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Mesenchymal Differentiation and Organ Distribution of Established Human Stromal Cell Lines in NOD/SCID Mice

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Key Words
Stromal cells · Mesenchymal stem cell · Osteoblast · Osteogenesis

Abstract
Two human stromal cell lines were established previously from bone marrow-derived primary long-term cultures by immortalization using the SV40 large T antigen and cellular cloning. After irradiation, the fibroblast-like cell lines L87/4 and L88/5 support hematopoietic differentiation of allogeneic cord blood cells in vitro. The stromal cells do not express CD34 and CD50, but some adhesion molecules and integrins, such as CD44, CD54 and CD58. Their expression profiles on RNA and protein levels are suggestive of their osteogenic potency. The quality and quantity of osteocalcin and osteopontin protein expression depended on the culture conditions. Expression of the osteogenic markers increased over time in culture, especially in cells growing in clusters. The stromal cells also expressed collagens I and V, but did not show any expression of collagens II and III. The potentially osteoblastic stromal cells were transplanted into NOD/SCID recipient mice by intravenous injection and were found in various mesenchymal organs up to 10 weeks after transplantation. Osteocalcin-positive human stromal cells could be detected in the bone marrow, thymus, liver, brain and gut of the recipient animals. In summary, there is evidence that human bone-marrow-derived stromal cells have to be considered mesenchymal progenitors, persistently expressing osteogenic markers in vitro and in vivo.

Introduction

In recent years, there has been increasing interest in the plasticity of hematopoietic stem cells. In traditional understanding, hematopoietic differentiation requires the support by growth factors provided by stromal cells. Stromal cells are the major source of essential growth factors and part of the stromal cell system which also consists of mesenchymal stem cells and its progeny, connective tissue cells and intercellular matrix proteins [1]. For many years, bone marrow was considered to consist of three

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individual cellular components: hematopoietic, endothelial and stromal cells. The mesenchymal stem cells within the stromal compartment can differentiate into various connective tissue lineages including osteogenic precursors [2].

Stromal cells were defined as a fibroblast-like, adherent cell population obtained by direct plating of bone marrow cells. The diversity of marrow stroma components depends on culture conditions and growth media. Using a standard colony-forming unit-fibroblast (CFU-F) assay, we generated a monoclonal stromal cell line from canine bone marrow after immortalization of the adherent cells with a human papilloma vIrus 16 E6E7 construct [3, 4]. From this CD34-negative cell line designated D064 we generated CD34-positive, nonadherent hematopoietic progenitors which also form other hematopoietic CFUs. D064 cells can also function as 'classical' stromal cells supporting the growth of allogeneic hematopoietic differentiation in vitro. This supports the notion that a stromal-like cell population can give rise to hematopoietic progeny. A similar statement was made by Huang and Terstappen in their controversial paper, proposing that a single CD34-positive stem cell gives rise to CD34-negative stromal cells as well as to committed hematopoietic precursors [5, 6].

Since most of the CD34-negative fibroblast-like stromal cells are predominantly quiescent and refractory to external signalling, only a very small number of them can differentiate into hematopoietic progenitors. The majority of CD34-negative stromal cells are not committed to hematopoiesis, but can maintain and fulfil other functions. Here we investigated, whether two well-established human stromal cell lines function as mesenchymal stem cells and display an osteogenic potency in vitro and in vivo.

Material and Methods

FACS Analysis

The established human stromal cell lines L87/4 and L88/5 cells were detached from culture flasks by collagenase/dispase (1 mg/ml, Boehringer, Mannheim), washed with culture medium, counted and adjusted to appropriate cell concentrations. 5–10 $\times$ 10^5 cells were used for FACS analysis. Cells were washed twice with IF buffer (2% FCS, 0.01% NaN_3 in PBS) and incubated with the antibodies to be analyzed for 30 min at 4°C. Excessive antibody was removed by washing the cells twice with cold IF buffer. Cells were then incubated for 30 min at 4°C with a secondary PE or FITC-labeled antibody, washed twice with cold IF buffer, resuspended in 1 ml IF buffer and analyzed in a flow-cytometer (Becton Dickinson, CellQuest).

Osteocalcin and Osteopontin m-RNA Detection by RT-PCR

One microgram total cellular RNA isolated from monolayers of L87/4 and L88/5 cells (Trizol, Gibco) was reverse transcribed by M-MuLV reverse transcriptase (Promega) in a 10-µl RT assay (1 m*M* dNTPs, 1 $\times$ RT buffer, 100 ng oligo$(dT)_{15}$, 10 U RNasin, 100 U M-MuLV RT) for 1 h at 42°C. PCR was performed in 25-µl reactions with 5 µl of the RT product, 5 pmol of each primer (osteocalcin sense: 5′-ATGAGAGCCCTCACACTCCTCG-3′; osteocalcin antisense: 5′-CCGGGCCGTAGAAGCGCCGATA-3′; osteopontin sense: 5′-CATTGACTCGAACGACTCTG-3′, osteopontin antisense: 5′-ACGGCTGTCCCAATCAGAAG-3′) and 0.75 U Taq Polymerase (Sigma). cDNA was amplified in 40 cycles (58°C annealing temperature) followed by a final extension step (72°C, 7 min) (thermocycler: Biozym). The amplified DNA was separated on a 1.2% agarose gel, stained with ethidium bromide and photographed. Verification of the yielded osteocalcin PCR product was performed by an internal restriction analysis.

Transplantation of L87/4 and L88/5 Cells into NOD/SCID Mice

NOD/SCID mice were irradiated with 2 Gy and transplanted 3 h later with 2 $\times$ 10^6 L87/4 or 2 $\times$ 10^6 L88/5 cells by slow injection into the tail vein. Control animals were irradiated without transplantation. 3, 6, and 10 weeks posttransplantation, 2 animals of each group were sacrificed, organs were removed and imbedded in paraffin, sectioned (1–2 µm) and analyzed immunohistochemically.

Immunohistochemical Investigation of Paraffin-Embedded Tissues

Transplanted mice were sacrificed 3, 6, and 10 weeks posttransplantation and tissues were immediately fixed overnight in buffered 4% formalin. Fixed tissues were embedded into paraffin and 1- to 2-µm sections were analyzed as follows: Deparaffinized slides were pretreated in TRS6 (target retrieval) solution in a microwave oven for 15 min at 800 W. Unspecific antibody-binding sites were blocked by incubating the slides in 'protein block serum free' (DAKO) solution for 10 min. Subsequently, slides were incubated with primary antibody (osteocalcin Biotrend, 1:300) for 60 min at room temperature and stained with an alkaline phosphatase coupled secondary antibody (enVision Labelled Polymer-AP, DAKO). Binding was visualized by incubating the slides in Fast Red® solution (Sigma) for 10 min.

Immunohistochemical Investigation of L87/4 and L88/5 Cells Grown on Chamber Slides

L87/4 and L88/5 stromal cells were grown to 70% confluency in Iscove's/10% FCS medium in chamber slides. Slides were fixed with 4% paraformaldehyde for 20 min and digested with proteinase K (1:1,000, Quiagen) for 15 min at room temperature. Unspecific antibody-binding sites were blocked by 50 min incubation in 20% FCS at room temperature. Binding of primary antibodies (table 1) was performed at 4°C overnight. Antibody binding was vizualized by the ABC-peroxidase method (Vector, Burlingame, Calif., USA) as described by the manufacturer. As positive controls for the primary antibodies, the SaOS-2 osteosarcoma cell line as well as primary cultures from human middle ear bone were analyzed. As negative controls primary endothelial cells, primary fibroblasts and CHO cells were used.

Table 1. Antibodies against osteogenic markers

Epitope	Manufacturer	Dilution
Osteocalcin	Biotrend	1:400
Collagen I	Quartett	1:50
Collagen II	Quartett	1:50
Collagen III	Quartett	1:75
Collagen V	Quartett	1:100

Results

Surface Expression of the Mesenchymal Stem Cell Markers in Human Stromal Cell Lines L87/4 and L88/5

To characterize the mesenchymal phenotype of L87/4 and L88/5 stromal cells, we analyzed the surface expression of known mesenchymal markers by FACS analysis. For the generation of these data both cell lines were grown in monolayer cultures without stimuli. As shown in table 2, L87/4 and L88/5 cells did not express CD50, a marker which has been described for stromal components giving rise to hematopoietic progeny, while CD50 is not found on mesenchymal progenitors. Quiescent L87/4 and L88/5 cells did not express typical differentiation markers like Mac1 (CD11b), CD34, or HLA-DR. Both cell lines were positive for the adhesion molecules ICAM-1 (CD54), LFA-3 (CD58) and HCAM (CD44), molecules known to be important for the interaction of mesenchymal stem cells with hematopoietic progenitors (table 2). According to their colony forming potential in soft agar [8], L87/4 and L88/5 cells expressed the surface markers CD10, CD13, and the CD29/CD49 family of integrins (table 2) which are characteristically found on CFU-F precursor cells.

Additionally, both cell lines produced a variety of growth factors (Il-1, IL-6, IL-7, IL-8, IL-11, LIF, M-CSF, SCF, G-CSF, GM-CSF) known to be essential for a function as feeder layers in long-term cultures of hematopoietic cells [7].

Osteogenic Potential of L87/4 and L88/5 Cell Lines

In order to analyze the mesenchymal differentiation potential of L87/4 and L88/5 stromal cells, we first performed RT-PCR reactions specific for the osteogenic marker genes osteocalcin and osteopontin. As shown in figure 1, both cell lines expressed osteocalcin as well as osteopontin mRNA.

Table 2. Immunophenotype of L87/4 and L88/5 cells

	L87/4	L88/5
Miscellaneous antigens		
CD10	++	+
CD13	+++	++
CD33, CD34, CD36, CD38, CD56	–	–
CD68	+	–
CD71	+++	++
HLKA-DR, glycophorin A	–	–
Fc-receptors		
CD16, CD23, CD32, CD64	–	–
Adhesion molecules		
CD11a, CD11b, CD11c, CD18	–	–
CD29	+++	+++
CD44	+++	+++
CD49b	++	+
CD49d	++	–
CD49e	+++	+++
CD49f	+++	+++
CD54	+++	++
CD58	+++	+++
CD50, CD61, CD62E, CD102, CD106	–	–

Osteocalcin protein expression was demonstrated by immunohistochemistry. As positive control we used the osteocalcin-expressing human osteosarcoma cell line SaOS-2 and primary cultures from middle ear bones. Depending on culture conditions, varying percentages of L87/4 and L88/5 cells stained positive for osteocalcin (fig. 2).

Cells belonging to the osteogenic differentiation pathway are expected to express collagens I and V while being negative for collagens II and III. To characterize the osteogenic potential of L87/4 and L88/5 stromal cells, we analyzed the expression of collagen I, II, III, and V immunohistochemically. To generate these data, the cell lines were grown in monolayers on chamber slides without any stimuli. The osteosarcoma cell line SaOS-2 as well as primary cultures of middle ear bones served as positive controls. As expected, SaOS-2 cells (fig. 3) and primary osteogenic cells stained positive for collagens I and V, while they did not show any signal for collagens II and III. Analyzed with these highly specific antibodies, L87/4 and L88/5 cells strongly express the typical osteogenic markers collagen I and V, while we did not get any signal on the cells with antibodies against collagens II and III (fig. 3).

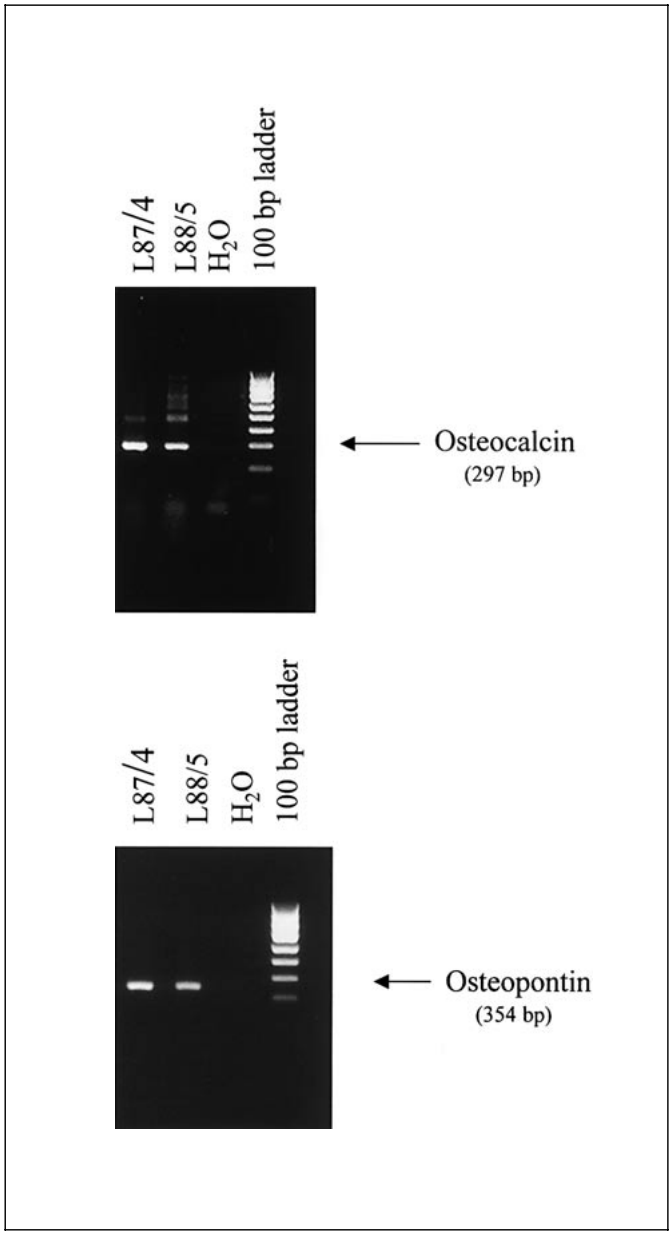

Fig. 1. Osteocalcin and osteopontin expresssion in L87/4 and L88/5 cells. 1 μg of total RNA of L87/4 and L88/5 cells was analyzed by RT-PCR using primers against osteocalcin and osteopontin. PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining.

Organ Distribution of L87/4 and L88/5 Cells in NOD/SCID Mice

Accumulating experimental evidence shows that mesenchymal stem cells are able to differentiate into a variety of organ-specific cell types after transplantation into NOD/SCID mice. To analyze the capacity of organ-specific homing and differentiation of L87/4 and L88/5 cells, we transplanted both cell lines into irradiated NOD/SCID mice. Organs of the transplanted mice were collected 3, 6 and 10 weeks posttransplantation, embedded into paraffin and stained immunohistochemically. To show the differentiation status of the human transplants in murine tissue background, we used a human-specific antibody against osteocalcin. Since osteocalcin-positive cells seemed to persist at an extremely low number, we also stained the slides with an antibody against SV40 large T antigen as an additional specific marker for the transplanted human cells. We could show that up to 10 weeks posttransplantation both cell lines persisted in the bone marrow, the brain, the liver, the thymus and the gut of the transplanted animals (fig. 4).

Discussion

The two well-established human stromal cell lines L87/4 and L88/5 are commonly used to support human hematopoietic cells in vitro [7, 8]. Some years ago, it was already reported that STRO-1^+ adult human bone marrow contains osteogenic precursors [9]. To test the hypothesis of a common population of stromal, mesenchymal stem cells and even hematopoiesis, we investigated the osteogenic potential of the two stromal cell lines mentioned above. The phenotypic analysis revealed an expression pattern common of established stromal cell lines, but, additionally, the expression of osteogenic markers such as osteocalcin and osteopontin in a certain number of the stromal cells [10, 11]. Expression of the osteogenic markers increased over time in culture, especially in cells growing in clusters. Both cell lines, L87/4 and L88/5, expressed type I and type V collagen, indicative of bone precursors [12, 13], while they did not show any expression of types II and III collagens. This is suggestive for traditional stromal cells having the potential to function as osteogenic progenitors. After transplantation into NOD/SCID mice, human stromal cells also persisted in various mesenchymal organs. Human osteocalcin-positive L87/4 and L88/5 cells were not only found in capillary vessels of the lung, liver and bone marrow, but were also well suited in the thymus and the central nervous system. Therefore, human bone marrow-derived stromal cells have to be considered mesenchymal precursors which can persist in various organ sites of the recipient animals. The importance of these data becomes clear in view of the current discussion about the plasticity of stem cells [14], since these pluripotent stem cells can be isolated from various tissues

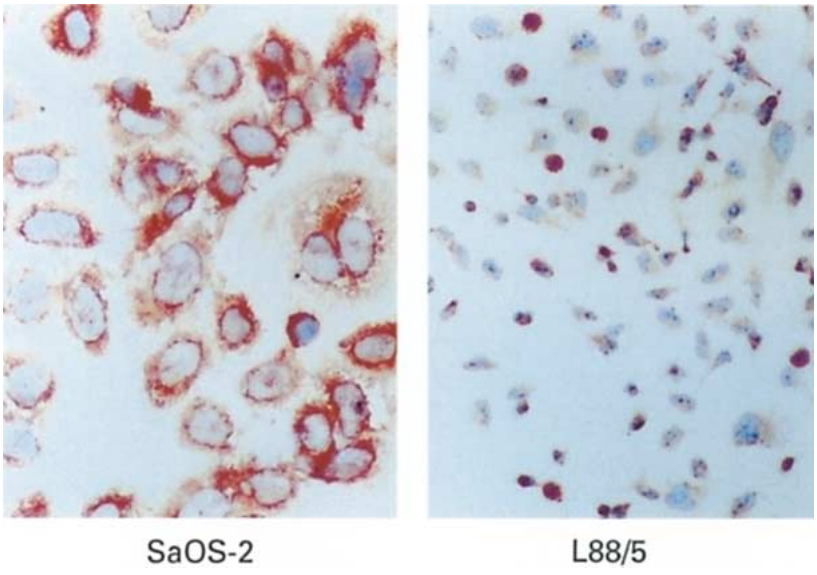

Fig. 2. Immunohistochemical staining of osteocalcin in SaOS-2 and L88/5 cells *SaOS-2* and L8875 cells were grown on chamber slides, fixed, air-dried and incubated with a polyclonal antibody against osteocalcin. Antibody binding was visualized by the ABC-peroxidase method. A high percentage (>80%) of SaOS-2 cells were positive for osteocalcin, while L88/5 cells showed variable protein expression, depending on the cell cycle status.

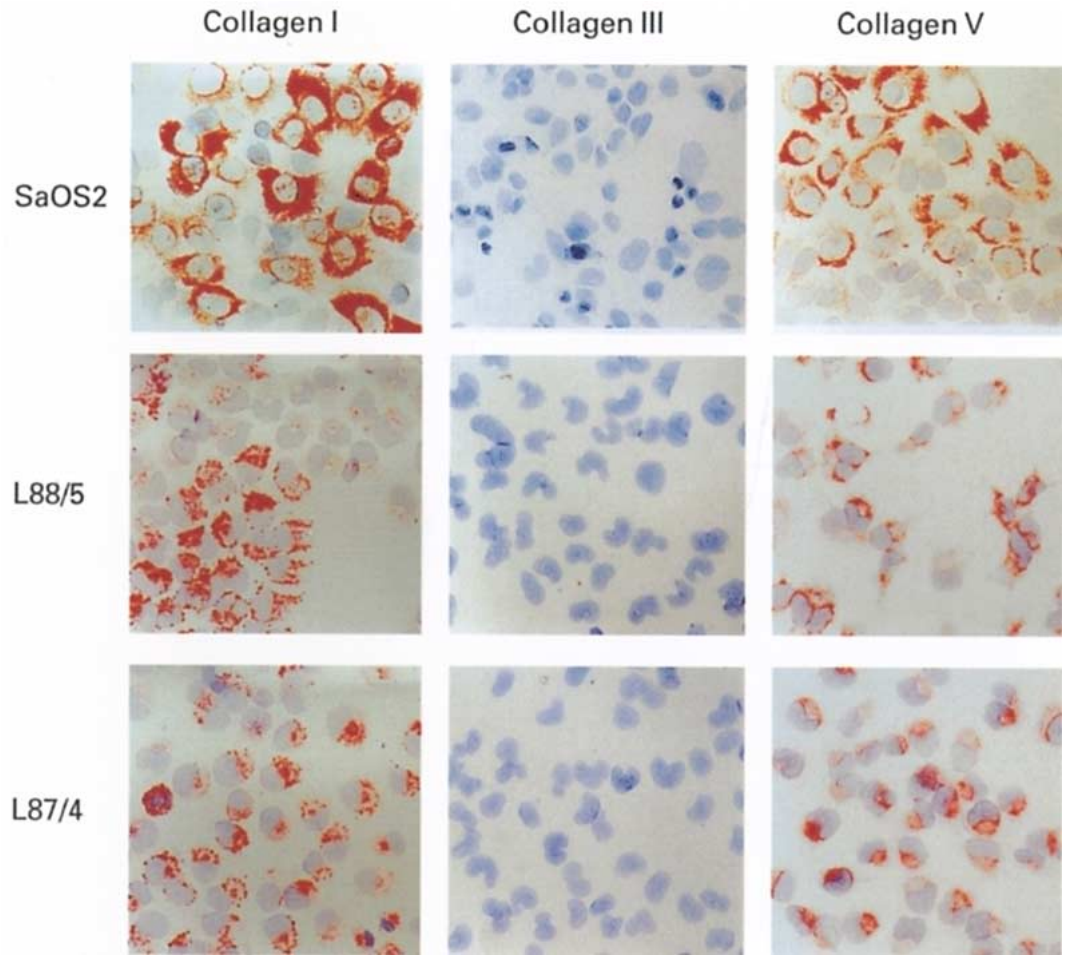

Fig. 3. Collagen expression of SaOS-2, L87/4 and L88/5 cells SaOS-2, L87/4 and L88/5 cells were grown in monolayer cultures on chamber slides, fixed in paraformaldehyde and air-dried. Cells were incubated with antibodies against collagens I, II, III and V and binding was visualized by the ABC-peroxidase method. All three cell lines showed a collagen expression pattern typical for cells of the osteogenic differentiation pathway, positive for collagens I and V, negative for collagens II and III.

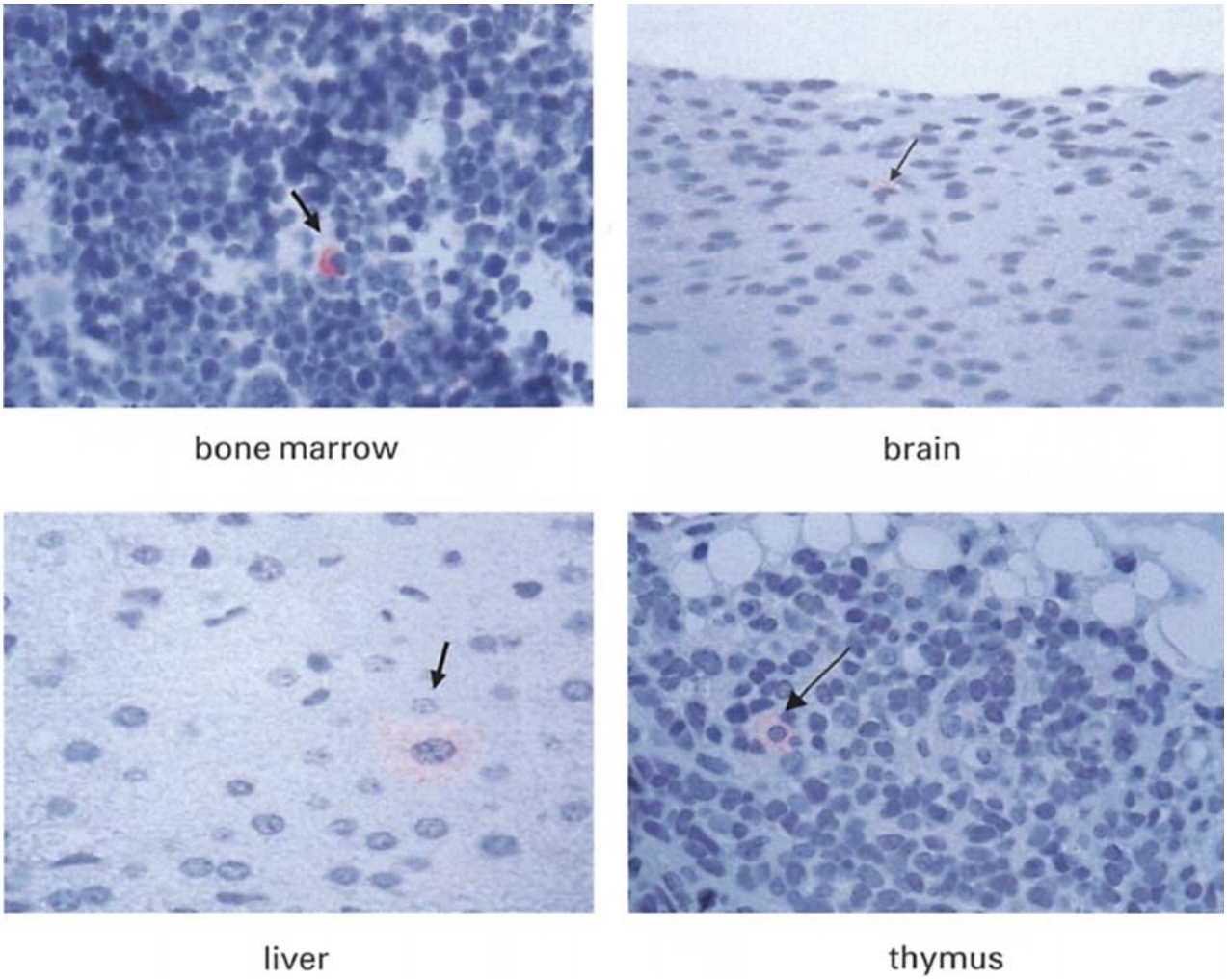

Fig. 4. Immunohistochemical detection of transplanted L87/4 cells in different tissues of NOD/SCID mice. NOD/SCID mice were transplanted with L87/4 cells and sacrificed 10 weeks posttransplantation. Murine tissues were imbedded into paraffin, sectioned and analyzed immunohistochemically. As indicated above (arrow), single osteocalcin-positive L87/4 cells were present in the bone marrow, thymus, liver and brain of the transplanted animals

and even peripheral blood where they exist as circulating stem cells [15–17].

As of yet, hematopoietic and repopulating stem cells have been identified by surface expression of CD34 antigen. There were some publications in recent years demonstrating that CD34-negative cells can also reconstitute the complete lympho-hematopoietic system of mice and sheep [18–22]. Although evidence is controversial , stromal cells as well as mesenchymal stem cells are considered to be CD34-negative [23]. Our own experiments showed that CD34-positive hematopoietic progenitors can be generated from CD34-negative, stroma-like precursors [24]. In culture, approximately 0.1% of the L87/4 and L88/5 stromal cells express CD34 even spontaneously [unpubl.]. The phenotype of a mesenchymal cell line seems to depend on cell culture conditions. Grown in monolayer cultures, our two cell lines do not express CD50 and CD34 while they are positive for CD44, CD49e and CD49f. This expression pattern is consistent with the phenotype of described mesenchymal cells [1]. According to other reports, mesenchymal stem cells express CD106 (VCAM), but are negative for CD49d. VCAM was not found in either cell line, L87/4 and L88/5, but the former was positive for CD49d. Depending on cell culture condi-

tions, different phenotypes of mesenchymal precursor cells could be observed, although the cell lines are morphologically and functionally closely related.

Recent reports suggested the clinical use of mesenchymal stem cells isolated from bone marrow [25]. Our results provide further evidence that even marrow stromal cell lines can be used to generate distinct mesenchymal organ tissue such as bone tissue, depending on cell culture conditions. Most of the growth factors which play a pivotal role in directing differentiation of quiescent stem cells towards specialization are produced in an autocrine and paracrine fashion by stromal cells [7, 26]. Therefore, stromal cells might be the essential link between mesenchymal stem cells and quiescent hematopoietic progenitors which we could identify as CD34-negative, fibroblast-like cells [27, 28]. It is even probable that there is a common progenitor cell to all three cell types, the function of which is determined by the ultimate demand within an organism.

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