SUPPLEMENTARY DATA

Supplementary Methods

Immunostainings

Formalin-fixed paraffin-embedded CMN tissue sections (5 µm) were obtained from the department of Pathology at Trousseau Hospital. Tissue sections were labeled with the following antibodies: Rabbit anti-human MITF (dilution 1:250, ab20663, Abcam, Cambridge, UK), mouse anti-human Nestin (dilution 1:100, ab22035, Abcam), mouse anti-human HMB45 (pre-diluted, ab51935, Abcam), mouse anti-human KI67 (dilution 1:100, NCL-L-Ki67-MM1, Novocastra, Wetzlar, Germany), rabbit anti-human CD44 (dilution 1:100, ab51037, Abcam), rabbit anti-human Oct4 (dilution 1:100, ab19857, Abcam), mouse anti-human Melan-A (dilution 1:100, ab3168, Abcam) and rabbit anti-human Sox10 (dilution 1:100, sc-17342, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Antigen retrieval was performed by incubating the slides with citrate buffer (pH=6) or EDTA buffer (pH=9) at 98°C for 20 min. Non specific antigen sites were blocked in PBS with 3% BSA with 10 or 20 % serum goat.

For MITF, Nestin, Ki67, CD44 and HMB45 stainings, endogenous peroxidase activity was blocked with 3 % H2O2 for 10 min. The sections were incubated with the different primary antibodies overnight in PBS with 3% BSA and 0.1% triton for intracellular staining. Staining was achieved using appropriate biotinylated goat anti-mouse or anti-rabbit secondary antibodies (BA9200, BA1000, Vector Laboratories, Burlingame, CA, USA) and Avidine Biotine complex (PK-6100, Vector Laboratories). Bound antibodies were visualized using aminoethylcarbazol (SK-4205, Vector Laboratories) as chromogen. Nuclei were counterstained with Mayer's haematoxylin (TA-125-MH, Thermoscientific, Waltham, MA, USA).

For Melan-A, Sox 10 and Oct-4 immunofluorescence, slides were incubated overnight with primary antibodies diluted with 3% BSA and 0.1% triton for intracellular staining. Sections were washed with PBS followed by secondary antibody goat anti-mouse Alexa 488 (dilution 1:100, A10680, Life Technologies, Carlsbad, CA, USA), or goat anti-rabbit Cy3 (dilution 1:1000, A10522, Life Technologies) for 45 min at room temperature. Slides were also incubated with DAPI (D9542, Sigma-Aldrich, Saint-Louis, MO, USA) for 5 min for nuclei staining and coverslipped using Fluoromount G (0100-01, Southern Biotech, Birmingham, AL, USA).

Negative controls were performed in parallel with the samples substituting the primary antibody with the equivalent isotype.

Photomicrographs were obtained under x20 objective using Nikon Eclipse 90i microscope (Nikon, Tokyo, Japan). Using Image J software, positive cells were counted in 100 µm2 randomized surface squares in both epidermis and dermis of each specimen. In each area, the number of positive cells was counted and reported to the total number of DAPI+ cells. Thus, results are expressed as a percentage of positive cells for each marker analyzed per total cells per 100 mm2 surface areas, both in the epidermis and dermis. Quantitations were done on various parts of the CMN and always in areas within the lesions, usually in the center: these lesions were dissected in a gridded fashion and 3 different sectors were analyzed; for each different sector of the lesions, 3 different areas of 100 µm2 were counted.

In vitro assays

Fresh CMN tissues were manually dissected into small pieces, before sequential enzymatic digestion in Dispase II (2%, 049420780001, Roche, Basel, Switzerland) overnight at 4°C, then in Collagenase I (3.5%, 17100-017, Life Technologies) for 1 hour at 37°C. Cells were filtered using 100 µm and 40 µm cell strainers to obtain a single-cell suspension.

For sphere formation assay, the nevocytic single cell suspension was seeded at a density of 50000 cells/ml in a 12-well plate in DMEM/F12 (31966021-317655027, Life Technologies) supplemented with EGF (100ng/mL, PHG0311, Life Technologies), bFGF (100 ng/mL, PHG0026, Life Technologies), B27 supplement (10889-038, Life Technologies) and insulin (5 ug/mL, I2643-25, Sigma-Aldrich). The number of colonies was counted per well. The diameter was also measured on "day in vitro" DIV7 and DIV13 using Image J software.

For serial passages, the culture medium was collected on DIV13, centrifuged at 500 rpm for 15 min. Spheres were dissociated using pro-accutase (A11105, Life Technologies) 10 min at 37°C, and single cells were seeded again.

The number and frequency of initiating cells was obtained as previously described (16). CMN cells were seeded at ratios of 2000, 500, 250, 100 and 10 cells per well. Using optical microscope (Olympus ULWCD 0.30, Tokyo, Japan), wells containing spheres were counted on DIV7 and 13. The frequency of initiating cells corresponding to 37% of negative wells according to the Poisson statistics was calculated with GraphPad Prism.

Immunocytofluorescence on CMN nevospheres was performed as previously described (16). The following antibodies were used: anti-Melan-A (mouse anti-human, dilution 1:100, Abcam), anti-Oct4 (rabbit anti-human, dilution 1:50, Abcam), as well as anti-mouse Alexa 488 and anti- rabbit Cy3 secondary antibodies.

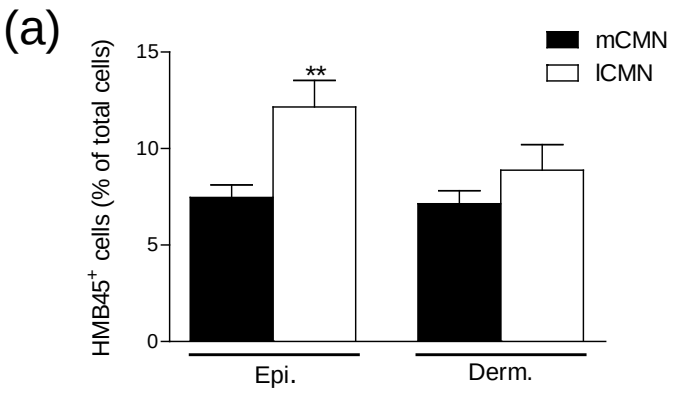
Supplementary Table 1. Clinical features and nevus categorization[a]

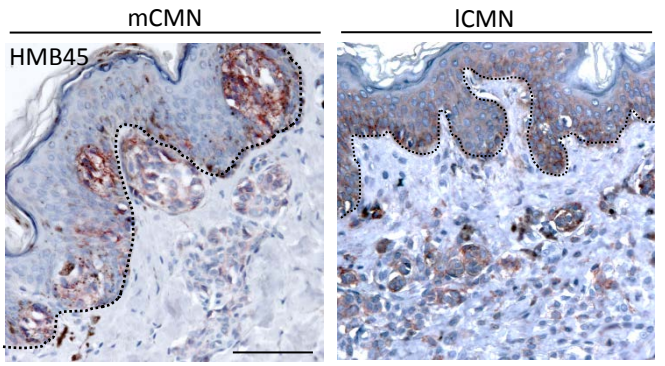
	Medium 1 nevus	Large/giant nevus
Median age[b]	45mo [5mo to 16y]	19.5mo[3mo to 5y]
Female	9/18 (50%)	6/10 (60%)
Nevus site		
Extremities	6/18 (33.3%)	2/10 (20%)
Trunk	7/18 (38.9%)	3/10 (30%)
Head	5/18 (27.8%)	5/10 (50%)
Number of satellites		
S0	16/18 (88.9%)	3/10 (30%)
S1	2/18 (11.1%)	6/10 (60%)
S2	0	1/10 (10%)
S3	0	0
Color heterogeneity		
C0	10/18 (55.6%)	1/10 (10%)
C1	8/18 (44.4%)	4/10 (40%)
C2	0	5/10 (50%)
Surface rugosity		
R0	17/18 (94.4%)	2/10 (20%)
R1	1/18 (5.6%)	4/10 (40%)
R2	0	4/10 (40%)
Dermal or subcutaneaous nodules		
N0	17/18 (94.4%)	6/10 (60%)
N1	1/18 (5.6%)	1/10 (10%)
N2	0	3/10 (30%)
Hypertrichosis		
H0	11/18 (61.1%)	1/10 (10%)
H1	3/18 (16.7%)	2/10 (20%)
H2	4/18 (22.2%)	7/10 (70%)

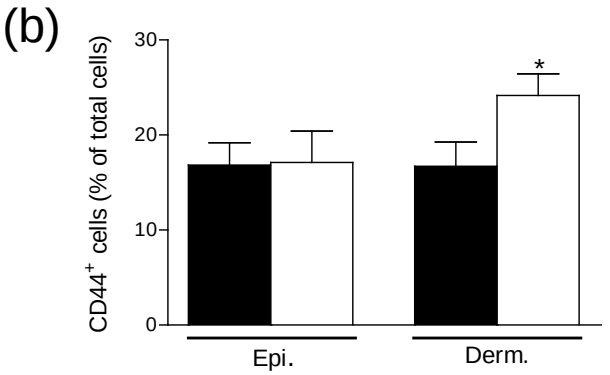
[a]Data are expressed as *n* (%) unless otherwise indicated ; nevus categorization as described in (1) : S0-S3: grading of nevus satellites; C0-C2: grading of color heterogeneity; R0-R2: grading of nevus rugosity; N0-N2: grading of nodules; H0-H2: grading of hypertrichosis.

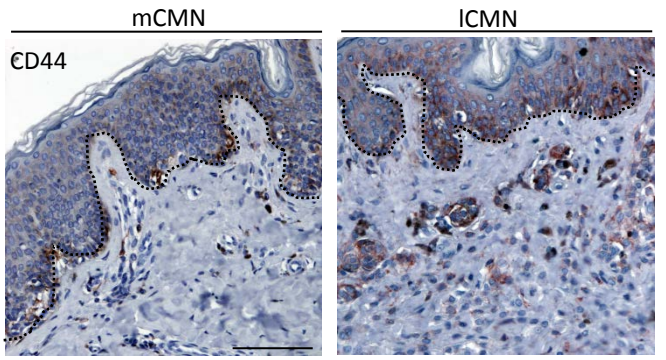
[b]Abbreviations : mo, month; y, year.

Supplementary Figure 1.



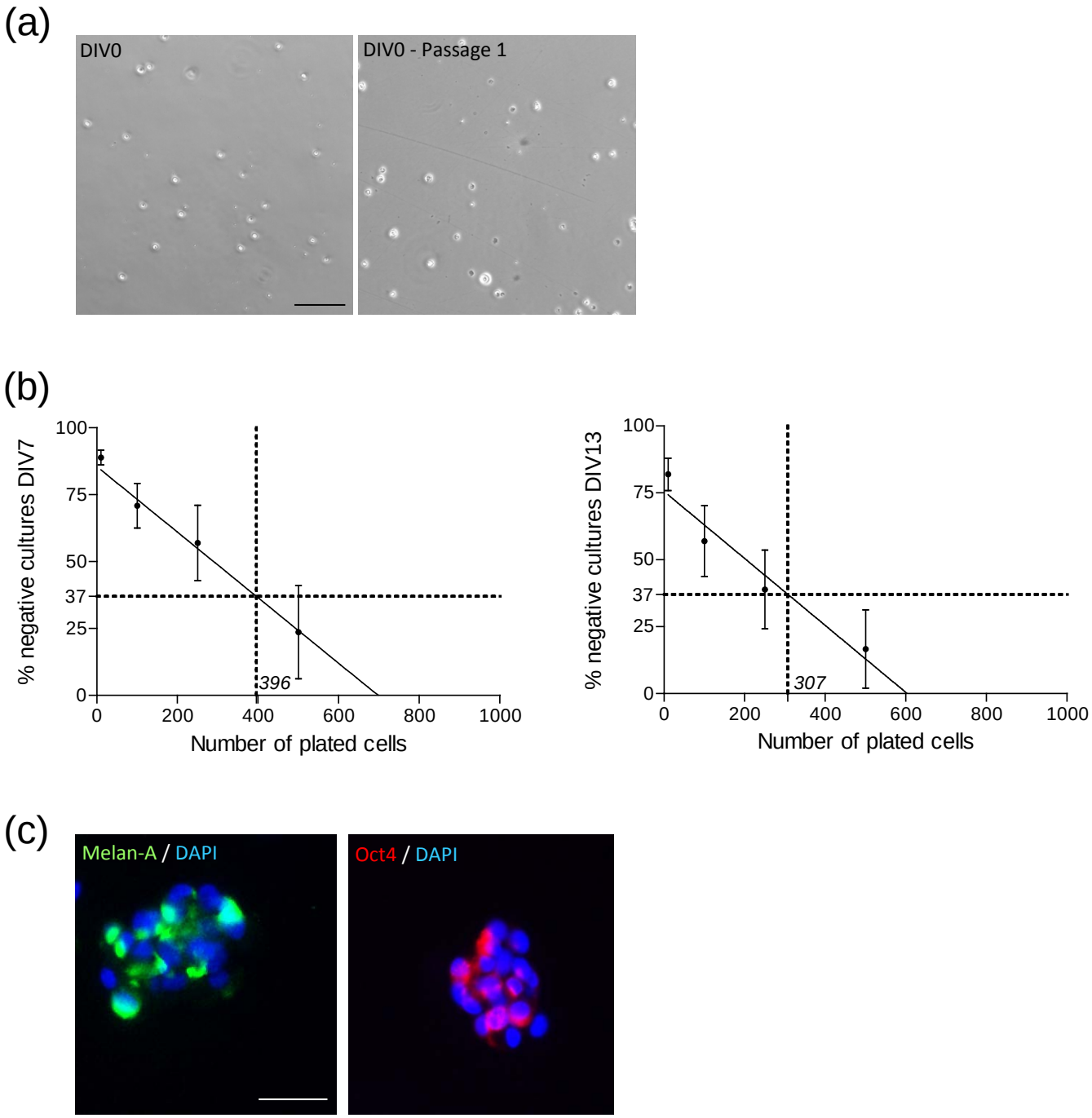






Human CMN display cells with progenitor/stem cell markers *in vivo*.
(a, b) Number of positive cells per total cells in epidermis and dermis of human medium (mCMN n=5) and large Congenital Melanocytic Nevi (lCMN n=5) after staining for HMB45 (a) and CD44 (b) and using AEC chromogen. Dotted line separates dermis from epidermis. * represents the difference between mCMN and lCMN. *p≤0.05, **p≤0.01 in Student's t-test.

Supplementary Figure 2.



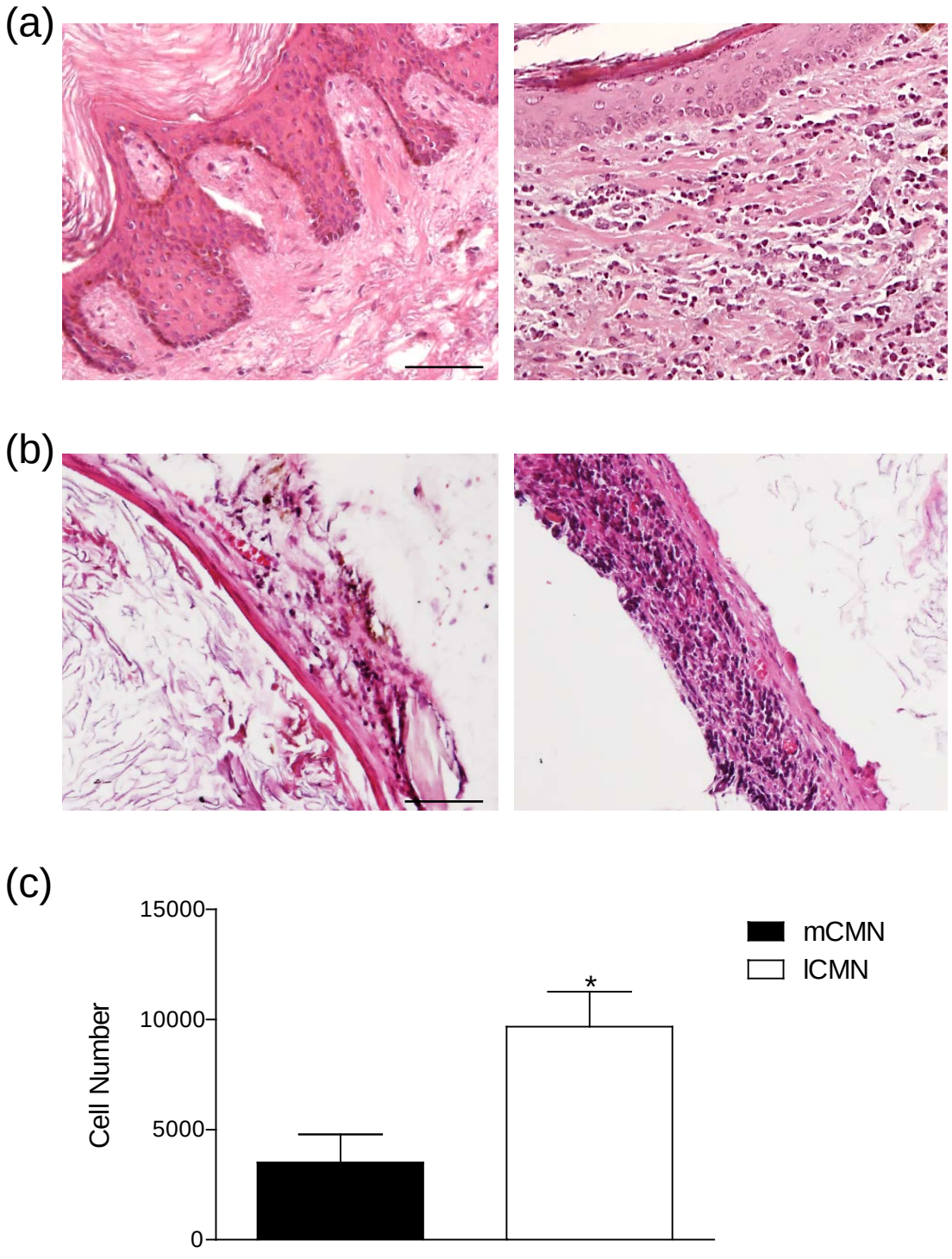
***In vitro* human CMN cells characteristics.**

(a) Photomicrographs showing single plated mCMN cells on DIV0 after tissue dissociation and after passage 1. Scale bar = 100 µm.

(b) Limiting dilution assays of lCMN cells plated at decreasing concentrations from 500 to 10 cells/well. Colonies numbers were counted on DIV7 and 13. 1/396 and 1/307 initiating cells were capable of forming colonies on DIV7 and DIV13 respectively (SEM bars are represented).

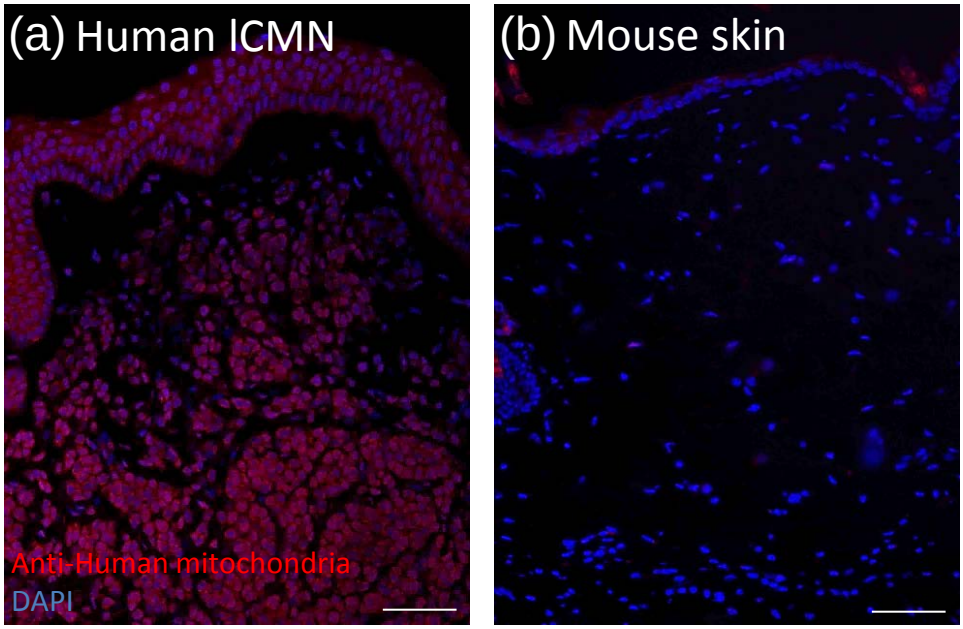
(c) Immunocytochemistry of mCMN nevospheres. Melan-A^{+} (left panel) and Oct4^{+} (right panel) were stained with Alexa488 and Cy3 secondary antibodies respectively. Scale bar = 50 µm.

Supplementary Figure 3.



Histology of xenografts 7 months postgrafting.
Both mCMN and lCMN xenografts displayed the same architecture as (a) the original grafted CMN tissues with (b) a surrounding pigmented outgrowth tissue (b). (c) Counting of nuclei counterstained with haematoxylin revealed higher cell numbers in lCMN (n=3) as compared to mCMN (n=3). Scale bar = 100 µm. * represents the difference between mCMN and lCMN. *p≤0.05 in Student's t-test.

Supplementary Figure 4.



Anti-Human mitochondria immunostainings on human lCMN (a) and mouse skin (b).
(a) Human lCMN was used as positive control : all cells were labeled with anti-human mitochondria antibody. (b) Conversely, no cells were labeled when using the same antibody on mouse skin. Scale bar = 50 µm.