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Merkel cells of human oral mucosa express the pluripotent stem cell transcription factor Sox2

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Summary. Merkel cells are neuroendocrine cells associated to a neural sensitive ending and localized primarily in the epidermis, although they are also found in oral mucosa. Sox2 or SRY-box2 is a key transcription factor important in the maintenance of embryonic neural crest stem cell pluripotency. Sox2 has been described in Merkel cells of skin and in Merkel cell carcinomas, but not specifically in oral Merkel cells. The aims of the present study were to analyze the density of Merkel cells in human oral mucosa and to study the expression of Sox2 in these cells. For these purposes, immunohistochemical analyses for Sox2 and CK20 (the best marker for Merkel cells) were automatically performed on sections of normal human oral mucosa. Double immunofluorescence for Sox2 and CK20 was also performed. To analyze the density of Merkel cells, CK20 positive cells were counted in each sample and the length of the epithelial apical edge was measured (cells/mm). Merkel cells, demonstrated by CK20 immunoreactivity, were found in 95% of oral mucosa specimens studied (n=21). Mean density of Merkel cells in oral mucosa was 1.71±2.34 cells/mm. Sox2 immunoreactivity was found in the nuclei of scattered cells located at the basal layer. Serial sections immunostained for Sox2 and CK20 showed that Sox2-positive cells of oral mucosa coexpressed CK20, confirming that they were Merkel cells. Immunofluorescence for Sox2 and CK20 showed colocalization of both markers, demonstrating that virtually all oral Merkel cells expressed Sox2. This transcription factor could play a role in Merkel cell maturation and maintenance.

Key words: Merkel cells, Sox2, Oral mucosa, Immunohistochemistry, Double immunofluorescence

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Introduction

In 1875, Friedrich Sigmund Merkel described a type of epidermal cell with a specific innervation that was called "Tastzellen" to indicate its tactile function (Merkel, 1875). In 1902, Tretjakoff proposed that these cells be denominated Merkel cells (Tretjakoff, 1902). Merkel cells are localized primarily in the epidermis of fingertips, but also in eyelids, tactile hair follicles and snout of different animals. They are also found in oral mucosa where they are abundant in lip, palate and especially in gingiva (Turner, 1983).

Merkel cells are neuroendocrine cells associated to a neural sensitive ending. A Merkel cell and a terminal neurite form the so-called Merkel cell complex, a slowly adapting mechanoreceptor. General markers for Merkel cells are chromogranin (Gauweiler et al., 1988), synaptophysin (García-Caballero et al., 1989a) and cytokeratin 20 (CK20). The latter is the most general and specific marker for these cells (Moll et al., 1995). They also contain numerous peptides (Tachibana, 1995; Tachibana and Nawa, 2002) as well as amines (García-Caballero et al., 1989b).

Merkel cell carcinoma was first described by Toker in 1972 as a trabecular carcinoma of skin (Toker, 1972). In 1978 it was renamed Merkel cell carcinoma due to its ultrastructural similarity with epidermal Merkel cells (presence of electrondense granules) (Tang and Toker, 1978). It is an aggressive dermal neoplasm, which appears more frequently in males (twice as often as in females) and the elderly (15 times more frequently than in people under 65 years of age). The most common localizations are sun-exposed skin of head and neck, followed by extremities. Diverse etiologic factors have been related with Merkel cell carcinomas: ultraviolet irradiation, immunosuppression and human Merkel cell polyomavirus, which was discovered in 2008 and is found in 80-85% of cases (Feng et al., 2008; Becker et al., 2009).

The first description of a Merkel cell carcinoma arising in oral mucosa was reported in 1988 (Mir et al., 1988). Up to now, only 26 cases of perioral and oral Merkel cell carcinomas have been reported (19 in males). They arise from lip (15 cases), buccal mucosa (3 cases), floor of mouth (3 cases), tongue (2 cases), posterior mandible, palatine tonsil and hard palate (one case each) (Islam et al., 2018). Unlike cutaneous Merkel cell carcinomas, oral Merkel cell carcinomas are not obviously related with ultraviolet irradiation nor with Merkel cell polyomavirus (Duncavage et al., 2009; Tanio et al., 2015). They usually have aggressive behavior with high tendency to recurrence and metastasis. Treatment includes surgery with sentinel lymph node biopsy, which can be followed by radiotherapy. Chemotherapy is reserved for recurrent or metastatic cases.

Sox2 or SRY-box2 (Sex determining region Y-box 2) is a key transcription factor important in the maintenance of embryonic neural crest stem cell pluripotency. It is a protein composed of 317 amino acids and encoded by the Sox2 gene of a single non-introns exon located on chromosome 3 in both humans and mice (Stevanovic et al., 1994). It is expressed throughout embryonic development; initially in the inner cell mass of the blastocyst and later in the ectodermal, endodermal and mesodermal derivatives, as well as in primordial germ cells. Sox2 expression is maintained in fetal and adult tissues derived from Sox2-positive fetal progenitor cells. It marks stem and progenitor cells and also differentiated cells in some cases (Sarkar and Hochedlinger, 2013).

Sox2 has been detected by immunolabeling techniques in Merkel cells of skin (Driskell et al., 2009; Laga et al., 2010; Lesko et al., 2013) and rat hard palate (Widera et al., 2009), as well as in Merkel cell carcinomas (Laga et al., 2010). However, to the best of our knowledge, Sox2 was not specifically reported in human oral Merkel cells, although one of the previous papers included a photograph of Sox2 in Merkel cells of human lip mucosa (Laga et al., 2010).

The aims of the present study were to analyze the density of Merkel cells in different regions of human oral mucosa and the expression of Sox2 in these cells.

Material and methods

Specimens

Samples of normal human oral mucosa, fixed in 10% neutral buffered formalin for 24 h and included in paraffin, were obtained from the files of the Pathology Department of the University Clinical Hospital of Santiago de Compostela. The research was conducted in accordance with Spanish law (RD 1301/2006; Ley 14/2007; RD 1716/2011 and Orden ECC 1414/2013), and following the ethical principles for medical research involving human material set by the 1964 Helsinki Declaration of the WMA and its later amendments. We studied 21 samples from 20 patients (10 men and 10 women) that corresponded to normal lip mucosa (n=9), gingiva (n=4), jugal mucosa (n=5) and palate (n=3). Mean age of patients was 65.3 years (range 34–93 years).

Immunohistochemical analysis

Four μm-thick sections were mounted on FLEX IHC microscope slides (Dako-Agilent, Glostrup, Denmark) and heated in an oven at 60°C for 1 h. Immunohistochemical analyses were automatically performed using an AutostainerLink 48 (Dako-Agilent). After deparaffinization and epitope retrieval in EnVision FLEX target retrieval solution (high pH) for 20 min at 97°C, the slides were allowed to cool in PT Link to 65°C and then in Dako-Agilent Wash Buffer for 5 min at room temperature (RT). The immunostaining protocol included the following steps: (1) EnVision FLEX peroxidase-blocking reagent (Dako-Agilent) for 5 min; (2) Sox2 rabbit monoclonal antibody (Novus Biologicals, Littleton, CO), at 1:5,000 for 20 min or CK20 mouse monoclonal (Dako-Agilent), ready to use for 20 min; (3) EnVision FLEX/HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse and anti-rabbit immunoglobulins) (Dako-Agilent) for 20 min; (4) substrate working solution (mix) (3,3'-diaminobenzidine tetrahydrochloride chromogen solution) (Dako-Agilent) for 10 min and (5) EnVision FLEX hematoxylin (Dako-Agilent) for 9 min.

Immunofluorescence analysis

Double immunofluorescence was performed for Sox2 and CK20. Sox2 rabbit monoclonal antibody was incubated at 1:4000 for 48 h at 4°C, followed by Alexa Fluor® 488 donkey anti-rabbit antibody (ThermoFisher Scientific, Rockford, IL) at 1:200 for 1h at RT; CK20 mouse monoclonal antibody (Dako-Agilent), ready to use for 15 min at RT, followed by sheep anti-mouse IgG $F(ab)_2$ Cy3 (Sigma), 1:200 for 1h at RT.

The slides were observed and photographed in a Provis AX70 microscope equipped with an Olympus DP70 camera (Olympus Corp., Tokyo, Japan).

Morphometric analysis

To calculate the density of Merkel cells in oral mucosa, CK20 positive cells were counted (using a 20x objective) and the length of the epithelial apical edge was measured employing a microscope stage micrometer (Graticules LTD, Tonbridge Kent, UK). The ratio between the number of Merkel cells and the epithelial length (cells/mm) was calculated for each oral region studied (the total number of tissue fragments analyzed in the 21 samples was 50).

Statistical analysis

Data on density of Merkel cells are shown as mean±SD. Analysis of variance was used to examine the differences among means obtained in each region of oral mucosa. Differences were considered statistically significant at $p<0.05$.

Results

Merkel cells, demonstrated by CK20 immunoreactivity, were found in 20/21 samples studied (95%). These cells appeared isolated or forming small clusters (Fig. 1). Density of Merkel cells showed high variability not only between different oral regions, but also between different samples from the same region and between different fragments of the same sample. Mean density of Merkel cells in oral mucosa was 1.71±2.34 cells/mm.

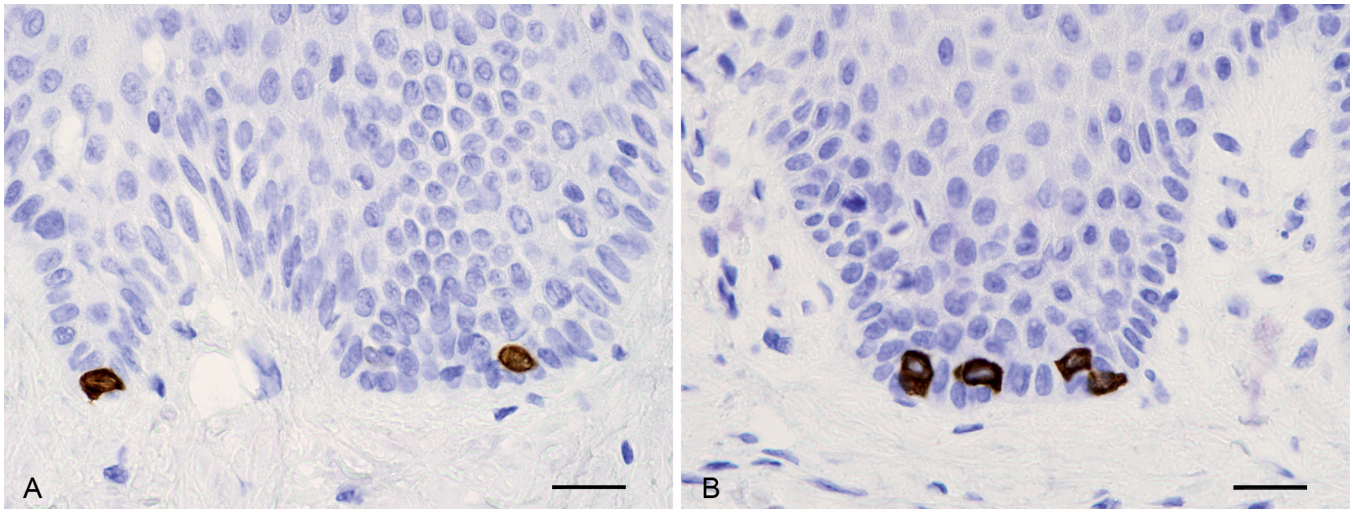

Fig. 1. In these sections immunostained for CK20, positive cells appear isolated **(A)** or forming clusters **(B)** at the base of rete ridges. Palate. Scale bars: 25 µm.

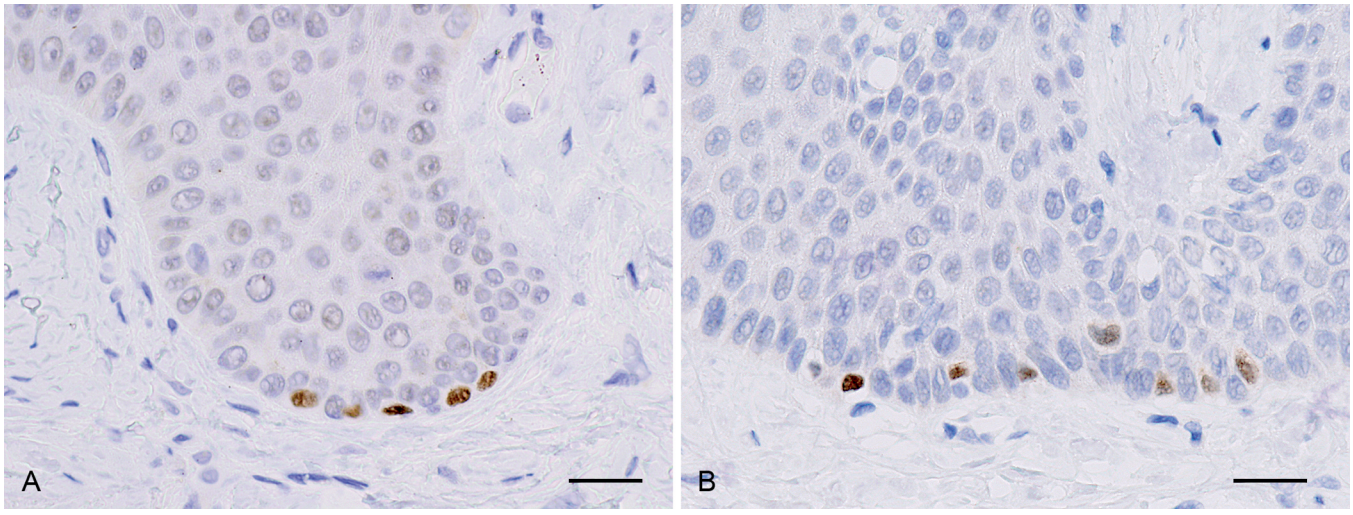

Fig. 2. Sox2 immunopositive cells in different areas of oral mucosa: Gingiva **(A)** and Palate **(B)**. Sox2 is expressed in the nuclei of Merkel cells. Scale bars: 25 µm.

The regions of oral mucosa with highest density of Merkel cells were gingiva (2.78±2.40) and jugal mucosa (2.78±3.51), followed by palate (2.01±2.98). Lip mucosa was the region with the least density (0.53±0.44) (Table 1). Interregional density differences were not statistically significant (p=0.243).

Sox2 immunoreactivity was found in the nuclei of some cells located in the basal layer of human oral mucosa epithelium. Immunostained cells were primarily located at the tips of rete ridges resembling Merkel cells (Fig. 2). Serial sections immunostained for Sox2 and

Table 1. Density of Merkel cells in human oral mucosa.

Merkel cells/mm	Samples analyzed	Mean	SD
Gingiva	4	2.78	2.40
Jugal mucosa	5	2.78	3.51
Palate	3	2.01	2.98
Lip mucosa	9	0.53	0.44
Total	21	1.71	2.34

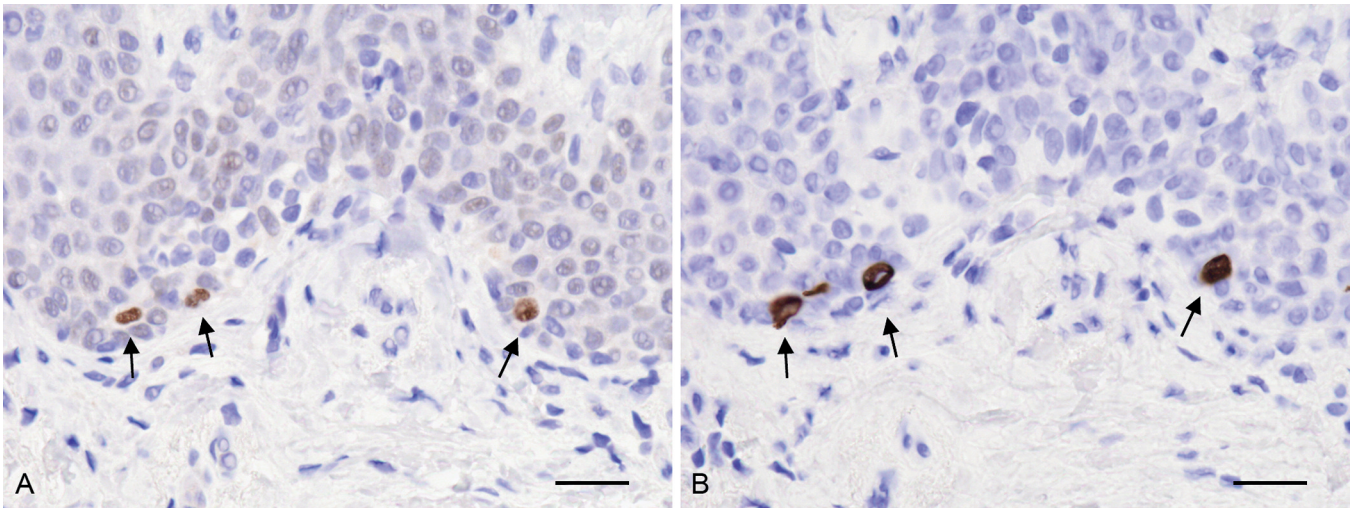

Fig. 3. Serial sections showing Merkel cells (arrows) positive for Sox2 **(A)** and CK20 **(B)**. See that Sox2 and CK20 were coexpressed by the same cells. Gingiva. Scale bars: 25 µm.

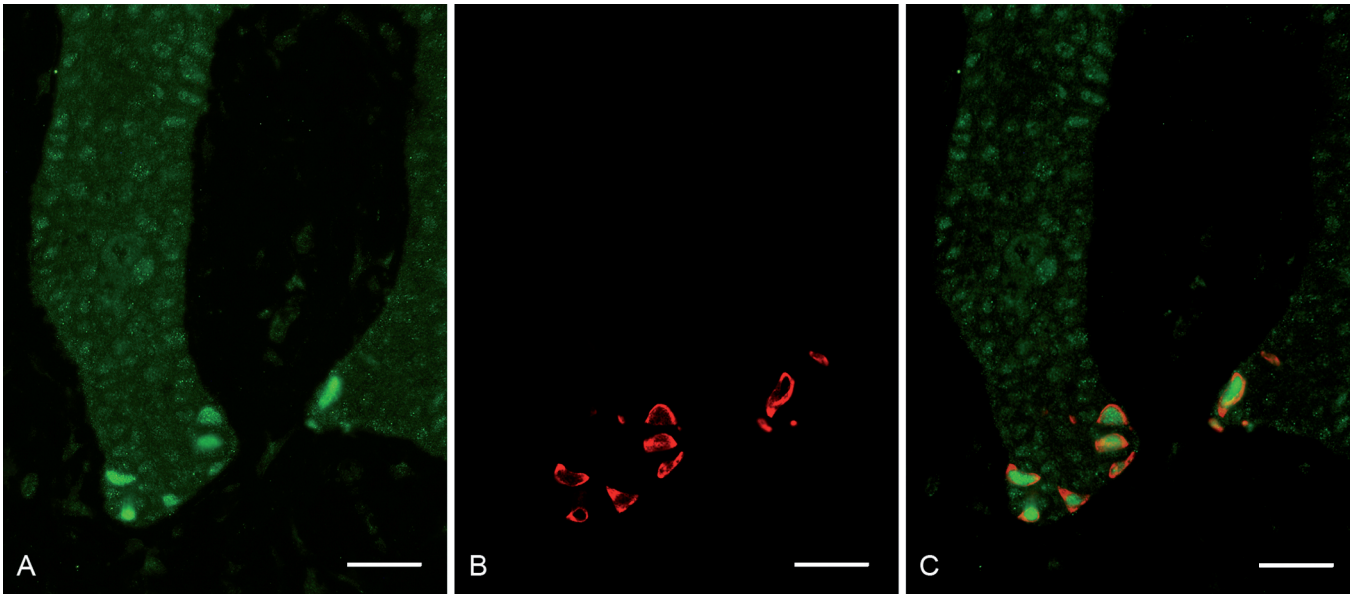

Fig. 4. Immunofluorescence showed double-labelled cells for Sox2 (green) and CK20 (red), confirming that virtually all Merkel cells express Sox2. Sox2 **(A)**, CK20 **(B)** and merge image **(C)**. Gingiva. Scale bars: 25 µm.

CK20 (the best immunohistochemical marker for Merkel cells) showed that Sox2-positive cells of the oral mucosa coexpressed CK20, demonstrating that they were in fact Merkel cells (Fig. 3).

Double immunofluorescence for Sox2 and CK20 was also performed. Positivity for Sox 2 (green) was found in nuclei (Fig. 4A) and for CK20 (red) in cytoplasms (Fig. 4B). Colocalization of both markers demonstrated that virtually all oral Merkel cells expressed Sox2 (Fig. 4C).

Discussion

The presence of Merkel cells in human oral mucosa was demonstrated for the first time by Fortman and Winkelmann in 1977. They usually appear isolated or forming small groups at the tip of rete ridges. Merkel cells are numerous in surface normal mucosa and cornified masticatory mucosa of mammals and they are similar to epidermal Merkel cells at the ultrastructural level (Halata et al., 2003).

Controversial findings regarding frequency and density of oral Merkel cells are evident. In our study, Merkel cells were demonstrated by CK20 in 95% of samples studied (n=21). However, other authors, analyzing surgical biopsies of oral mucosa also by CK20, found Merkel cells only in 50% of the normal biopsies analyzed (n=14). They reported higher frequency in lichen planus (Merkel cells in 71% of samples), chronic aspecific inflammation (79%), and mucosa overlying tumors (80%). These authors showed higher density of Merkel cells in palate mucosa (mean 14 cells/case), followed by gingiva (13 cells/case), and buccal mucosa (9 cells/case), but without statistical significance (Righi et al., 2006). Our study showed the highest Merkel cell density in gingiva, as was previously shown in primates (Turner, 1983), and jugal mucosa and the lowest density in lip mucosa. No statistically significant differences were found between the different regions analyzed, probably due to the great variability in Merkel cell density found not only between different samples of each region, but also between different fragments of the same sample.

The current paper demonstrated that human oral Merkel cells showed Sox2 immunopositivity, as was previously described for human epidermal Merkel cells (Laga et al., 2010) and for Merkel cells of rat hard palate (Widera et al., 2009). Sox2 immunoreactivity was found in the nuclei of Merkel cells localized in the different regions of oral mucosa, as was demonstrated by serial sections immunostained with Sox2 and CK20 and by double immunofluorescence techniques. In contrast to Laga et al. (2010), who found that some epidermal Merkel cells did not express Sox2, our results demonstrated that virtually all oral Merkel cells showed Sox2 positivity.

Sox2 is expressed in stem and progenitor cells, and in some cases also in differentiated cells (Sarkar and Hochedlinger, 2013). This transcription factor could play a role in Merkel cell maturation (Perdigoto et al., 2014). Merkel cells arise from epidermal stem cells and require the transcription factor *Atoh1* for initial differentiation (Maricich et al., 2009; Morrison et al., 2009; Van Keymeulen et al., 2009). Although *Atoh1* expression has been demonstrated to be sufficient to produce new Merkel cells in the epidermis (Ostrowski et al., 2015), Isl1 and Sox2 co-regulate the subsequent maturation steps by sustaining expression of *Atoh1* (Perdigoto et al., 2014). Ablation of Sox2 has been found to result in a loss of Merkel cells, suggesting that Sox2 is critical for Merkel cell specification and maintenance (Bardot et al., 2013). In adult mice, Van Keymeulen et al. (2009) demonstrated that epidermal Merkel cells are maintained, at least in part, through differentiation of epidermal stem cells.

During mouse and human aging there is a widespread decrease in Sox2 expression that has been suggested as a biomarker for aging (Carrasco-García et al., 2019). Moreover, this decrease in Sox2 expression could be functionally linked to the process of aging since it has been reported that the exhaustion of Sox2-positive cells leads to premature aging in a mouse model of induced repetitive partial depletion of Sox2 cells (Vilas et al., 2018). Interestingly, in aged mice, palatal Merkel cells are reduced in number at key time-points that correlate with impaired oral abilities, such as swallowing and mastication (Moayedi et al., 2018). It would also be of interest to explore the levels of Sox2 expression in oral Merkel cells during aging.

Sox2 expression has been reported in a number of neoplasms, sometimes contributing to the malignant phenotype (Wuebben and Rizzino, 2017). Merkel cell carcinomas of the skin have been reported to show nuclear Sox2 expression (Laga et al., 2010), however it remains unknown whether a similar expression is also present in oral Merkel cell carcinomas and the putative contribution of Sox2 to these tumors.

In summary, this paper demonstrated for the first time that virtually all Merkel cells of human oral mucosa express the transcription factor Sox2. Further research is warranted to ascertain the precise role of Sox2 in oral Merkel cells and its expression in oral Merkel cell carcinomas.

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