Expression of mesenchymal stem cell marker CD90 on dermal sheath cells of the anagen hair follicle in canine species

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The dermal sheath (DS) of the hair follicle is comprised by fibroblast-like cells and extends along the follicular epithelium, from the bulb up to the infundibulum. From this structure, cells with stem characteristics were isolated: they have a mesenchymal origin and express CD90 protein, a typical marker of mesenchymal stem cells. It is not yet really clear in which region of hair follicle these cells are located but some experimental evidence suggests that dermal stem cells are localized prevalently in the lower part of the anagen hair follicle.

As there are no data available regarding DS stem cells in dog species, we carried out a morphological analysis of the hair follicle DS and performed both an immunohistochemical and an immunocytochemical investigation to identify CD90+ cells. We immunohistochemically evidenced a clear and abundant positivity to CD90 protein in the DS cells located in the lower part of anagen hair follicle. The positive cells showed a typical fibroblast-like morphology. They were flat and elongated and inserted among bundles of collagen fibres. The whole structure formed a close and continuous sleeve around the anagen hair follicle. Our immunocytochemical study allowed us to localize CD90 protein at the cytoplasmic membrane level.

Key words: CD90, mesenchymal stem cells, hair follicle, dog.

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The hair follicle represents an important stem cell niche in the skin. It contains dermal and epithelial stem populations that display distinct properties and localization. While epithelial stem cells reside in the middle region of the hair follicle outer root sheath (Schneider *et al.,* 2009; Lyle *et al.,* 1998; Cotsarelis *et al.,* 1990), dermal stem cells are located in the dermal sheath (DS) (Jahoda, 2003; Jahoda and Reynolds, 2001).

The dermal sheath, or fibrous root sheath, is a layer of dense connective tissue that extends along the hair follicle, from the bulb up to the infundibulum. In the anagen hair follicle, it is comprised of mesenchymal cells located among collagen and elastic fibres. The cells are flat and elongated while collagen fibres form a circular inner layer and a longitudinal outer layer in the lower part of hair follicle (VonTscharner and Suter, 1994; Jahoda *et al.,* 1992). At the base of the hair follicle, the DS is connected to the dermal papilla (Scott *et al.,* 2000). The basement membrane, or glassy membrane, separates the DS from the epithelial component of the hair follicle (Scott *et al.,* 2000).

Follicular dermal stem cells have a mesenchymal origin and share many properties common to bone marrow-derived mesenchymal stem cells (MSCs) (Hoogduijn *et al.,* 2006). They express the MSC cell-surface marker CD90, show a high colony forming unit ability and can differentiate into several mesenchymal lineages, such as osteoblasts, adipocytes, chondrocytes and myocytes (Hoogduijn *et al.,* 2006; Jahoda *et al.,* 2003). They also express neuroprogenitor markers (Hoogduijn *et al.,* 2006) and, finally, they can repopulate the haematopoietic system (Lako *et al.,* 2002). In the literature, we can find different information about stem cell localization: the whole dermal sheath, the peri-bulbar dermal sheath, the dermal papilla (Hoogduijn *et al.,* 2006, McElwee *et al.,* 2003,

Gharzi *et al.,* 2003, Jahoda *et al.,* 2003.)

CD90 (Thy-1) is a small GPI-anchored protein localized in the outer leaflet of the cell membrane (Low and Kincade, 1985). This protein is present in a large number of tissues and cells, even if a great species variation has been described (Mansour Haeryfar, 2004; Tokugawa *et al.,* 1997; McKenzle and Fabre, 1981). CD90 plays a role in cell-cell interaction events, including intracellular adhesion and cell recognition during development (Saalbach *et al.,* 2000; Morris, 1985), and is considered an important stem cell marker; for this last reason it is commonly used to identify mesenchymal stem cells *in vitro* (Kern *et al.,* 2007; Yoshimura *et al.,* 2006; Le Blanc and Ringdén, 2006; Pittenger *et al.,* 1999). Furthermore, it has been identified in other kinds of stem cells such as haematopoietic progenitor cells (Craig *et al.,* 1993) and hepatic progenitor cells in the human fetal liver (Masson *et al.,* 2006).

The hair follicle is the focus of increasing interest because it contains well defined stem cell populations that exhibit various developmental properties. We retain that in dogs, as already demonstrated in other species (Hoogduijn *et al.,* 2006; Zhang *et al.,* 2006; Jahoda *et al.,* 2003; Lako *et al.,* 2002), this organ may be a suitable and accessible source for both epithelial and mesenchymal stem cells that may be isolated and *in vitro* cultured. Since it is possible to take skin samples without injuring the patient, we chose the hair follicle to study and identify stem cells with the future purpose of using them in regenerative medicine.

Dogs are affected by several skin diseases and some of them may be related to alterations of somatic stem cells. We retain that the study of hair follicle stem cell biology may improve our knowledge of etiology and pathogenesis of these skin diseases.

In previous works we investigated the stem cells in dog hair follicles; we identified the location of putative epithelial stem cells at the isthmus and described the bulge-like region (Pascucci *et al.,* 2006; Mercati *et al.,* 2008). To the authors' knowledge, there are no data available neither concerning the localization of DS stem cells nor concerning the expression of CD90 in the hair follicle as regards the canine species. Therefore, in this study, we described the morphological characteristics of DS cells and examined the immunohis-tochemical localization of CD90 protein in dog hair follicles with both light and transmission electron microscopy. The aim of our study is to observe the dermal sheath cells encompassing the hair follicle and to determine where CD90+ cells reside. CD90 is one of the main markers used to identify mesenchymal stem cells and it has been observed in stem cells isolated from the dermal sheath of hair follicles (Hoogduijn *et al.,* 2006). For this reason, we suppose that CD90 protein can help us to identify the hair follicle dermal stem compartment in dog.

Materials and Methods

Sample collection

Skin samples were obtained by excisional biopsy from the dorsal neck, from the cheek and from the abdominal region of healthy dogs; the skin was devoid of primary or secondary cutaneous lesions. Dogs of different breeds, but all characterized by a smooth coat, were selected: a Belgian shepherd, a Dalmatian, a Labrador retriever, a Border Collie, and three mixed breeds. Their ages ranged from 3 to 9 years.

Basic histology

The skin samples were fixed in a 10% neutral buffered formalin solution, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Five µm thick sections were processed for staining with haematoxylin and eosin and then observed under a light microscope.

Ultrastructural studies

Immediately after excisional biopsy, the tissue samples were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 4 hours at room temperature, and post-fixed in 2% osmium tetroxide in 0.1M phosphate buffer for 3 hours. Then, the specimens were dehydrated in graded ethanol, embedded in Epon 812 (Electron Microscopy Sciences) and cured at 60°C for 48 hours.

Semithin sections were cut for light microscope and stained with methylene blue in borax; 90 nm ultrathin sections were mounted on 200-mesh gold grids, counterstained with uranyl acetate and lead citrate and analyzed by a transmission electron microscope (Philips EM208).

Immunohistochemistry

Sections processed as for basic histology were mounted on poly-L-lysine-coated slides and dried at 37°C.

Immunohistochemistry was performed using standard techniques. In dewaxed sections the endogenous peroxidase activity was quenched with 10' incubation in a peroxidase-blocking solution (3% H_2O_2). Then, the sections were treated for 10 min with 0.05% trypsin solution for antigen retrieval and blocked with 1:10 normal goat serum for 30'. Subsequently, the sections were incubated with 1:100 mouse monoclonal anti-CD90 antibody (VMRD) for 24 hours at room temperature and, then, with 1:200 goat anti-mouse biotin conjugate antibody (Zymed). The Vectastain ABC kit (Vector Laboratories) and DAB cromogen (Dako Cytomation) were used to visualize the immunohistochemical reaction. Negative control sections were produced by omission of the primary antibody.

Immunocytochemistry

After fixation for 1 hour at room temperature in 4% paraformaldehyde and 0.1% glutaraldehyde buffered by 0.1M phosphate saline solution (pH 7.4), the specimens were dehydrated and embedded in Bioacryl resin (Bio-optica, Milano, Italy). Semithin sections (1 µm thick) were stained with methylene blue in borax for light microscopic examination to identify the region of our interest. Ultrathin sections (90 nm) were subsequently cut and mounted on parlodion-coated 200-mesh nickel grids (Sigma), treated with 1% BSA in 0.1M TBS pH 7.4 for 5 min at room temperature and, then, incubated for 2 h at room temperature in a humid chamber with mouse monoclonal anti-CD90 antibody (VMRD) diluted 1:20 in a solution of 0.1 M TBS containing 1% BSA and 1% Normal Goat serum. After several washes in TBS to remove the excess antibody, the grids were incubated for 1 h at room temperature with goat anti-mouse antibody conjugated with 10 nm gold particles (Aurion) diluted 1:40 in 0.1M TBS pH 7.4 plus 1%BSA. After several washes in TBS and counterstaining with uranyl acetate, the sections were examined with a Philips EM 208 transmission electron microscope.

Sections in which the primary antibody was omitted were used as negative control.

Results

Morphological evaluation

At the light microscopical level, we could describe the DS of the lower part of the anagen hair follicles as a thin layer closely adjacent to the outer root sheath (Figure 1a, 1c, 4a). It was rich in dermal flat cells with lengthened nuclei; the cells were arranged along the follicular wall in a parallel way. The DS appeared as a distinct and clearly recognizable structure because in the lower part of the hair follicle it was encompassed by adipocytes. In the medium and upper regions, the DS was contiguous with the dermis and the dermal cells seemed to be less numerous. In semithin sections, the DS cells were clearly recognizable as a continuous layer of flattened cells whose cytoplasm appeared as a slender and threadlike frame that exactly followed the epithelium profile (Figure 1a, 1c).
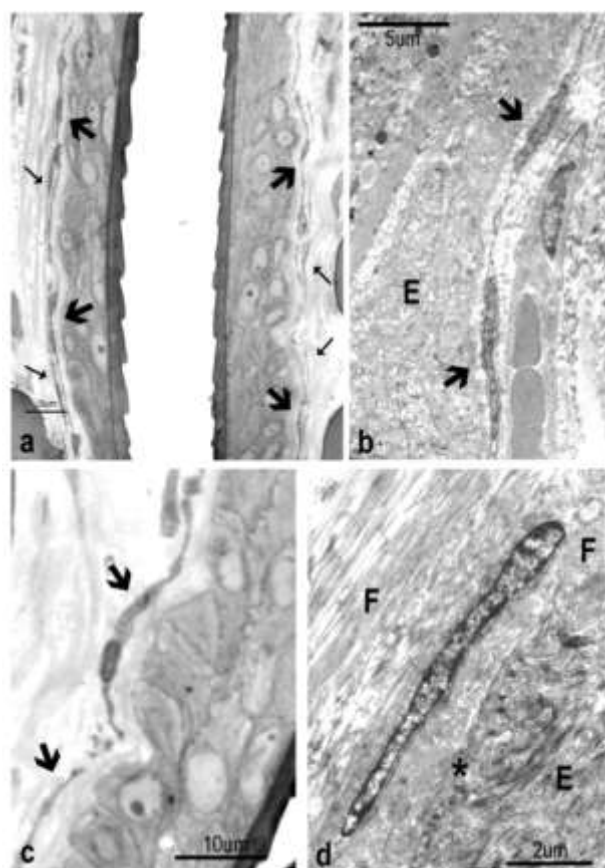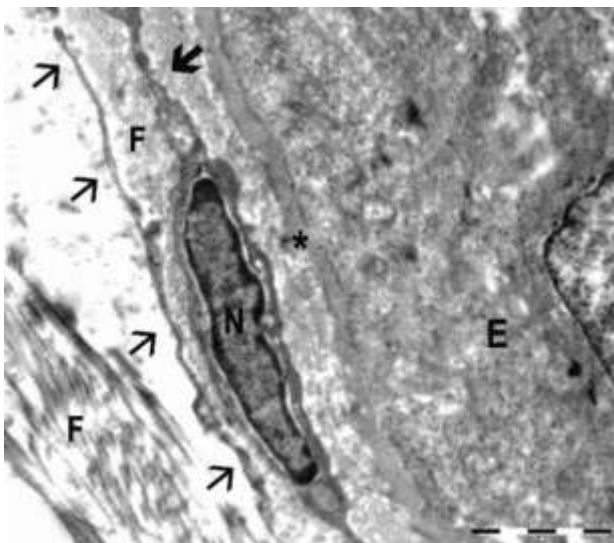

Figure 1. The soprabulbar and bulbar region of the anagen hair follicle. The dermal sheath cells are located all along the hair follicle, are very thin and flattened with elongated nuclei. The cytoplasm appears as a threadlike frame that occasionally overlaps among adjacent cells (thin arrow). Semithin (a-c) and ultrathin sections (b-d). Arrows = dermal sheath cells; E = epithelial cells of the follicle; *basal membrane; F, collagen fibres. Bar =10 µm (a-c), 5 µm (b), 2 µm (d).

Transmission electron microscopical observations showed that the DS consisted of a layer of fibrous connective tissue and a basal membrane in close apposition with the follicular epithelium (Figure 1d, 2). In the connective tissue it was possible to recognize the dermal cells as being elongated and slender, flattened against the follicular epithelium and parallely aligned to the follicular wall (Figure 1b). The nuclei of the dermal cells appeared regular and elongated with a distinctive layer of marginal heterochromatin adjacent to the membrane (Figure 1d, 2). The cells were comprised among bundles of collagen fibres that presented a circular and longitudinal arrangement; the circular layer was the nearest to the basal membrane (Figure 1d). The cytoplasm of dermal cells was very thin and attenuated (Figure 2). The cell processes were inserted among the fibres and it seemed that, as a whole, the cells formed an unbroken sleeve around the follicle. It was possible to observe a large number of hemidesmosomal junctions between the basal cells of the outer root sheath and the basal membrane.

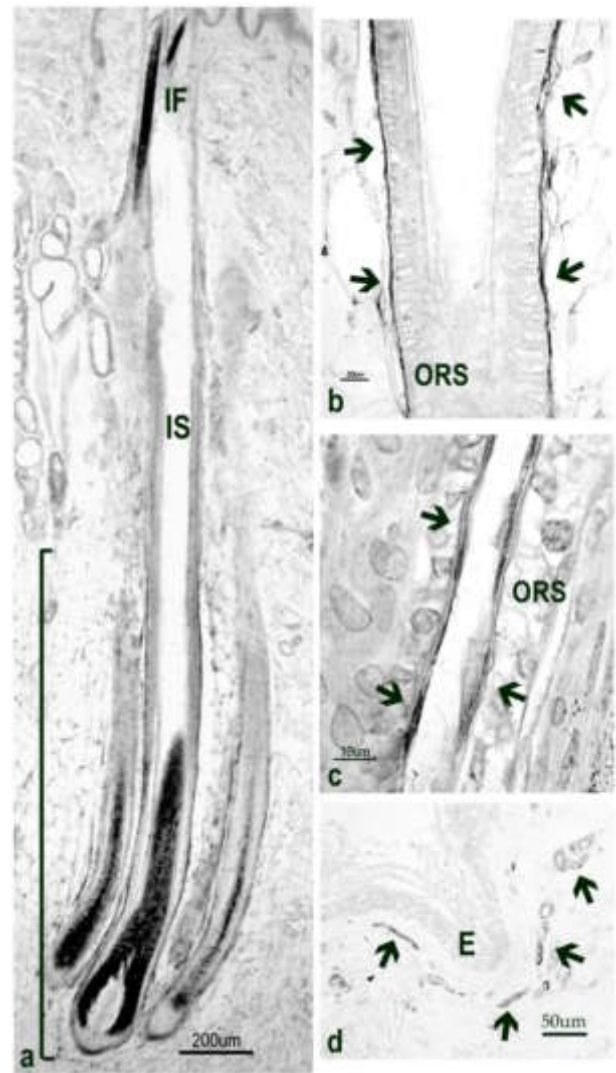### In situ expression of CD90 in the hair follicle dermal sheath

Immunohistochemistry

The immunohistochemical investigations, performed on the skin specimens, permitted us to evidence the expression of CD90 antigen in the perifollicular DS (Figure 3). The CD90+ area was quite large with regard to the hair follicle extension (Figure 3a). This area appeared as a continuous line around the hair follicles (Figure 3b, 3c), but only the bulbar and the soprabulbar regions were clearly encompassed by an intense staining. The reactivity sometimes stopped in a rather sharp way between the isthmic and the soprabulbar region; however, it sometimes seemed to involve the most prossimal part of the isthmus as well. We also observed some
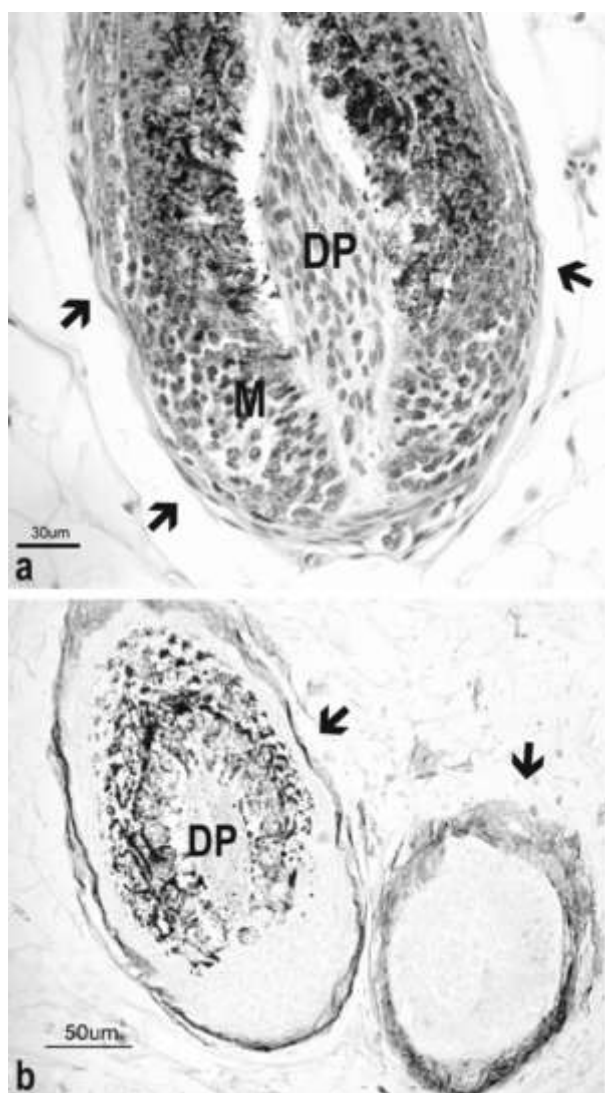

Figure 2. A dermal sheath cell extends along the hair follicle and is parallely aligned with the epithelium profile. It is possible to observe the thicker central part, where the nucleous (N) is located, and the thinner peripherical part (big arrow). The thin arrows point to the threadlike cytoplasm of another cell that is located at the side. E = epithelial cells of the follicle; *basal membrane; F, collagen fibres. Bar = 2 μm.

Figure 3. Immunohistochemical staining for CD90 protein. (a) An entire primary and two secondary hair follicles. The bulbs are well-developed and present the onion-shaped thickening typical of the anagen phase. The soprabulbar and the bulbar regions of all follicles are positively stained for CD90 protein (square bracket). (b-c) Soprabulbar region, higher magnification. The positive staining (arrows) appears as a sharp outline along the epithelium profile. (d) The microvessels below the epidermis are CD90 positive (arrows). Epidermis not stained. ORS, outer root sheath; E = epidermis; IF, infundibulum; IS, Isthmus. Bar =200 μm (a), 20 μm (b), 10 μm (c), 50 μm (d).

CD90^{+} mesenchymal cells around the bulb of anagen hair follicles but detached from the DS. The dermal papilla cells were negative to CD90 antibody (Figure 4b). All primary and secondary hair follicles showed clear immunohistochemical positivity (Figure 3a). No reaction for CD90 was detected in the epithelial structures of the skin, such as the follicular epithelium, the epidermis, sebaceous or sweat glands. The blood vessels scattered in the dermis appeared positive to CD90 (Figure 3d).

Immunocytochemistry

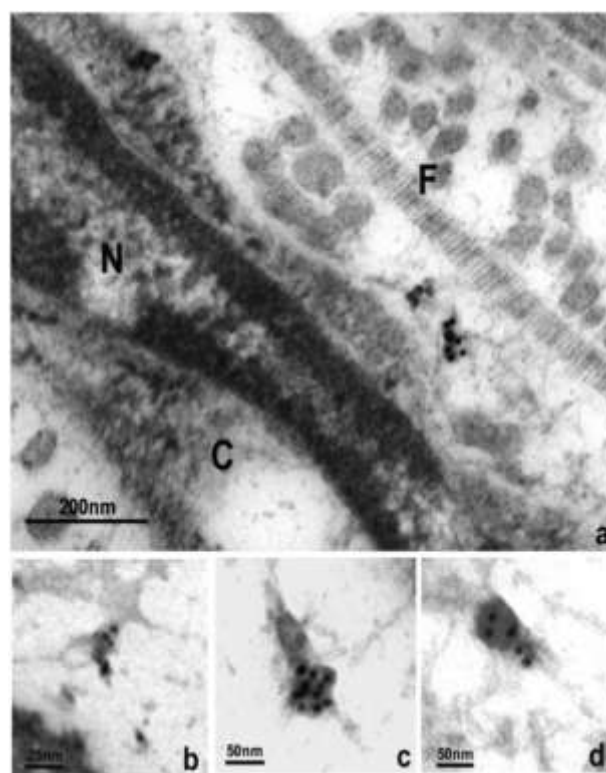Immunocytochemical observations evidenced clusters of gold particles, localized at the level of the cytoplasmic membrane of DS cells (Figure 5). Positivity was not uniformly scattered along the membrane but showed a focal expression with a distribution all along the cell. We observed positivity in the lower regions of anagen hair follicles.

Discussion

Mesenchymal stem cells arouse great interest because of their potentiality in the treatment of several diseases in man as well as in domestic animals and, at the moment, it is possible to obtain these cells from a great range of tissutal source besides hair follicles (Kern *et al.,* 2007; In't Anker *et al.,* 2003; O'Donoghue *et al.,* 2003; Shi *et al.,* 2003; Zuk *et al.,* 2002; Erices *et al.,* 2000). Each source presents some peculiar characteristics that distinguish it from others such as morphology, stem cell concentration, cell differentiation capacity, etc.

The dermal sheath surrounding the outer root sheath contains progenitor and stem cells, but it is

Figure 4. The bulb region. (a) Morphological features of anagen (H&H). (b) Dermal sheath cells are clearly recognizable as a dark line around the bulb (CD90 immunohistochemical staining). Arrows = dermal sheath; DP, dermal papilla; M, matrix. Bar =30 μm (a), 50 μm (b).

Figure 5. Immunocytochemical assay for CD90 protein. The colloidal gold particles are grouped in clusters and show a focal distribution along the cytoplasmic membrane. (a) A gold aggregate in the cell body and (b, c, d) in the cytoplasmic processes, higher magnifications. C, dermal sheath cell; N, nucleus; F, collagen fibres. Bar =200 nm (a), 25 nm (b), 50 nm (c-d).

not yet really clear where these cell populations reside: on the basis of functional characteristics, it seems that these cells are localized in the lower part of hair follicles; nevertheless, it is also possible to obtain multipotent cells from every region of the hair follicle dermal sheath (Hoogduijn *et al.,* 2006; McElwee *et al.,* 2003; Jahoda *et al.,* 2003; Lako *et al.,* 2002).

Given the potentiality to use hair follicle stem cells in the therapy of some skin diseases, in this report we proposed an investigation regarding hair follicle dermal stem cells in dog species.

Dog coat is a mosaic comprised of primary and secondary hair follicles that are in different stages of the hair cycle at any one time (Scott *et al.,* 2000). This fact can cause some morphological differences among hair follicles that reveal themselves also in the connective compartment and in the spatial distribution of dermal cells.

Since dog hair types vary greatly, we selected only dogs with smooth coats, avoiding curly coats, to standardize our work plan. As it is known (Scott *et al.,* 2000), straight hairs are produced by straight follicles while curly hairs are produced by curly follicles. On the basis of this consideration, we chose only smooth coats to obtain whole longitudinal sections of the cut hair follicles and to observe, at the same time, the entire structure and morphology of the organ.

As we did not want to evaluate age differences, we chose a group of animals whose ages ranged from three to nine years. We retain that this group is homogeneous enough because in this period the dog is considered an adult at the height of its physical and sexual development. We did not select younger or older dogs.

We first carried out morphological investigations on dermal sheath anagen hair follicles and evidenced a structure with typical characteristics as described by other authors (Scott *et al.,* 2000; VonTscharner and Suter, 1994; Jahoda *et al.,* 1992). We identified DS as a thin structure closely adjacent to the outer root sheath and rich in dermal flat cells. By transmission electron microscopy, we observed slender and attenuated cells located among bundles of collagen fibres and parallely aligned to the follicular wall; the whole structure formed a close and continuous sleeve around the anagen hair follicle. The cells presented a typical fibroblastic morphology that is also described in anagen VI mouse hair follicles (Tobin *et al.,* 2003).

The nuclei were rather euchromatic and had a regular and elongated shape. Our observation evidenced a cell population with a uniform appearance.

Immunohistochemical investigation allowed us to evidence CD90 expression in dog skin: the protein was expressed only in the cells with a mesenchymal origin while all epithelial structures resulted negative. Most of the positive cells were located in the perifollicular DS of anagen hair follicles, especially in the bulbar and the soprabulbar regions; despite this, in the dermal papilla there were no cells positive to CD90. Also the infundubulum and the isthmus were negative: the positivity sometimes stopped in a rather sharp way between the isthmic and the soprabulbar region; however, we could sometimes observe positive cells in the lower part of the isthmus. We ascribed these differences to the changeable morphological features of hair follicles (Scott *et al.,* 2000). We also observed clear positivity to CD90 antigen in the endothelial cells of the vascular structures localized in the dermis. As it is known, CD90 protein promotes the adhesion of leukocytes and monocytes to activated endothelial cells during the inflammatory process (Wetzel *et al.,* 2004; Saalbach *et al.,* 2000). However, this protein was also detected on microvascular endothelial cells of noninflamed human skin by immunohistochemical technique (Mason *et al.,* 1996; Fivenson *et al.,* 1992). CD90 was evidenced on endothelial cells of human fetal liver as well (Masson *et al.,* 2006).

Immunocytochemical investigation gave the same pattern of CD90 localization in anagen hair follicles as immunohistochemical technique. CD90 was localized in the dermal cells at the cytoplasmic membrane level. It was distributed all along the cell with a focal expression that is a typical pattern of distribution of the membrane proteins. The immunohistochemical investigation showed CD90 positivity as a continuous line around the hair follicle, while the more accurate immunocytochemical observation revealed a focal expression of the protein.

The precise biological function of CD90 protein is not yet clear (Mansour Haeryfar, 2004); it probably has a complex role in multiple biological processes (Rege and Hagood, 2006). Even if CD90 is considered a marker of mesenchymal stem cells, it is expressed by several kinds of cells including fibroblasts. It is known that these cells exhibit func-

tional and phenotypic heterogeneity among organs and also within tissues (Silvera *et al.,* 1991; Jordana *et al.,* 1988; Elias *et al.,* 1987; Azzarone and Macieira-Coelho, 1982), and that they have a role beyond architectural support. On the basis of CD90 expression, the fibroblasts of some tissues were divided into groups with different morphological and functional characteristics (Koumas *et al.,* 2001, 2003; Borrello and Phipps, 1996; Smith *et al.,* 1995; Derdak *et al.,* 1992). Furthermore it has been hypothesized that CD90+ fibroblasts are actively involved in wound healing (Koumas *et al.,* 2001, 2003).

On the basis of these considerations, we retain that the clear localization of CD90+ cells we noted in dog skin specimens is of some interest, and suppose that this result may provide further evidence for biological differences existing among mesenchymal cells of the dermis. The lower part of anagen hair follicles is the only region where CD90+ cells are localized while the dermal sheath cells of the upper hair follicle, such as the mesenchymal cells scattered in the dermis, result CD90−. The localization is so specific that CD90 may also be considered as being a marker of this region.

Some authors have recently described the multilineage differentiation potential of fibroblasts and their homology with mesenchymal stem cells (Lorenz *et al.,* 2008; Lysy *et al.,* 2007; Chen *et al.,* 2007). They observed that skin fibroblasts are able to differentiate towards mesodermal and endodermal lineages, and provide arguments that these cells share mesenchymal stem cells features both at phenotypic and functional levels.

The CD90 positive area is included among the regions where cells with stem characteristics and with a regenerative capacity towards an amputated bulb reside, as described in the literature (Hoogduijn *et al.,* 2006; McElwee *et al.,* 2003; Jahoda *et al.,* 2003; Lako *et al.,* 2002). For this reason we suppose that the CD90+ population likely includes stem cells. However, the area we observed was quite large with regard to hair follicle extension and it is probable that a CD90+ population may comprise other kinds of cells such as progenitor and endothelial cells. Furthermore, to support our hypothesis, it is important to point out that the dermal papilla cells are the only CD90− cells in the lower part of the anagen hair follicle. A migration of cells from the dermal sheath to the papilla during anagen has been described and it has

been hypothesized that dermal papilla cells derive from dermal sheath cells (Tobin *et al.,* 2003). Even if they share the same mesenchymal origin, the immunohistochemical differences we observed allowed us to distinguish the two cell populations; they may present distinct functions and may be sited at two different levels of differentiation.

As described by other authors, the division of stem cells gives rise to a hierarchy of cells gradually moving outside the stem compartment, and showing a progressive reduction of proliferative and differentiation potential (Taylor *et al.,* 2000). This may be the role of the CD90+ dermal sheath cells and the CD90 negative dermal papilla cells respectively.

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