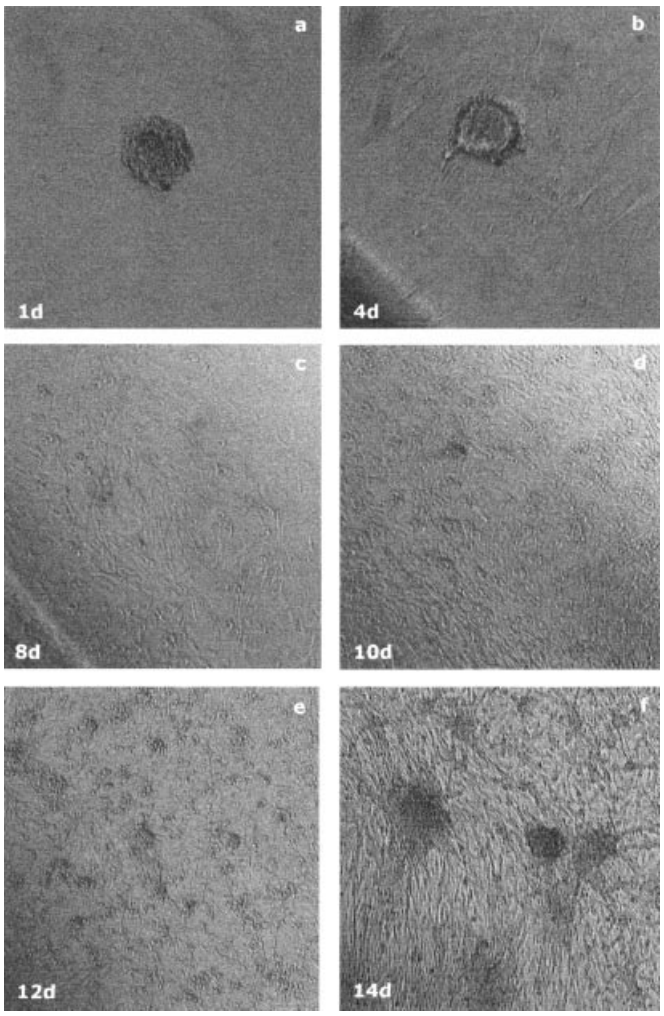


**Figure 1.** Dermal papilla cells (DPC) in culture. (a) Isolated DPC on day 1 (×10). (b) First outgrowth of cells is seen on day 4. (c) DPC on day 8 forming a monolayer. (d, e) Typical aggregative growth pattern is clearly visible on days 10 and 12. (f) Cells form multilayered conglomerates on day 14 (×25 original magnification).

ring around the nucleus was visible (Fig. 2b). During the next two passages, an intense staining appeared in the whole cytoplasm (Fig. 2d). From the fifth passage on the staining intensity decreased (Fig. 2f) and disappeared gradually to the ninth passage.

In DFB, only a few, more outspread cells showed weak positive staining for collagen IV and for laminin especially in higher passages (Fig. 2g,h).

Within the hair follicles of control skin sections, immunoreactivity for collagen IV as well as for laminin was present in the dermal papilla and in the connective tissue sheath (Fig. 3a,b). The basal membrane of fat cells and endothelial cells of blood vessels were clearly positive, whereas fibroblasts in the surrounding dermal tissue were negative for laminin and collagen IV staining.

## DISCUSSION

Dermal papilla cells have been cultured from a wide variety of species, including rat,[2] sheep[3] and red deer.[4] They are believed to be a useful model system for studying many aspects of hair growth.[5] Especially in human hair research, valuable insights into the mechanism of hair growth have been gained by investigating, for example, the influence of steroid hormones on *in vitro* dermal papilla cell growth,[6,7] steroid metabolism in dermal papilla cells from different body sites[8,9] and cell to cell signalling in response to various stimuli.[10,11]

We report here for the first time a method for the culture of canine dermal papilla cells. They showed typical cell morphology (flattened, polygonal cells with multiple cytoplasmic processes) and growth characteristic of dermal papilla cells. Similar to human cell lines, canine DPC showed an aggregative growth pattern and the formation of pseudopapillae.[2,12–14]

These specific growth characteristics made them clearly distinguishable from dermal fibroblasts, which had a spindle-shaped morphology and grew as branching, parallel lines, which remained as monolayers until confluence.

Dermal papilla cells are highly specialized fibroblasts, which have the capability of inducing hair growth in afollicular skin[15] and of producing basal membrane components *in vivo* (e.g. glycosaminoglycans,[16] laminin and collagen IV,[14,17] perlecan[17]). Human[12,13,18] and rat[15,19] dermal papilla cells retain their special capabilities for a certain time *in vitro*.

By the positive staining of control skin sections, we were able to demonstrate that canine dermal papilla cells *in vivo* have the ability to synthesize collagen IV and laminin. Collagen IV and laminin expression

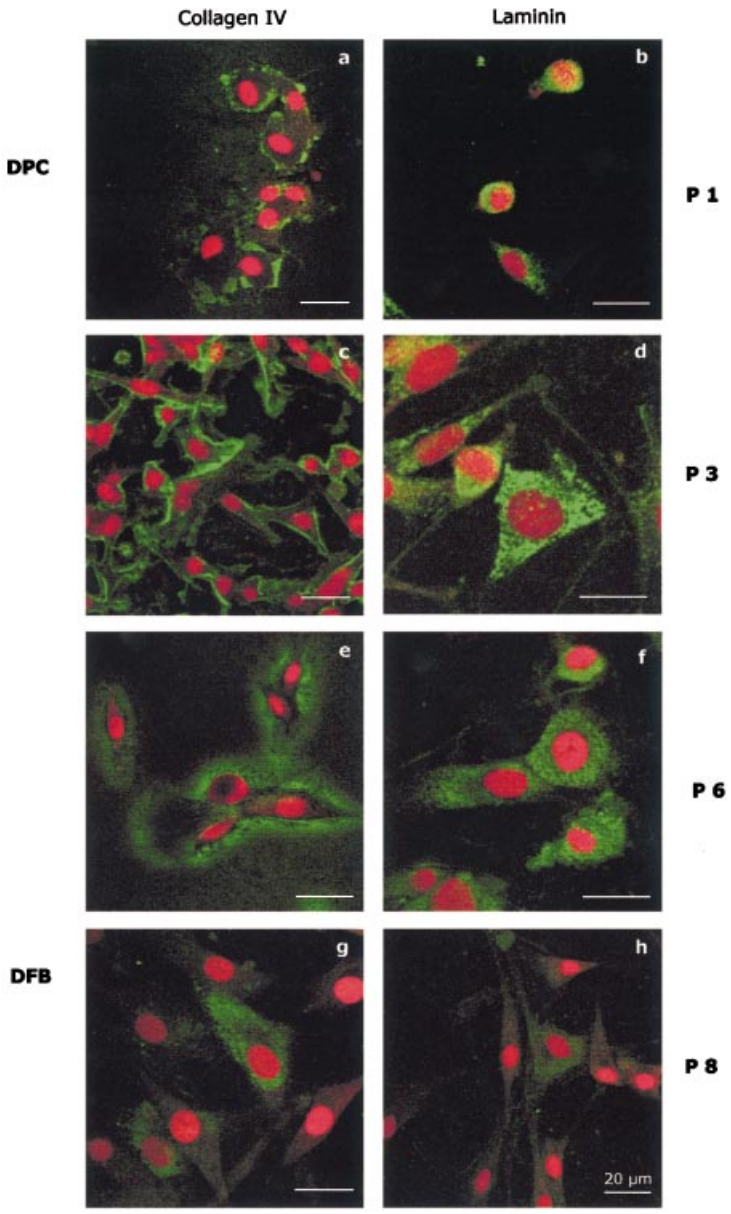


**Figure 2.** Fluorescent microscopical detection of collagen IV and laminin. (a–f) Dermal papilla cells, passage 1, 3 and 6. (g, h) Dermal fibroblasts, passage 8. Collagen IV was detected mainly surrounding the cell border, whereas laminin was found intracellularly. Both collagen IV and laminin were seen only in a few more outspread dermal fibroblasts and the staining was very weak.

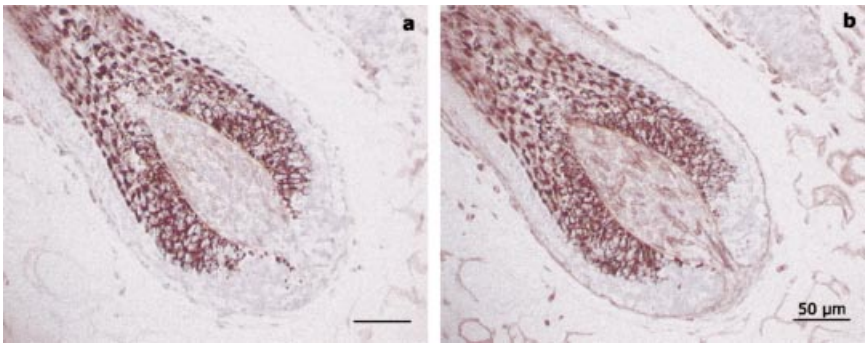


**Figure 3.** Detection of (a) collagen IV and (b) laminin in control skin samples. Note the positive staining around the cells of the dermal papilla.

*in vitro* could be observed up to the ninth passage in all examined passages of DPC. These results indicate clearly, that canine dermal papilla cells *in vitro* have similar capabilities to the corresponding cells *in vivo* during low passages. For DFB, *in vitro* expression of collagen IV[12] and laminin[20] is described as well. In our study, only occasional dermal fibroblasts in higher passages were able to express those basement membrane components.

William's medium E with the addition of 10% (v/v) fetal calf serum was used to culture dermal papilla cells from wool follicles of sheep.[21] Under these conditions, up to 16 passages were possible without significant change in the morphology. In our culture system the addition of 10% (v/v) canine serum was essential.

During higher passages, DPC lost some of their specific functions, as already described for rat cells.[19] We noticed a gradual decrease in the proliferation

rate as well as a decrease of collagen IV and laminin expression from the fifth passage on. As the investigated morphological parameters did not change during the first four passages, we suggest the use of dermal papilla cells up to the fourth passage for further investigations. Only at the third passage is the number of cells large enough to enable comparative studies on a large number of parallel experiments. This culture of canine dermal papilla should be a valuable tool for investigations of the canine hair growth.

## ACKNOWLEDGEMENTS

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## REFERENCES

1. Jahoda, C., Oliver, R. F. The growth of vibrissa dermal papilla cells *in vitro*. *British Journal of Dermatology* 1981; **105**: 623–7.
2. Almond-Roesler, B., Schön, M., Schön, M. P. *et al.* Cultured dermal papilla cells of the rat vibrissa follicle. Proliferative activity, adhesion properties and reorganization of the extracellular matrix *in vitro*. *Archives of Dermatological Research* 1997; **289**: 698–704.
3. Withers, A. P., Jahoda, C. A., Ryder, M. L. *et al.* Culture of wool follicle dermal papilla cells from two breeds of sheep. *Archives of Dermatological Research* 1986; **279**: 140–2.
4. Randall, V. A., Thornton, M. J., Messenger, A. G. *et al.* Hormones and hair growth: variations in androgen receptor content of dermal papilla cells cultured from human and red deer (*Cervus elaphus*) hair follicles. *Journal of Investigative Dermatology* 1993; **101**: 114–20S.
5. Randall, V. A. The use of dermal papilla cells in studies of normal and abnormal hair follicle biology. *Dermatologic Clinics* 1996; **14**: 585–94.
6. Arai, A., von Hintzenstern, J., Kiesewetter, F. *et al. In vitro* effects of testosterone, dihydrotestosterone and estradiol on cell growth of human hair bulb papilla cells and hair root sheath fibroblasts. *Acta Dermato-Venereologica* 1990; **70**: 338–41.
7. Kiesewetter, F., Arai, A., Schell, H. Sex hormones and antiandrogens influence *in vitro* growth of dermal papilla cells and outer root sheath keratinocytes of human hair follicles. *Journal of Investigative Dermatology* 1993; **101**: 98–105S.
8. Thornton, M. J., Laing, I., Hamada, K. *et al.* Differences in testosterone metabolism by beard and scalp hair follicle dermal papilla cells. *Clinical Endocrinoology (Oxford)* 1993; **39**: 633–9.
9. Hamada, K., Thornton, M. J., Laing, I. *et al.* The metabolism of testosterone by dermal papilla cells cultured from human pubic and axillary hair follicles concurs with hair growth in 5 alpha-reductase deficiency. *Journal of Investigative Dermatology* 1996; **106**: 1017–22.
10. Thornton, M. J., Hamada, K., Messenger, A. G. *et al.* Androgen-dependent beard dermal papilla cells secrete autocrine growth factor(s) in response to testosterone unlike scalp cells. *Journal of Investigative Dermatology* 1998; **111**: 727–32.
11. Hembree, J. R., Harmon, C. S., Nevins, T. D. *et al.* Regulation of human dermal papilla cell production of insulin-like growth factor binding protein-3 by retinoic acid, glucocorticoids, and insulin-like growth factor-1. *Journal of Cellular Physiology* 1996; **167**: 556–61.
12. Chiu, H. C., Chang, C. H., Chen, J. S. *et al.* Human hair follicle dermal papilla cell, dermal sheath cell and interstitial dermal fibroblast characteristics. *Journal of the Formosan Medical Association* 1996; **95**: 667–74.
13. Messenger, A. G., Senior, H. J., Bleehen, S. S. The *in vitro* properties of dermal papilla cell lines established from human hair follicles. *British Journal of Dermatology* 1986; **114**: 425–30.
14. Messenger, A. G., Elliott, K., Temple, A. *et al.* Expression of basement membrane proteins and interstitial collagens in dermal papillae of human hair follicles. *Journal of Investigative Dermatology* 1991; **96**: 93–7.
15. Inamatsu, M., Matsuzaki, T., Iwanari, H. *et al.* Establishment of rat dermal papilla cell lines that sustain the potency to induce hair follicles from afollicular skin. *Journal of Investigative Dermatology* 1998; **111**: 767–75.
16. Taylor, M., Ashcroft, A. T., Westgate, G. E. *et al.* Glycosaminoglycan synthesis by cultured human hair follicle dermal papilla cells: comparison with non-follicular dermal fibroblasts. *British Journal of Dermatology* 1992; **126**: 479–84.
17. Couchman, J. R. Hair follicle proteoglycans. *Journal of Investigative Dermatology* 1993; **101**: 60–4S.
18. Katsuoka, K., Schell, H., Hornstein, O. P. *et al.* Comparative morphological and growth kinetics studies of human hair bulb papilla cells and root sheath fibroblasts *in vitro*. *Archives of Dermatological Research* 1986; **279**: 20–5.
19. Jahoda, C. A., Horne, K. A., Oliver, R. F. Induction of hair growth by implantation of cultured dermal papilla cells. *Nature* 1984; **311**: 560–2.
20. Woodley, D. T., Stanley, J. R., Reese, M. J. *et al.* Human dermal fibroblasts synthesize laminin. *Journal of Investigative Dermatology* 1988; **90**: 679–83.
21. Bates, E. J., Hynd, P. I., Penn, N. M. *et al.* Serum-free culture of wool follicles: effects of nutrients, growth factors and hormones. *British Journal of Dermatology* 1997; **137**: 498–505.

**Résumé** Des biopsies cutanées ont été prélevées sur des chiennes, les follicules pileux primaires ont été isolés et les papilles dermiques ont été disséquées. Après incubation dans des boîtes de culture contenant un milieu C100 supplémenté en Amniomax, les cellules de la papille dermique (DPC) ont poussé à confluence à 3 semaines. Ensuite, elles ont été repiquées tous les 7 jours. Des cultures de fibroblastes dermiques (DFB) ont été établies en utilisant des cultures de derme interfolliculaire dans un milieu sans sérum, et ont atteint la confluence en 10 jours. Elles ont été repiquées tous les 5 jours. the dermal papilla cells (DPC) grew to confluence within 3 weeks. Pour l'immunohistochimie, les cellules ont été cultivées sur des cover slips pendant 24 heures, fixées et marquées avec des anticorps contre le collagène IV et la laminine. Les DPC ont montré une pousse avec aggrégation, et la

formation de pseudopapilles. Un marquage intensif pour le collagène IV et la laminine a pu être observé jusqu'au sixième passage. Les DFB ont poussé en lignes parallèles, et ont présenté un marquage faible pour le collagène IV et la laminine.

**Resumen** Se tomaron biopsias cutáneas de hembras y se disecaron los folículos primarios y la papila dérmica. Después de su incubación en medio Amniomax C100 suplementado en placas de 24 pocillos, las células de papila dérmica (DPC) crecieron hasta la confluencia en 3 semanas. Posteriormente, se subcultivaron cada 7 días. Los cultivos de fibroblastos dérmicos (DFB) se establecieron por cultivos de explantación de la dermis interfolicular en medio libre de suero, en el que alcanzaron la confluencia a los 10 días. Se subcultivaron cada 5 días. Para inmunohistoquímica, las células fueron cultivadas en cubreobjetos durante 24 h., fijadas y teñidas con anticuerpos contra colágeno IV y laminina. Las DPC mostraron un patrón de crecimiento agregativo y formación de pseudopapilas. Se observó una tinción intensa para colágeno IV y laminina hasta el sexto pase. Las DFB crecieron como líneas remificadas y paralelas y mostraron solamante una débil tinción para colágeno IV y laminina.

**Zusammenfassung** Hautbiopsien von Hündinnen wurden entnommen, die Primärhaarfollikel isoliert und die dermalen Papillae reseziert. Nach Inkubation in supplementiertem Amniomax C100 Medium in Kulturplatten konfluierten die Dermalpapillarzellen (DPZ) innerhalb von 3 Wochen. Danach wurden sie alle 7 Tage subkultiviert. Kulturen von dermalen Fibroblasten (DFB) wurden durch Explant-Kultur von interfollikulärer Dermis in serumfreiem Medium etabliert und konfluierten innerhalb von 10 Tagen. Sie wurden alle 5 Tage subkultiviert. Zur immunhistochemischen Bewertung wurden Zellen auf Deckgläsern für 24 Stunden kultiviert, fixiert und mit Antikörpern gegen Kollagen IV und Laminin gefärbt. DPZ zeigten ein aggregatives Wachstumsschema und Formation von Pseudopapillae. Intensive Färbung von Kollagen IV und Laminin konnte bis zur 6. Passage gesehen werden. DFB wuchsen in verzweigten, parallelen Linien und zeigten nur schwache Färbung für Kollagen IV und Laminin.