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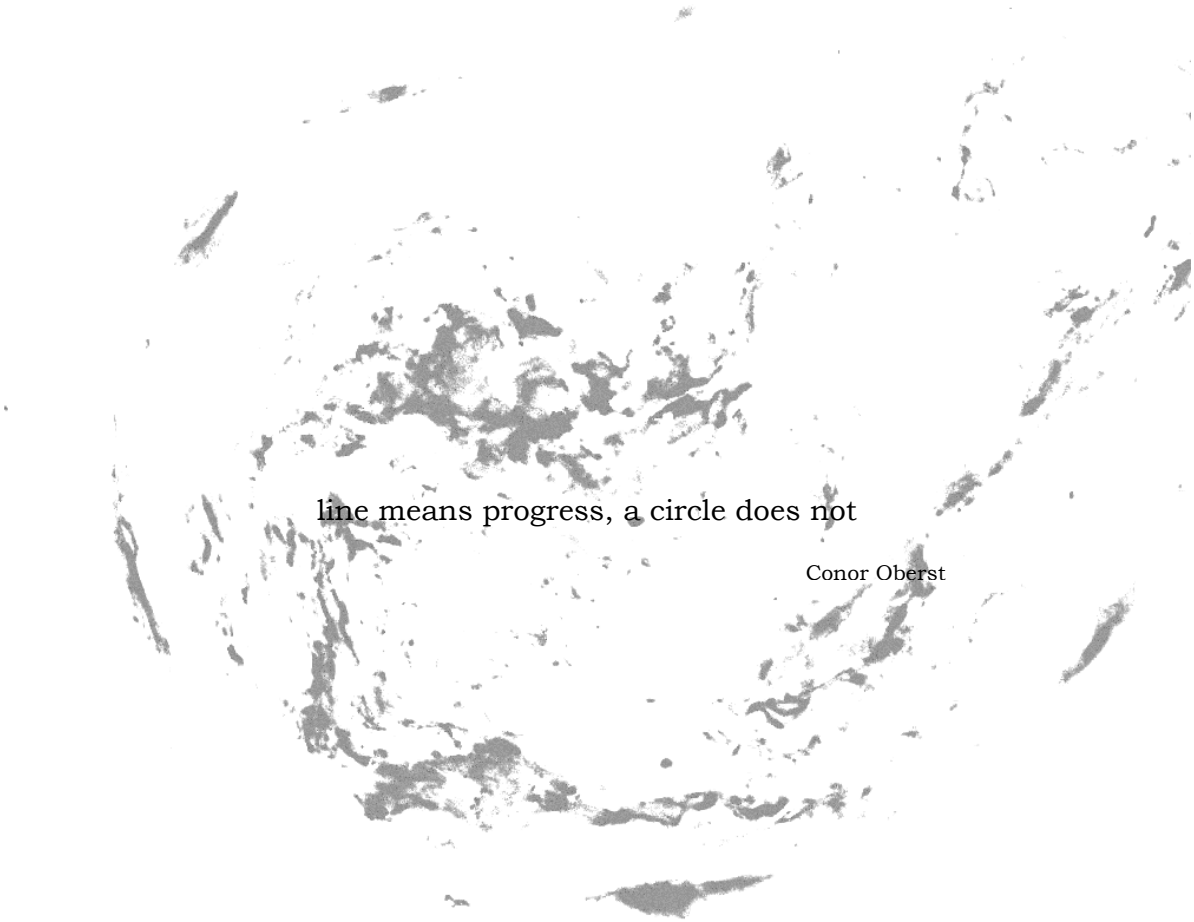

line means progress, a circle does not

Conor Oberst

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1 DANKSAGUNG

Ich möchte mich herzlich bei Ao. Univ.-Prof. Dr. Adelheid Elbe-Bürger bedanken, die mir diese Diplomarbeit möglich gemacht und mich so sehr mit ihrem Wissen und ihrer Kompetenz unterstützt und gefordert hat. Danke für deinen ansteckenden Enthusiasmus für dieses, dein Metier, und für deine schier endlose Geduld.

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Philipp

2 KURZFASSUNG

Stammzellen sind Körperzellen, die sich durch symmetrische oder asymmetrische Teilung entweder unendlich vermehren, oder in verschiedene Nachkommen ausdifferenzieren können. Sie stellen die funktionellen Elemente für therapeutische Ansätze zur Krankheitsbehandlung dar und finden in den verschiedensten Bereichen der Stammzelltransplantation und bei der Herstellung von Geweben durch „Tissue Engineering" ihren Einsatz. Für diese Anwendungen sind die Charakterisierung, sowie die Entwicklung von Methoden zur Definition und Trennung von Stamm- und Vorläuferzellen notwendig. Mehrere Studien haben über die Existenz dermaler multipotenter Stammzellen berichtet, ohne allerdings deren genaue Lokalisation in der Dermis nachweisen zu können. Ziel dieser Arbeit war es daher, Stammzellen in der humanen Haut zu identifizieren. Mittels Immunofluoreszenz-Färbungen von Gefrierschnitten humaner Vorhaut und Analyse am konfokalen Laser-Scanning Mikroskop konnten wir durch Kombination verschiedenster Oberflächenmarker wie CD34, CD133, oder CD318, die für Stammzellen beschrieben wurden, tatsächlich Zellen identifizieren, die solche Marker exprimieren. Der Stammzellcharakter dieser Zellen wird in funktionellen Experimenten noch zu prüfen sein. Der Erhalt primitiver, aktiv zirkulierender Stammzellen aus einem einfach zugänglichen Gewebe wie der Haut könnte die Effizienz vieler aktueller Therapien erheblich verbessern.

3 ABSTRACT

The commonly used criteria that define stem cells are sustained proliferation, multipotency and self-renewal. They are the functional elements of reparative medicine and tissue engineering. For potential therapeutic utility, the characterization and establishment of methods to separate defined populations of stem and progenitor cells of other than the commonly used sources is of great interest. For this reason we investigated human skin for the presence of stem cells of different lineages. Compared to other stem cell sources, skin is easy accessible, and it contains stem cells which can differentiate into tissues of all three germ layers. Furthermore, the extraction of small amounts of skin tissue is simple and without any risk, and can be pursued into adulthood. Despite the fact that multipotent dermal stem cells have been described, so far to our knowledge, no one has traced these cells in the dermis. Using multi-colour immunofluorescence staining of cryostat sections of human foreskin with subsequent analysis with confocal laser scanning microscopy, we demonstrate that subsets of putative stem/progenitor cell populations can be characterized by combination of various cell surface markers, such as CD34, CD133 or CD318. The availability of pure, primitive, actively cycling stem cells from an easily accessible source, such as skin, may improve the efficiency of a number of gene therapy protocols.

4 ABBREVIATIONS

7-AAD	7-amino-actinomycin D
Ab	Antibody
AF	Alexa Fluor
APC	Allophycocyanine
ß-gal	ß-galactosidase
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of differentiation
CLA	Common leukocyte antigen
CLP	Common lymphoid progenitor(s)
CLSM	Confocal laser scanning microscopy
DDT	Dichloro-diphenyl-trichloroethane
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DNase I	Desoxyribonuclease I
E	Erythrocyte
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorter/sorting
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
GB	Gitschier's buffer
GMP	Myelomonocytic progenitor(s)
HFSC	Hair follicle stem cell(s)
HLA	Human leukocyte antigen
Hox	Homeobox
HPRT	Hypoxanthine guanine phosphoribosyltransferase

HSC	Hematopoietic stem cell(s)
Ig	Immunoglobulin
K14	Keratin 14
Lin	Lineage
LT-HSC	Long-term hematopoietic stem cell(s)
mAb	Monoclonal antibody
Meg	Megakaryocyte
MEP	Megakaryocyte-erythrocyte progenitor(s)
Min	Minute(s)
MPP	Multipotent progenitor(s)
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell(s)
NSC	Neural stem cell(s)
O/N	Overnight
PAL-E	Pathologische Anatomie Leiden-Endothelium
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Paraformaldehyde
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SC	Stem cell(s)
Sca-1	Stem cell antigen-1
Sec	Second(s)
SKP	Skin-derived precursor(s)
SP	Side population
ST-HSC	Short-term hematopoietic stem cell(s)

5 INTRODUCTION

5.1 STEM CELLS

Stem cells have been defined as populations of cells that undergo symmetric and asymmetric divisions to either self-renew or differentiate into multiple kinds of differentiated progeny[a] [1-3]. Research in the stem cell field started out of findings by the Canadian scientists Ernest A. McCulloch and James E. Till in the 1960s, who demonstrated the presence of self-renewing cells in mouse bone marrow (BM) [4].

Stem cells can be distinguished from each other based on the tissue of origin, their bias in differentiation ability, the stage of development at which they exist, and the genes they express [2] (TABLE 1).

TABLE 1. TISSUE-SPECIFIC STEM CELLS.

ECTODERM	MESODERM
Epidermal stem cells	Hematopoietic stem cells
Neural stem cells	Mesenchymal stem cells
Neural crest stem cells	Umbilical cord blood stem cells
Hair follicle stem cells	Umbilical cord matrix stem cells
	Cardiac muscle stem cells
	Skeletal muscle stem cells
	Multipotent adult precursor cells
ENDODERM	**Germ CELLS**
Pancreatic islet stem cells	Primordial stem cells
Hepatic oval stem cells	

(adapted from Cai et al. [2])

Stem cells are basically divided in two main categories: embryonic stem cells and adult stem cells. The abilities of unlimited expansion and pluripotency make embryonic stem cells a potential source for regenerative medicine and tissue replacement after injury or disease [3;4]. Adult stem cells or somatic stem cells are undifferentiated cells and reside in peripheral blood and nearly every tissue, including the brain, BM, kidney, epithelia of the digestive system, skin, retina, muscles, pancreas, and liver. Adult stem cells are able to produce identical daughter cells during a relatively large number of cell divisions. This feature is often referred to as self-renewal or clonogenicity. Another property of adult stem cells is their ability to give rise to precursors of mature, and then terminally

[a] STAGES OF DIFFERENTIATION POTENTIAL OF A STEM CELL

TOTIPOTENCY	the fertilized oocyte forms the embryo and the trophoblast of the placenta
PLURIPOTENCY	embryonic stem cells differentiate into cells of all three germ layers: endoderm, ectoderm and mesoderm
MULTIPOTENCY	most tissue-based stem cells produce a limited range of differentiated cell lineages appropriate to their location
OLIGOPOTENCY	cells, such as epidermal stem cells, that can contribute to a more restricted subset of cell lineages usually within a single tissue
UNIPOTENCY	ability to generate one single cell type

differentiated cells with specified morphological characteristics and function. In mature tissues, adult stem cells play a crucial role in maintaining local homeostasis by replacing dead or damaged cells as well as in the process of tissue remodeling. Many adult stem cells may be better classified as progenitor cells, due to their limited capacity for cellular differentiation. Specific multipotent or even unipotent adult progenitors may have potential utility in regenerative medicine. They have mainly been studied in humans and rodents [3;5-8].

5.2 STEM CELL PLASTICITY

Stem cell plasticity refers to the ability of adult stem/progenitor cells to acquire mature phenotypes that are different from their tissue of origin [9]. However, most studies published so far have not definitively proved that the greater potency of adult stem cells can be ascribed to a single cell that can differentiate into the tissue of origin and one or more additional tissues. Some investigators have suggested that transdifferentiation[b] of stem cells or cell-cell fusion underlies these events and therefore care should be taken in the interpretation of data. Beyond the ability to differentiate into lineages of blood cells, hematopoietic stem cells (HSC) might have sufficient plasticity to give rise to other nonhematopoietic cells, such as muscle cells, neurons, hepatocytes, adipocytes, osteoblasts, and others. HSC and multipotent progenitors (MPP) can be mobilized into the circulation following treatment with a variety of natural or molecularly engineered cytokines, chemokines, and adhesion molecule antagonists [4;10-15].

5.3 STEM CELL NICHE

Stem cell niches are composed of microenvironmental cells that nurture stem cells and enable them to maintain tissue homeostasis. An appropriate dialog occurs between stem and niche cells in order to fulfil lifelong demands for differentiation and maintenance of stem cells. Niche cells provide a sheltering environment that isolates stem cells from differentiation stimuli, apoptotic stimuli, and other stimuli that would challenge the stem cell reserves. The niche also safeguards against excessive stem cell proliferation that could lead to cancer. Stem cells proliferate periodically to produce progenitor or transit amplifying cells that are committed to produce mature cell lineages. Maintaining a balance of stem cell quiescence and activity is a hallmark of a functional niche [7;16;17].

[b] **TRANSDIFFERENTIATION** describes the conversion of a cell of one tissue lineage into a cell of an entirely distinct lineage, with concomitant loss of the tissue specific markers and function of the original cell type together with acquisition of markers and function of the transdifferentiated cell [3].

5.4 HEMATOPOIETIC STEM CELLS

HSC are the best studied adult stem cells and the most primitive cell type in the hematopoietic lineage [15]. They self-renew for lifetime and give rise to short-term reconstituting subsets of HSC, with limited self-renewal capacity, and then to MPP that do not self-renew.

Differentiation progresses with segregation of myeloid and lymphoid lineages through the generation of common myeloid progenitors (CMP) [18] and common lymphoid progenitors (CLP) [19]. Both CLP and CMP undergo further lineage segregation, with CMP giving rise to myelomonocytic progenitors (GMP) and megakaryocyte/erythrocyte progenitors (MEP) that separate granulocyte and macrophage production from red blood cell and blood cell production [15;18;20-22]. This model states that only the most primitive long-term reconstituting subsets of HSC (LT-HSC) self-renew for life and give rise to short-term reconstituting subsets of HSC (ST-HSC), with limited self-renewal capacity, and then to MPP that do not self renew [23-25]. In the mouse, multipotent hematopoietic activity is contained in a small fraction of the BM that lacks expression of mature hematolymphoid-associated cell-surface markers (lineage markers) and displays high levels of the stem cell-associated markers c-Kit and Sca-1 ($Lin^-c\text{-}Kit^+Sca\text{-}1^+$ or KLS). The KLS fraction can be further subdivided into functionally distinct subpopulations based on the expression levels of different markers, such as Thy1. Loss of Thy1 and gain of Flk2 in the KLS fraction correlates with decreased self-renewal ability and increased proliferation. It has been reported that in mice, Flk2-expressing KLS cells might not be fully multipotent because they failed to generate significant Megakaryocyte (Meg) and Erythrocyte (E) progenies (MegE potential) in the assays used in those studies. Conclusions from those reports suggested that early progenitor populations might sequentially lose lineage potential as they differentiate from LT-HSC, starting with loss of MegE potential. Recently, it has been demonstrated, that $Flk2^+$ ST-HSC and MPP retain MegE potential at the population and clonal levels in vivo. These results indicate that Flk2 expression by early progenitors is not at the expense of full multipotency and support a model of hematopoietic development with segregation of myeloid and lymphoid lineages from multipotent progenitors, loss of lineage potential occurs later during differentiation into the oligolineage-commited progenitor populations [26] (FIG. 1.).

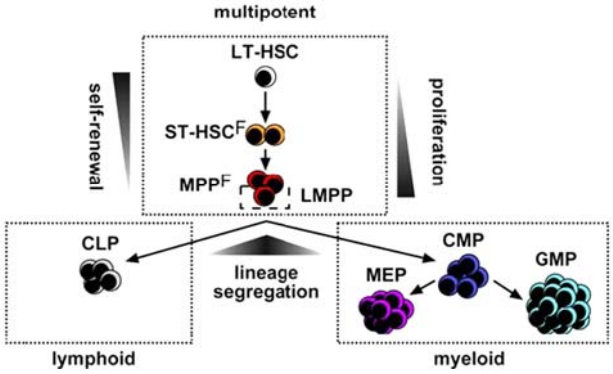

FIGURE 1. BLOOD LINEAGE DEVELOPMENT. As LT-HSC differentiate into ST-HSC and then to MPP, they progressively lose their self-renewal ability, increase their proliferation, and change their gene expression programs to leave the stem cell niche and undergo lineage differentiation while remaining multipotent. The lymphoid-primed multipotent progenitors (LMPP) represent a more committed subset of MPP. (adapted from Eckfeldt et al. [1])

It is possible to distinguish among HSC, progenitors, and more mature cells within the $CD34^+$ population by their different patterns of expression of other markers. For example, the CD38 antigen is expressed at intermediate to high levels on more than 90% of $CD34^+$ cells from most sources of hematopoietic cells. $CD34^+CD38^-$ cells have the ability for sustained multilineage hematopoietic reconstitution after transplantation into immunodeficient mice [22]. Other markers that are absent or only weakly expressed on HSC and primitive progenitor cells but highly expressed on certain types of lineage-committed progenitor cells include CD33, CD71, HLA-DR, and CD45RA. These markers can be used to subdivide $CD34^+$ cells into lineage committed and multipotent fractions. There is also evidence for the existence of $CD34^-$ cells with HSC and lymphopoietic potential in human cord blood and adult hematopoietic sources. The phenotype of $CD34^-$ HSC has been characterized by the additional absence of CD38 and lineage-specific cell surface antigens as well as the expression of CD133 [27].

The term HSC has at times been used to include a miscellany of precursor cells ranging from multipotential self-regenerating cells to lineage-restricted progenitors with little capacity for self-generation. It is probable that the stem cells of other tissues also vary widely in their multipotentiality and proliferative capacity [28].

5.4.1 THE HSC NICHE

In adults, the majority of HSC reside in the BM, where they are intimately associated with mesenchymal cells that constitute the hematopoietic microenvironment and which produce stimulatory and inhibitory factors that regulate stem cell development. Bone and marrow are intrinsically linked with HSC, and their primitive progeny are located proximal to the endosteal surface of trabecular bone. Osteoblasts are required for this localization

[7;11;16;17]. The niche's milieu allows it to retain stem cell daughters, but expel terminally differentiating daughters [7]. Its complex environment comprises many different cell types, which together secrete a specialized extracellular matrix. The effects of these supporting cells are mediated through specific molecular interactions, some of which could be used to promote HSC expansion in vitro [21]. Of particular interest for possible clinical applications of stem cell expansion are genes that (i) encode transcription factors (e.g. HOXB4, GATA1/2, SCL/TAL1), (ii) signaling molecules (e.g. WNT, Notch, Ang-1), and (iii) genes that regulate the cell cycle (e.g. p18, p21, p27).

5.4.2 SIDE POPULATION CELLS

In the population of hematopoietic cells are cells that manifest a specific profile with the use of Hoechst 33342 dye. These are so called side population cells and, when isolated from BM, they differentiate into various types of cells different from hematopoietic lineages. Side population cells have been detected in many tissues (e.g. small intestine, skeletal muscle) and in different species including humans. BM-derived side population cells are highly enriched for long-term repopulating cells, engraft in irradiated recipients and show multi-lineage reconstitution. In skin, cells with a side population phenotype are localized in the epidermis, with a few in the dermis and express high levels of α6-integrin, β1-integrin, Sca-1, Keratin 14 and 9 and low levels of E-cadherin, CD3 and CD71 [3;15;27;29].

5.5 MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSC) can give rise to BM stroma, bone, cartilage, tendon, fat, and, at least in rodents, skeletal muscle [30]. Their origin is either the cambium layer of periosteum or BM; furthermore muscle, fat, and synovium provide a limited source. MSC have a large capacity for self-renewal while maintaining their multipotency. MSC are the basic cellular unit of embryologic bone formation. Secondary bone healing mimics bone formation with proliferation of MSC, and their subsequent differentiation into components of fracture callus. Bone regeneration can be achieved by autologous transplantation of MSC, combined with strategies of osteogenesis, osteoinduction, osteoconduction, and osteopromotion. However, the capacities of cells to proliferate and differentiate are known to decrease with the age of the donor, as well as the time in culture. Although these cells do not have hematopoietic differentiation potential, they might be able to support HSC expansion in vitro or may be used to increase engraftment of HSC in vivo. MSC are known to derive from individual clonogenic cells, termed colony-forming unit-fibroblasts, which have fibroblastic appearance, characteristic prominent nucleoli, but are extremely rare in human peripheral blood. In the presence of osteogenic stimuli such as

ascorbic acid, inorganic phosphate and dexamethasone, MSC differentiate into osteoblasts. Addition of transforming growth factor-beta differentiates MSC into chondrocytes [7;31-34].

A mesenchymal-related cell type, termed multipotent adult progenitor cell, was isolated from humans, rat and mouse, which grows on a fibronectin-coated surface. Multipotent adult progenitor cells could proliferate without senescence and differentiated into mesodermal, neuroectodermal and endodermal cell types [35-37]. Furthermore, CD271, CD140b, CD340, CD349 and many more molecules were described as MSC-specific. These markers are suitable for the prospective isolation of highly purified BM MSC, which can be used as an improved starting population for transplantation in various diseases [38]. Most recently, clonal, multipotent MSC have been isolated from human postnatal dermal tissue. The progeny of a single MSC could be differentiated into adipogenic, osteogenic, and myogenic lineages [39].

5.6 THE HEMANGIOBLAST

Endothelial and hematopoietic cells arise in close spatial and temporal association from a common mesodermal progenitor in the yolk sac, the hemangioblast [40]. It has been conceptually accepted that the hemangioblast is a transient cell population, present only during embryogenesis, but recent studies in postnatal mice and humans provide evidence that the hemangioblast is also present in the postnatal stage and that adult HSC or their progenitors have a similar capacity as they can also contribute to the maintenance and repair of the vascular systems. For example, human $CD133^+$ cells from granulocyte colony stimulating factor mobilized peripheral blood can differentiate into both hematopoietic and endothelial cells in cultures. Further characterization of the hemangioblast, both embryonic and postnatal, will be critical for a better understanding of the molecular events involved in hematopoietic and endothelial cell differentiation [41;42].

5.7 THE FIBROCYTE

Fibrocytes are BM-derived mesenchymal progenitors that coexpress HSC antigens, markers of the monocyte lineage and fibroblast products. These cells contribute to the new population of fibroblasts and myofibroblasts that emerge at the tissue site during normal or aberrant wound healing, in ischemic or inflammatory fibrotic processes, and as part of the stromal reaction to tumor development. Fibrocytes were found to express CD34, CD45 and several markers of the monocyte lineage, such as CD14. Recent data suggest that fibrocytes mature from an immature subpopulation of $CD14^+$ peripheral blood mononuclear cells, which can differentiate into a number of cells different from

macrophages and dendritic cells. This subpopulation may represent an intermediate stage of differentiation of precursors of the monocyte lineage into mature fibroblasts and myofibroblasts at the tissue sites. Fibrocytes can be distinguished from circulating or tissue-resident MSC/multipotent mesenchymal stromal cells, because the latter cells express fibroblast products but are $CD90^+$ and do not express CD34 or CD45. Human MSC can express CD34 and CD45, but remain negative for monocyte markers. Mature fibrocytes produce more collagens and fibronectin than fibroblasts, express the myofibroblast marker α-smooth muscle actin (α-SMA), and downregulate expression of CD34 and CD45 [43].

5.8 THE CELL SURFACE PROFILE OF HUMAN HSC

There are several markers whose expression changes at different rates as primitive hematopoietic cells differentiate. By targeting various combinations of markers, it has become possible to subdivide this functionally heterogeneous mixture of cells into more homogeneous subpopulations.

5.8.1 LINEAGE MARKERS

One distinguishing characteristic of stem cells is the absence of differentiation markers. In the HSC field these markers have been summarized as lineage markers (Lin). Lack of expression of a number of Lin markers, which include all of the mature hematopoietic cell types (T cells, B cells, natural killer cells, monocytes, and granulocytes), can be used to distinguish immature cells from the more abundant differentiated cells in most populations of hematopoietic cells. Selection of Lin^- cells typically gives a 20- to 500-fold enrichment of HSC depending on the combination of Lin markers used. Examples of the Lin markers commonly used to isolate human Lin^- cells are Glycophorin A, CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD19, CD20, CD38, CD56, and CD66b. MSC are Lin^- for osteoblast, chondrocyte, and muscle markers; neural stem cells are negative for astrocyte, oligodendrocyte, neuronal, peripheral neuron, mesenchymal markers and Schwann cells; and embryonic stem cells are Lin^- for ectodermal, endodermal, and mesodermal markers [2;15;22].

5.8.2 STEM / PRECURSOR CELL MARKERS

5.8.2.1 CD13

CD13 (aminopeptidase N, gp150) is a type II integral membrane protein composed of 967 amino acids, and expressed on the cell surface as a homodimer. The protein is

expressed during hematopoiesis at several different stages of myeloid differentiation and, like CD33 and CD123, on long-term repopulating cells from human cord blood and BM. Under steady state conditions, macrophages, myelomonocytes, interdigitating dendritic cells, immature mast cells, granulocytes, some large granular lymphocytes, fibroblasts, osteoclasts, perineurium of peripheral nerves, central nervous system synapses, endothelial cells, placenta, renal proximal tubules, prostate secretory cells, small intestine, and bile duct canaliculi are anti-CD13 reactive. When expressed on $CD34^{+}c\text{-}kit^{+}$ cells, it becomes a marker of a progenitor cell population that includes mast cell, monocyte and mast cell/monocyte (bipotential) precursors. CD13 autoantibodies are strongly associated with chronic graft versus host disease after BM transplantation [44;45].

5.8.2.2 CD14

CD14 [lipopolysaccharide (LPS) receptor, monocyte differentiation antigen] is a pattern recognition receptor that detects antigenic molecules on the surface of bacteria (lipoteichoic acid on gram positive, LPS on gram negative), myobacteria (glycolipids), and fungi (mannans), as part of the innate immune system. A soluble form of this protein is secreted by liver cells and monocytes; in low concentrations it confers LPS-responsiveness to cells which are otherwise CD14 negative. CD14 is detectable on macrophages/monocytes, granulocytes, Langerhans cell-like cells, dendritic cells and B cells [44;46;47]. Circulating $CD14^{+}CD34^{low}$ cells, but not $CD14^{+}CD34^{-}$ cells, proliferate in response to stem cell growth factors, and exhibit clonogenicity and multipotency, as shown by their ability to differentiate not only into endothelial cells, but also into osteoblasts, adipocytes, or neural cells. The use of $CD14^{+}CD34^{low}$ cells, which exhibit both phenotypic and functional features of stem cells, may be useful in improving cell-based therapies of vascular and tissue damage [48].

5.8.2.3 CD31

CD31 [platelet endothelial cell adhesion molecule, (PECAM-1)] belongs to the immunoglobulin superfamily, and is a cell adhesion molecule that plays a key role in leukocyte trafficking across endothelium and removal of aged neutrophils from the body. It may mediate the signal-transduction pathways in spermatozoa and acts as a minor histocompatibility antigen in HLA-identical stem cell transplantation. CD31 is a superior marker for angiogenesis and can detect vascular endothelium associated antigen. Furthermore, it has been used to measure angiogenesis, which reportedly predicts tumor recurrence. The CD31 molecule appears on endothelial cells (at cell junctions) including glomeruli, alveolar wall capillaries, hepatic, lymphatic and splenic sinuses, platelets, megakaryocytes, monocytes, macrophages, Kupffer cells, osteoclasts, granulocytes, T cells, natural killer cells, plasma cells, fibroblasts, brown fat, trophoblasts and spermatozoa. CD31 and CD34 can be used as markers for myeloid progenitor cells that

recognize different subsets of myeloid leukemia infiltrates (granular sarcomas). CD31 has been used as a marker for benign and malignant human vascular disorders, and myeloid leukemia infiltrates in normal BM [44;49;50].

5.8.2.4 CD34

CD34 was the first differentiation marker to be recognized on primitive human hematopoietic cells and is still the most commonly used marker to obtain enriched populations of human HSC and progenitors for research or clinical use. Cell surface expression of this protein has become the distinguishing feature used as the basis for the enumeration, isolation and manipulation of human HSC, because CD34 is downregulated as cells differentiate into more mature cells. Human and murine CD34 homologues are highly conserved in their protein coding regions. The cytoplasmic domains of the human and mouse proteins share 90% amino acid identity, the transmembrane and C-terminal regions of the extra-cellular domains are also well conserved, with 73-82% amino acid identity. The majority of human cells capable of producing multilineage hematopoietic engraftment in myeloablated recipients express CD34. But even in a highly purified population of $CD34^+$ cells (>90%), the frequency of cells possessing progenitor activity is typically <20% and the frequency of HSC is much lower (<0.1%). CD34 is expressed by dendritic interstitial cells, dermal dendrocytes, endometrial stroma, endoneurium, fibroblasts in breast, hair endothelial cells of blood vessels, endothelial cells of some lymphatics, fibrocytes, hematopoietic progenitor cells (pluripotent and erythroid-, lymphoid- and monomyeloid committed cells), follicle cells, lymphocyte (B and T) precursors, neural stem cells in the central nervous system, perivascular stroma, umbilical cord blood; but also outside the hematopoietic system on vascular endothelial cells and some fibroblasts (e.g. breast fibroblasts and vascular adventitial fibroblastic cells in stomach) [15;22;27;44;45;49-52].

5.8.2.5 CD45

CD45 [common leukocyte antigen (CLA), T220 and B220 in mice] is a protein tyrosine phosphatase located in certain hematopoietic cells. Different subsets of hematopoietic cells express different CD45 isoforms due to variable exon splicing, which can change in response to cytokines: CD45RA (naive/resting T cells, medullary thymocytes), CD45RO (memory/activated T cells, cortical thymocytes). CD45 is an essential regulator of T and B cell antigen receptor-mediated activation, and also required for thymic selection. Mutations with loss of CD45 cause severe combined immunodeficiency with a defect in cell function or development, lymphopenia, and deficiency in humoral and cell-mediated immunity. CD45 is expressed by hematopoietic cells (including monocytes, macrophages, mast cells) except mature red blood cells and their immediate progenitors, platelets and megakaryocytes; dendritic cells, fibrocytes, thymus (medullary thymocytes) [44;50].

5.8.2.6 CD90

CD90 is a 25-35 kDa glycosylphosphatidylinositol-binding glycoprotein expressed on fibroblasts, human BM and cord blood $CD34^+$ hematopoietic progenitor cells [53]. Furthermore, it is widely used as a mouse HSC marker (Thy-1). $CD90^+$ cells from cultured neonatal normal human epidermal keratinocytes show higher proliferation and colony-forming abilities than $CD90^-$ cells and, thus seems to be a marker for human keratinocyte stem cell-enriched populations in cultured keratinocytes. $CD34^+CD90^+$ cells include HSC that serve as autologous grafts to replace the BM in patients with malignancies [44;54].

5.8.2.7 CD117

HSC and progenitor cells express receptors for hematopoietic growth factors that are responsible for regulating their survival and proliferation, although the expression of many of these (e.g. receptors for IL-3, GMCSF, and IL-6) is upregulated as they differentiate into late precursor cells and mature blood cells. One exception is CD117 (c-kit), the receptor for stem cell factor. CD117 mediated signal transduction is critical for the normal development and survival of haematopoietic progenitor cells (expressed on ~2/3 of $CD34^+$ cells), mast cells, interstitial cells of Cajal (intestinal pacemaker cells), melanocytes and germ cells. Upon differentiation, CD117 expression is lost with the exception of mast cells, melanocytes and interstitial cells of Cajal [55]. However, the expression level of CD117 on $CD34^+$ cells is usually high enough to allow its use in hematopoietic cell isolation methods [27;44;45].

5.8.2.8 CD133

Other selection markers for human hematopoietic cells have been identified with expression patterns that overlap with, but are not identical to, those of CD34. One such marker is CD133 (AC133, Prominin-1) – a member of the prominin family gene products. CD133 was first described as a marker for human HSC and has recently been proposed as a universal marker for tissue stem/progenitor cells. It is expressed on a majority of, but not all, $CD34^+$ cells. These include immature progenitors, monocyte/granulocyte progenitors and some erythroid progenitors. It also appears to be expressed on $CD34^-$ HSC identified in human cord blood. Furthermore, CD133 is expressed on neural and endothelial stem cells, other primitive cells such as retina, villous and extravillous cytotrophoblasts, and syncytiotrophoblasts [15;27;44;51;52;56-58].

5.8.2.9 CD318

CD318 (CUB-domain containing protein 1, CDCP1), has an expression pattern on adult human BM cells similar to that of CD133. It is expressed on HSC subsets, epithelial cells, and on cells with a phenotype reminiscent of MSC and neural cells. It is not expressed on

mature blood cells and was originally identified as a gene that is over-expressed in colorectal tumors and the K562 erythroleukemia cell line. The expression of CD318 on human hematopoietic cells is restricted to a subset of $CD34^+$ cells including those with NOD/SCID repopulating activity, $CD34^+CD38^-$ BM stem/progenitor cells, but not mature peripheral blood cells. Similar to CD34 and CD133, CD318 may thus be useful for the enrichment of human HSC and progenitor cells, but not for their discrimination. Furthermore, CD318 is unique in its property to recognize cells with phenotypes reminiscent of mesenchymal and neural progenitor cells [27;59].

5.8.2.10 THE SIGNALLING LYMPHOCYTE ACTIVATION MOLECULE (SLAM) FAMILY OF RECEPTORS

The location and identity of differentiated cells are often defined by the differential expression of individual families of cell surface receptors. Thus, it is necessary to use complex combinations of markers to purify HSC. SLAM family receptors, including CD150, CD244, and CD48, are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness. The SLAM family is a group of 10-11 surface receptors that regulate the proliferation and activation of lymphocytes. While the SLAM family is very well-investigated in the murine system, less is known about these proteins in the human system [16;60-62].

5.9.2.10.1 CD150

CD150 is 4- to 17-fold upregulated in HSC as compared to multipotent hematopoietic progenitors and $CD45^+$ BM cells.

5.9.2.10.2 CD244

CD244 is differentially expressed among HSC and progenitors, thus, HSC are $CD244^-$, whereas some multipotent hematopoietic progenitors are $CD244^+$.

5.9.2.10.3 CD48

CD48 is a ligand for CD244 and expressed at significantly higher levels on $CD45^+$ cells as compared to HSC, or multipotent hematopoietic progenitors. CD48 is expressed by restricted B lineage and myeloerythroid lineage progenitors.

5.9 SKIN

As the skin is the largest and most accessible organ, it provides an attractive target for therapeutic gene repair. Skin is composed of three primary layers: a multilayered epidermis of ectodermal origin, the underlying dermis of mesodermal origin, and the hypodermis (subcutaneous adipose layer, FIG. 2). The **epidermis** can be subdivided into the following strata: corneum, lucidum, germinativum, spinosum and basale. It consists of a stratified squamous epithelium composed of keratinocytes, associated hair follicles, sebaceous glands and sweat glands. The predominant cell type in the epidermis is the keratinocyte. Stratification of the epidermis commences during embryonic development and is a process that continues to occur throughout the life of the organism. The **dermis** contains a number of structures including blood vessels, nerves, hair follicles, smooth muscle, glands and lymphatic tissue. Additionally, it contains fibroblasts secreting collagen, elastin and ground substance providing support and elasticity of the skin. The dermal papillary layer is outermost and extends into the epidermis to supply it with nutrients. The dermal reticular layer is denser and is continuous with the hypodermis. The **hypodermis** consists of a network of collagen and fat cells. Normal skin contains BM–derived cells (such as epidermal Langerhans cells, dermal dendritic cells, macrophages, mast cells, and T cells) that are involved in host defence and inflammatory processes, including wound healing [3;63].

Significant progress has been made in corrective gene therapy of inherited skin diseases,

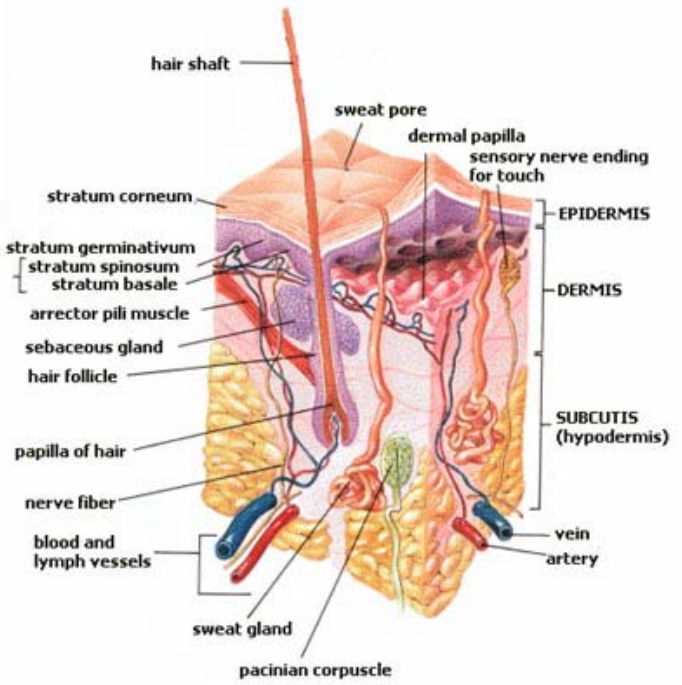

FIGURE 2. SCHEMATIC OVERVIEW OF HUMAN SKIN LAYERS.

(adapted from www.wikipedia.org)

including vector technology, targeted gene expression, gene replacement, and the availability of appropriate animal models for a variety of candidate diseases. In addition, an increased understanding of the uptake and trafficking mechanisms inside keratinocytes has evolved. The translation into clinical trials and the control of immune responses represent challenges for further researches [64].

5.10 EPIDERMAL STEM CELLS

The epidermis and its associated structures arise from two stem cell populations within the hair follicle and interfollicular regions, one, in the basal layer of skin, giving rise to stratified skin layers. A second, the hair follicle stem cell (HFSC), resides in a region of the outer root sheath (bulge), and is responsible for the regeneration of hair and sebaceous glands. At the start of the hair cycle, stem cells are stimulated to proliferate and exit at the base of the bulge. The receding follicle carries the dermal papilla from the base of the hair bulb up to the bulge and cyclic alternation in the microenvironment stimulates label-retaining cells to exit the niche, divide rapidly and produce a new hair. In response to injury, bulge label-retaining cells exit the niche and move upward to repair the skin [65;66]. Expression profiling of bulge stem cells has identified 97–157 genes that are differentially expressed in bulge cells, including fibroblast growth factor 1 and its receptor, transforming growth factor activators, Bmp and Wnt pathway inhibitors. ß-catenin causes de novo follicle morphogenesis and, eventually, skin tumors. Increase in ß-catenin levels accelerate the transition from the resting to the growth phase of the hair cycle. The transient activation of ß-catenin in adult epidermis may lead to new follicles derived from existing follicles, interfollicular epidermis, and sebaceous glands, but not from HFSCs within the bulge region. Conditional ablation of Bmpr1a results in hair follicle defects. Activation of the Wnt pathway and inhibition of the Bmp pathway appear to be necessary for functional ß-catenin/Lef1 transcriptional complexes [17;63].

5.11 DERMAL STEM CELLS

The adult hair follicle dermal papilla and dermal sheath cells are developmentally active cell populations with a proven role in adult hair follicle-cycling activity and unique inductive powers. Cultured papilla and sheath cells lines have extended proliferative capabilities and can be directed to lipid and bone differentiation [67].

The first evidence for the existence of stem cells in the dermis has been provided by Toma and colleagues. They have isolated, expanded and characterized a multipotent precursor cell from mammalian dermis, termed skin derived precursor (SKP) [5]. Recently, they have isolated a similar precursor cell from neonatal human foreskin tissue. In the

presence of fibroblast growth factor and epidermal growth factor these dermal cells proliferate and form spheres in vitro. Addition of transforming growth factor-β during culture increased the formation and proliferation of SKP. The human SKP could be maintained in culture for long periods of time and still differentiated into neural cells (neurons, glia) or mesodermal cells (smooth muscle cells, adipocytes)[6]. Evidence has been provided that human SKP apparently derive from an endogenous multipotent, neural crest-related precursor within human foreskin. SKP were described as being distinct from MSC and to represent the discovery of a new, multipotent adult stem cell [17;63]. Dermal but not epidermal compartments of the adult hair follicle have some HSC activity, suggesting that the capacity to produce hematopoietic cells locally in the skin could play a role in wound healing and immune surveillance. Recently, hair follicle derived dermal stem cells were isolated, which show a fibroblastic morphology and are $CD44^+CD73^+CD90^+CD34^-$. The population has the capacity to differentiate into various mesenchymal lineages, such as osteoblasts, adipocytes, chondrocytes, and myocytes [68].

As a highly accessible source, capable of being discretely isolated, the follicle has important potential as a stem cell source for tissue engineering and cell therapy purposes. The most obvious therapeutic use of such multipotent adult human precursors is cell transplantation. Perhaps equally important is the possibility that expandable adult stem cell populations could be used to generate human cell types on an individual basis for screening or discovery research and may have implications for certain pathologies in which abnormal differentiation occurs in the skin.

5.12 CROSS-TISSUE-SPECIFIC STEM CELL COMPARISONS

Despite the shortcomings in gene-expression evaluation for many adult stem-cell populations, comparison between the molecular signatures of HSC, neural stem cells (NSC), bulge stem cells and gastrointestinal stem cells have identified several classes of genes that might be expressed in all tissue specific stem cells. For instance, 15–30% of genes that are expressed specifically in HSC, but not in hematopoietic progenitor cells, are also specifically expressed in NSC-enriched populations; and 14% of genes that are expressed in bulge stem cells are also expressed in HSC and neural stem cells. Among these are genes involved in Wnt signalling (for example, Tcfs and Fzd7), Notch signalling (for example, Hes1), adhesion (for example, Itga6) and transcriptional regulation (for example, Bmi1) [1].

6 OBJECTIVE OF THE THESIS

The true potential of stem cells will only be realized through continued effort to increase basic scientific understanding at all levels, such as the development of adequate methods to achieve a functional phenotype, and attention to safety issues associated with careful control of cell localization, proliferation and differentiation. As the phenotype of skin stem cells is unknown, the aim of my diploma thesis was to characterize and localize mammalian stem cells in murine and human dermis.

7 RESULTS & DISCUSSION

7.1 MURINE STUDIES – ANALYSIS OF DONOR CELL ENGRAFTMENT IN TISSUES OF RECONSTITUTED MICE

In their recent publication, Meindl et al. from our laboratory have tested dermal cell suspensions, cryosections, and whole mounts in newborn and adult mice for HSC marker expression [69]. Phenotypic analysis revealed that a small population of $CD45^+$ cells and a large population of $CD45^-$ cells expressed CD34, CD117, and stem cell antigen-1 (Sca-1) molecules. When cultivated in selected media supplemented with hematopoietic cytokines, total dermal cells, Lin^-, and/or highly enriched phenotypically defined cell subsets produced hematopoietic and nonhematopoietic colonies. When injected into lethally irradiated recipient mice, a small percentage of newborn dermal cells was able to migrate into hematopoietic tissues and the skin and survived through the 11-month monitoring period. To distinguish between host and donor cells, Meindl et al. have used C57BL/6 ROSA26 (CD45.2) mice as donors which express ß-galactosidase (ß-gal) systemically. Dermal cells isolated from these mice were injected into congenic mice expressing the CD45.1 allele, allowing analysis of donor and host cells for defined populations (FACS analysis, ß-gal expression). Briefly, lethally irradiated mice (CD45.1) were transplanted with total dermal cells, Lin^-, $CD34^+$, $CD90^+$ and $CD45^+$ dermal cells from newborn C57BL/6 ROSA26 (CD45.2) mice together with syngeneic (CD45.1) BM cells. Recipient mice were sacrificed at selected time points (8-44 weeks), and chimerism was evaluated by flow cytometry and ß-gal detection. $CD45.2^+$ donor cells and ß-gal activity were observed in hematopoietic tissues (thymus, spleen, lymph nodes, liver and BM) and in skin of mice injected with purified $CD45^+$ and Lin^- dermal cells.

ß-gal is encoded by the lacZ gene, which can be detected on DNA and RNA levels by polymerase chain reaction technique (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR), respectively. In parallel to the phenotypic analysis of recipient mice we performed RT-PCRs of hematopoietic tissues and the skin of mice with primers specific for the lacZ gene [70] to strengthen the results.

In the first series of experiments, skin, thymus, spleen, lymph nodes, liver and BM from C57BL/6 ROSA26 and C57BL/6 mice, were tested for lacZ gene expression on genomic (FIG. 3A) and RNA levels (FIG. 3B). Equal amounts of DNAse-treated total RNA were used for first-strand cDNA synthesis. The lacZ gene was detected only in ROSA26, but not C57BL/6 mice, proving that only ROSA26 mice carry the lacZ gene (positive controls). C57BL/6 mice were used as negative controls.

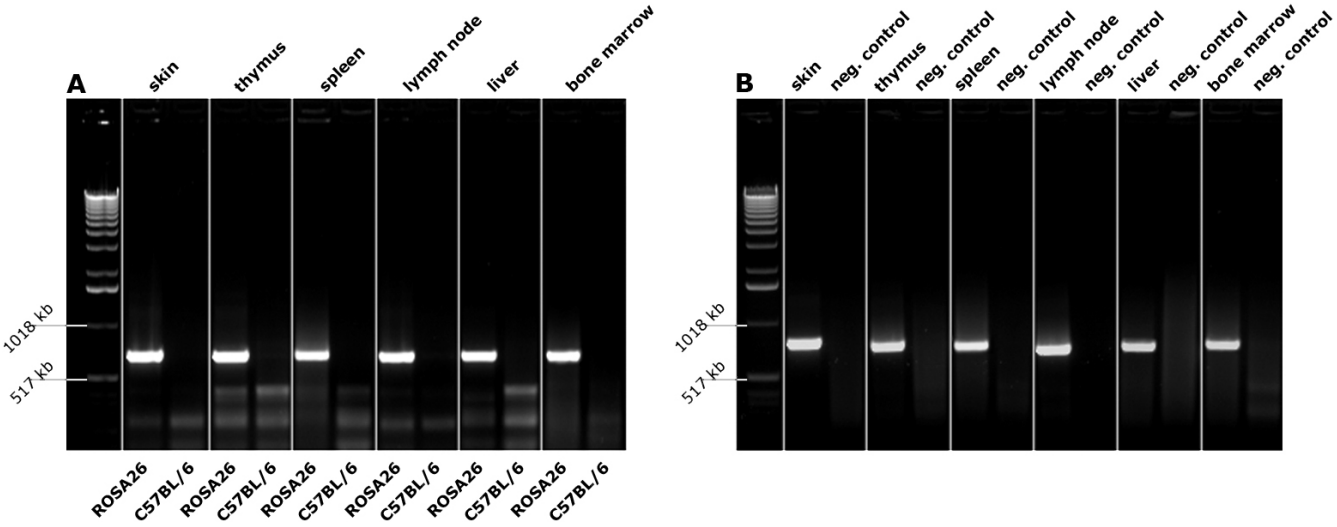

FIGURE 3. DETECTION OF LACZ IN C57BL/6 ROSA26 MICE, BUT NOT IN C57BL/6 MICE. A. lacZ PCR of DNA isolated from skin and hematopoietic tissue of C57BL/6 ROSA26 mice (n = 2) and C57BL/6 mice (n = 2), indicating the lack of the lacZ gene in C57BL/6 mice. **B.** lacZ RT-PCR of either cDNA or RNA (negative controls) isolated from skin and hematopoietic tissues of C57BL/6 ROSA26 mice (n = 2). Forty five cycles were performed for lacZ primers.

7.1.1 LACZ WAS NOT DETECTABLE IN RECONSTITUTED MICE

To detect lacZ mRNA, and thus the presence of donor cells, in hematopoietic tissues and skin of reconstituted CD45.2^{+} mice (TABLE 2), RT-PCR was performed. To verify successful RNA isolation and cDNA synthesis, PCRs with primers specific for the expressed housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) were conducted for each sample. Equal amounts of cDNA were used for all PCR reactions. As expected, HPRT mRNA was detected in all samples (data not shown), whereas lacZ was only detectable in the positive control mice (ROSA26; n=2). We have screened 25 reconstituted mice for lacZ expression, using primers described by Sibilia et al. [70]. Surprisingly, in none of the tested organs we detected lacZ gene expression, regardless of the source of the injected donor cells (FIG. 4A). Consequently, we decided to test lacZ primers with different sequences such as those described by Lako et al. [66] (FIG. 4B). Again, except for the positive controls, we failed to observe lacZ gene expression in any of the reconstituted mice (FIG. 4C).

TABLE 2. OVERVIEW: INVESTIGATED MICE AND TRANSPLANTED CELL TYPES.

CELL TYPE TRANSPLANTED	CELLS INJECTED / MOUSE	CELL TYPE TRANSPLANTED	CELLS INJECTED / MOUSE
adult dermis	$1x10^6$	$CD90^-$	$5x10^4$
$CD34^-$	$1x10^4$	$CD90^+$	$5x10^4$
$CD34^+$	$1x10^4$	Lin^-	$1x10^6$
$CD45^+$	$1x10^4$	newborn dermis	$1x10^6$

Reconstituted mice were tested for lacZ mRNA levels using 2 different primer pairs. Purified populations were isolated from newborn C57BL/6 ROSA 26 mice (see also **TABLE 6.**).

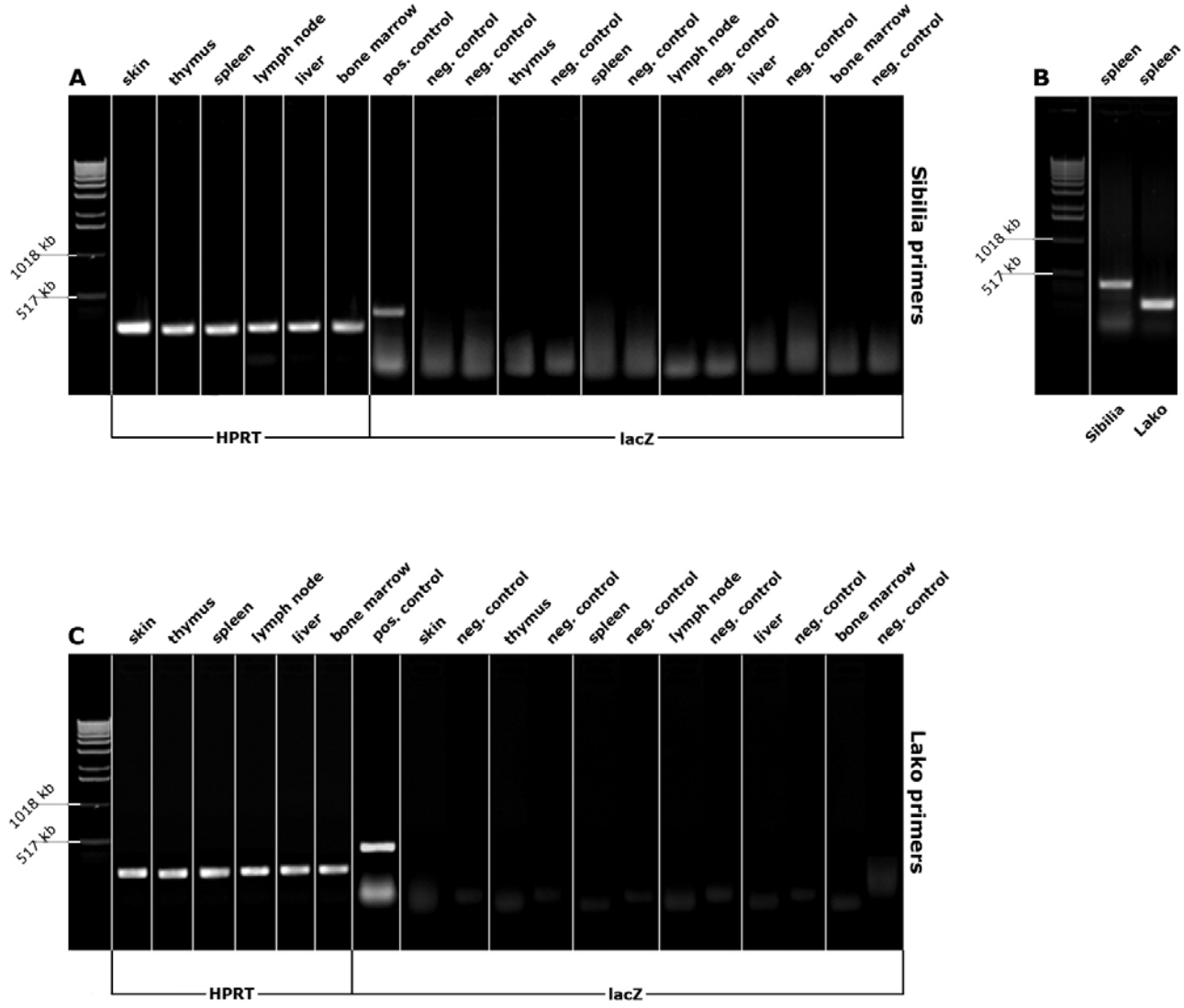

FIGURE 4. LACZ EXPRESSION ANALYSIS OF RECIPIENT MICE BY RT-PCR. A. RT-PCR analysis of cDNA generated from RNA isolated from skin and hematopoietic tissues of irradiated C57BL/6 mice reconstituted with $CD34^+$ cells using primers from Sibilia et al. [70]. **B.** Comparison of lacZ primer pairs using cDNA from a positive control revealed a difference in the size of the lacZ PCR product. **C.** RT-PCR analysis of cDNA generated from RNA of irradiated C57BL/6 mice reconstituted with dermal cells from adult C56BL/6 ROSA26 mice using Lako primers [66]. In both experiments (**A** & **C**) the lacZ gene was only detected in positive controls. Forty five cycles were performed for lacZ primers.

In their reconstitution assays, Meindl et al. [68] injected cells isolated from C57BL/6 ROSA26 mice carrying the lacZ reporter gene into lethally irradiated C57BL/6 mice. A small percentage of the injected cells was able to migrate into hematopoietic tissues and the skin and survived through a 11-month monitoring period. $CD45.2^+$ donor cells were identified by flow cytometry, and ß-galactosidase activity was detected in cells on cytospins. To confirm these data we thought to additionally apply a molecular approach. Using the RT-PCR technology we expected to detect the lacZ reporter gene in specific organs of the reconstituted mice. Total RNA was isolated from skin, thymus, spleen, lymph nodes, liver and BM, transcribed to cDNA and examined for the lacZ mRNA expression with two different lacZ primer pairs. The lacZ fragment could not be detected in any of the investigated tissues, suggesting that this method may not be sensitive enough to detect transplanted cells in the tissues indicated.

For the establishment of the lacZ-specific RT-PCR method we tested the polymerase, primers, PCR-mix and the PCR programme with cDNA obtained from organs of a C57BL/6 ROSA26 mouse. lacZ was detected in all tissues of this mouse using either of the primer pairs. To control the successful RNA isolation and cDNA synthesis we additionally performed a PCR, specific for the housekeeping gene HPRT. We could demonstrate the presence of HPRT in all samples obtained from either the control mice, or the reconstituted mice. Furthermore, to exclude contamination with genomic DNA in our PCR procedure we carried out lacZ and HPRT PCRs with the RNA samples and all components of the PCR mix separately. As expected, neither lacZ nor HPRT were detected in any of the samples.

We used established lacZ systems acquired from studies by Sibilia et al. [70] and Lako et al. [66]. The systems differed from each other in the sequences of the primers, as well as in the size of the lacZ fragment received from RT-PCR. The results we gained from tests with this system were negative.

Our failure to demonstrate the lacZ reporter gene in the recipient mice can be explained by the low frequency of donor cells in the investigated tissues. Another explanation might be that we have not analyzed freshly isolated, but frozen tissue. Furthermore, intensive inquiries resulted in a sparse list of publications which described the use of the lacZ reporter gene system. In comparison to many other reporter genes in mice, the use of the lacZ gene seems to be quite unpopular. The application of another reporter gene may be more successful.

7.2 HUMAN STUDIES – EXPRESSION OF STEM CELL MARKERS IN HUMAN SKIN

There are several markers whose expression changes at different rates as primitive hematopoietic and non-hematopoietic cells differentiate. By targeting various combinations of markers, it has become possible to subdivide this functionally heterogeneous mixture of cells into more homogeneous subpopulations. It hast been demonstrated that the murine skin contains $CD45^+$ and $CD45^-$ populations of cells that can be distinguished by the expression of several markers, including stem cell markers, some of which might represent candidate stem/progenitor cells [69]. Based on these observations and the fact that the skin rapidly changes with regard to the cellular composition during the first years of life [71], we have decided to investigate skin tissue from young donors (6 months up to 4 years after birth), speculating to find precursors and stem cells in a higher frequency compared to adults. We performed multi-colour immunofluorescence staining of cryostat sections and flow cytometry of total single cell suspensions from human foreskin with subsequent analysis using confocal laser scanning microscopy (CLSM). Selected cell surface marker combinations were employed to identify and localize hematopoietic and non-hematopoietic stem cell candidates. All experiments were performed with human foreskin obtained from routine circumcisions. The use of human foreskin has the advantage of easy accessibility, which also makes it ethically defensible in comparison to biopsies from other body sites. Moreover, foreskin is hairless and, thus, assumed to be devoid of the already well described hair follicle stem cells, implying that dermal cells expressing stem cell markers do not represent these cells.

7.2.1 CD34

Cell surface expression of CD34 has become the distinguishing feature used as the basis for the enumeration, isolation and manipulation of human HSC. It is downregulated as cells differentiate into more mature cells. Confocal microscopy and flow cytometry analysis revealed that the dermis contains a high number of cells expressing CD34 (FIG. 5A, B). While the majority of $CD34^+$ cells were $CD45^-$, we consistently observed a small population of $CD34^+CD45^+$ cells (FIG. 5B). In repeated experiments we could localize $CD34^+$ cells in the papillary and the reticular dermis, arranged in clusters or spread in an irregular fashion over the whole dermis, thus, confirming and extending findings by Meindl et al. who have shown that a small population of $CD45^+$ cells expressed CD34 in murine skin [69]. The isolation and cultivation of $CD34^+CD45^+$ cells from the human system might lead to similar results, and, thus, would represent ideal vehicles for genetic

manipulation and gene therapy. When cultivated in selected media, these murine $CD34^+CD45^+$ cells produced hematopoietic and nonhematopoietic colonies.

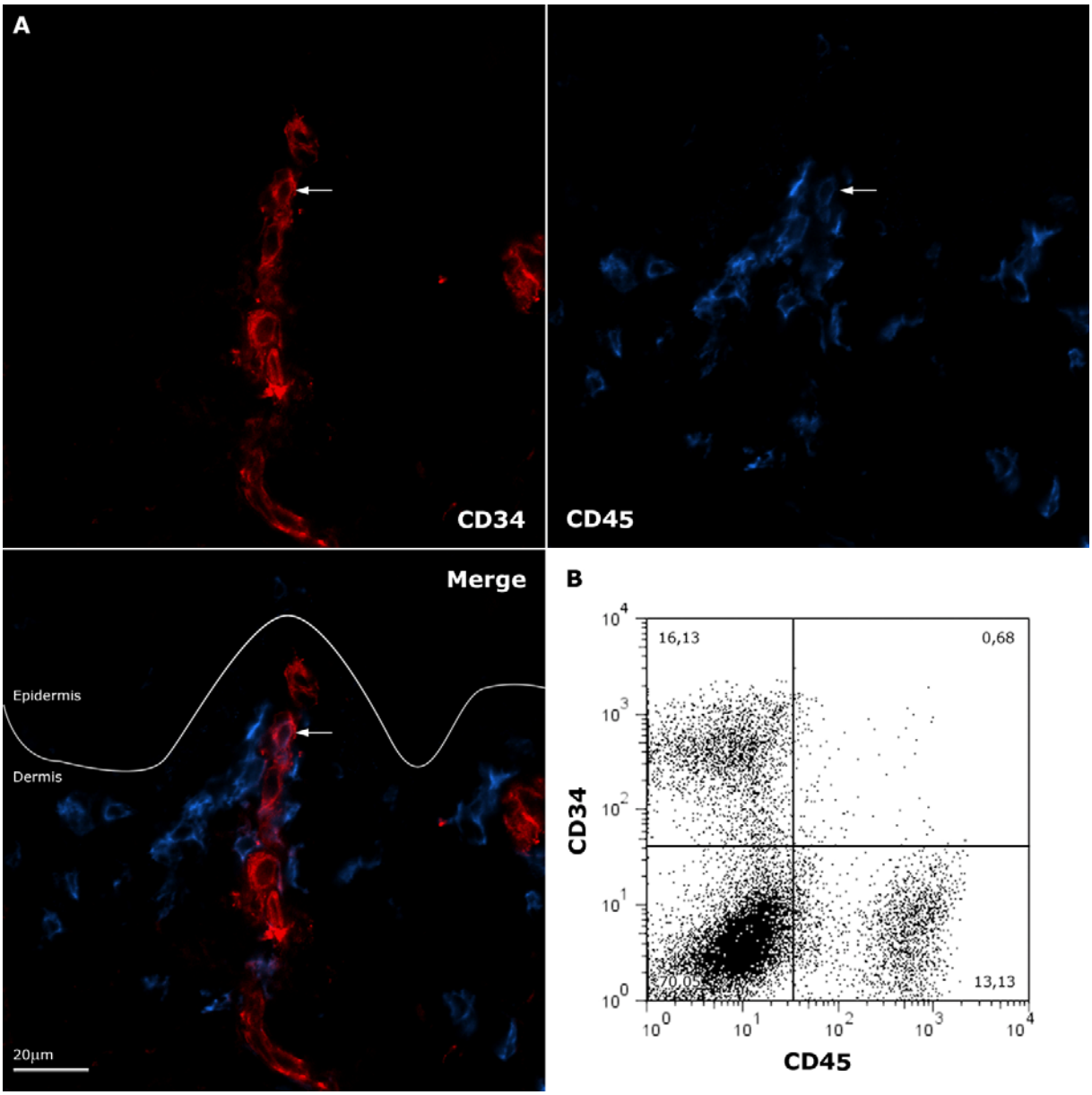

FIGURE 5. HUMAN SKIN HARBOURS $CD34^+CD45^+$ CELLS. A. Immunofluorescence analysis of cryostat sections from human foreskin (2 year-old). Sections were exposed to PE-anti-CD34 and APC-anti-CD45 double labeling. Arrow indicates one $CD34^+CD45^+$ cell. **B.** FACS analysis of human foreskin (3 year-old). Total dermal single cell suspension was stained for the indicated markers. Numeric values indicate percentage among living cells. Dead cells positive for 7-AAD were excluded and 30.000 events per sample were acquired.

Using stem cell markers, such as CD13 and CD90, we identified a subset of $CD34^+CD13^+CD90^+$ cells. CD13 is expressed during hematopoiesis at several different stages of myeloid differentiation and on LT-repopulating cells. When expressed on $CD34^+$ cells, it becomes a marker of a progenitor cell population that includes mast cell, monocyte and mast cell/monocyte precursors. CD90 is known to stain fibroblasts as well as stem cell-enriched cell populations. Triple-staining for $CD34^+CD13^+CD90^+$ cells on whole mounts allowed a clear visualization of blood and lymphatic vessels ($CD34^+$) (FIG. 6).

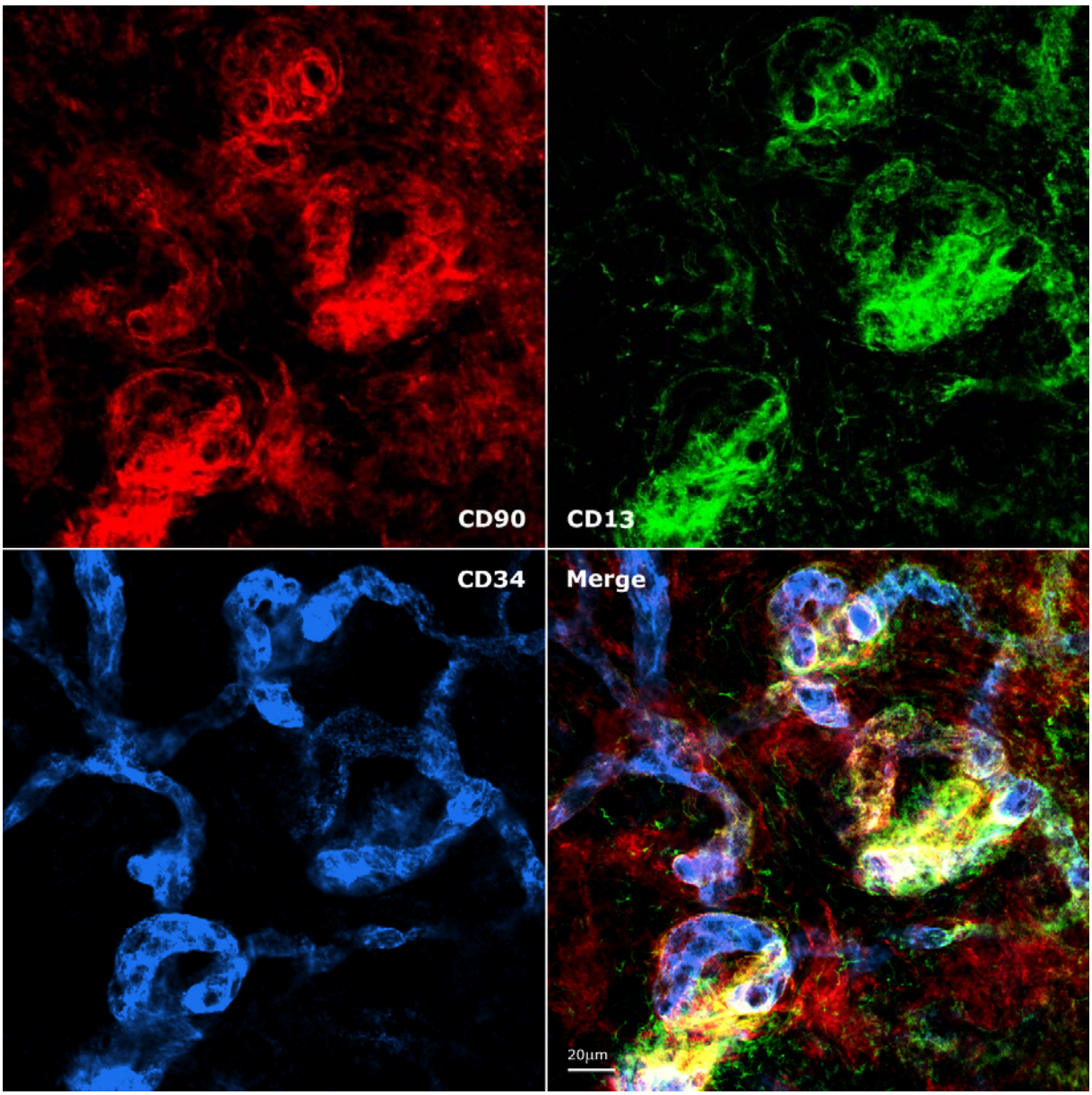

FIGURE 6. EXPRESSION OF CD90, CD13 AND CD34 IN HUMAN SKIN. Immunofluorescence analysis of whole mounts (3 year-old). Samples were exposed to PE-anti-CD90, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

The disadvantage of the whole mount staining compared to cryostat sections is that the identification of single cells expressing certain markers is more difficult. In cryostat sections we also localized $CD34^+CD13^+CD90^+$ candidate stem/progenitor (Fig. 7). These data were confirmed by flow cytometry. We found that approximately 80% of $CD34^+CD90^+$ cells coexpress CD13 (Fig. 8).

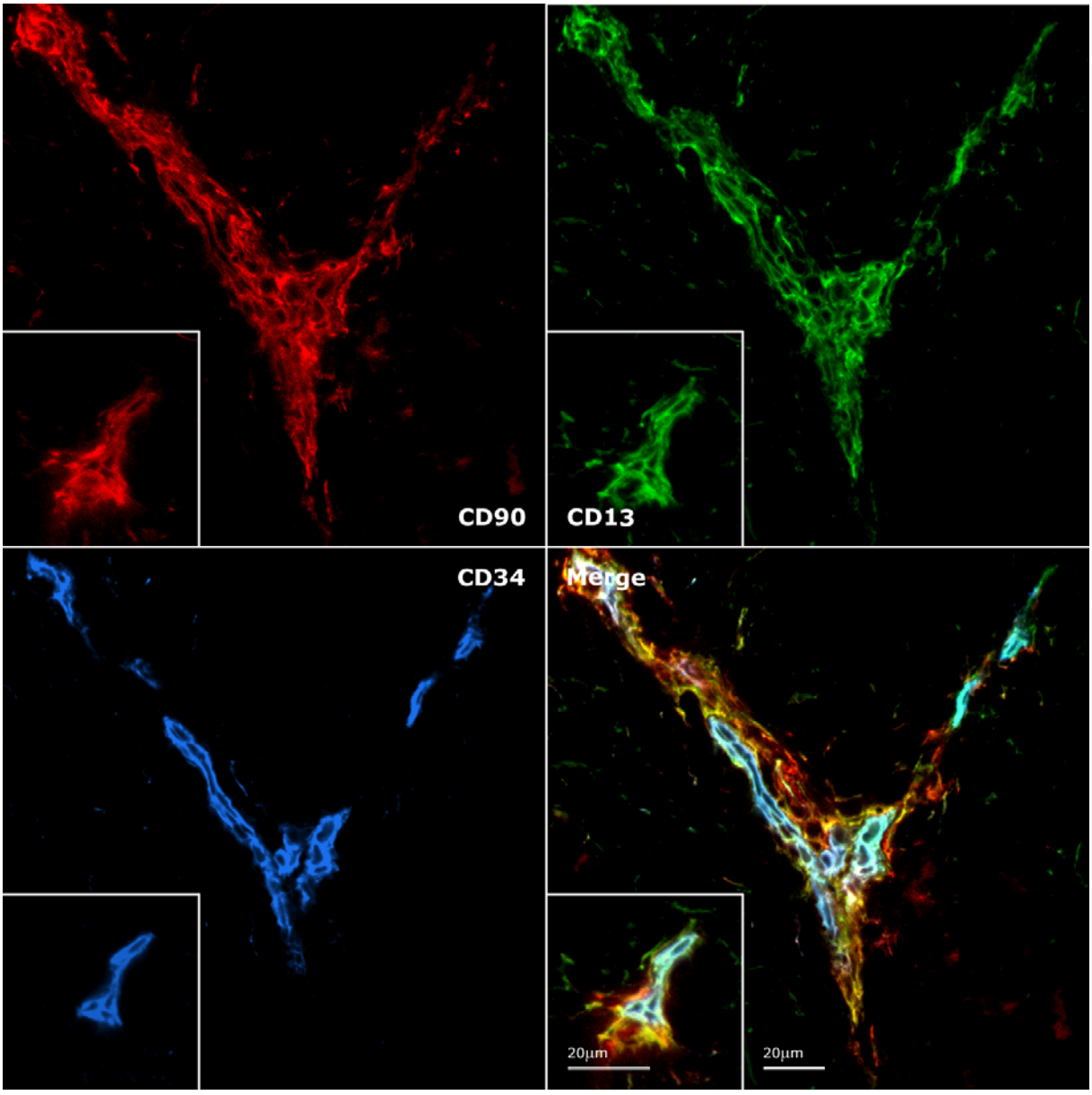

Figure 7. Human skin harbours $CD90^+CD13^+CD34^+$ cells. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Samples were exposed to PE-anti-CD90, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

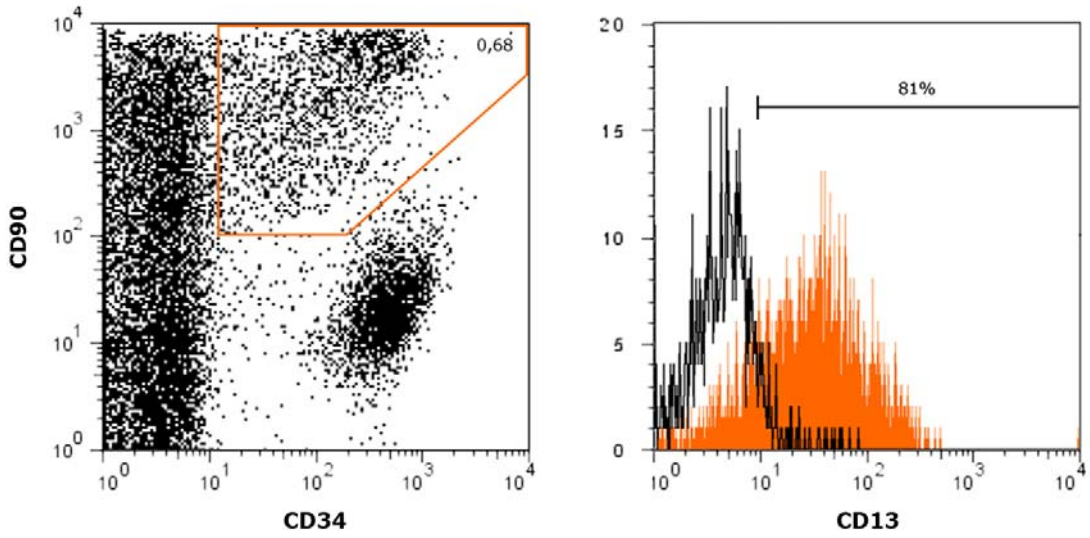

FIGURE 8. HUMAN SKIN HARBOURS CD90⁺CD34⁺ CELLS, MOST OF WHICH COEXPRESS CD13. Total dermal cells (human foreskin of a 3-year old child) were stained for the indicated markers and analyzed by flow cytometry. Numeric values indicate percentage among mononuclear cells. Dead cells positive for 7-AAD were excluded and 10.000 events per sample were acquired. The orange rectangle represents the gate for detection of the third antibody analyzed in the histogram. The closed (orange) profile in the histogram illustrates the reactivity of $CD90^+CD34^+$ cells with an anti-CD13 mAb, the open profile represents an irrelevant, isotype-matched control mAb.

In addition to $CD34^+CD45^+CD13^+$ cells, we identified $CD34^-CD45^+CD13^+$, $CD34^+CD45^-CD13^-$, $CD34^-CD45^+CD13^-$ and $CD34^-CD45^-CD13^+$ cells (**Fig. 9** and **Fig. 10**). While the $CD34^+CD45^+CD13^+$ cell population indicates undifferentiated cells, $CD34^-CD45^+CD13^+$ cells represent differentiated cells.

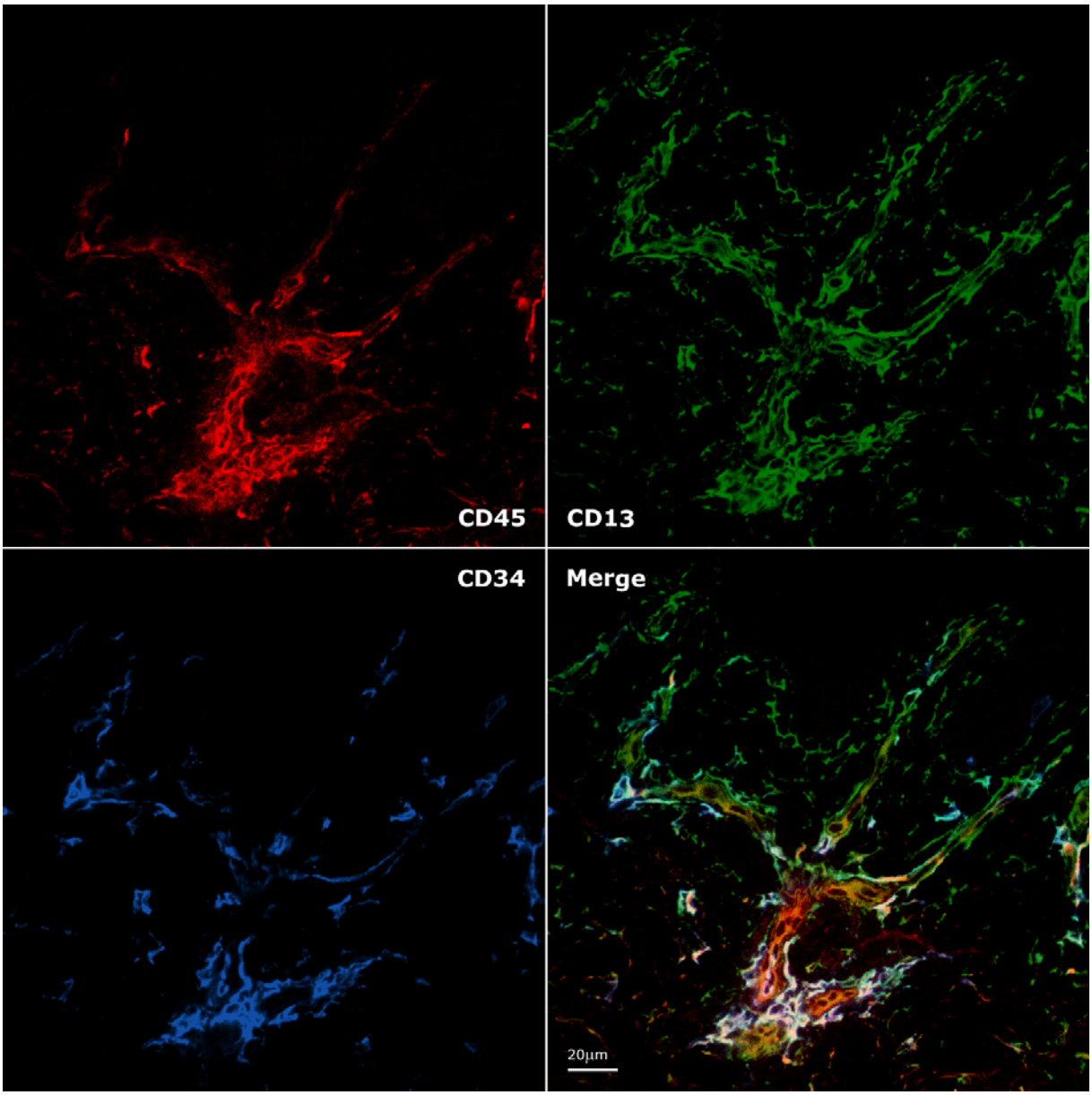

Figure 9. Expression of CD45, CD13 and CD34 in human skin. Immunofluorescence analysis of cryostat sections from human foreskin (4 year-old). Sections were exposed to purified-anti-CD45+Biotin+Streptavidin-Texas Red, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

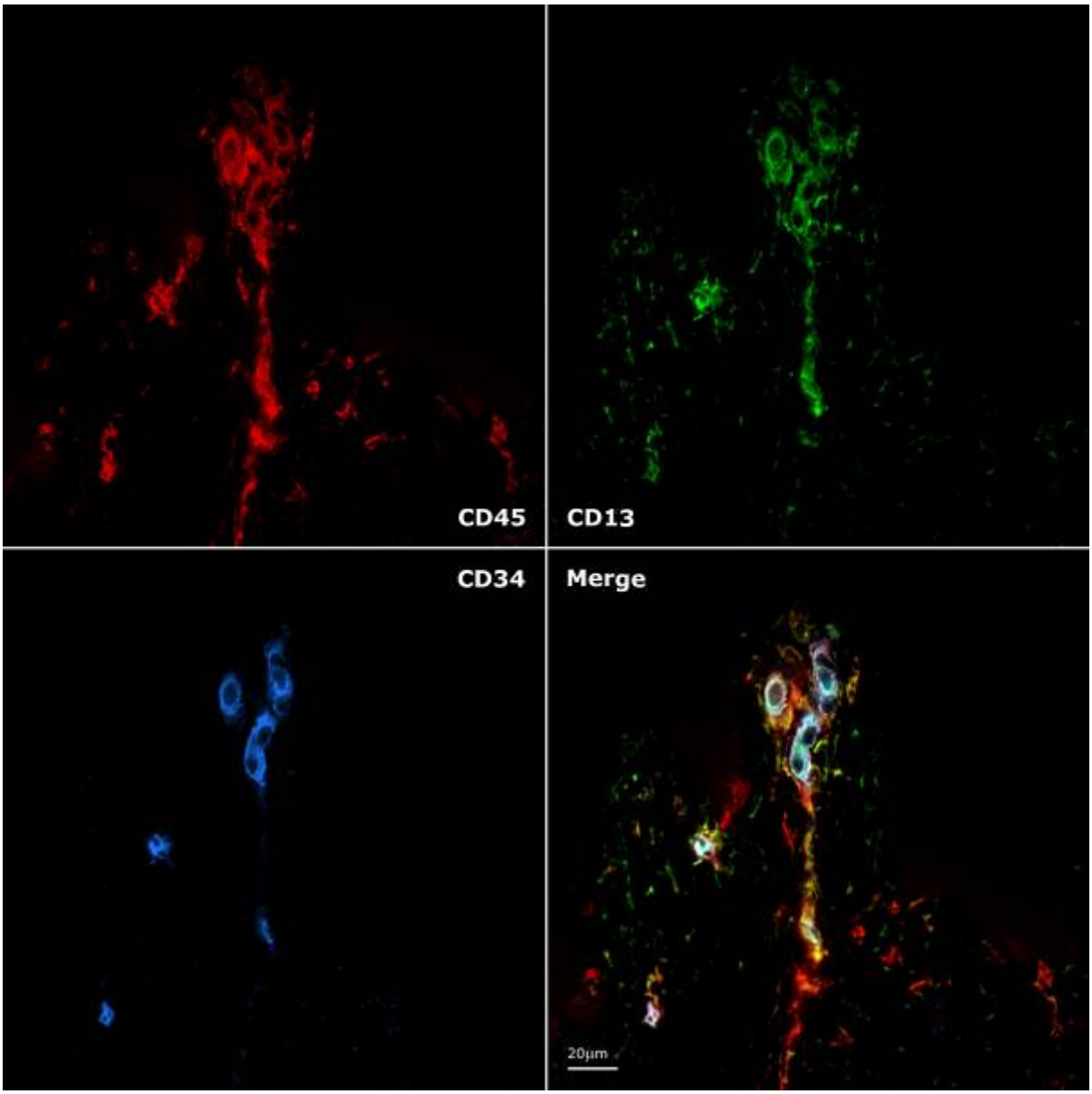

FIGURE 10. EXPRESSION OF CD45, CD13 AND CD34 IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD45+Biotin+Streptavidin-Texas Red, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

To distinguish between lymphatic and blood vessels, we introduced the Pathologische Anatomie Leiden-endothelium (PAL-E) mAb for our investigations. It recognizes a protein expressed exclusively by endothelial cells that line blood capillaries and small veins, with exception of those in the brain. Tumor blood vessels and the high endothelial venules in lymph nodes are particularly PAL-E reactive. In contrast, PAL-E is non-reactive with lymphatic capillary endothelial cells and with the arterial endothelium. PAL-E has been used extensively to determine if small vessels in the skin and elsewhere are of blood or lymphatic origin [72;73]. By a combination of CD34, PAL-E and CD90, we could not only distinguish between blood ($CD34^+PAL\text{-}E^+$) and lymphatic vessels ($CD34^+PAL\text{-}E^-$), but also identified $CD34^+CD90^+PAL\text{-}E^+$ cells (**Fig. 11**, white arrows).

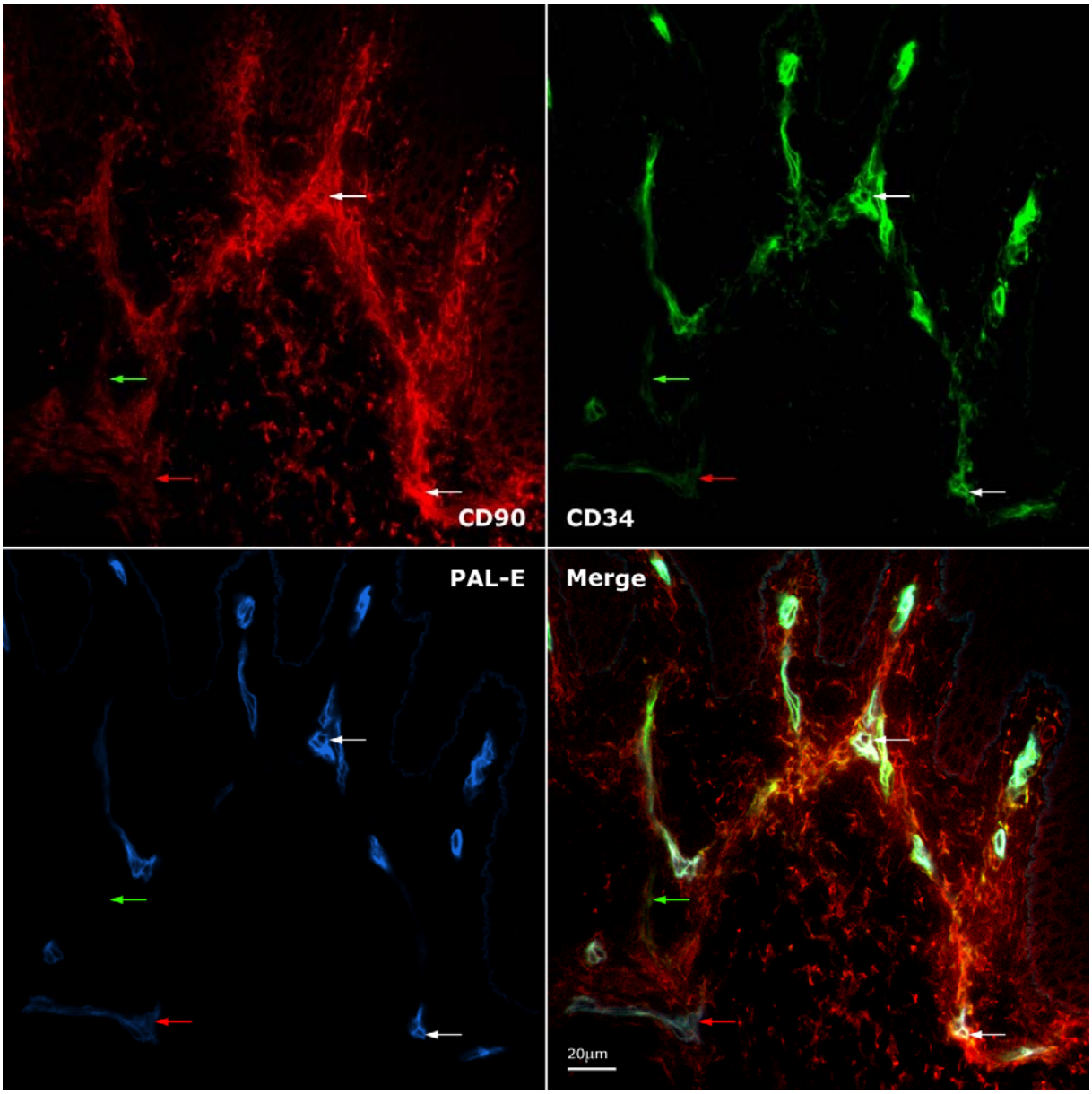

FIGURE 11. EXPRESSION OF CD90, CD34 AND PAL-E IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to PE-anti-CD90, FITC-anti-CD34 and APC-anti-PAL-E triple labeling. White arrows indicate $CD34^+CD90^+PAL\text{-}E^+$ cells, red arrows indicate $CD34^+PAL\text{-}E^+$ blood vessels, whereas green arrows indicate $CD34^+PAL\text{-}E^-$ lymphatic vessels.

Staining for CD34 and CD14 showed that $CD14^+$ cells are often surrounded by $CD34^+$ cells and vessels in the papillary dermis (FIG. 12).

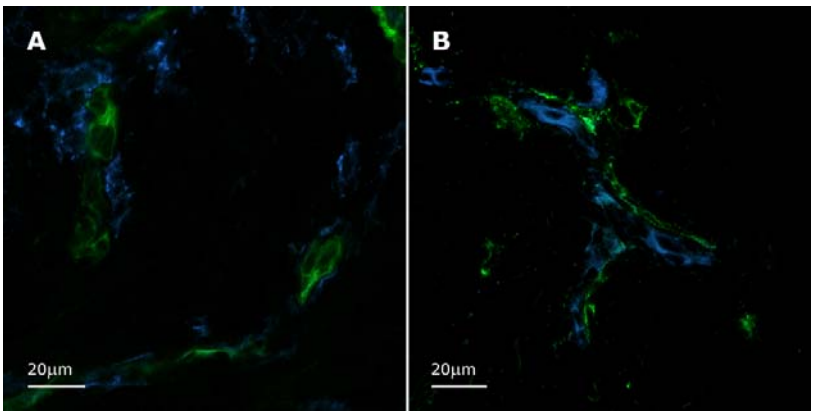

FIGURE 12. EXPRESSION OF CD14 IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to FITC-anti-CD34 and APC-anti-CD14 double labelling.

Triple staining for CD34, CD14, and CD31 revealed coexpression of CD34 and CD14 on certain cells, but not CD31 (FIG. 13), and coexpression of CD34 and CD31, but not CD14 (FIG. 14). CD31 appears on endothelial cells and can be used for measuring angiogenesis. In combination with CD34, CD31 is also a marker for myeloid progenitor cells. CD31 is normally found on endothelial cells, platelets, macrophages and Kupffer cells, granulocytes, T cells, natural killer cells lymphocytes, megakaryocytes, fibroblasts, osteoclasts, and neutrophils. Thus, it is conceivable that $CD14^+CD34^+$ cells represent candidate stem/progenitor cells, as their appearance is quite rare and it has been shown that the macrophage/monocyte marker CD14 is also detectable on some $CD34^+$ cells. These $CD14^+CD34^+$ cells exhibit clonogenicity and multipotency, as shown by their ability to differentiate not only into endothelial cells, but also into osteoblasts, adipocytes, or neural cells [48]. $CD34^+CD31^+$ cells might represent endothelial cells or myeloid progenitors, though an endothelial character is more likely, due to the relative high frequency in the dermis.

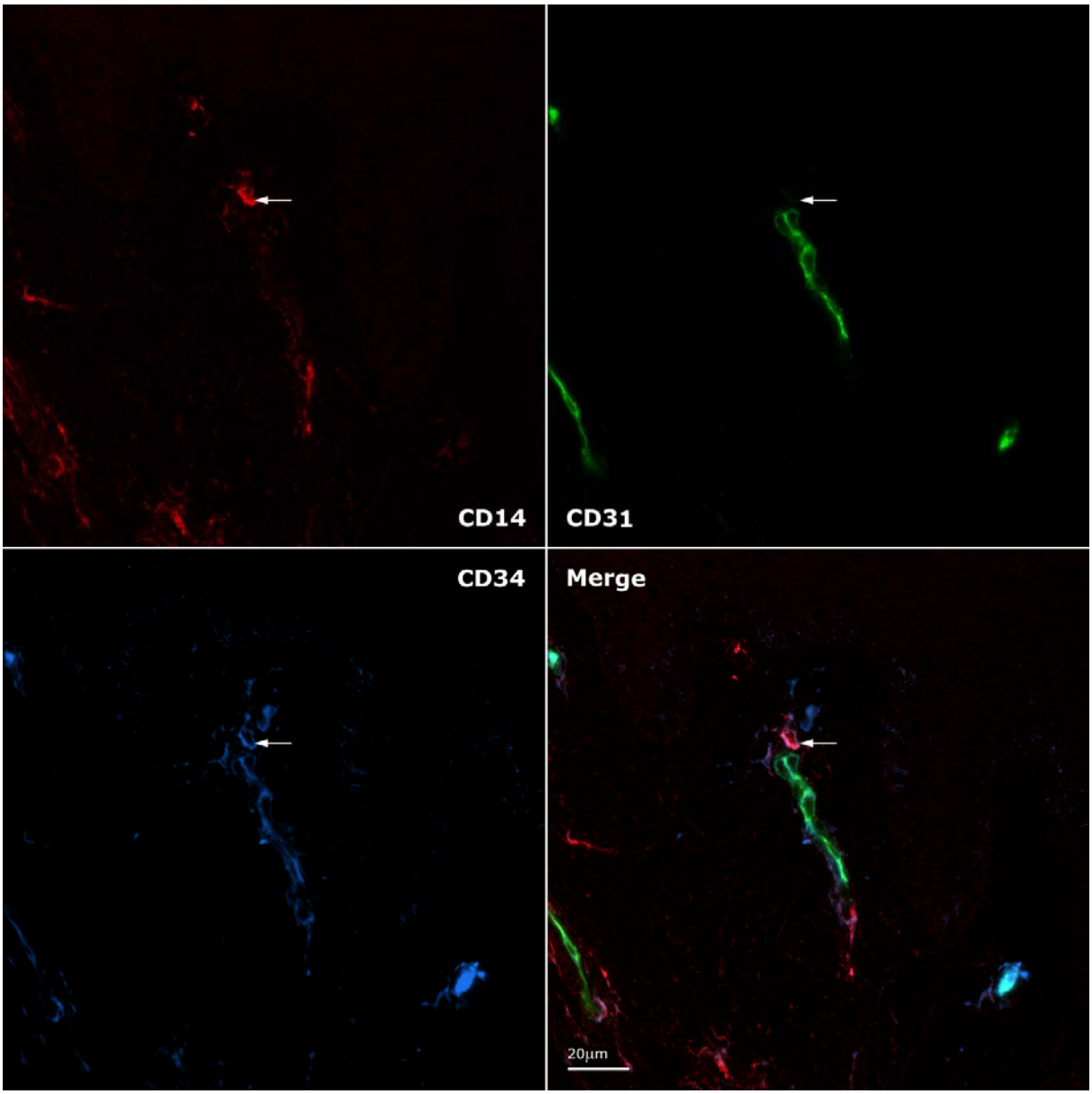

FIGURE 13. EXPRESSION OF CD14, CD31 AND CD34 IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD14+Biotin+Streptavidin-Texas Red, FITC-anti-CD31 and APC-anti-CD34 triple labeling. Arrow indicates a $CD14^{+}CD34^{+}CD31^{-}$ cell.

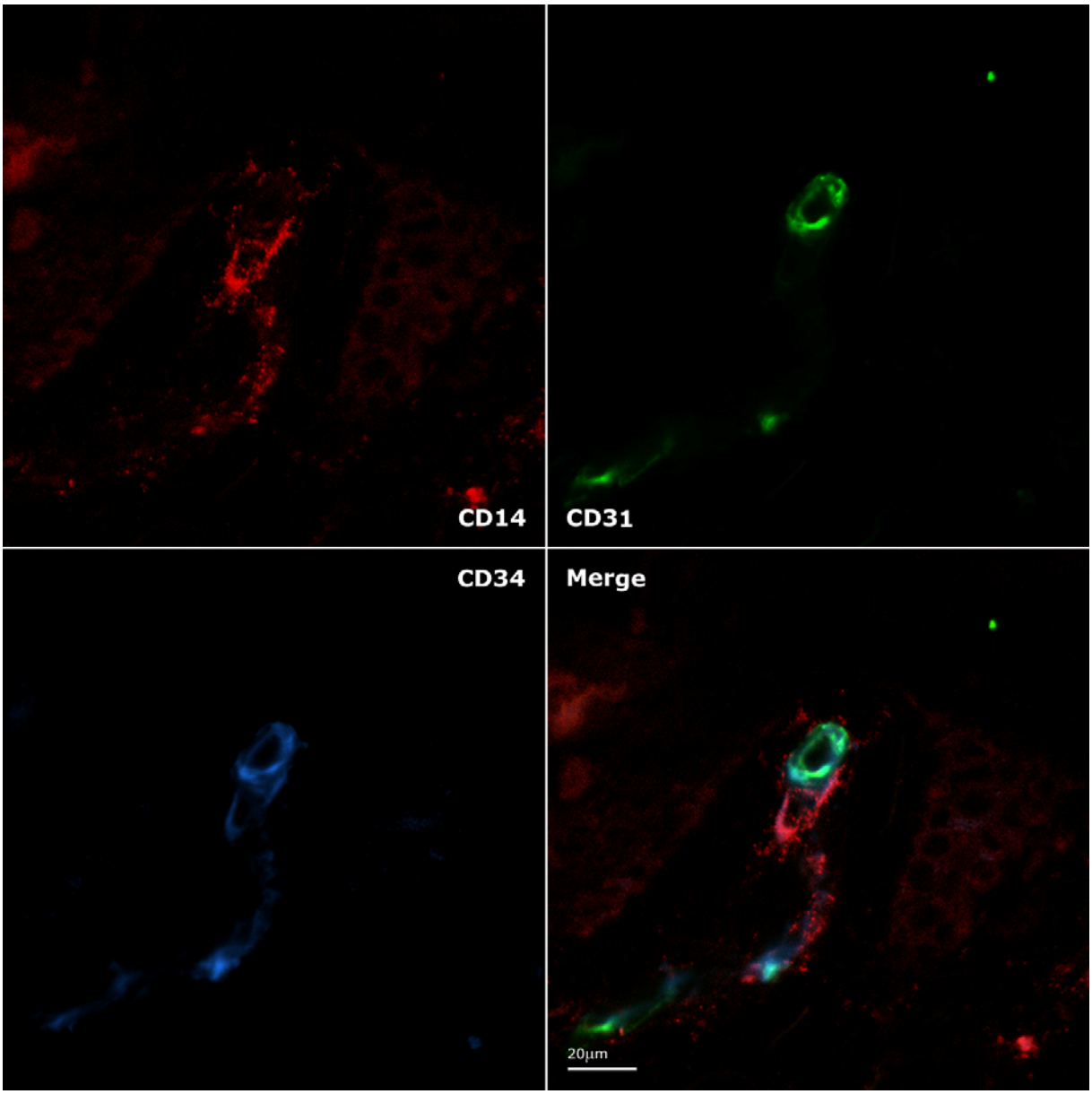

FIGURE 14. HUMAN SKIN HARBOURS CD31⁺CD34⁺ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD14+Biotin+Streptavidin-Texas Red, FITC-anti-CD31 and APC-anti-CD34 triple labeling.

7.2.2 CD117

CD34 is the most common marker for the selection of HSC. In peripheral blood, 60–80% of $CD34^+$ cells coexpress CD117, another marker for stem cell separation. The expression level of CD117 on $CD34^+$ cells is usually high enough to allow its use in hematopoietic cell isolation methods. Furthermore, CD117 is an important cell surface marker for the identification of certain types of hematopoietic progenitors in the BM. Some of the BM stem cells that express CD117 but not CD34, generate angiogenic cytokines and differentiate into endothelial cells [74]. Additionally, mast cells, epidermal melanocytes, and interstitial cells of Cajal in the digestive tract express CD117. Using confocal microscopy analysis, we could identify $CD117^+$ cells localized both in the papillary and the reticular dermis (FIG. 15).

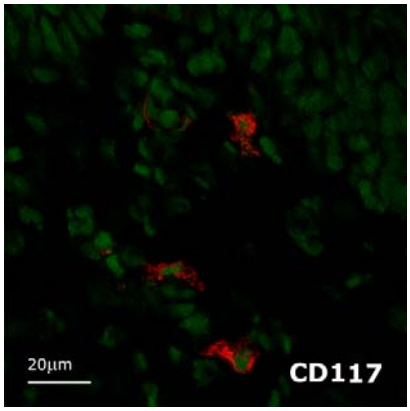

FIGURE 15. EXPRESSION OF CD117 IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to PE-anti-CD117 and Sytox Green (nucleic acid staining).

For their further characterization we included CD45 in our staining protocol. Occasionally, we found $CD117^+CD45^+$ cells which most likely represent mast cell progenitors (FIG. 16A). The majority of $CD117^+$ cells surprisingly failed to express CD45 (FIG. 16B). As the presence of so high numbers of melanocytes in the dermis appears unlikely [75], it is possible that the levels of CD45 are below those detectable by immunofluorescence.

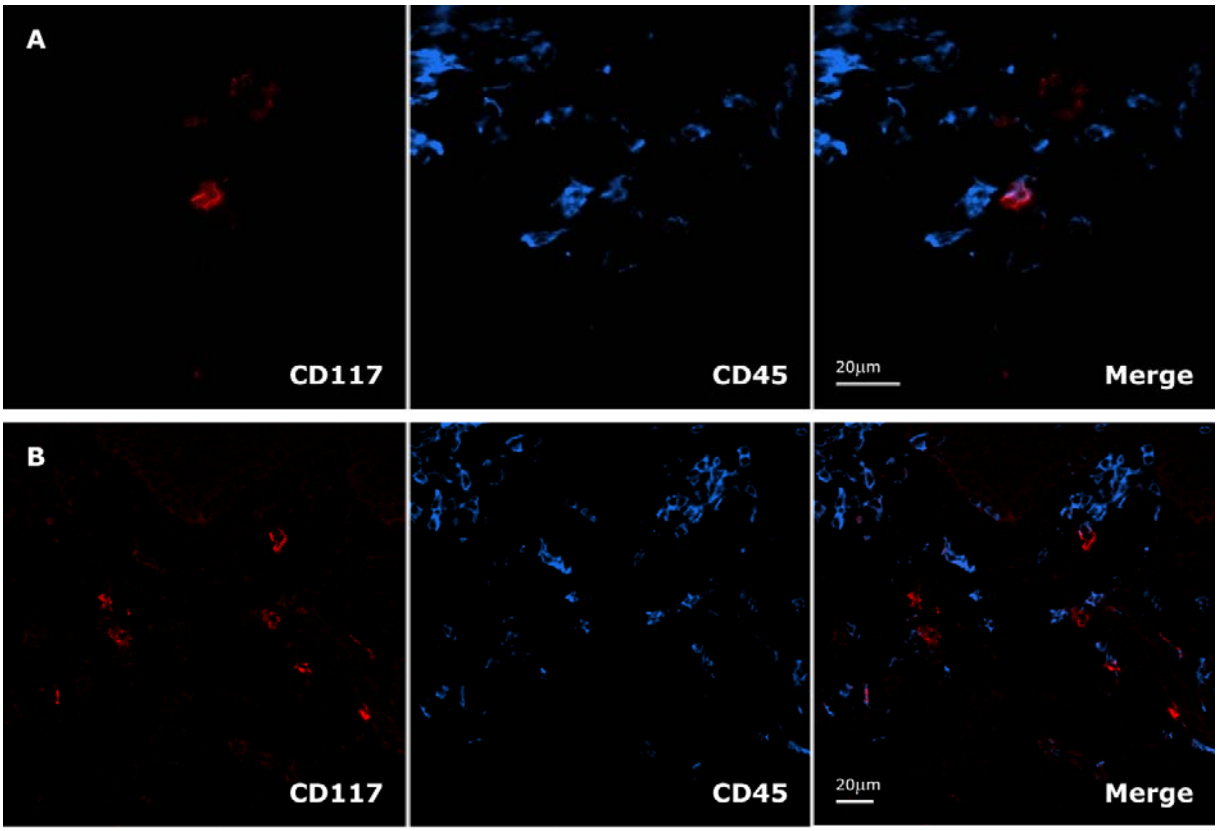

FIGURE 16. EXPRESSION OF CD117 AND CD45 IN HUMAN SKIN. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to PE-anti-CD117 and APC-anti-CD45 double labeling.

7.2.3 CD133

CD133 was originally described as a marker for human HSC and has recently been proposed as a universal marker for tissue stem/progenitor cells [57]. Its expression pattern overlaps with, but is not identical to that of CD34. CD133 is expressed on a majority of, but not all, $CD34^+$ cells. These include immature progenitors, monocyte/granulocyte progenitors and some erythroid progenitors. It also appears to be expressed on $CD34^-$ HSC identified in human cord blood [58]. In our studies, we identified a few $CD133^+$ cells in the human papillary dermis (FIG. 17).

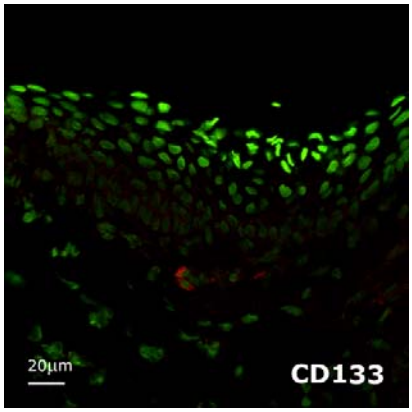

FIGURE 17. EXPRESSION OF CD133 IN HUMAN SKIN. Immunofluorescence analysis of sections from human foreskin (3 year-old). Cryostat sections were exposed to purified-anti-CD133+Biotin+Streptavidin Texas Red and Sytox Green (nucleic acid staining).

Double labeling of CD133 and CD34 revealed some $CD133^{+}CD34^{+}$ cells in the human dermis (**Fig. 18**).

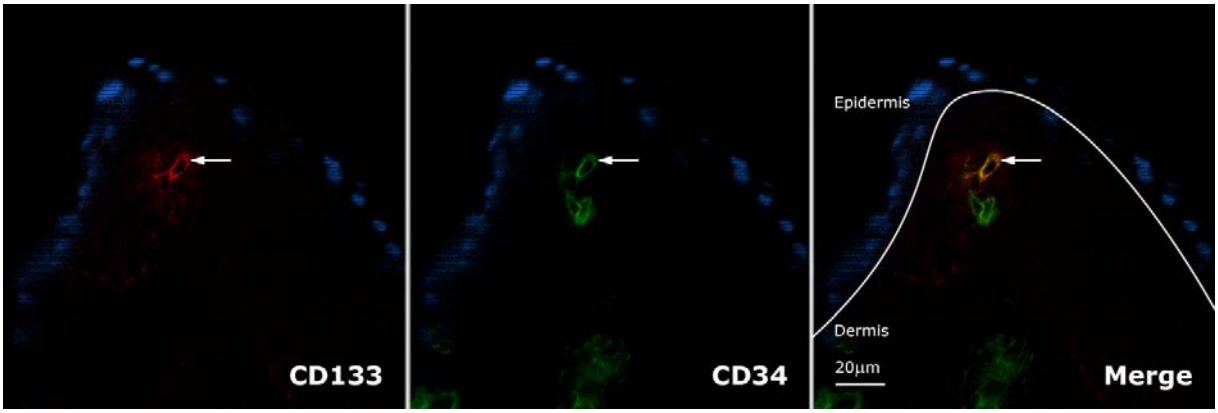

Figure 18. Human skin harbours $CD133^{+}CD34^{+}$ cells. Immunofluorescence analysis of cryostat sections from human foreskin (1 year-old). Sections were exposed to purified-anti-CD133+Biotin+Streptavidin Texas Red, FITC-anti-CD34 and Dapi (nucleic acid staining).

We also encountered $CD133^{+}CD45^{+}CD34^{-}$ cells (**Fig. 19**). This finding is in concordance with a previous observation showing that $CD133^{+}CD34^{-}$ HSC are present in cord blood and imply that dermal $CD133^{+}CD45^{+}CD34^{-}$ cells may represent candidate HSC.

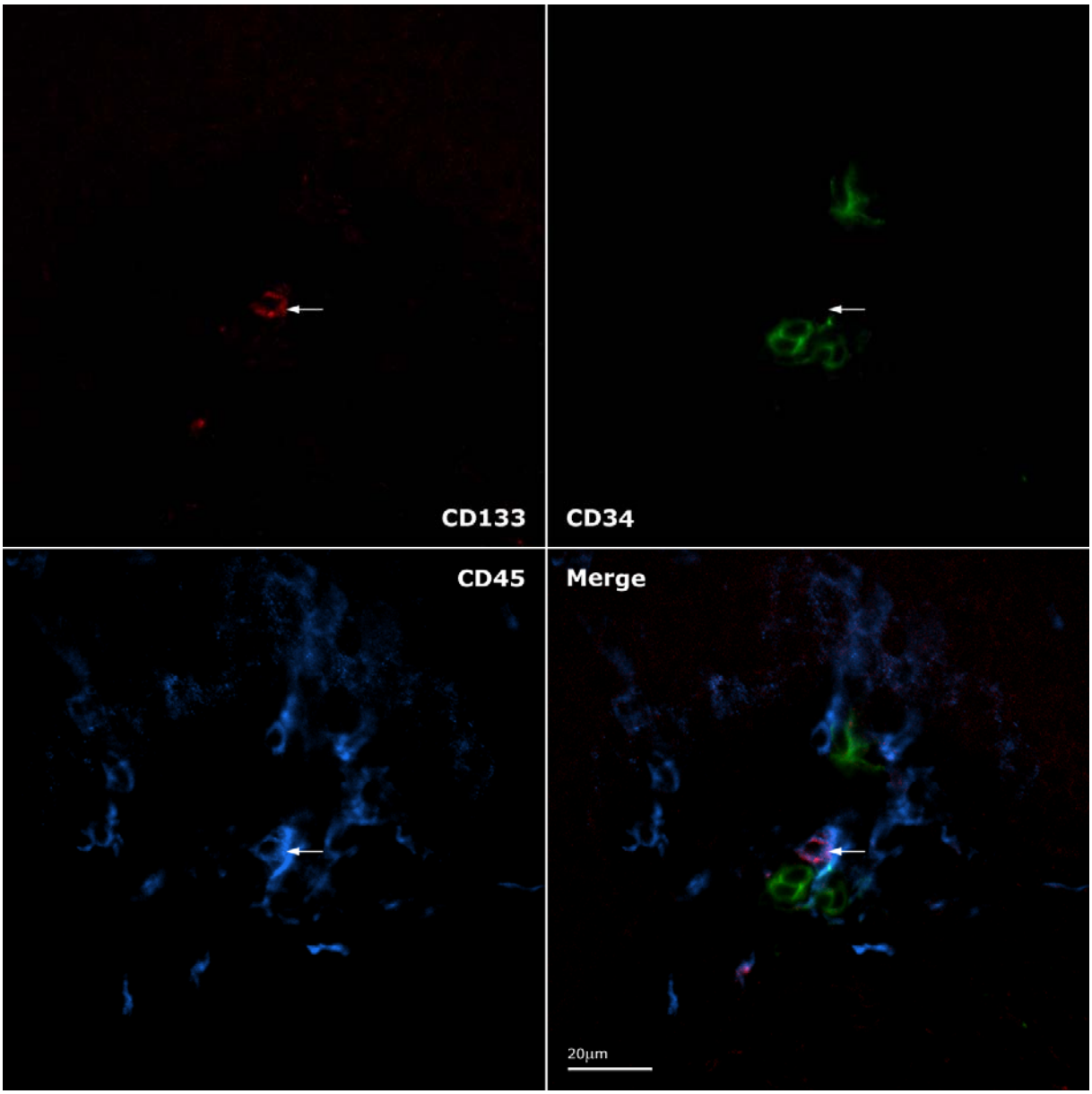

FIGURE 19. HUMAN SKIN HARBOURS CD133$^+$CD45$^+$CD34$^-$ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD133+Biotin+Streptavidin Texas Red, FITC-anti-CD34 and APC-anti-CD45 triple labelling. Arrows indicate a CD133$^+$CD45$^+$CD34$^-$ cell.

7.2.4 CD150

Purification of HSC has always been a problem, because no definitive marker was known. Recently, purification of murine HSC has improved due to the introduction of the SLAM family receptors. CD150, CD244, and CD48 are differentially expressed among hematopoietic progenitors in a way that correlates with progenitor primitiveness. In mice CD150 is expressed on long-term repopulating cells. It has most recently been shown, that CD150 family molecules are also present on human HSC [76]. As CD150 expression has never been tested on human skin cells, we included this receptor in our search for stem cells in the human dermis. Indeed, we could identify $CD150^{+}CD34^{+}$ (FIG. 20A) and $CD150^{+}CD45^{-}$ cells (FIG. 20B). Moreover, a few cells express CD150, CD34 and CD45, implying a stem cell character (FIG. 21, arrow).

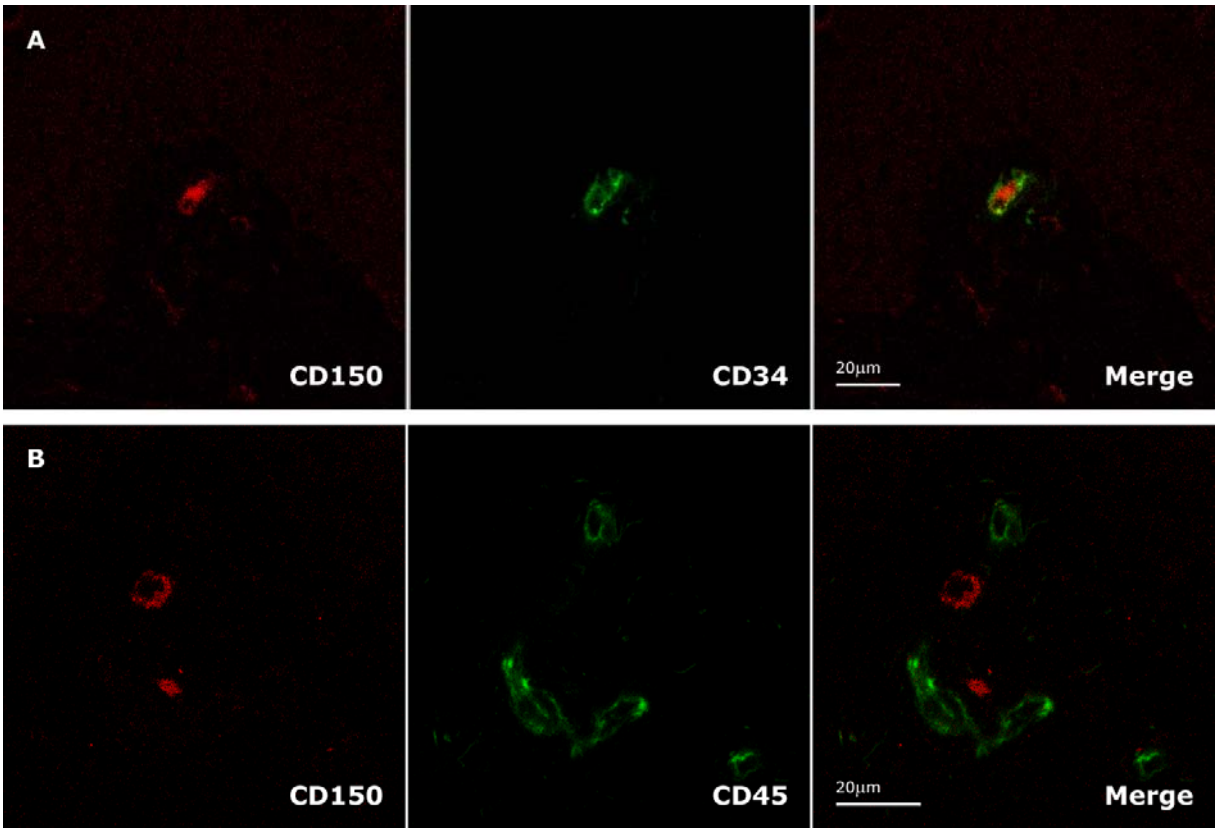

FIGURE 20. FEW DERMAL CELLS EXPRESS CD150. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD150+Biotin+Streptavidin Texas Red and FITC-anti-CD34 double labeling (**A**) or purified-anti-CD150+Biotin+Streptavidin Texas Red and FITC-anti-CD45 double labeling (**B**).

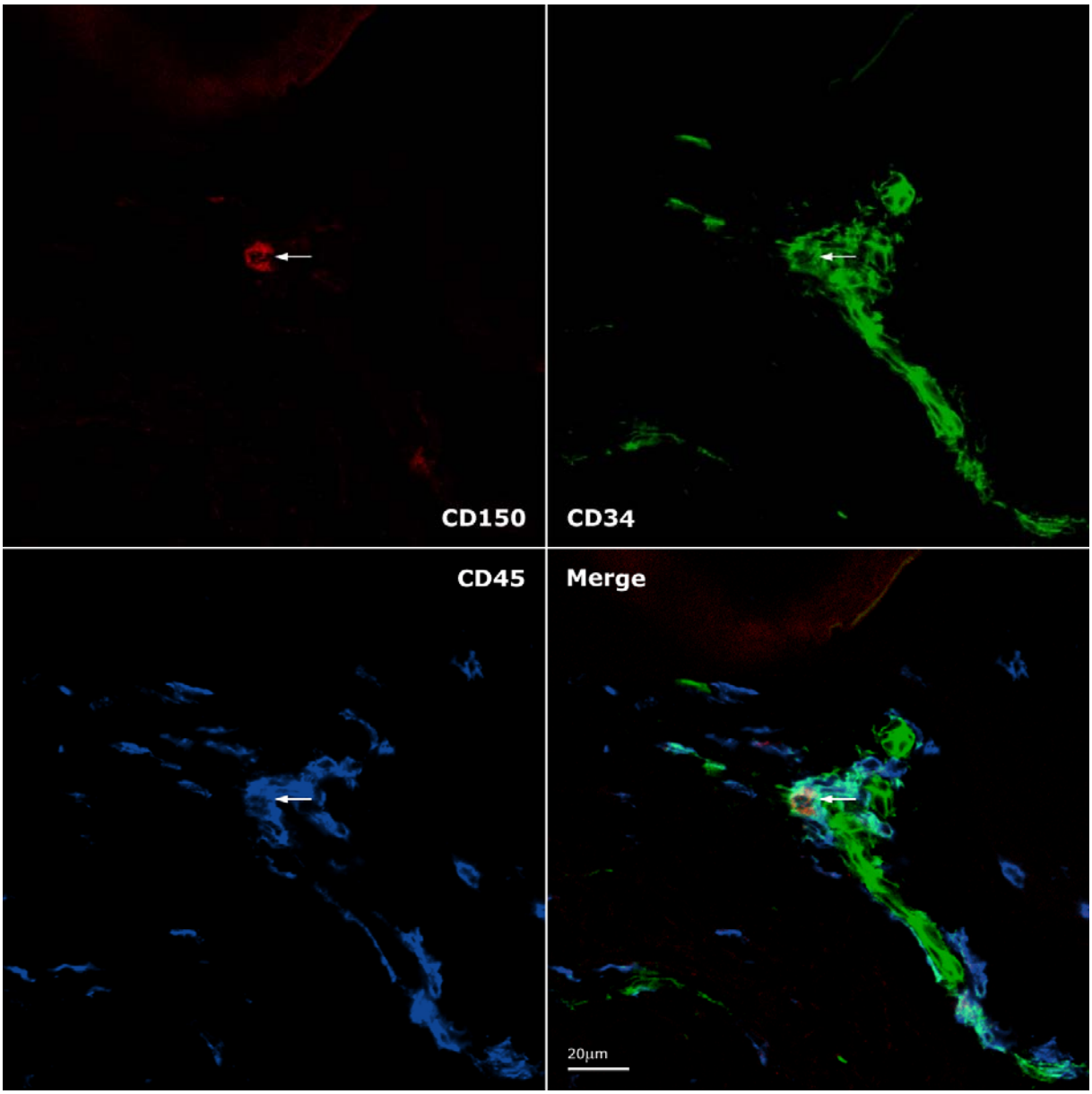

FIGURE 21. HUMAN SKIN HARBOURS CD150$^+$CD34$^+$CD45$^+$ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD150+Biotin+Streptavidin Texas Red, FITC-anti-CD34 and APC-anti-CD45 triple labeling. Arrow indicates a CD150$^+$CD34$^+$CD45$^+$ cell.

7.2.5 CD318

CD318 is expressed on HSC, epithelial cells, and on cells with a phenotype reminiscent of MSC. The expression of CD318 on normal human hematopoietic cells is restricted to a subset of $CD34^+$ cells. Similar to CD34 and CD133, CD318 may thus be useful for the enrichment of human HSC and progenitor cells, but not for their discrimination.

We performed staining for $CD318^+$ cells in human skin, and by employing various markers we could identify certain $CD318^+$ cell subsets. In first attempts we localized solitary $CD318^+$ cells spread over the dermis in low counts, preferentially located in the papillary dermis. Furthermore, we found that all that all keratinocytes expressed CD318 (**Fig. 22A**), what is consistent with a most recent finding [77]. Approximately 9% of total dermal cells expressed CD318 and, as determined by flow cytometry, 0.39% of total dermal cells coexpress CD318 and CD34 (**Fig. 22B**).

According to our immunofluorescence staining data, it appeared quite unlikely that all $CD318^+$ single positive cells are from the dermis. Reasoning that keratinocytes might

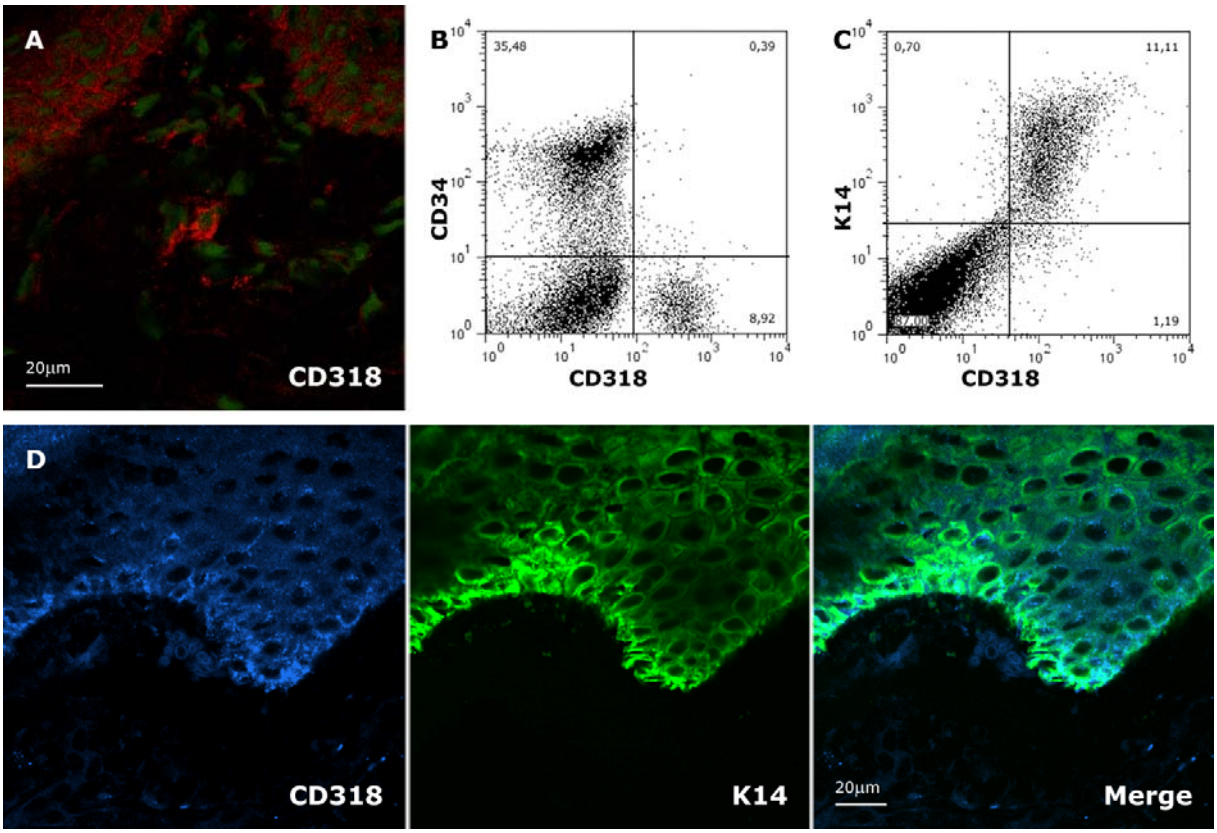

Figure 22. Human skin harbours different $CD318^+$ subsets. A. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin Texas Red and Sytox Green (Nucleic acid staining). **B, C.** FACS analysis of human foreskin (3 year-old). Total dermal single cell suspension was stained for the indicated markers. **D.** Immunofluorescence analysis of cryostat sections from human foreskin (1 year-old). Sections were exposed to AF647-anti-CD318 and FITC-anti-K14 double labeling.

have contaminated our dermal cell preparations, we used the K14 mAb, an antigen that is expressed in basal keratinocytes, to better define the $CD318^+$ population. Indeed, we

found that all $K14^+$ keratinocytes coexpressed CD318, showing that epithelial cells contaminate our dermal cell suspensions (Fig. 22c, d). Thus, CD318 is also an excellent surface marker for keratinocytes and clearly visualizes all epidermal layers (Fig. 23).

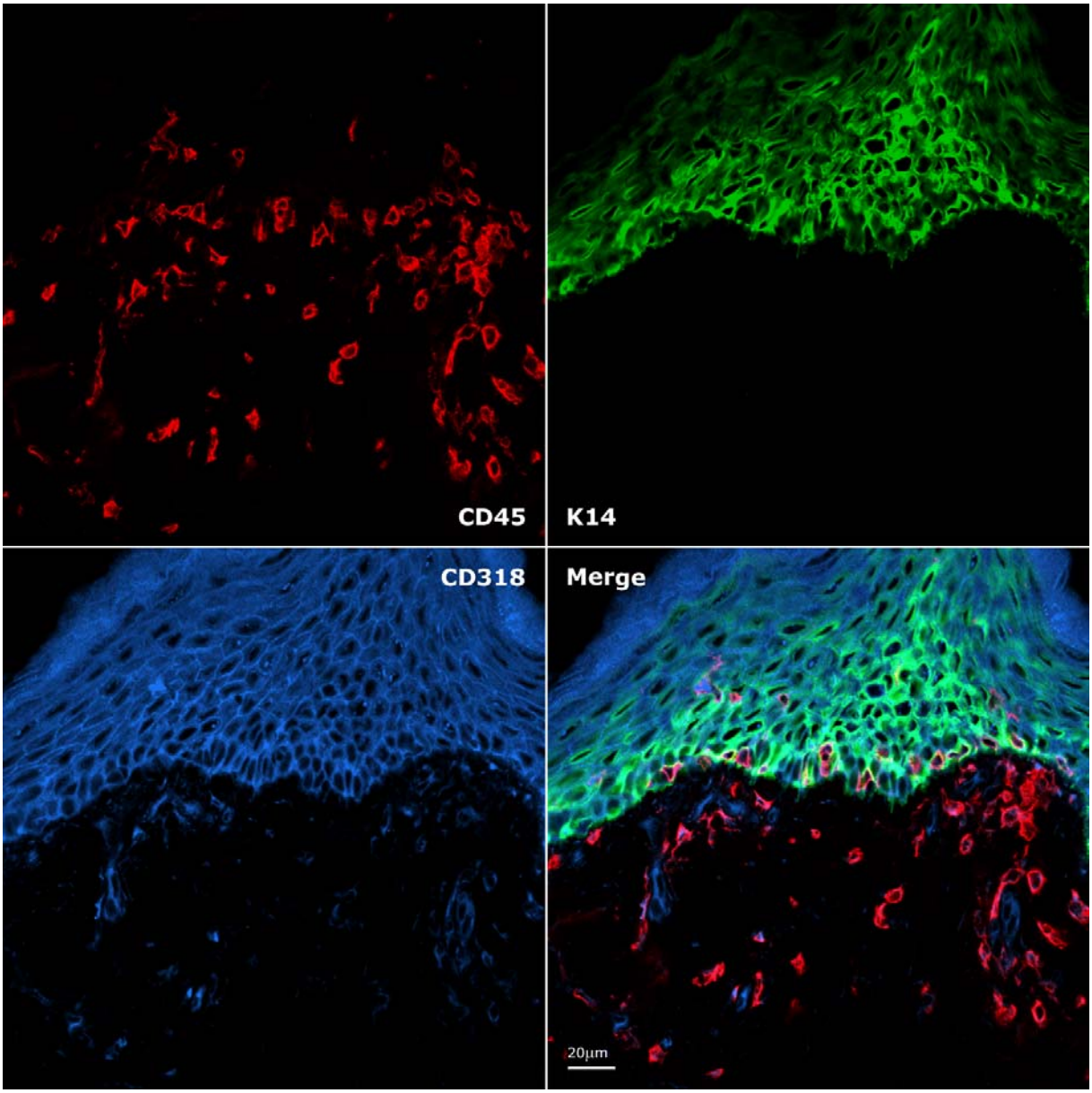

Figure 23. CD318 clearly visualizes the total epidermis. Immunofluorescence analysis of cryostat sections from human foreskin (1 year-old). Sections were exposed to AF555-anti-CD45, FITC-anti-K14 and AF647-anti-CD318 triple labeling.

To test whether $CD318^+CD34^+$ cells may represent HSC candidates, we included a CD45 mAb in our staining protocol. Indeed, we identified few $CD318^+CD45^+CD34^-$ (**Fig. 24**, arrows) and $CD318^+CD45^+CD34^+$ cells (**Fig. 25**).

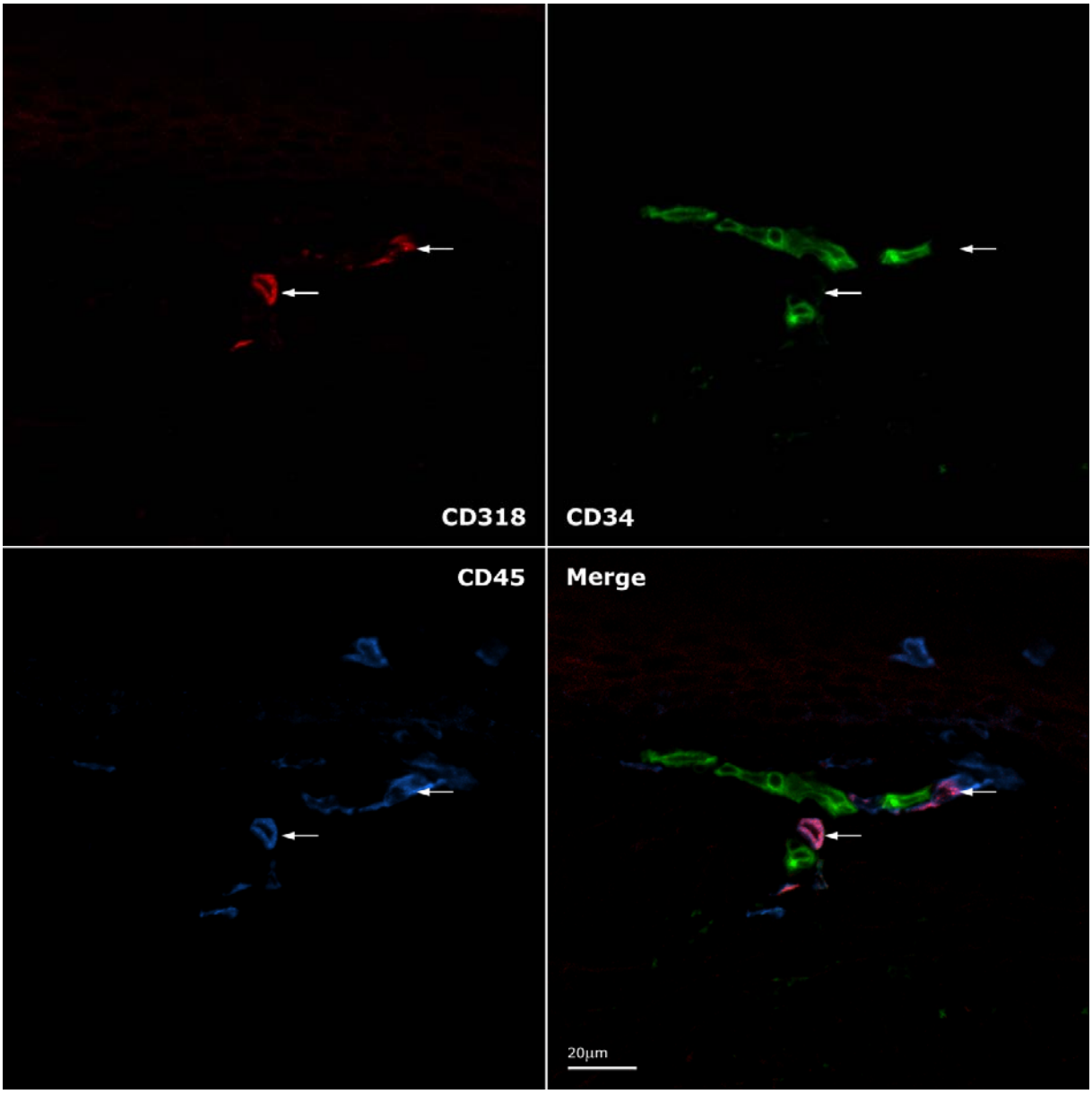

Figure 24. Human skin harbours $CD318^+CD45^+CD34^-$ cells. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-C318+Biotin+Streptavidin-Texas Red, FITC-anti-CD34 and APC-anti-CD45 triple labeling. Arrows indicate $CD318^+CD45^+CD34^-$ cells.

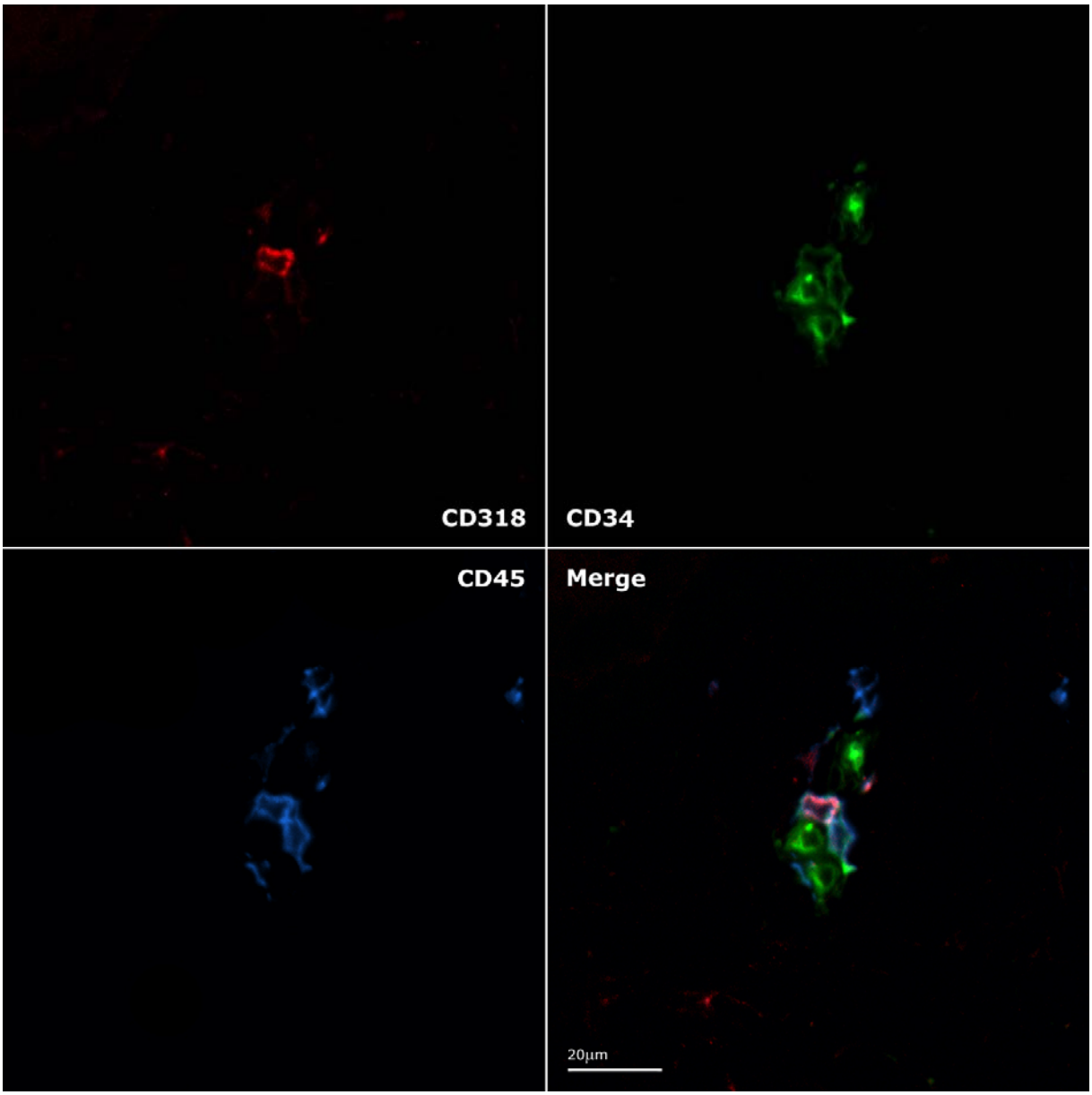

FIGURE 25. HUMAN SKIN HARBOURS CD318$^+$CD45$^+$CD45$^+$ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD34 and APC-anti-CD45 triple labeling.

So far our results imply that some $CD318^+$ dermal cells are closely linked to $CD34^+$ cells. To better characterize their nature (i.e. endothelial progenitors) we combined mAb for CD34 and CD31, and, furthermore, PAL-E, to distinguish between lymphatic and blood vessels. Immunofluorescence analysis showed that the $CD34^+CD318^-$ cells are part of blood vessels, as they expressed CD31 and PAL-E (**Figs. 26, 27, 28,** and **29**). We also found $CD31^+$PAL-E^- lymphatic vessels (**Fig. 30**). Occasionally $CD318^+CD31^+$PAL-E^+ cells, were found.

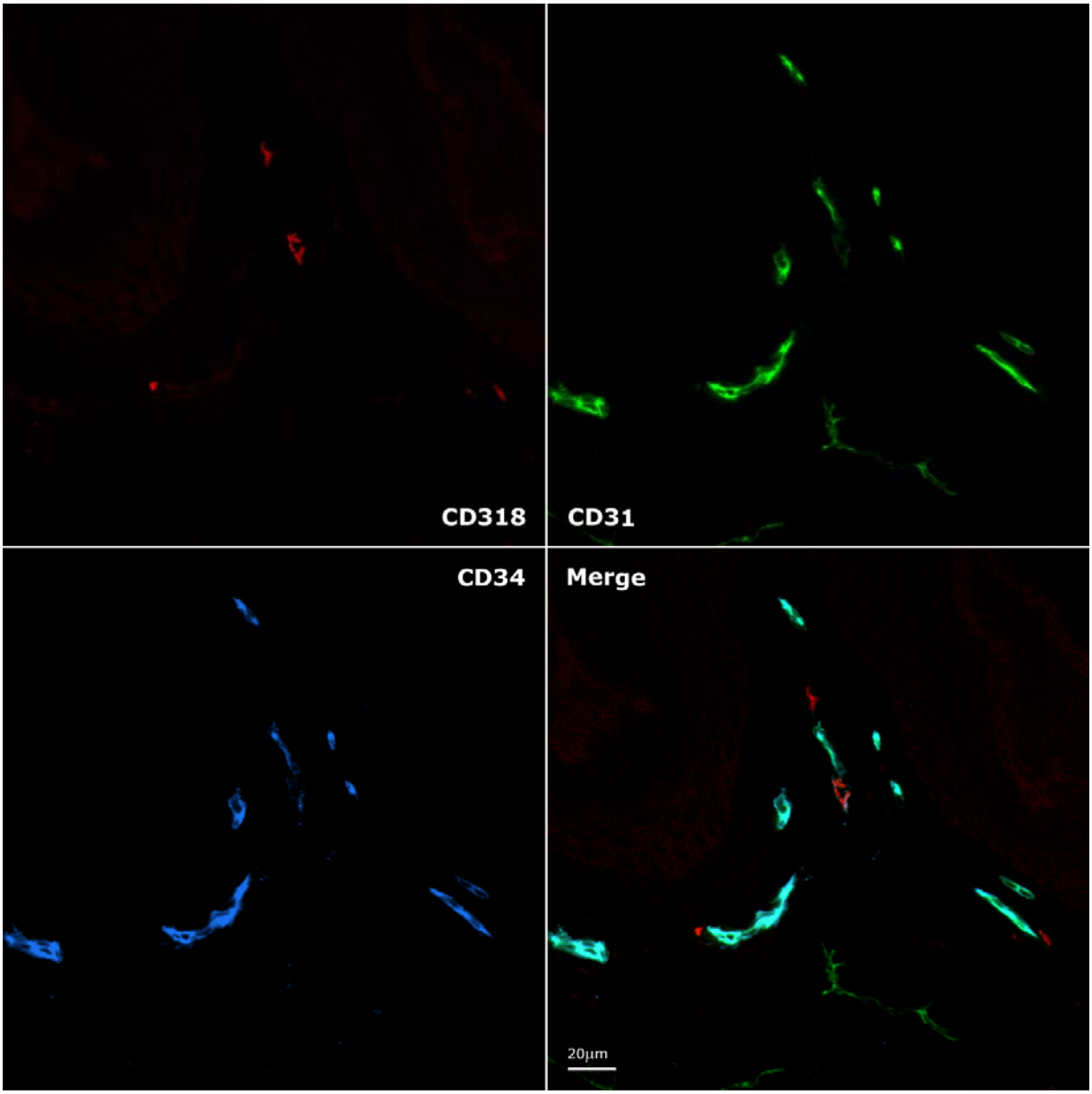

Figure 26. $CD318^+$ cells do not coexpress CD31 and CD34 in Human Skin. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD31 and APC-anti-CD34 triple labeling.

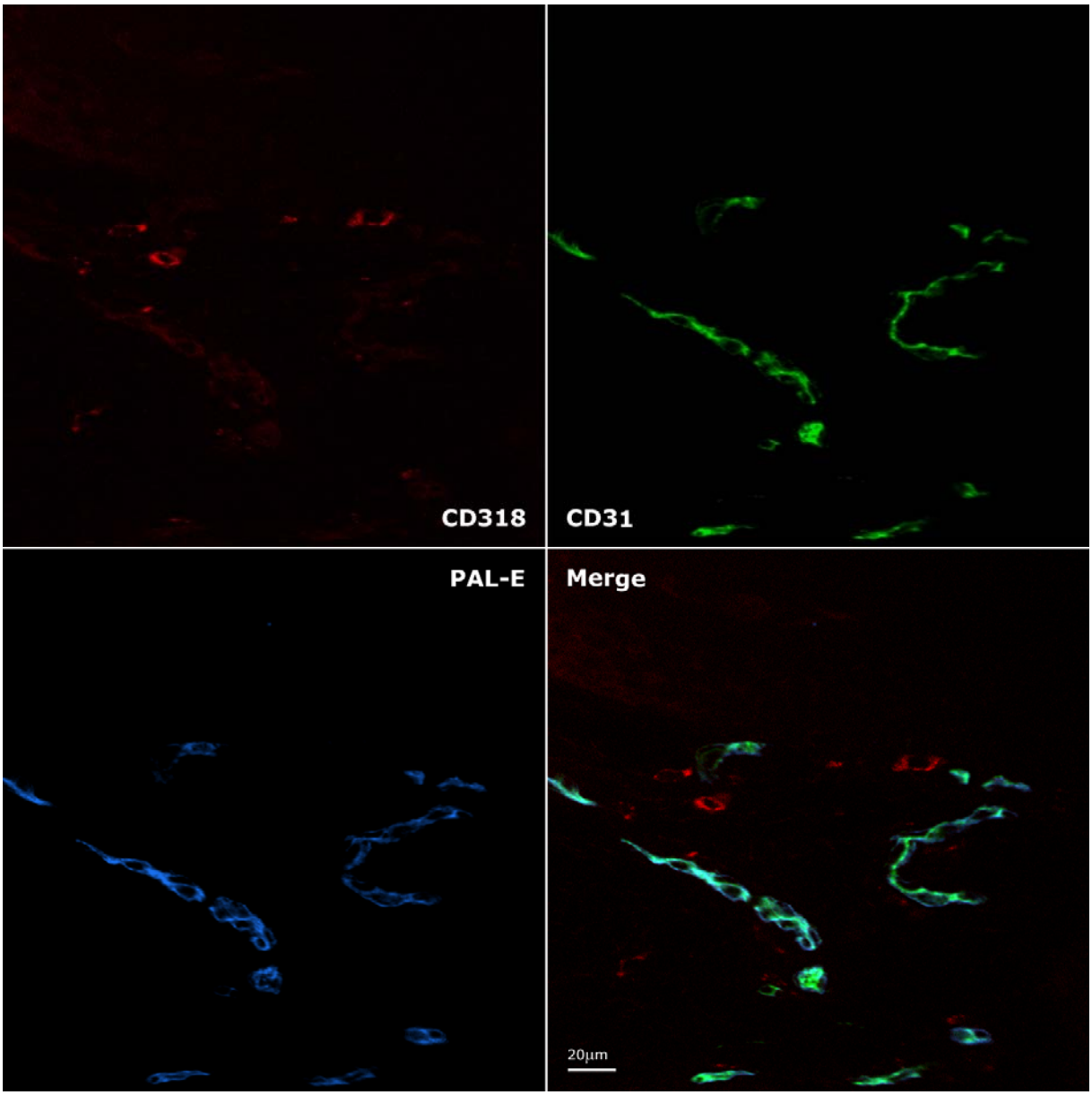

FIGURE 27. CD318⁺ CELLS DO NOT COLOCALIZE WITH CD31⁺PAL-E⁺ HUMAN DERMAL CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD31 and APC-anti-PAL-E triple labeling.

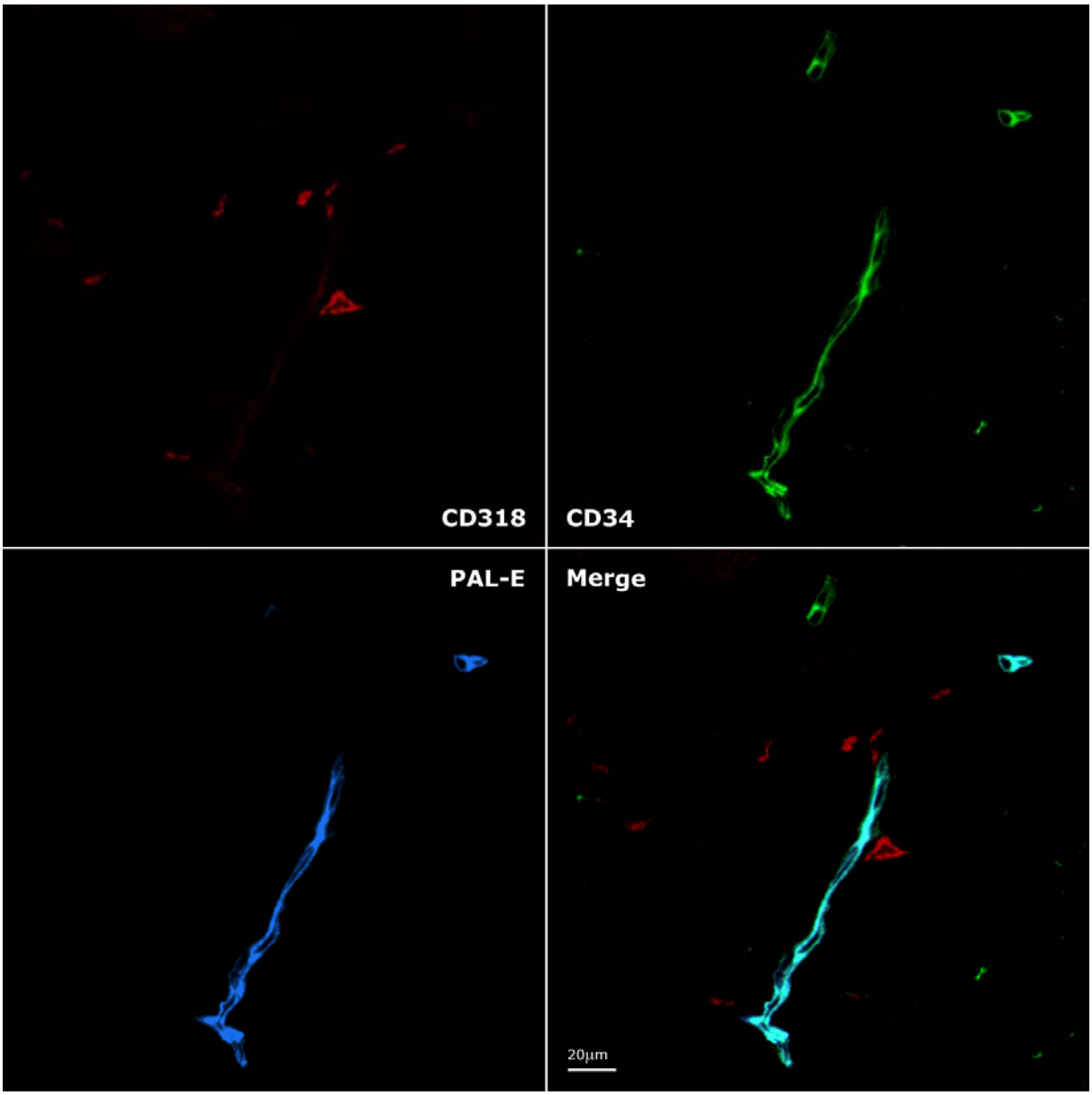

FIGURE 28. MOST OF THE CD318⁺ CELLS DO NOT EXPRESS CD34 AND PAL-E. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD34 and APC-anti-PAL-E triple labeling.

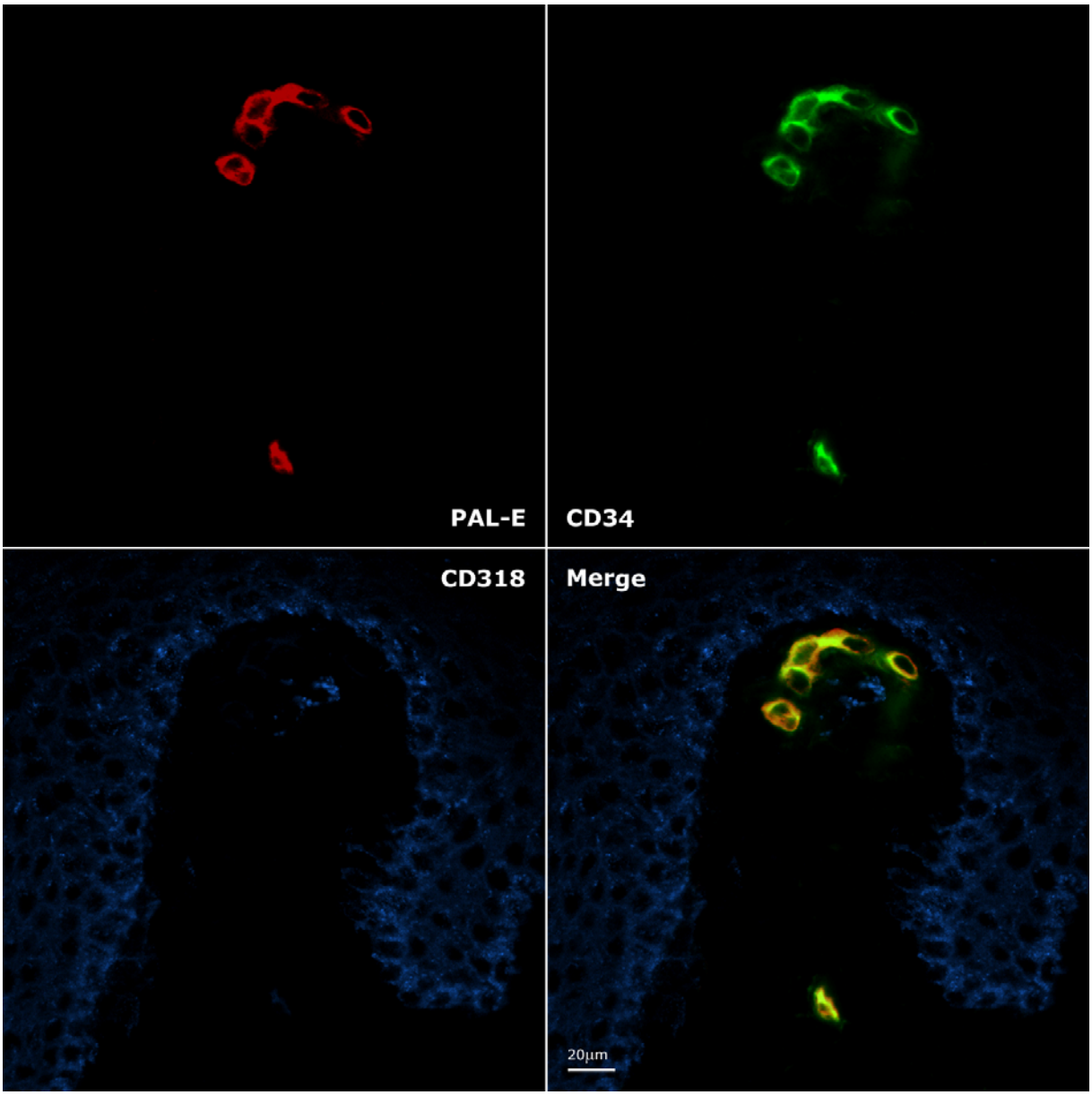

FIGURE 29. A FEW CD318⁺ CELLS COEXPRESS CD34 AND PAL-E. Immunofluorescence analysis of cryostat sections from human foreskin (1 year-old). Sections were exposed to PE-anti-PAL-E, FITC-anti-CD34, AF647-anti-CD318 triple labeling.

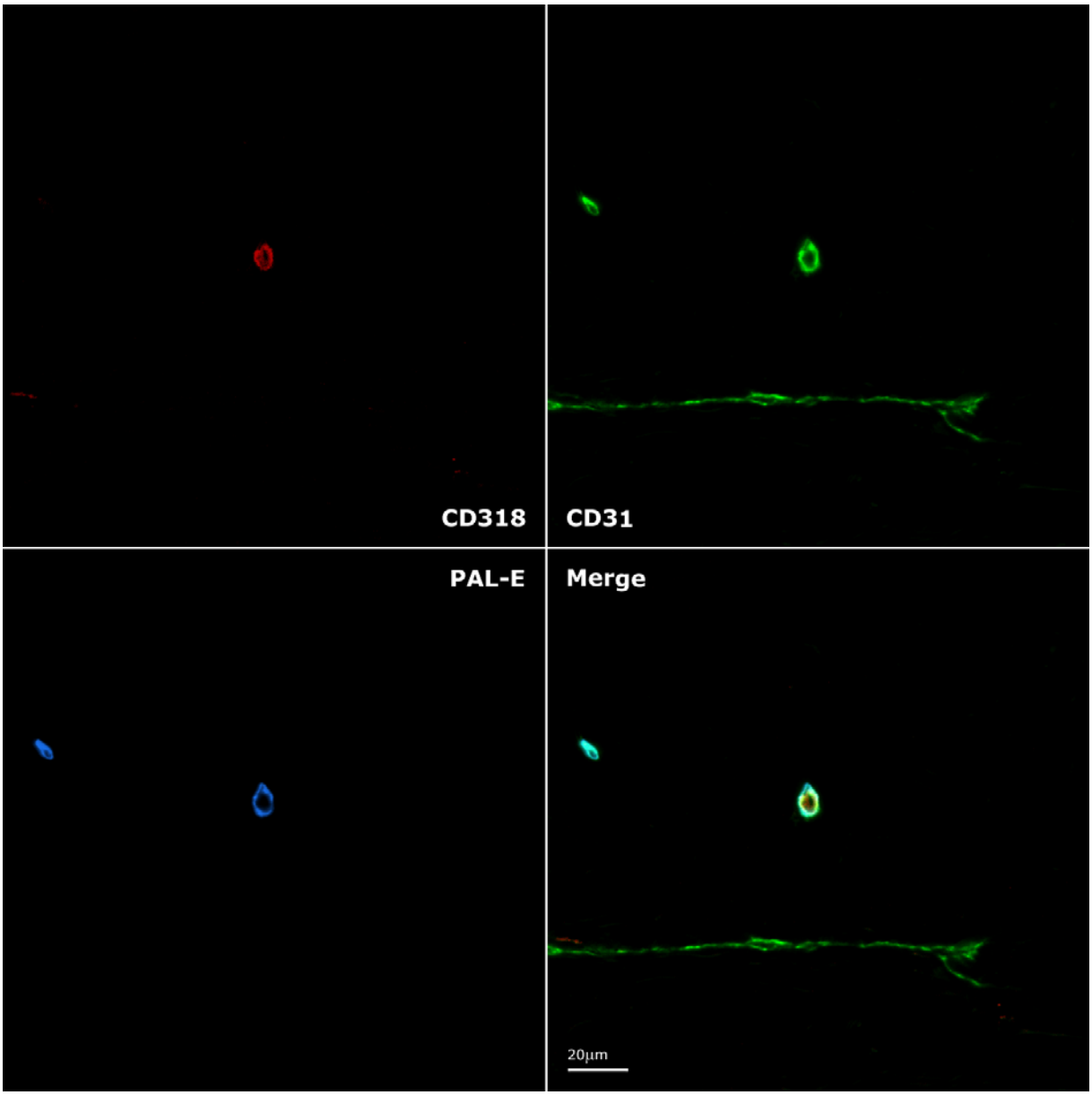

FIGURE 30. A FEW CD318⁺ CELLS COEXPRESS CD34 AND PAL-E. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD31 and APC-anti-PAL-E triple labeling.

Further analysis of $CD318^+CD34^+$ cells revealed coexpression of CD13. As $CD13^+$ cells have been shown to have LT-repopulating capacity, $CD318^+$ cells may also have this ability [59] (**Fig. 31** and **Fig. 32**). In conclusion, similar to CD34 and CD133, CD318 may be useful for the enrichment of human HSC and progenitor cells, but not for their discrimination.

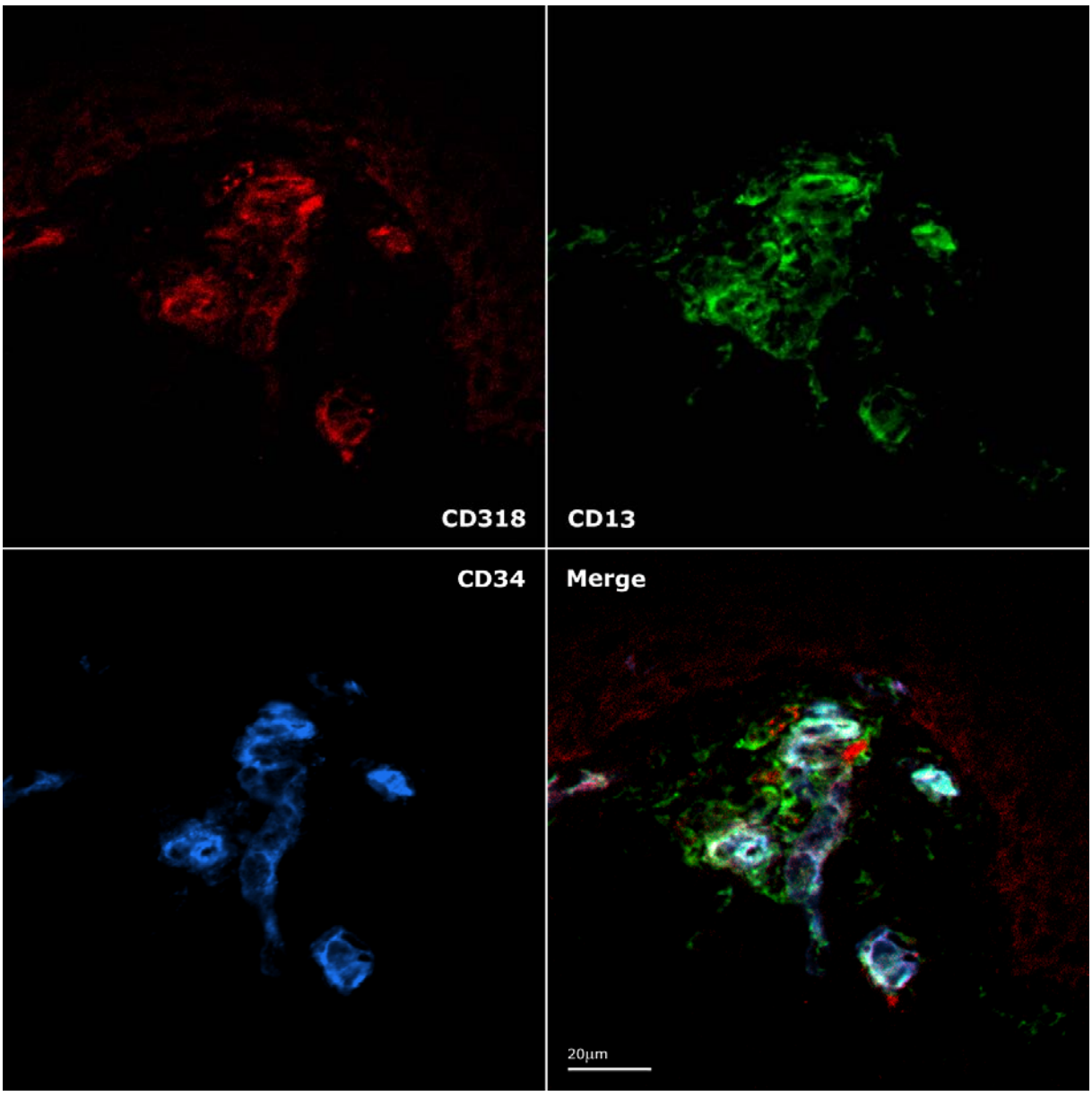

Figure 31. Human Skin harbours $CD318^+CD13^+CD34^+$ cells. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

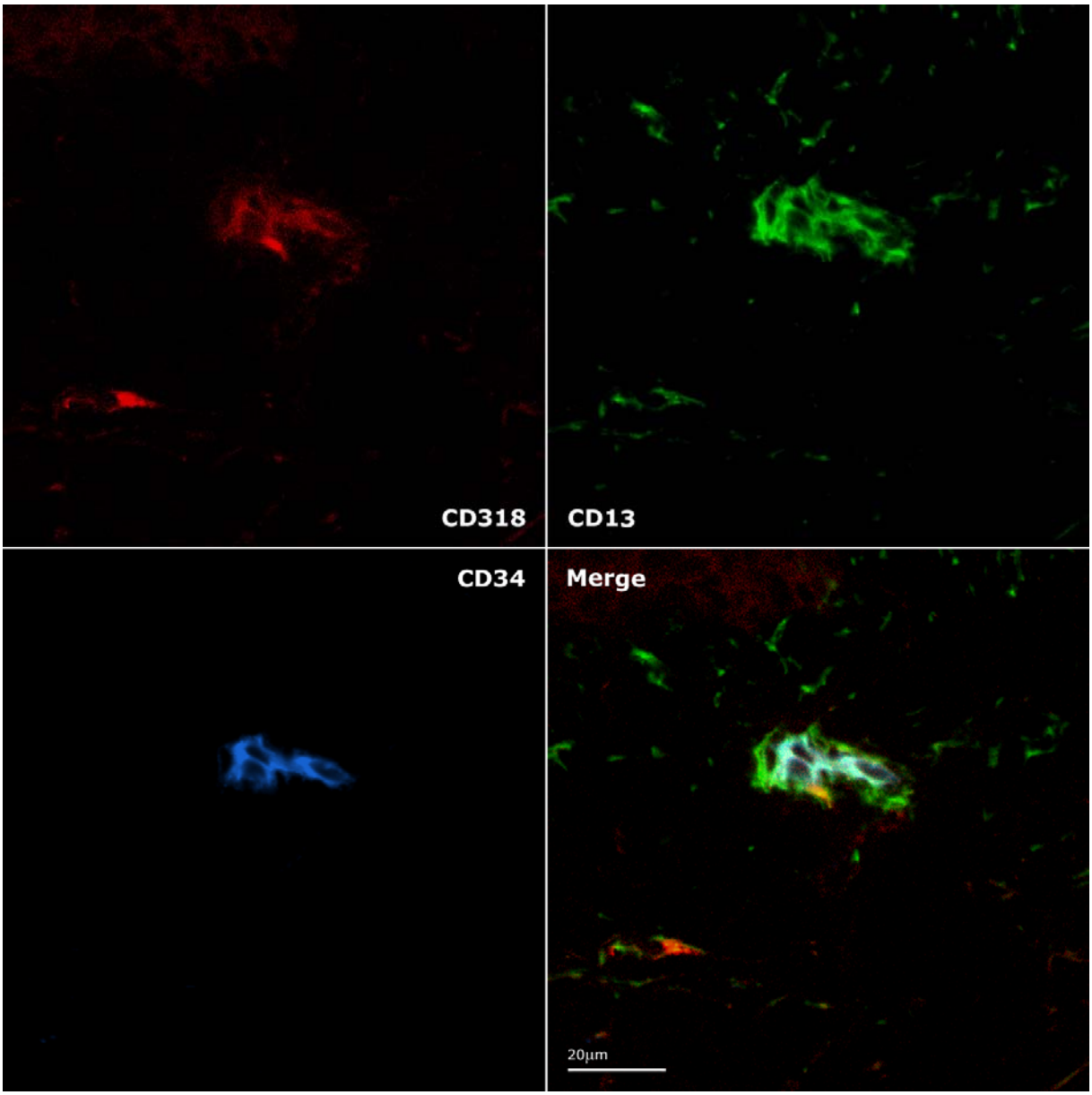

FIGURE 32. HUMAN SKIN HARBOURS CD318⁺CD13⁺CD34⁺ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Cryostat sections were exposed to purified-anti-CD318+Biotin+Streptavidin-Texas Red, FITC-anti-CD13 and APC-anti-CD34 triple labeling.

A frequently used marker for myofibroblasts and vascular smooth muscle cells is α-smooth muscle actin (α-SMA) [78]. Immunohistochemical and immunoelectron microscopy studies revealed the presence of α-SMA in fibroblasts located in the connective tissue sheath of human anagen hair follicles. α-SMA was detected only in fibroblasts located in the innermost layer of the transverse collagenous fibres. Since α-SMA is located in cells with contractile potential, this newly identified layer may play an important role in the morphological changes of the lower portion of the hair follicle during the hair growth cycle [79]. The combination of the markers CD318, CD34 and α-SMA revealed that distinct cells in the dermis coexpress these proteins (FIG. 33). Interestingly, α-SMA$^+$ cells appear quite regularly, even though only few hair follicles are present in foreskin. Human fetal cord blood contains a population of cells that may differentiate toward both an endothelial and a smooth muscle phenotype in culture. For the identification of these cells, the expression of α-SMA was used. Following culture in an endothelial cell-promoting environment, CD34$^+$ isolates produced endothelial-like cells that expressed alpha-SMA [80]. Thus, it is conceivable that CD318$^+$α-SMA$^+$CD34$^+$ population may also have the potential to form both hematological and endothelial cells (FIG. 34).

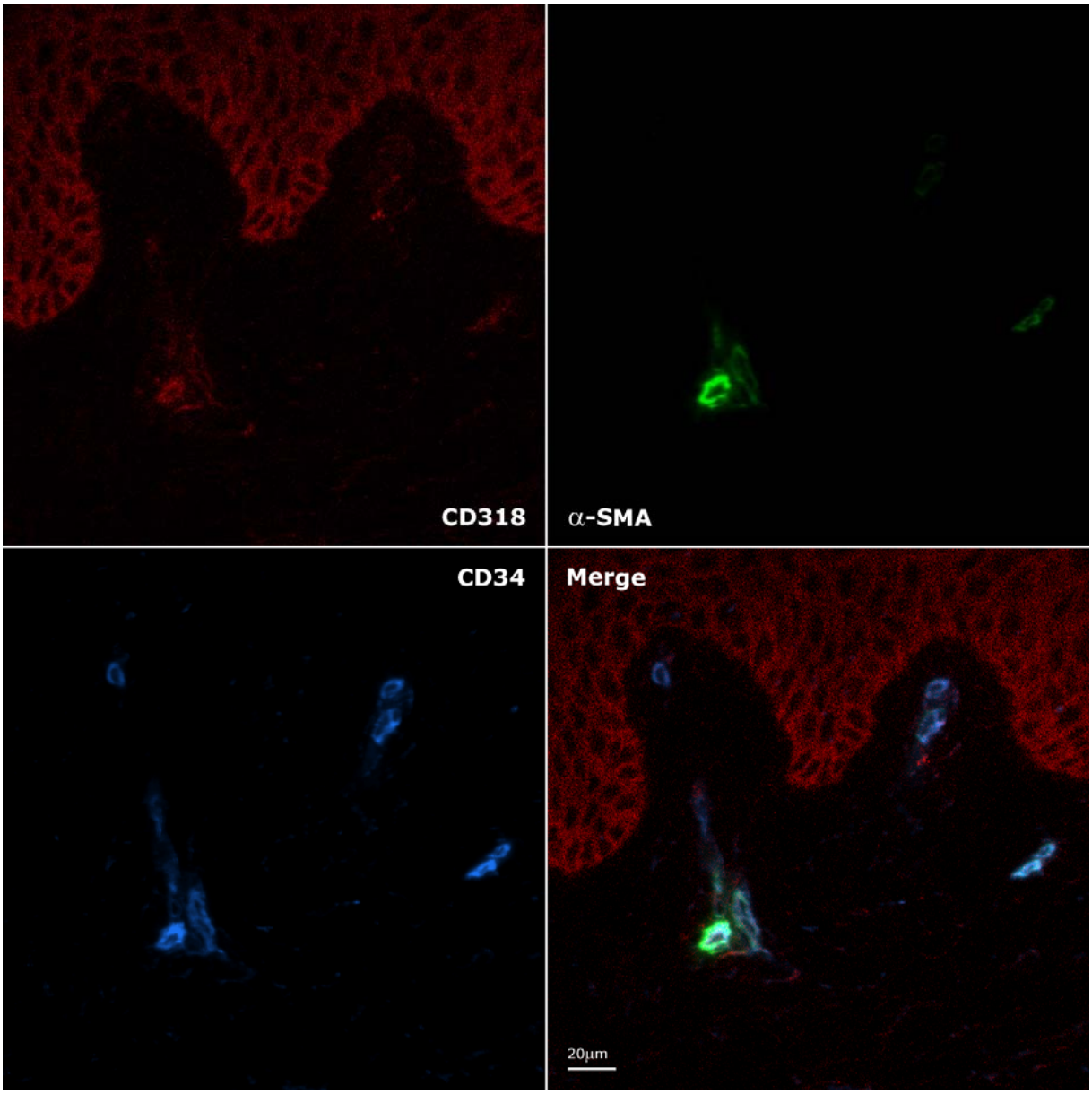

FIGURE 33. HUMAN SKIN HARBOURS CD318$^+$α-SMA$^+$CD34$^+$ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318/Biotin/Streptavidin-Texas Red/FITC-anti-α-SMA/APC-anti-CD34 triple labeling.

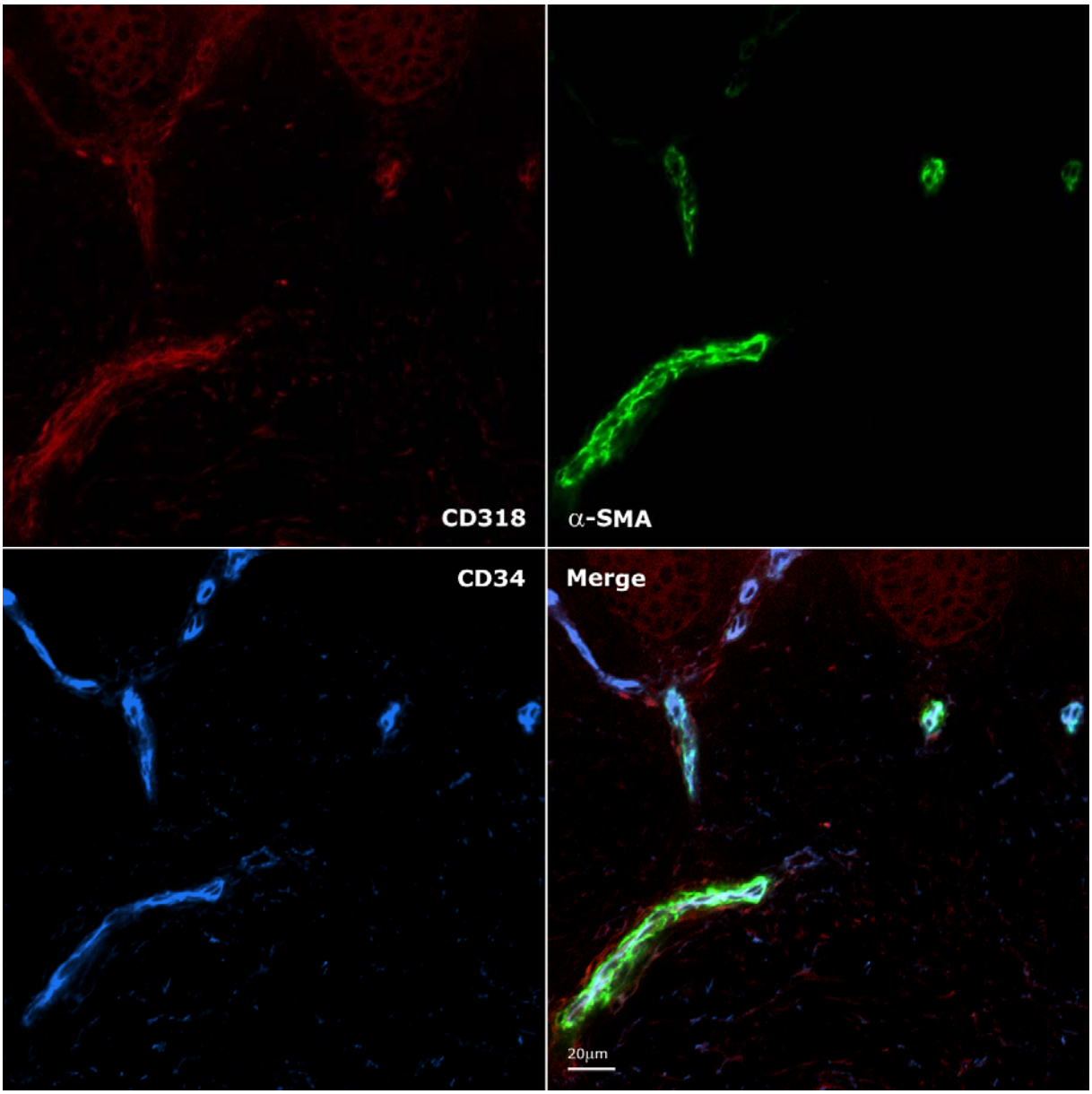

FIGURE 34. HUMAN SKIN HARBOURS CD318$^+$α-SMA$^+$CD34$^+$ CELLS. Immunofluorescence analysis of cryostat sections from human foreskin (3 year-old). Sections were exposed to purified-anti-CD318/Biotin/Streptavidin-Texas Red/FITC-anti-α-SMA/APC-anti-CD34 triple labeling.

8 MATERIALS & METHODS

8.1 APPARATUSES AND INSTRUMENTS

CO_2 incubator (Heraeus, Vienna, Austria)

Confocal laser scanning microscope (CLSM 510, Zeiss, Jena, Germany)

Cooling centrifuges (Heraeus)

Centrifuge 5415 R (Eppendorf, Hamburg, Germany)

Electrophoresis chamber (Sub Cell GT, Bio-Rad Laboratories, Hercules, CA, USA)

Fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson, San Jose, CA, USA)

Forceps

Freezers (-20°C, -80°C)

Fridges

ImmEdge pen (Vector Laboratories, Inc., Burlingame, CA, USA)

JungCM1800 Cryostat (Leica Microsystems, Wetzlar, Germany)

Laminar flow (Holten, Allerod, Denmark)

LSM Image Browser (Zeiss)

LumiAnalyst-software (Roche, Penzberg, Germany)

Microcentrifuges (Eppendorf)

Pipetaid (Costar, Cambridge, MA, USA)

Pipetmen (2 µl, 20 µl, 200 µl, 1 ml; Gilson, Middleton, WI, USA)

Power supply (Power Pac 300 power supply, Bio-Rad Laboratories)

PTC 200 – Peltier Thermal Cycler (MJ Research Inc., Waltham, MA, USA)

Scale (Sartorius, Vienna, Austria)

Scalpels

Scissors

Stainless steel metal mesh

Transilluminator (Lumi-Imager, Boehringer-Mannheim, Mannheim, Germany)

U-2000 spectrophotometer (Hitachi, San Jose, CA, USA)

Vortex Genie 2 (Lactan, Graz, Austria)

8.2 PLASTIC MATERIAL

Cell strainer (70 µm; Falcon, Lincoln Park, New Jersey, USA)

Culture dishes (35 mm; Stem Cell Technologies, Vancouver, Canada)

Eppendorf tubes (1.5 ml, 2 ml; Eppendorf)

Gloves

Microscope glass cover slips (24x50 mm; Chance proper LTD, Warley, UK)

Microscope slides (ChemMate Capillary Gap Microscope Slides, DakoCytomation, Glostrup, Denmark)

Microtubes for flow cytometry (Micronic, Lelystad, The Netherlands)

PCR reaction tubes (Eppendorf)

Petri dishes for tissue culture (100 x 20 mm; Corning, NY, USA)

Pipettes (5 ml, 10 ml, 25 ml and 50 ml; Corning)

Polypropylene tubes (15 and 50 ml; Falcon)

Sterile tips (1-100 and 200-1000 µl; Corning)

Swabs

Tissue culture plates (Nunc, Roskilde, Denmark)

8.3 REAGENTS

1 kb DNA ladder (Invitrogen, Carlsbad, CA, USA)

2-propanol (Merck, Dortmund, Germany)

7-amino-actinomycin D (Sigma, Saint Louis, MO, USA)

Acetone (p.a.; Merck)

Agarose

Ammonium chloride solution (0.8% NH_4Cl/0.1 mM EDTA; Stem Cell Technologies)

Ammoniumthiocyanate (3.8% in PBS)

Antibodies (see TABLE 5.)

Betaisodona (10%, Mundipharma, Vienna, Austria)

Bovine serum albumin (BSA, Sigma)

Chloroform

DEPC-treated water

Desoxyribonuclease I (DNase I, from bovine pancreas, Sigma)

Dimethylsulfoxide (DMSO)

Dispase II (neutral protease, grade II, Roche, Basel, Switzerland)

DNAse buffer

dNTP mix (Invitrogen, Carlsbad, CA, USA)

Ethanol (Merck)

Ethidium bromide

Ethylenediaminetetraacetic acid (EDTA, Merck)

FACS buffer (PBS supplemented with 2% FCS, 0.01% NaN3 and 0.5 mM EDTA)

Fluorescent mounting medium (Dako, Vienna, Austria)

Isoamylalcohol

Liberase Blendzyme 3 (10.7 Wünsch units in 20 ml PBS; Roche)

Methanol (Merck)

OCT compound (Tissue Tek, Zoeterwoude, The Netherlands)

Phenol

Phosphate-buffered saline (PBS 1x, Gibco Life Technologies, Carlsbad, CA, USA)

Second-step and third-step reagents (see TABLE 5.)

SuperScript II RT kit (Invitrogen)

Taq polymerase II (Roche)

TRIS-acetate/EDTA (TAE)

TRIzol reagent (Invitrogen)

8.4 MEDIA, SERA AND SUPPLEMENTS

Fetal calf serum (FCS, PAA Laboratories, Linz, Austria)

Goat serum (Sera-Lab Ltd., Sussex, England)

Mouse serum (self made)

8.5 EXPERIMENTAL ANIMALS

C57BL/6 (CD45.1; Charles River Wiga GmbH, Sulzfeld, Germany and Centre de Developèment des Techniques Avanceés pour l´expérimentation animale, Orléans, France), and C57BL/6 ROSA-26 (CD45.2) [81] (The Jackson Laboratory, Bar Harbor, Maine) mice were bred and maintained at the local animal facility. Newborn mice were obtained from timed pregnancies, taking the appearance of a vaginal plug as day 0 of gestation. All mouse procedures were performed with consent from the Institutional Committee of Animal Experimentation of the Medical University of Vienna and by the Austrian Ministry of Science and Education (GZ 66.009/177-Pr/4/2002, GZ 66.009/81-BrGT/2004).

To distinguish between host and donor cells, C57BL/6 ROSA-26 (CD45.2) mice were used as donors that express ß-galactosidase systemically. Consequently, these donor cells can be tested for ß-gal expression by their "blue" appearance. Donor cells were injected into congenic mice expressing the CD45.1 allele, thus, allowing analysis of donor and host cells for defined populations. Briefly, adult (8- to 12-week old) female C57BL/6 (CD45.1) mice were given sterile, acidified water (pH 3.0) ad libitum for 2 weeks. Subsequently, mice were exposed to 7.5 Gray total body γ-irradiation in a split dose with a 3 h interval. Five hours after irradiation, inocula containing total dermal cells ($1x10^7$/mouse), Lin$^-$ dermal cells ($1x10^7$/mouse), CD34$^-$, CD34$^+$, CD90$^-$, CD90$^+$ or CD45$^+$ dermal cells ($1x10^3$-$1x10^4$/mouse) from C57BL/6 ROSA-26 newborn mice (TABLE 5) or for control purposes, BM cells ($1x10^7$/mouse) from C57BL/6 ROSA-26 adult mice were injected intravenously into the tail vein or intraperitoneally using a syringe fitted with a 25-gauge needle, in the presence or absence of syngeneic BM (1:10). Control mice received 50 µl PBS. At least 3 mice per group were used for each experimental condition. Total dermal cells from adult mice but less so from newborn mice often failed to reconstitute intravenously transplanted recipient mice because of cellular aggregation causing fatal embolic formation. To determine donor chimerism, tail-vein blood samples were first analyzed 6 weeks after transplantation and then every second month. Additionally, recipient mice were sacrificed at selected time points (8-44 weeks), and cells from BM, spleen, liver, lymph nodes, thymus, epidermis and dermis were double-stained with anti-CD45.2 and a panel of mAb against Lin markers to detect donor-derived-cells to monitor engraftment (FIG. 35).

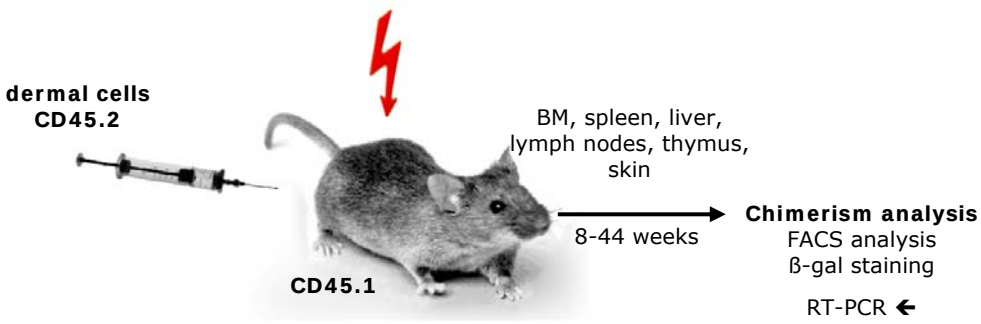

FIGURE 35. EXPERIMENTAL DESIGN.

(modified with permission from Meindl et al. [69])

8.6 TOTAL DNA ISOLATION

Mouse tissue (stored at -20°C) was incubated in 500 µl tail buffer (50 mM Tris-HCl pH 8, 100 mM EDTA, 100 mM NaCl, 1% SDS) plus 25 µl Proteinase K shaking O/N (overnight) at 55°C. After mixing for 5 min, 170 µl of saturated NaCl (6 M) were added, and mixed again. Samples were centrifuged at 16.1 rcf (relative centrifugal force) for 10 min, supernatants (without top phase and pellet) were transferred to new tubes. Five hundred µl of isopropanol were added, the sample mixed for 2 min, and spinned at 16.1 rcf for 5 min. The remaining pellet was washed with 1.5 ml of 70% EtOH and spinned at 16.1 rcf for 5 min. Washed pellet was resuspended in 300–400 µl TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA), according to the size of pellets. Samples were incubated, gently shaking, for two hours at 37°C, and stored at 4°C until use.

8.7 TOTAL RNA ISOLATION

Mouse tissue (stored at -20°C) was homogenized in 1-1.5 ml Trizol and then incubated for 5 min at room temperature (RT). Per 1 ml Trizol 200 µl trichlormethane/chloroform were added and mixed for 10 sec. After 10 min of centrifugation at 16.1 rcf, the aqueous phase was transferred into a fresh tube and mixed with 500 µl of isopropanol. Samples were incubated (10 min, RT) and then centrifuged at 16.1 rcf for 10 min. The RNA pellet was washed with 1 ml 70% ethanol, mixed and centrifuged (5 min, 4°C). The pellet was air-dried, resuspended in DEPC-H_2O and incubated (5 min, 65°C). Samples were stored shock-frozen at -20°C until use.

8.8 DNASE TREATMENT

RNA samples were mixed with 5 µl 10x DNAse buffer, 2 µl DNAse I, 1 µl RNAse inhibitor and with DEPC-H_2O up to a total volume of 50 µl. The mix was incubated (25 min, RT) and subsequently 50 µl DEPC-H_2O and 50 µl of cholorform/isoamylalcohol/phenol (24:1:25) were added. The RNA solution was centrifuged at 16.1 rcf for 5 min. The aqueous phase was transferred and mixed with 2.5x volume EtOH and $^1/_{10}$ volume 3M sodiumacetate. After 5 min centrifugation at 4°C, the RNA pellet was washed with 0.5 ml 70% EtOH and again centrifuged. The pellet was resuspended in 20 µl DEPC-H_2O. The concentration of isolated total RNA of each sample was quantified by measuring the optical density at 260 nm with a spectrophotometer at a dilution of 1:100. Samples were stored shock-frozen at -20°C until use.

8.9 CDNA SYNTHESIS

For the reverse transcriptase reaction an amount of 2 µg of total RNA, 1 µl dNTP mix, 3 µl of random hexamers and DEPC-H_2O were mixed to a total volume of 10 µl. The samples were incubated (5 min, 65°C) and then put on ice for 1 min. Nine µl of the reaction mix (2 µl 10x RNA buffer, 4 µl 25 mM $MgCl_2$, 2 µl 0.1 M DDT, 1 µl RNAse OUT) were pipetted into the RNA samples and incubated (2 min, RT). One µl of SuperScript II RT was added to the reaction mix, except for the RT^- control, and incubated (10 min, 25°C), and subsequently for 50 min at 42°C. The reaction was terminated at 70°C (15 min). To remove the RNA 1 µl of RNAse H was added and incubated (20 min, 37°C). The cDNA samples were diluted 1:1 with DEPC-H_2O.

8.10 POLYMERASE CHAIN REACTION (PCR)

8.10.1 PREPARATION OF THE PCR MASTER MIX

Twenty three µl of the PCR master mix consisted of 5.5 µl dH_2O, 2.5 µl 10x GB (670 mM Tris-HCl pH8, 166 mM $(NH_4)_2SO_4$, 67 mM $MgCl_2$, 50 mM ß-mercaptoethanol, 67 µM EDTA), 2.3 µl DMSO, 2.5 µl 10x DNTPs, 5 µl primer 1, 5 µl primer 2, 0.2 µl aTaq polymerase.

8.10.2 REACTION SET-UP

Equivalents of the above master mix were pipetted into 0.5 ml PCR tubes prior to the addition of template cDNA (2 µl per reaction).

8.10.3 THERMAL PROTOCOL

The PCR amplification profile employed 45 cycles of denaturation (1 min, 94°C), primer annealing (1 min, 58°C) and primer extension (1 min 30 sec, 65°C) with a final extension step (10 min, 65°C). The PCR products were analyzed immediately or stored O/N at 4°C.

8.10.4 SEQUENCES OF PRIMERS FOR DETERMINATION OF mRNA EXPRESSION

Primers for RT-PCR were purchased from VBC-Genomics, Vienna, Austria (TABLE 3.).

TABLE 3. PRIMER SEQUENCES.

TARGET	PRIMER PAIRS	
lacZ[c]	5 – CGC CAG CTG GCG TAA TAG CG – 3	5 – CTG TCC TGG CCG TAA CCG AC – 3
lacZ[d]	5 – CGC TCA CAT TTA ATG TTG ATG AAA GC – 3	5 – TCC AGA TAA CGT CCG TCA CTC CAA – 3
HPRT[e]	5 – TTG CTC GAG ATG TGA TGA AGG A – 3	5 – AAA GTT GAG AGA TCA TCT CCA CCA A – 3

8.10.5 AGAROSE GEL ELECTROPHORESIS

PCR products were analyzed by electrophoresis on a 1% agarose gel in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA). Agarose was melted for 60 sec, shaken and heated for additional 60 sec. After cooling, 1% of ethidium bromide was added, the gel was poured into a gel tray, and allowed to set for 20 min. The comb was removed, the gel placed into an electrophoresis chamber and filled with 1% TAE. Five µl of gel loading dye was added to 25 µl of PCR product and loaded onto the gel. To determine the length of PCR products a 1 kb ladder was included on the gel. Gels were run at 95 V to 135 V depending on the size of the gel and visualized with an UV transilluminator and analyzed with the LumiAnalyst-software.

8.11 PREPARATION OF HUMAN SKIN

Skin was obtained from routine circumcisions of volunteers after informed consent (EK Nr. 555/2005). Foreskin was disinfected (betasiodona/PBS, 10 min) and rinsed with 70% ethanol before further use. Skin was spread, epidermal side down, on a petri dish, and muscle and adipose tissue was scraped away with a scalpel blade.

[c] Complete coding regions for gene of interest were obtained from Sibilia et al. [70].
[d] Complete coding regions for genes of interest were obtained from Lako *et al.* [66].
[e] Complete coding regions for gene of interest were obtained from Sibilia et al. [70].

8.12 Preparation and fluorescence staining of dermal sheets

Foreskin was cut into small pieces (3 x 3 mm) and placed dermal side down on 3.8% ammoniumthiocyanate (25 min, 37°C). After separating epidermis and dermis, tissues were washed with PBS. Dermal sheets were cut into even smaller pieces and fixed in 2% PFA (30 min, RT). For fluorescence staining dermal sheets were incubated with primary Ab (O/N, 4°C, or 1 h, 37°C). When the mAb was purified, second- and third-step reagents were applied (1 h, RT), and blocked with an adequate serum (5% serum in PBS). After washing in PBS, sheets were incubated with secondary and tertiary Ab (O/N, 4°C, or 1 h, 37°C). Specificity of staining was confirmed using isotype-matched control mAb. Dermal sheets were mounted and analyzed with CLSM.

8.13 Preparation and fluorescence staining of cryosections

Foreskin was cut into small stripes (10 x 3 mm), rolled in and shock-frozen in OCT. Five µm sections were mounted on capillary gap microscope slides, fixed in acetone (10 min, ice-cold) and stored at -20°C or stained immediately.

Sections were encircled with an ImmEdge pen and washed in PBS. If necessary, sections were blocked with adequate sera. Primary Ab was incubated (O/N, 4°C, or 1h, 37°C). If Ab was purified, second- and third-step reagents were applied (1 h, RT), and blocked with adequate serum (5% serum in PBS). After washing in PBS, skin sections were incubated with secondary and tertiary Ab (O/N, 4°C, or 1 h, 37°C). Specificity of staining was confirmed using isotype-matched control mAb. Nucleic acid staining was performed with either Dapi or Cytox reagents. Slides were mounted with fluorescent mounting medium, analyzed with a CLSM and stored at 4°C.

8.14 CONFOCAL LASER SCANNING MICROSCOPY

To identify and reveal co-localization of certain proteins of interest on dermal/epidermal cells, CLSM was performed. The advantage of confocal microscopy is the ability to collect light from a single plane. This is accomplished by imaging objects through a small pinhole aperture, thereby preventing detection of all light except for that originating from a thin optical section. Fluorescence recordings were performed by means of a confocal laser scanning setup, equipped with an argon laser, exciting at 458, 477, 488 and 514 nm, a helium/neon laser, single excitation 543 nm, and a second helium/neon laser, excitation 633 nm (TABLE 4.).

TABLE 4. PEAK EXCITATION AND EMISSION WAVELENGTHS OF FLUOROCHROMES USED FOR CLSM.

FLUOROCHROME	EXCITATION	EMISSION	EXCITATION LASER
Alexa Fluor 546	556 nm	573 nm	HeNe 1 (458 nm)
Alexa Fluor 555	555 nm	565 nm	HeNe 1 (458 nm)
Alexa Fluor 647	650 nm	668 nm	HeNe 2 (633 nm)
APC	650 nm	660 nm	HeNe 2 (633 nm)
FITC	495 nm	520 nm	Ar (488 nm)
PE	470 nm	576 nm	HeNe 1 (543 nm)

Lasers required for excitation of individual fluorochromes are indicated.

TABLE 5. ANTIBODIES, SECOND-STEP AND THIRD-STEP REAGENTS.

AB SPECIFICITY	CLONE	IG CLASS	WORKING DILUTION	SOURCE*	CONJUGATE
CD13	5-390	mouse IgG1	4.5 µg/ml	O. Majdic	FITC, PE
CD13	5-623	mouse IgG1	2.5 µg/ml	O. Majdic	FITC, PE
CD14	M5E2	mouse $IgG2_a$	5 µg/ml	BD Biosciences	purified
CD14	TüK4	mouse $IgG2_a$	4 µg/ml	Caltag	APC
CD31	WM59	mouse IgG1	1 µg/ml	Serotec	FITC
CD34	8G12	mouse IgG1	0.5 µg/ml	BD Biosciences	PE
CD34	581	mouse IgG1	5 µg/ml	BD Biosciences	purified
CD34	581	mouse IgG1	2 µg/ml	Immunotech	purified
CD34	AC136	mouse $IgG2_a$	1:100	Miltenyi	APC, FITC
CD45	2D1	mouse IgG1	0.5 µg/ml	BD Biosciences	FITC, purified
CD45	HI30	mouse IgG1	1:100	BD Biosciences	PE
CD45	J33	mouse IgG1	1 µg/ml	Immunotech	APC
CD90	5E10	mouse IgG1	1 µg/ml	BD Biosciences	PE
CD90	F15-42-1	mouse IgG1	2 µg/ml	Serotec	purified
CD117	YB5.B8	mouse IgG1	4 µg/ml	BD Biosciences	PE, purified
CD133/1	AC133	mouse IgG1	1 µg/ml	Miltenyi	purified
CD150	A12	mouse IgG1	5 µg/ml	BioLegend	PE
CD150	A12	mouse IgG1	1 µg/ml	BD Biosciences	purified
CD318	CUB1	mouse $IgG2_b$	2 µg/ml	BD Biosciences	AF 647, PE
CD318	CUB1	mouse $IgG2_b$	undiluted	H. J. Bühring	purified
Keratin 14	polyclonal	rabbit IgG	1 µg/ml	Covance	FITC
PAL-E	PAL-E	mouse $IgG2_a$	1:100	Fitzgerald	purified
Smooth Muscle Actin	1A4	mouse $IgG2_a$	7 µg/ml	Sigma	FITC
AB SPECIFICITY	**IG CLASS**		**WORKING DILUTION**	**SOURCE**	**CONJUGATE**
Goat anti-mouse	$F(ab')_2$ IgG (H+L)		5 µg/ml	Caltag	FITC
Goat anti-mouse	$F(ab')_2$ IgG (H+L)		10 µg/ml	Invitrogen	Alexa 546, Alexa 647
Goat anti-mouse	goat $F(ab')_2$ IgG+IgM (H+L)		7 µg/ml	Medac	Biotin
Streptavidin			2 µg/ml	Amersham	Texas Red
NUCLEIC ACID STAINING					
Dapi			1:4	Vectashield	detection via UV light
Sytox			$1:10^6$	Molecular Probes	Green, Orange

*AMERSHAM, Piscataway, NJ, USA; BIOLEGEND, San Diego, CA, USA; BD BIOSCIENCES, San Jose, CA, USA; HANS-JÖRG BÜHRING, Department of Internal Medicine, University of Tübingen, GER; CALTAG, Burlingame, CA, USA; FITZGERALD, Concord, MA, USA; IMMUNOTECH, Marseille, F; INVITROGEN, Carlsbad, CA, USA; OTTO MAJDIC, Institute of Immunology, Medical University of Vienna, AUT; MEDAC, Hamburg, GER; MILTENYI, Bergisch Gladbach, GER; MOLECULAR PROBES, Eugene, OR, USA; SEROTEC, Oxford, UK; SIGMA, Saint Louis, MO, USA; VECTASHIELD, Burlingame, CA, USA.

8.15 PREPARATION OF HUMAN DERMAL CELLS FOR FACS ANALYSIS

Sterilized foreskin was placed on 25% dispase (O/N, 4°C). After separating dermis from epidermis, dermis was cut into small pieces and digested in liberase (90 min, 37°C). Digested tissue was sifted through a steel mesh, and subsequently filtered through a cell strainer. Cells were washed in normal cell culture medium (RPMI 1640 medium was supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 10 µg/ml gentamycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 µM 2-ME, and 0.002% antibiotic-antimycotic), centrifuged and counted.

For FACS analysis, dermal cells were diluted in 100 µl FACS buffer. Antibodies were added in the proper concentrations and incubated (30 min, ice). After centrifugation, supernatant was discarded and cells resuspended.

For two and three-color analyses, dermal cells ($5x10^5$/sample) were resuspended in FACS buffer and incubated with APC-, FITC-, PE-conjugated mAbs directed against selected human antigens. To block non-specific binding of mAb, cells were incubated with adequate serum prior to the staining procedures. Specificity of staining was confirmed using isotype-matched control mAb. Dead cells were excluded by 7-AAD (1 µg/ml) uptake. To detect intracellular antigens, such as K14, dermal cells were permeabilized with Cytofix/Cytoperm (250 µl/ml; 24°C, 20 min). After washing with 0.1% saponin/PBS, cells were either stained with a non-conjugated or direct labeled mAb (30 min, 4°C). Non-conjugated mAb were visualized by a second-step reagent (30 min, 4°C). Appropriate isotype mAb were used as controls. After washing with 0.1% saponin/PBS, subsequent staining was performed as described above. The staining procedure was performed in microtubes (100 µl cells/sample).

Fluorescence was measured with a FACSCalibur flow cytometer, and data were analyzed with Cell Quest software.

9 SUPPLEMENTAL DATA

TABLE 6. ANALYZED MURINE ORGAN SAMPLES.

SAMPLE #	EXPERIMENT	ORGAN	CELL TYPE TRANSPLANTED	ISOLATION OF…
1	Exp 16.07.2003 / 19.04.2004	Spleen	NB dermis	DNA & RNA
2	Exp 16.07.2003 / 19.04.2004	Thymus		DNA & RNA
3	Exp 16.07.2003 / 19.04.2004	Skin		DNA & RNA
4	B2 SP (neg. control)	Spleen		DNA & RNA
5	R2 SP (pos. control)	Spleen		DNA & RNA
6	Exp 28.01.2004 / 21.04.2004	Spleen	$CD45^+$	DNA & RNA
7	Exp 28.01.2004 / 21.04.2004	BM		DNA & RNA
8	Exp 28.01.2004 / 21.04.2004	Skin		DNA & RNA
9	Exp 28.01.2004 / 21.04.2004	Lymph Node		DNA & RNA
10	Exp 11.02.2004 / 23.04.2004	Spleen (Sp B)	$CD34^-$	DNA & RNA
11	Exp 11.02.2004 / 23.04.2004	BM (BM B)		DNA & RNA
12	Exp 11.02.2004 / 23.04.2004	Skin (SK B)		DNA & RNA
13	Exp 11.02.2004 / 23.04.2004	Lymph Node (LN B)		DNA & RNA
14	B2 BM (neg. control)	BM		DNA & RNA
15	R2 BM (pos. control)	BM		DNA & RNA
16	Exp 11.02.2004 / 23.04.2004	Skin (Sk A)	$CD34^+$	RNA
17	Exp 11.02.2004 / 23.04.2004	Spleen (Sp A)		RNA
18	Exp 11.02.2004 / 23.04.2004	Thymus (Th A)		RNA
19	Exp 11.02.2004 / 23.04.2004	Lymph Node (LN A)		RNA
20	Exp 11.02.2004 / 23.04.2004	BM (BM A)		RNA
21	Exp 11.02.2004 / 23.04.2004	Liver (L A)		RNA
22	Exp 13.08.2003 / 06.04.2004	Skin (SK A)	$CD90^+$	RNA
23	Exp 13.08.2003 / 06.04.2004	Spleen (Sp A)		RNA
24	Exp 13.08.2003 / 06.04.2004	Thymus (Th A)		RNA
25	Exp 13.08.2003 / 06.04.2004	Lymph Node (LN A)		RNA
26	Exp 13.08.2003 / 06.04.2004	BM (BM A)		RNA
27	Exp 13.08.2003 / 06.04.2004	Liver (L A)		RNA
28	Exp 18.11.2003 / 02.04.2004	Skin (SK A)	Lin^-	RNA
29	Exp 18.11.2003 / 02.04.2004	Spleen (Sp A)		RNA
30	Exp 18.11.2003 / 02.04.2004	Thymus (Th A)		RNA
31	Exp 18.11.2003 / 02.04.2004	Lymph Node (LN A)		RNA
32	Exp 18.11.2003 / 02.04.2004	BM (BM A)		RNA
33	Exp 18.11.2003 / 02.04.2004	Liver (L A)		RNA
34	B2 L (neg. control)	Liver		RNA
35	R2 L (pos. Control)	Liver		RNA
36	Exp 13.08.2003/06.04.2004	Skin (SK B)	$CD90^-$	RNA
37	Exp 13.08.2003/06.04.2004	Spleen (Sp B)		DNA & RNA
38	Exp 13.08.2003/06.04.2004	Thymus (Th B)		RNA
39	Exp 13.08.2003/06.04.2004	Lymph Node (LN B)		RNA
40	Exp 13.08.2003/06.04.2004	BM (BM B)		RNA
41	Exp 13.08.2003/06.04.2004	Liver (L B)		RNA
42	Exp 13.05.2003/09.04.2004	Skin (SK A)	NB dermis	RNA
43	Exp 13.05.2003/09.04.2004	Spleen (SP A)		DNA & RNA
44	Exp 13.05.2003/09.04.2004	Thymus (Th A)		RNA
45	Exp 13.05.2003/09.04.2004	Lymph Node (LN A)		RNA
46	Exp 13.05.2003/09.04.2004	BM (BM A)		RNA

47	Exp 13.05.2003/09.04.2004	Liver (L A)		RNA
48	Exp 14.11.2003/13.04.2004	Skin	adult dermis	RNA
49	Exp 14.11.2003/13.04.2004	Spleen		DNA & RNA
50	Exp 14.11.2003/13.04.2004	Thymus		RNA
51	Exp 14.11.2003/13.04.2004	Lymph Node		RNA
52	Exp 14.11.2003/13.04.2004	BM		RNA
53	Exp 14.11.2003/13.04.2004	Liver		RNA
54	Exp 21.04.2003/22.04.2004	Skin	Lin^-	RNA
55	Exp 21.04.2003/22.04.2004	Spleen		DNA & RNA
56	Exp 21.04.2003/22.04.2004	Thymus		RNA
57	Exp 21.04.2003/22.04.2004	Lymph Node		RNA
58	Exp 21.04.2003/22.04.2004	BM		RNA
59	Exp 21.04.2003/22.04.2004	Liver		RNA
60	Exp 21.01.2004/15.04.2004	Skin (SK A)	Lin^-	RNA
61	Exp 21.01.2004/15.04.2004	Spleen (SP A)		DNA & RNA
62	Exp 21.01.2004/15.04.2004	Thymus (Th A)		RNA
63	Exp 21.01.2004/15.04.2004	Lymph Node (LN A)		RNA
64	Exp 21.01.2004/15.04.2004	BM (BM A)		RNA
65	Exp 21.01.2004/15.04.2004	Liver (L A)		RNA
66	B2 SP (neg. control)	Spleen		DNA & RNA
67	R2 SP (pos. control)	Spleen		DNA & RNA
68	B2 L (neg. control)	Liver		RNA
69	R2 L (pos. Control)	Liver		RNA
70	Exp 16.07.2003/19.04.2004	Lymph Node	NB dermis	RNA
71	Exp 16.07.2003/19.04.2004	BM		RNA
72	Exp 16.07.2003/19.04.2004	Liver		RNA
73	Exp 28.01.2004/21.04.2004	Thymus	$CD45^+$	RNA
74	Exp 28.01.2004/21.04.2004	Liver		RNA
75	Exp 23.07.2003/09.04.2004	Skin (SK B)	Lin^-	RNA
76	Exp 23.07.2003/09.04.2004	Spleen (SP B)		RNA
77	Exp 23.07.2003/09.04.2004	Thymus (TH B)		RNA
78	Exp 23.07.2003/09.04.2004	Lymph Node (LN B)		RNA
79	Exp 23.07.2003/09.04.2004	BM (BM B)		RNA
80	Exp 23.07.2003/09.04.2004	Liver (L B)		RNA
81	Exp 07.05.2003/02.04.2004	Skin	Lin^-	RNA
82	Exp 07.05.2003/02.04.2004	Spleen		RNA
83	Exp 07.05.2003/02.04.2004	Thymus		RNA
84	Exp 07.05.2003/02.04.2004	Lymph Node		RNA
85	Exp 07.05.2003/02.04.2004	Bonde Marrow		RNA
86	Exp 07.05.2003/02.04.2004	Liver		RNA
87	Exp 11.02.2004 / 23.04.2004	Thymus (Th B)	$CD34^-$	RNA
88	Exp 11.02.2004 / 23.04.2004	Liver (L B)		RNA
89	Exp 16.07.2003/30.03.2004	Skin (SK C)	NB demis	RNA
90	Exp 16.07.2003/30.03.2004	Spleen (SP C)		RNA
91	Exp 16.07.2003/30.03.2004	Thymus (TH C)		RNA
92	Exp 16.07.2003/30.03.2004	Lymph Node (LN C)		RNA
93	Exp 16.07.2003/30.03.2004	BM (BM C)		RNA
94	Exp 16.07.2003/30.03.2004	Liver (L C)		RNA
95	Exp 21.01.2004/15.04.2004	Skin (SK B)	Lin^-	RNA
96	Exp 21.01.2004/15.04.2004	Spleen (SP B)		RNA
97	Exp 21.01.2004/15.04.2004	Thymus (TH B)		RNA
98	Exp 21.01.2004/15.04.2004	Lymph Node (LN B)		RNA
99	Exp 21.01.2004/15.04.2004	BM (BM B)		RNA

100	Exp 21.01.2004/15.04.2004	Liver (L B)		RNA
101	Exp 23.04.2003/16.03.2004	Skin	adult dermis	RNA
102	Exp 23.04.2003/16.03.2004	Spleen		RNA
103	Exp 23.04.2003/16.03.2004	Thymus		RNA
104	Exp 23.04.2003/16.03.2004	Lymph Node		RNA
105	Exp 23.04.2003/16.03.2004	Bonde Marrow		RNA
106	Exp 23.04.2003/16.03.2004	Liver		RNA
107	Exp 18.11.2003 / 04.02.2004	Skin	Lin^-	RNA
108	Exp 18.11.2003 / 04.02.2004	Kidney		RNA
109	Exp 18.11.2003 / 04.02.2004	Thymus		RNA
110	Exp 18.11.2003 / 04.02.2004	Lymph Node		RNA
111	Exp 18.11.2003 / 04.02.2004	Bonde Marrow		RNA
112	Exp 18.11.2003 / 04.02.2004	Liver		RNA
113	Exp 10.09.2003/05.04.2004	Skin	$CD90^+$	RNA
114	Exp 10.09.2003/05.04.2004	Spleen		RNA
115	Exp 10.09.2003/05.04.2004	Thymus		RNA
116	Exp 10.09.2003/05.04.2004	Lymph Node		RNA
117	Exp 10.09.2003/05.04.2004	Bonde Marrow		RNA
118	Exp 10.09.2003/05.04.2004	Liver		RNA
119	Exp 11.08.2003/18.03.2004	Skin (SK A)	$CD90^-$	RNA
120	Exp 11.08.2003/18.03.2004	Spleen (SP A)		RNA
121	Exp 11.08.2003/18.03.2004	Thymus (TH A)		RNA
122	Exp 11.08.2003/18.03.2004	Lymph Node (LN A)		RNA
123	Exp 11.08.2003/18.03.2004	BM (BM A)		RNA
124	Exp 11.08.2003/18.03.2004	Liver (L A)		RNA
125	Exp 29.04.2004	Skin	Lin^-	RNA
126	Exp 29.04.2004	Spleen		RNA
127	Exp 29.04.2004	Thymus		RNA
128	Exp 29.04.2004	BM		RNA
129	Exp 29.04.2004	Liver		RNA
130	Exp 11.08.2003/18.03.2004	Skin (SK B)	$CD90^-$	RNA
131	Exp 11.08.2003/18.03.2004	Spleen (SP B)		DNA & RNA
132	Exp 11.08.2003/18.03.2004	Thymus (TH B)		RNA
133	Exp 11.08.2003/18.03.2004	Lymph Node (LN B)		RNA
134	Exp 11.08.2003/18.03.2004	BM (BM B)		RNA
135	Exp 11.08.2003/18.03.2004	Liver (L B)		RNA
136	Exp 29.04.2004	Skin (SK C)	Lin^-	RNA
137	Exp 29.04.2005	Spleen (SP C)		DNA & RNA
138	Exp 29.04.2006	Thymus (TH C)		RNA
139	Exp 29.04.2007	Lymph Node (LN C)		RNA
140	Exp 29.04.2008	BM (BM C)		RNA
141	Exp 29.04.2009	Liver (L C)		RNA
142	Exp 29.04.2010	Skin (SK B)	Lin-	RNA
143	Exp 29.04.2011	Spleen (SP B)		DNA & RNA
144	Exp 29.04.2012	Thymus (TH B)		RNA
145	Exp 29.04.2013	Lymph Node (LN B)		RNA
146	Exp 29.04.2014	BM (BM B)		RNA
147	Exp 29.04.2015	Liver (L B)		RNA
148	Exp 16.07.2003/30.03.2004	Skin (SK A)	NB dermis	RNA
149	Exp 16.07.2003/30.03.2005	Spleen (SP A)		DNA & RNA
150	Exp 16.07.2003/30.03.2006	Thymus (TH A)		RNA
151	Exp 16.07.2003/30.03.2007	Lymph Node (LN A)		RNA
152	Exp 16.07.2003/30.03.2008	BM (BM A)		RNA

153	Exp 16.07.2003/30.03.2009	Liver (L A)		RNA
154	Exp 16.07.2003/30.03.2010	Skin (SK B)	NB dermis	RNA
155	Exp 16.07.2003/30.03.2011	Spleen (SP B)		DNA & RNA
156	Exp 16.07.2003/30.03.2012	Thymus (TH B)		RNA
157	Exp 16.07.2003/30.03.2013	Lymph Node (LN B)		RNA
158	Exp 16.07.2003/30.03.2014	BM (BM B)		RNA
159	Exp 16.07.2003/30.03.2015	Liver (L B)		RNA

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