

Immunohistochemical evaluation of intermediate filament nestin in dog hair follicles

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Summary. Hair follicles (HFs) are self-renewing structures that reconstitute themselves through the hair cycle. They maintain reservoirs of stem cells (SC) that are thought to reside in the bulge area, a region localized in the lowermost permanent portion of HFs. In mice and humans, HF bulge cells express nestin and present stem features as pluripotency. Nestin is a class VI intermediate filament protein; it was first described as a specific marker of CNS stem cells, but recent studies suggest that it may represent a more general stem cell marker (Wiese et al., 2004; Hoffman, 2006).

Bulge cell characteristics have mainly been studied in mice and humans, but recently, a bulge-like region was identified also in dog HFs (Pascucci et al., 2006). In this work we investigate the presence and localization of nestin in dog HFs with the aim of evaluating its expression and to correlate it with the location of the bulge-like region.

Immunostaining of skin samples collected from healthy dogs was performed by using a rabbit anti-nestin polyclonal antibody. The presence of a population of immunoreactive cells was revealed in the hair follicle middle region, at the arrector pili muscle insertion level. An immunohistochemical signal was detected only in primary hair follicles throughout the hair cycle.

These observations led us to conclude that nestin positive cells are located in the bulge-like region of dog HFs and strengthen our hypothesis regarding the correlation between this region and the dog HF stem compartment.

Key words: Nestin, Stem cells, Hair follicle, Dog

Introduction

Somatic stem cells, characterized by extensive growth and differentiation potential, receive considerable attention because of their possible use in regenerative medicine and gene therapy. The development of new strategies for clinical applications will require a readily available source of cells and, among other tissues, hair follicles may represent a suitable source of pluripotent epithelial stem cells.

The hair follicle (HF) is an appendage of mammalian skin characterized by complex morphological and biological characteristics. It appears as an invagination of the epidermis into the dermis in which it is possible to describe three main regions: the infundibulum is the upper third and it is continuous with the epidermis; the isthmus is the middle part and extends from the sebaceous gland duct inlet to the attachment of the arrector pili muscle; the lowest part of the HF extends from the attachment of the arrector pili muscle to the dermal papilla. The infundibulum and the isthmus represent the permanent portion of the HF (Scott et al., 2000).

While in humans HFs are simple, dog skin presents compound HFs where a primary HF is surrounded by several secondary HFs. The primary HF is bigger than the others and extends into the hypodermis; it is associated with the sebaceous gland, the sweat gland and the arrector pili muscle. The secondary HFs are smaller and confined to the dermis; only the sebaceous gland may be present. Near the surface, at the infundibulum level, the secondary HFs joint and their hairs emerge through a common follicular opening (Dellmann and Eurell, 2000).

The hair follicle is an extremely dynamic structure characterized by a cyclical activity in which a growth phase, called anagen, is followed by a regressive phase, the catagen, and then, by a resting one, the telogen. This peculiar activity has the purpose of growing and

maintaining the hair shaft.

Each hair cycle is characterized by structural modifications involving mainly the lowest part of the HF, which, during the regressive phase, involutes and reduces itself until reaching the isthmus region. In the subsequent anagen, the entire lower follicle regenerates and forms a new hair (Paus and Cotsarelis, 1999). The continuous regeneration of the hair follicle depends on the presence of somatic stem cells localized in a specific area of the outer root sheath (ORS), called the bulge region. This area, approximated by the insertion point of the arrector pili muscle, marks the lowermost permanent portion of the hair follicle, namely the isthmus, and represents a niche that is well protected from environmental insults (Cotsarelis et al., 1990; Lyle et al., 1998). During the foetal period it appears as a prominent protrusion, although it progressively becomes smaller until it remains a slight swelling in the adult (Cotsarelis et al., 1990).

The bulge region has been extensively investigated to prove the stem characteristics of its cells. Bulge cells are slow-cycling, pluripotent and show a high proliferative capacity. They were detected in the ORS basal layer, between the arrector pili muscle insertion point and the sebaceous gland (Morris et al., 2004; Blanpain et al., 2004; Ohyama et al., 2006; Hoffman, 2006).

The identification of specific markers that would enable the isolation of a pure cell population is one of the major goals in the study of stem cells. The potential candidates, proposed as hair follicle stem cell markers, include cytokeratin 15 (Lyle et al., 1998, 1999; Liu et al., 2003; Morris et al., 2004), CD34 (Trempey et al., 2003; Morris et al., 2004), S100A4, S100A6 (Ito and Kizawa, 2001; Morris et al., 2004), CD200, PHLDA1, follistatin, frizzled homolog1 (Ohyama et al., 2006), nestin (Li et al., 2003).

Nestin is a class VI intermediate filament protein, originally identified as a marker of neuronal stem and progenitor cells (Frederiksen and McKay, 1988; Cattaneo and McKay, 1990; Lendahl et al., 1990), but recent studies demonstrate a wider range of expression and suppose that it may be a common marker of multi-lineage stem cells (Wiese et al., 2004; Hoffman, 2006).

In mouse hair follicles, nestin positive cells were localized in the ORS bulge region (Li et al., 2003). It has been demonstrated that, *in vivo*, nestin positive bulge cells can give rise to hair follicle ORS as well as to a perifollicular vascular network (Amoh et al., 2004); moreover, they can extensively differentiate into neurons after transplantation to the subcutis of nude mice (Amoh et al., 2005a). *In vitro*, these cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes (Amoh et al., 2005a). Finally, vibrissae bulge stem cells, implanted into the gap region of a severed sciatic nerve, differentiated into Schwann cells and greatly enhanced the rate of nerve regeneration and the restoration of nerve function in transgenic mice (Amoh et al., 2005b). All together, these studies

demonstrate the pluripotency of nestin positive cells localized at the bulge level of mouse hair follicles (Hoffman, 2006).

Yu et al., (2006) showed that also human hair follicles contain a stem cell population located in the bulge area that can differentiate into neurons, smooth muscle cells and melanocyte lineage in induction medium. These cells generated *in vitro* three-dimensional sphere-like structures expressing nestin protein.

Most of the studies regarding the bulge region and its possible role as a stem cell niche have been performed on rodent and human species. However, in a recent work, we identified a bulge-like region in dog hair follicles by combining the localization of stem cell marker CD34 with the morphological characteristics of this region (Pascucci et al., 2006). At first, a slight swelling of the ORS in the region of the isthmus was observed in a large number of primary hair follicles. This thickening topographically corresponded to the bulge region described in humans and mice. In addition, we showed the existence of a subpopulation of CD34 positive keratinocytes localized in the middle part of the HFs and clearly detectable throughout the hair follicle cycle; the positive area was characteristically associated with the thickening of the follicular wall. Neither the thickening of the follicular wall nor the CD34 positivity were observed in the secondary HFs (Pascucci et al., 2006).

In the present work, we carried on an investigation on dog hair follicle stem cells, evaluating the expression and the immunohistochemical localization of nestin with the aim of correlating the presence of this protein with our previous results regarding CD34 localization, and to increase the possibility of considering the positive area a suitable dog HF stem compartment.

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 five healthy male and five healthy female dogs; the skin was devoid of primary or secondary cutaneous lesions. Dogs of different breeds and ages were selected: a belgian shepherd, a dalmatian, a labrador retriever, a border collie, a boxer, a beagle, a miniature pinscher and three mixed breeds; their ages ranged from 3 to 9 years.

Immunohistochemistry

Tissue samples were fixed in a 10% neutral buffered formalin solution, dehydrated, cleared in xylene and embedded in paraffin wax. Five μ m thick sections were cut, mounted on poly-L-lysine-coated slides and dried at 37°C.

Immunohistochemistry was performed using

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standard techniques. Skin sections were deparaffinised with xylene, rehydrated and digested for 10 minutes with 0.1% trypsin solution or exposed to microwaves for 5' at 750 Watts in 0.1M citrate buffer, pH 6.0, for antigen retrieval. After incubation for 10' in 3% H₂O₂ to reduce endogenous peroxidase activity, the sections were blocked with 1:10 normal goat serum for 30' and then incubated with 1:1000 rabbit polyclonal anti-nestin antibody (Abcam) for 1 Hour at room temperature. Subsequently, sections were incubated with 1:200 goat anti-rabbit IgG biotin conjugate antibody (Zymed

Laboratories) and the bound primary antibody was visualized by using the Vectastain ABC kit (Vector Laboratories). The reaction was developed using DAB as chromogen (Dako Cytomation). Negative control sections were produced by omission of the primary antibody.

Results

Nestin positive cells were identified in the isthmus region. The signal extended for a short distance between

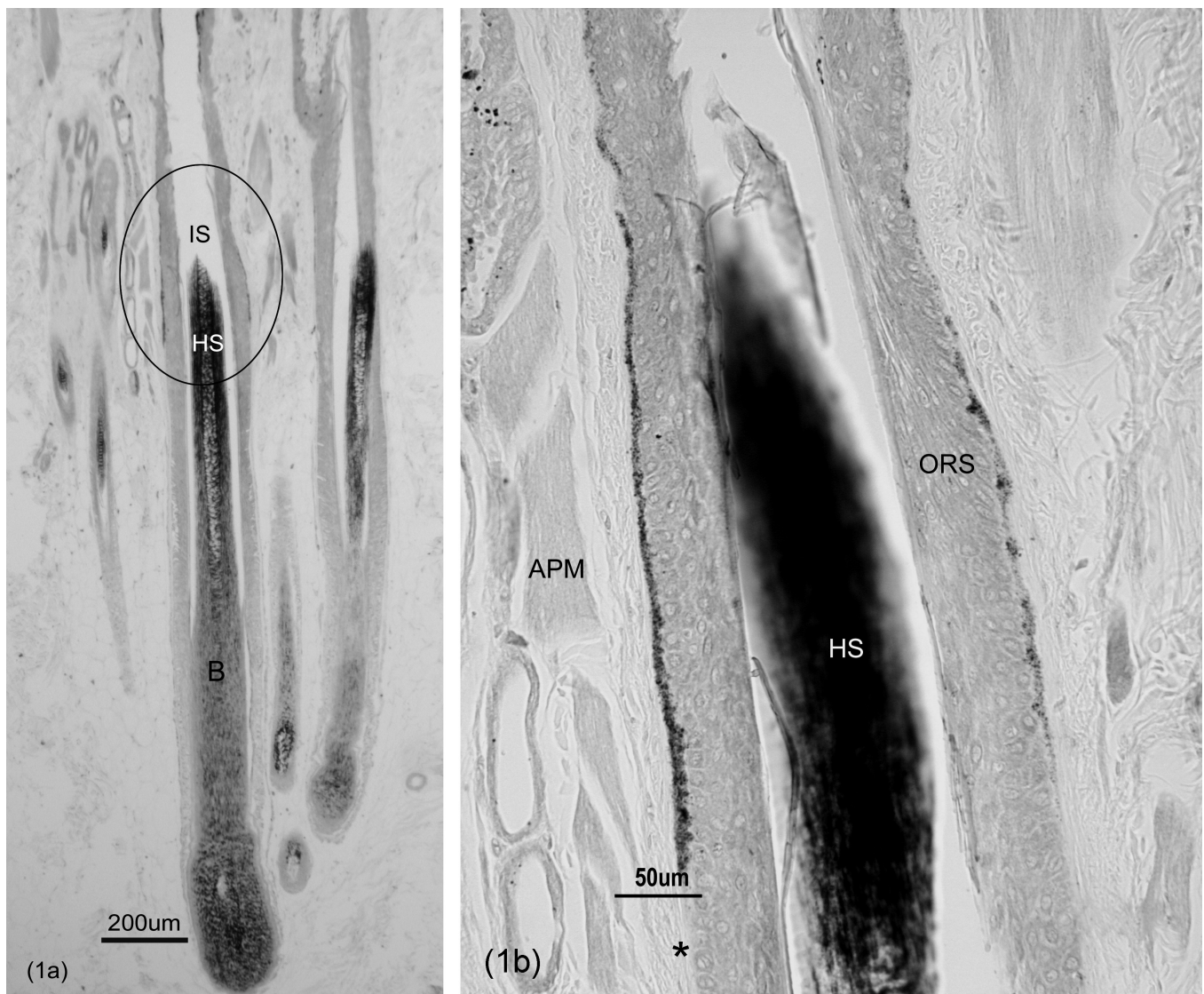


Fig. 1. Low and high magnification of anagen HFs in a longitudinal section. **a.** In the central hair follicle, it is possible to see the isthmus and the bulb regions; their boundary is indicated by the insertion of the arrector pili muscle. The proximal end of the bulb presents the typical onion-shaped thickening with dermal papilla surrounded by hair matrix. The circle encompasses the area where nestin positive cells are located; note that this area is quite small and the bulb region is negative. **b.** High magnification of nestin positive area. A strong immunohistochemical signal labels the basal layer of the isthmus region. The arrector pili muscle extends along the hair follicle and its insertion point is detectable just below the immunoreactive region. IS: isthmus; HS: hair shaft; B: bulb; ORS: outer root sheath; APM: arrector pili muscle; the asterisk labels the insertion point of the arrector pili muscle.

the arrector pili muscle and the sebaceous gland; in particular, it began at the level of the arrector pili muscle insertion point and ended before reaching the opening of the sebaceous gland. This cell population appeared quite small in comparison with the whole hair follicle (Fig. 1a).

The nestin positive cells belonged to the ORS basal layer while there were no signals in the ORS suprabasal layer or in the inner root sheath (Fig. 1b).

Except for isthmus, the other regions of the hair follicle, namely the infundibulum, the lowest part and the hair shaft, as well as the dermal papilla cells, were always devoid of any signal.

Nestin positive cells were detected only in the primary hair follicles, while the secondary ones were negative (Fig. 2b); moreover, in the primary hair follicles the signal persisted, at isthmus level, throughout the hair follicle cycle, both during the growing phase and the regressive phase (Fig. 3).

No immunohistochemical staining for nestin was observed in other structures of the skin, such as the epidermis or sebaceous gland. However, we were able to observe rare positive cells scattered in the connective tissue or localized in the interfollicular area.

No important differences were noted among skin samples belonging to the different body regions or the different breeds tested.

Omission of the primary antibody did not produce any signal, confirming the specificity of the reaction.

Discussion

Nestin is an intermediate filament protein expressed in several embryonal, foetal and adult tissues of mice, rats and humans (Wiese et al., 2004). During embryogenesis, nestin labels cells that migrate and proliferate while, in adults, it is mainly localized in areas of regeneration (Wiese et al., 2004); in several tissues it is considered a marker of stem and progenitor cells. In mouse, nestin positive cells have been identified in the ORS of the bulge region that represents the hair follicle stem compartment (Li et al., 2003). These cells present stem characteristics, such as *in vitro* and *in vivo* pluripotency (Amoh et al., 2004, 2005a,b; Hoffman, 2006).

In the present work, we provide evidence of the expression of stem cell marker nestin in dog hair follicles and show the localization of nestin positive cells in the bulge-like region.

We performed an immunohistochemical study that allowed us to observe the expression of nestin protein in a small cell population of the isthmus region, namely, the middle part of the HF. Nestin positive cells were localized in the ORS basal layer and extended for a short

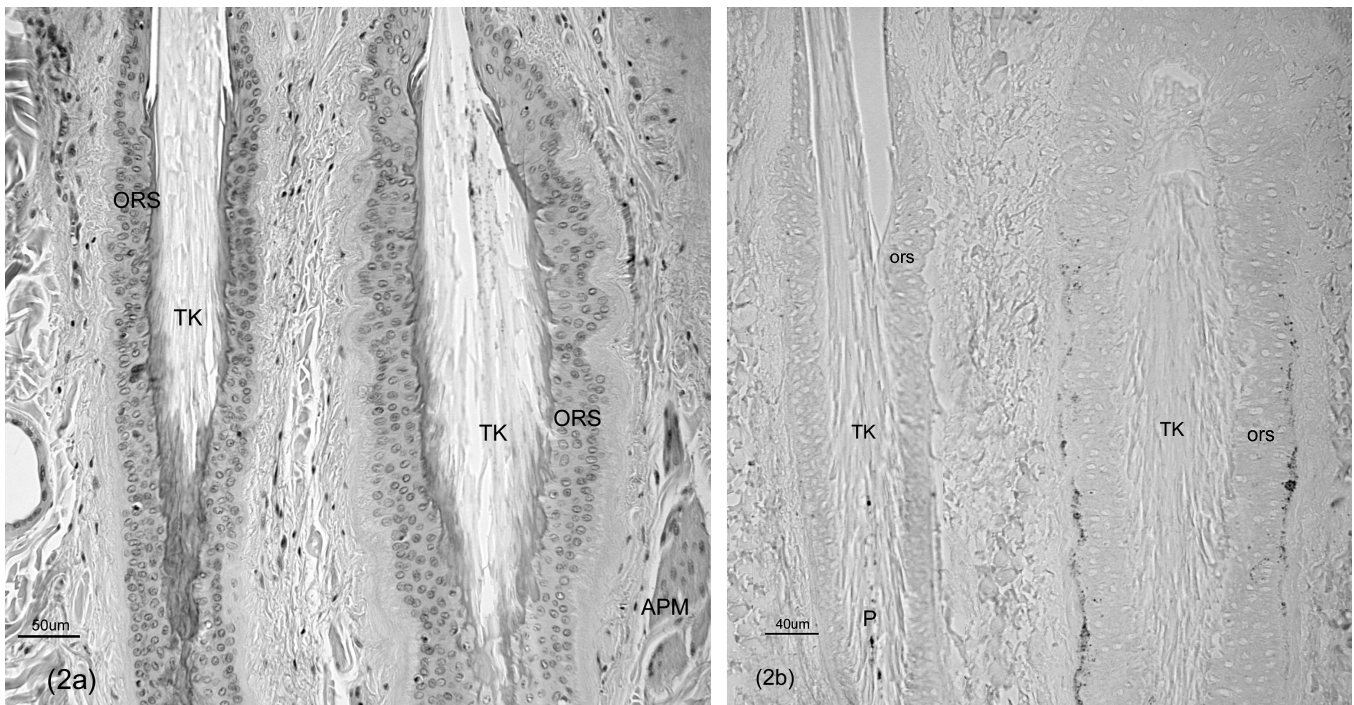


Fig. 2. Consecutive sections of primary and secondary hair follicles in a regressive phase. **a.** It is possible to recognize the irregular edge of the ORS and typical trichilemmal keratinization (E-E). **b.** Immunostaining with anti-nestin antibody. The primary HF, on the right, presents intense immunohistochemical positivity, while the secondary HF is negative. ORS: outer root sheath; P: pigment granules; TK: trichilemmal keratinization; APM: arrector pili muscle.

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tract between the arrector pili muscle insertion point and the sebaceous gland. This is the region where hair follicle stem cells are thought to reside (Cotsarelis et al., 1990; Lyle et al., 1998; Ohyama et al., 2006; Pascucci et al., 2006).

It is important to note that nestin positive cells represented a stable population, restricted to the permanent portion of the hair follicle and not involved in the degenerative processes occurring during the catagen phase. This is demonstrated by the observation that the immunohistochemical signal persisted in the middle part of the HF throughout the hair follicle cycle, and never appeared below the arrector pili muscle insertion point, namely the lowest part of the HF.

We also observed that nestin protein was expressed

only in primary hair follicles, while the secondary ones were negative. This result could confirm a previous hypothesis, asserting that since hair follicle stem cells can regenerate all epithelial lineage of hairy skin (suggesting the existence of a pilosebaceous epithelial unit), in canine compound hair follicles a unique stem compartment, located in primary hair follicles, could be the source of stem cells even for secondary follicular units within the same group (Pascucci et al., 2006).

In a previous work, we demonstrated that mouse hair follicle stem cell marker CD34 glycoprotein is expressed in dog hair follicles in a discrete subpopulation of keratinocytes residing in a bulge-like region. We supposed that CD34+ keratinocytes were potentially related to the hair follicle stem compartment, and that

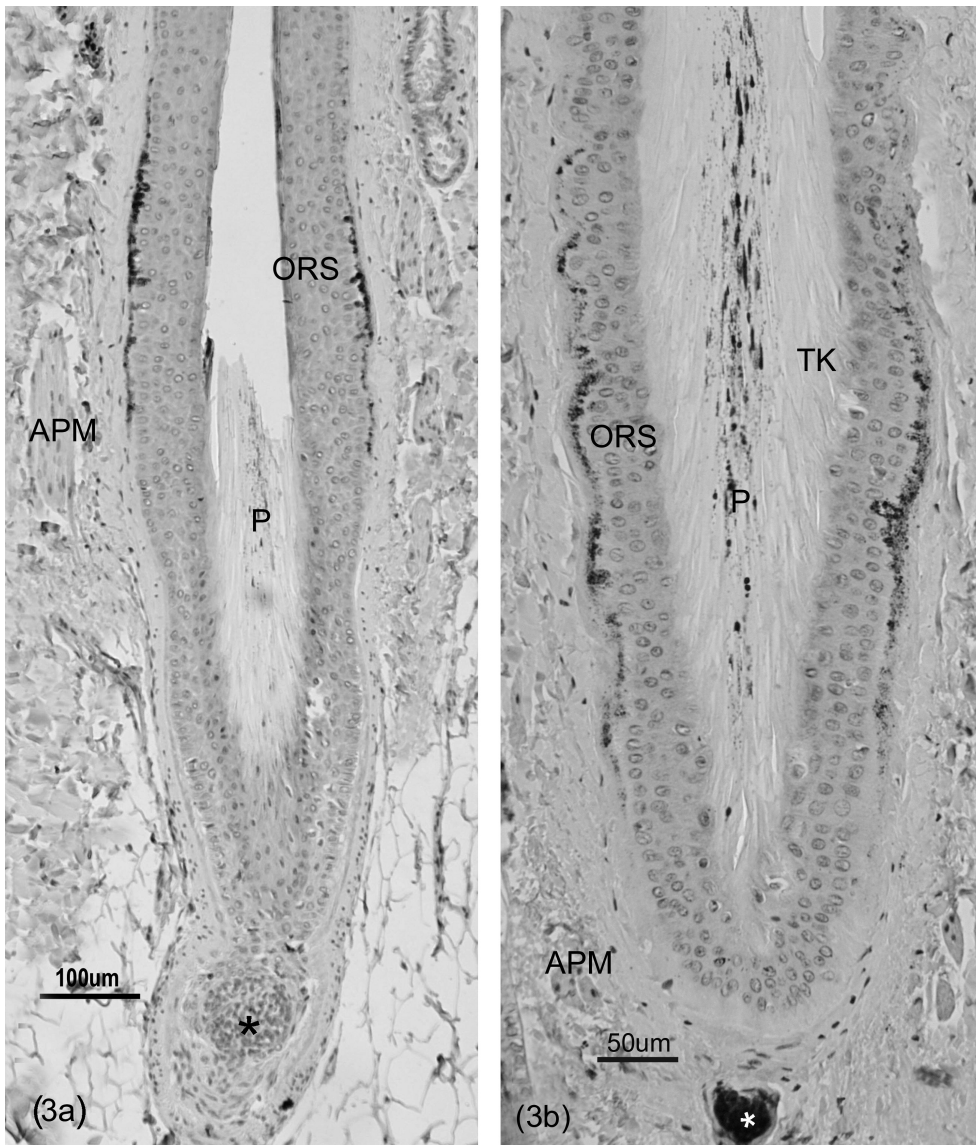


Fig. 3. Catagen (a) and telogen (b) HFs. In catagen, the bulb is shrinking and presents a circular dermal papilla surrounded by a hair germ. In telogen, the ORS edges are irregular, the hair shows a trichilemmal keratinization and the dermal papilla cells are densely packed in a hemispherical condensation. Note that, in both HFs, the nestin positive area is not affected by the degenerative process that occurs during the regressive phase and persists, at the isthmic level, throughout the hair follicle cycle. ORS: outer root sheath; APM: arrector pili muscle; P: pigment granules; TK: trichilemmal keratinization; the asterisk labels the dermal papilla.

CD34 glycoprotein may be considered as a reliable marker of follicular stem cells in canine species (Pascucci et al., 2006). In this work we have noted that nestin protein, in dog hair follicles, is localized similarly to CD34 glycoprotein. In particular, the expression of both molecules was observed in the follicular isthmus region and, in both cases, positive cells were detected only in primary hair follicles. Nevertheless, nestin positive cell populations presented a smaller extension with regard to CD34 positive cells, suggesting that nestin could be a marker of more undifferentiated cells, while CD34 glycoprotein could mark more differentiated transit amplifying cells. In a stem cell lineage, in fact, the loss of stem functional capabilities does not occur at the level of the ancestral cell, but one or two generations down the lineage, with a gradual loss of stemness until a critical cut-off point (Potten and Booth, 2002). The division of bulge stem cells gives rise to a hierarchy of transit amplifying cells moving outside the stem compartment, and showing a progressive reduction of proliferative potential and differentiation flexibility (Taylor et al., 2000). In regard to these remarks, both nestin protein and CD34 glycoprotein may be considered as markers of bulge stem cells that are localized at different levels of the stem lineage, and with different stem functional properties and phenotypic features.

Our results present some differences from those previously reported by Wang et al. (2006) in human skin samples. They observed the presence of nestin protein not only in hair follicle ORS, but also in hair follicle inner root sheath, in all layers of epidermis and, weakly, in the sebaceous gland. There could be a resemblance between mouse and dog bulge region molecular markers that does not exist in humans; therefore, nestin could show the same pattern of localization in dog and mouse hair follicles but another pattern in humans. We retain that these discrepancies may reflect biological and structural diversities between species. A different correlation between these species can also be observed with CD34 glycoprotein, which represents a bulge cell marker in dogs and mice, but not in humans, where it is localized below the attachment of the arrector pili muscle, outside the stem compartment (Poblet et al., 1994; Ohyma et al., 2006).

In conclusion, our study shows that nestin is expressed in dog hair follicles and that its location is strictly correlated with the area previously described as the bulge-like region, making this protein a suitable marker to identify it. Even if further investigations are necessary, including colony-forming efficiency tests or label retaining experiments, these results confirm those obtained in our previous work and add new data to our investigation about the bulge-like region. Moreover, the corresponding characteristics of localization between nestin and CD34 support our hypothesis of considering this area as a dog hair follicle stem compartment.

Hair follicles may be an easily accessible source of pluripotent stem cells that could be used for clinical applications. Our investigation regarding nestin protein

adds important elements in the knowledge of molecular mechanisms regulating the cell's fate and the biology of hair follicles in the canine species, and may make the identification of a pure stem population easier.

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