Fates of blood:
Studies on stem cell differentiation potential
and B lymphocyte generation
in chimeric cattle

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Academic dissertation

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“Omnis cellula e cellula.”

Rudolf Virchow and Robert Remak, 1855
Abstract

Stem cells exist in most adult tissues. Some of these somatic stem cells may be more plastic than expected, not limited to generating new cells for the tissue of their origin. While such findings suggest a revolution in regenerative medicine, they remain controversial. Most stem cell research is based on transplantation experiments of isolated and often cultured cells in mice, and the results may be affected by the manipulation of the animals and the transplanted cells.

Here, naturally chimeric twin cattle were used to investigate the differentiation potential of stem cells in a non-manipulated large mammal. Due to conjoined placental circulations, blood of the bovine twins is mixed for most of the fetal period, and circulating stem cells are effectively exchanged. We developed powerful methods for tracing their progeny in various tissues of freemartin cattle, females born as a twin to a bull. While from 10% to 90% of the hematopoietic system in freemartins was donor-derived, donor contribution to non-hematopoietic tissues was in most cases minor. Thus, hematopoietic stem cells and other cell types circulating in fetal blood do not generate significant numbers of non-hematopoietic cells in the development, growth and physiological turnover of bovine tissues. However, they may be important in tissue repair and regeneration, as suggested by increasing numbers of donor-derived cells in newly forming granulation tissue.

Chimeric cattle were also used to investigate the generation of bovine B lymphocytes, which occurs differently than in the commonly studied human and mouse, and is poorly understood. The results indicate that the ileal Peyer’s patch determines the peripheral B cell pool in young cattle, and is likely responsible for the production of the preimmune antibody repertoire by post-rearrangement strategies.

To facilitate a direct analysis of bovine stem cells, the first antibodies against bovine CD34 were generated. The CD34 glycoprotein is commonly used as a marker for hematopoietic progenitors and endothelial cells in the human and mouse. CD34 mRNA was found to be alternatively spliced and widely expressed in cattle tissues. Using the new antibody, the protein was detected in most blood vessel endothelia, primitive hematopoietic cells and some non-hematopoietic cell types.
Acknowledgements

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Helsinki, 6th December 2006

[Signature]
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1. List of original publications

This thesis is based on the following publications:


These publications are referred to in the text by their Roman numerals. Some unpublished material is presented (V).

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### 2. Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>Bmi1</td>
<td>B lymphoma Mo-MLV insertion region 1 (an oncogene)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD34</td>
<td>hematopoietic progenitor antigen CD34</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>complementarity-determining region</td>
</tr>
<tr>
<td>CSF3</td>
<td>colony-stimulating factor 3 (synonym: G-CSF, granulocyte colony-stimulating factor)</td>
</tr>
<tr>
<td>CXCL4</td>
<td>chemokine (C-X-C motif)</td>
</tr>
<tr>
<td>DIG</td>
<td>digoxigenin</td>
</tr>
<tr>
<td>DNP</td>
<td>dinitrophenyl</td>
</tr>
<tr>
<td>F&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>antibody fragment containing the antibody-binding regions</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FM</td>
<td>freemartin</td>
</tr>
<tr>
<td>GALT</td>
<td>gut-associated lymphoid tissue(s)</td>
</tr>
<tr>
<td>GSL</td>
<td><em>Griffonia simplicifolia</em> lectin</td>
</tr>
<tr>
<td>HSC</td>
<td>hematopoietic stem cell</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin (antibody)</td>
</tr>
<tr>
<td>IgH</td>
<td>immunoglobulin heavy chain</td>
</tr>
<tr>
<td>IgM</td>
<td>a class of immunoglobulins forming pentamers (macroglobulins)</td>
</tr>
<tr>
<td>IH</td>
<td>immunohistochemistry</td>
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<tr>
<td>ISH</td>
<td>in situ hybridization</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KDR</td>
<td>kinase insert domain receptor (synonym: VEGFR2, vascular endothelial growth factor receptor 2)</td>
</tr>
<tr>
<td>Kit&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>expressing high levels of the kit oncogene</td>
</tr>
<tr>
<td>lin&lt;sup&gt;+&lt;/sup&gt;</td>
<td>lineage negative; depleted of lineage commitment markers</td>
</tr>
<tr>
<td>lin&lt;sup&gt;lo&lt;/sup&gt;</td>
<td>expressing low levels of lineage commitment markers</td>
</tr>
<tr>
<td>LSK</td>
<td>the lin&lt;sup&gt;lo&lt;/sup&gt; Sca1&lt;sup&gt;+&lt;/sup&gt; Kit&lt;sup&gt;hi&lt;/sup&gt; fraction</td>
</tr>
<tr>
<td>LT-HSC</td>
<td>long-term repopulating hematopoietic stem cell</td>
</tr>
<tr>
<td>MACS</td>
<td>magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MAPC</td>
<td>multipotent adult progenitor cell</td>
</tr>
<tr>
<td>ML-I</td>
<td>mistletoe lectin I</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>mesenchymal stem cell</td>
</tr>
<tr>
<td>NBT/BCIP</td>
<td>nitroblue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline (a buffer solution)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer's patch</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog, a tumour suppressor</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription – PCR</td>
</tr>
<tr>
<td>Sca1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>stem cell antigen-1 positive</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SLAM</td>
<td>signalling lymphocyte activation molecule</td>
</tr>
<tr>
<td>SP</td>
<td>side population (effluxing the Hoechst dye 33342)</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate (a buffer solution)</td>
</tr>
<tr>
<td>SSPE</td>
<td>saline-sodium phosphate-EDTA (a buffer solution)</td>
</tr>
<tr>
<td>ST-HSC</td>
<td>short-term repopulating hematopoietic stem cell</td>
</tr>
<tr>
<td>Tek</td>
<td>endothelial-specific receptor tyrosine kinase (synonym: Tie2)</td>
</tr>
<tr>
<td>Y&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Y chromosome positive</td>
</tr>
</tbody>
</table>
3. Introduction

Stem cells have been a focus of intense research and publicity for the last decade. They are changing our understanding of development, physiology and pathophysiology (Joseph and Morrison, 2005; Clarke and Fuller, 2006). In addition to the embryo, adult tissues also contain stem cells; even the central nervous system is continuously renewed by them, contrary to long-held dogma (Gross, 2000). Intriguingly, some of these somatic stem cells may be more plastic than expected, not limited to generating cells for the tissue of their origin (Blau et al., 2001). Such discoveries suggest a revolution in regenerative medicine, stimulating public interest (Weissman, 2000). Yet, stem cells are also a controversial issue in many ways. Some of the exciting findings have been problematic to reproduce (Goodell, 2003). In the research models typically used, the manipulation of cells and animals may affect the results. The interpretation of experimental data is further complicated by poorly understood biological processes like cell fusion (Wagers and Weissman, 2004).

The present work aimed to dissect the differentiation potential of stem cells in a large, long-lived mammal, where tissue maintenance and regenerative processes might differ from the commonly studied small rodents. Cattle twins provide a unique chimeric model without artificial manipulation. Blood of bovine twins is mixed for most of the fetal period, and circulating progenitors, including hematopoietic stem cells, are effectively exchanged (Lillie, 1917; Owen, 1945). The progeny of donor-derived cells may then be traced by genetic markers (Wilkes et al., 1981). However, technical limitations have previously prevented an effective analysis of their contribution to non-hematopoietic tissues. We have now developed methods making this possible.

Chimeric cattle also were instrumental in early immunological research (Brent, 1997). Here, they offered a view on the generation and maintenance of the bovine B lymphocyte pool, a process strikingly different from that of the more commonly studied mouse and poorly understood.

In this thesis, I review the current literature on somatic stem cells, cattle twins, and the production of ruminant B lymphocytes. I then present our research on the fates of circulating cells, on the generation of B cells in cattle and on the expression of the hematopoietic progenitor antigen CD34 in bovine tissues.
4. Review of the literature

4.1. Somatic stem cells

4.1.1. “The stem cell is the origin of life”

A stem cell is defined as being capable of self-renewal and multilineage differentiation on a single-cell basis (Weissman, 2000). The zygote, the fertilized egg, is the ultimate stem cell; it is totipotent, able to generate all cells of the body, as well as the placenta and the fetal membranes (Harvey, 1651; Seidel, 1952).

The early mammalian embryo forms a hollow sphere of cells, and in most species an inner cell mass is positioned on one side of the ring (Eakin and Behringer, 2004). These inner cells are pluripotent; each may produce any cell of the body, but not the placental structures (Gardner and Rossant, 1979). They can be isolated and cultured as embryonic stem cells, which are able to proliferate indefinitely, and can be induced to differentiate into specific cell types (Evans and Kaufman, 1981).

As the development of the individual proceeds, cells gradually differentiate, losing potential and acquiring specialized functions. Our bodies mostly consist of terminally differentiated cells, such as mature neurons, erythrocytes, or keratinocytes of the skin, which are unable to proliferate (Lipinski and Jacks, 1999). Yet, stem cells also exist in adult mammals. Lost cells are in many tissues continuously replaced by somatic stem cells (Fuchs and Segre, 2000). These typically are tissue-specific, generating cells for the tissue in which they reside. Somatic stem cells are therefore considered multipotent, capable of generating several cell types, oligopotent or even unipotent, depending on the case (Sell, 2004).

The actual stem cells typically cycle relatively rarely (Cheshier et al., 1999; Bickenbach and Mackenzie, 1984). An asymmetric cell division renews the stem cell and creates a committed daughter (Ho, 2005). The daughter cell has restricted self-renewal capacity and potency, but often proliferates rapidly before the terminal differentiation. These progenitor or precursor cells produce the huge numbers of new cells needed for tissue maintenance, such as an estimated trillion blood cells daily in man (Akashi et al., 2000; Raff, 2003; Ogawa, 1993). Therefore, they are also called transit amplifiers.
A stem cell is the beginning of an individual, and stem cells renew the tissues throughout life. Thus, the stem cell indeed is the origin of life, as phrased poetically by Sell (2004). It can also be an origin of death: cancer appears to be largely a stem cell disease (Clarke and Fuller, 2006), and stem cells may have a major role in aging (van Zant and Liang, 2003). In this review, however, I focus on somatic stem cells in a healthy animal. I introduce some of the best characterized stem cell types and methods for identifying or enriching them. Evidence of somatic stem cell plasticity and currently emerging general principles in stem cell biology are discussed.

4.1.2. Hematopoietic stem cells

Hematopoietic stem cells (HSCs) are the most studied stem cells, and widely used in the clinic for treatment of leukemias, other cancers, and congenital immunodeficiencies (Kondo et al., 2003). Their existence was proposed already in 1906 by Alexander Maximov, in his hematopoietic theory (Maximow, 1906). Proof arrived decades later, when the use of nuclear weapons inspired the investigations of radiation effects on animals, and HSC failure was shown to be a cause of radiation death (Weissman, 2000). Lethally irradiated animals could be rescued by spleen or bone marrow transplants from normal animals (Jacobson et al., 1951; Lorenz et al., 1952; Nowell et al., 1956; Ford et al., 1956). These were soon shown to contain multipotent hematopoietic progenitors (Till and McCulloch, 1961; Becker et al., 1963).

HSC is a multipotent somatic stem cell: a single HSC is able to generate all blood cell lineages (Osawa et al., 1996). A subset of HSCs is capable of life-long self-renewal, and these are called long-term hematopoietic stem cells (LT-HSCs; Jones et al., 1990; Smith et al., 1991). They give rise to short-term HSCs (ST-HSCs), which are also multipotent but have a reduced self-renewal potential (Smith et al., 1991; Osawa et al., 1996; Morrison et al., 1997). ST-HSCs generate non-renewing multipotent progenitors (Morrison et al., 1997) or, according to a recent alternative model (Fig. 1), common myeloid progenitors and lymphoid-primed multipotent progenitors lacking significant megakaryocyte and erythrocyte potentials (Adolfsson et al., 2005). Mature blood cell lineages are finally produced through further committed progenitor stages.
Figure 1. Two proposed models for hematopoietic lineage development.

For brevity, the final stages are omitted from the alternative model. LT-HSC: long-term HSC; ST-HSC: short-term HSC; MPP: multipotent progenitor; LMPP: lymphoid-primed multipotent progenitor; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; GMP: granulocyte/macrophage progenitor; MkEP: megakaryocyte/erythroid progenitor; B: B cell; T: T cell; NK: natural killer cell. Based on Adolfsson et al. (2005).

Hematopoietic stem cells can be enriched by fluorescence-activated cell sorting (FACS), using monoclonal antibodies against cell surface antigens (Muller-Sieburg et al., 1986). In addition, HSCs in several species actively efflux certain fluorescent dyes, allowing isolation of the so-called side population (SP), highly enriched for LT-HSCs (Goodell et al., 1996; Goodell et al., 1997; Zhou et al., 2001b). Some of these strategies are summarized in Table I. The markers in use are, however, neither directly related to stem cell-like properties nor completely stem cell-specific (Zipori, 2004). Definitive proof for the “stemness” of an isolated cell population is provided by functional assays. Long-term self-renewal and complete blood reconstitution capacity are difficult to analyse in vitro, as the hematopoietic microenvironment is poorly understood. While
Somatic stem cells

several in vitro and xenograft models have been developed, only allograft transplantation of candidate stem cell populations to lethally irradiated recipients is considered an unequivocal test (Kondo et al., 2003). HSCs have thus been most thoroughly characterized in mice. Even there, the precise phenotypes of the various hematopoietic cell populations, and subsequently also the hematopoietic fate map, remain somewhat uncertain, as shown in Fig. 1. In mammals other than mouse and man, HSC phenotypes are poorly characterized, but they have been enriched using lineage depletion (Leon et al., 2005) or Kit positivity (Le Guern et al., 2003).

In the developing embryo, the hematopoietic and cardiovascular organ systems are the first to appear, being essential beyond the early postimplantation period (Copp, 1995). The earliest hematopoietic and endothelial progenitors are detected in the yolk sac, at embryonic day 7.25 in the mouse (Ferkowicz and Yoder, 2005). They appear to have a common precursor, the hemangioblast (Huber et al., 2004; Bailey et al., 2004). The yolk sac first produces primitive erythrocytes, which are initially nucleated and express embryonic globins; later, it also generates hematopoietic progenitors and erythrocytes of the adult type (McGrath and Palis, 2005). In the embryo proper, definitive HSCs are generated in the aorta-gonad-mesonephros region from day 10.5 (Dieterlen-Liévre and Martin, 1981; Medvinsky and Dzierzak, 1996; de Bruijn et al., 2000); the placenta has recently been recognized as another significant source (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). HSCs soon colonize the liver, which becomes the main hematopoietic organ for most of the fetal life (Johnson and Moore, 1975; Houssaint, 1981; Morrison et al., 1995; Ema and Nakauchi, 2000). Bone marrow is seeded by circulating LT-HSCs only shortly before birth, at day 17.5, migrating in a chemotactic response to the stromal cell-derived chemokine CXCL12 and the kit ligand (Christensen et al., 2004). In the mouse, at least, the seeding occurs gradually, with low numbers of HSCs constitutively circulating in the fetal bloodstream. Engraftment to the marrow is dependent on the calcium-sensing receptor, suggesting that it is guided by the high extracellular calcium concentrations associated with bone modelling (Adams et al., 2006).

In larger mammals, the timing of the changes in the hematopoietic environment is somewhat different. Hematopoietic activity is seen in the bone marrow from 10-15 weeks' gestation in human fetuses (Charbord et al., 1996) and from the 4th month in cattle (Rüsse, 1991).
**Table 1.** Some strategies for enrichment of adult hematopoietic stem cells.

The marker profile changes during ontogeny. For phenotypes of embryonic and fetal HSCs, see Mikkola and Orkin (2006).

<table>
<thead>
<tr>
<th>Marker(s)</th>
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<tr>
<td>CD34</td>
<td>Transmembrane glycoprotein commonly used as a marker to enrich human HSCs for transplantation (Berenson et al., 1991; Kondo et al., 2003). In murine HSCs, the expression is variable (Ito et al., 2000). Human and murine CD34 expression may be differently regulated (Okuno et al., 2002), but CD34+ HSCs also exist in the human (Zanjani et al., 1998; Bhatia et al., 1998). The CD34+ fraction is very heterogeneous and contains large numbers of committed progenitors (Bhatia et al., 1997).</td>
</tr>
<tr>
<td>CD34+KDR+</td>
<td>Highest achieved purity of repopulating human HSCs, about 20% (Ziegler et al., 1999).</td>
</tr>
<tr>
<td>CD133 (prominin-1)</td>
<td>A recently published marker possibly unifying human CD34+ and CD34- HSCs (Gallacher et al., 2000).</td>
</tr>
<tr>
<td>lin−/lo Sca1+ Kithi</td>
<td>Also called the LSK fraction. Contains virtually all murine HSC activity; represents approximately 0.05-0.1% of all bone marrow cells (Spangrude et al., 1988; Ikuta and Weissman, 1992). The population is heterogeneous, and only about 20% of the cells are capable of long-term reconstitution after single-cell transplantation (Osawa et al., 1996).</td>
</tr>
<tr>
<td>Side population (SP)</td>
<td>Cells that actively efflux the vital dye Hoechst 33342 by ABC transporters (Zhou et al., 2001b). This property allows in several species the isolation of a population that is enriched for LT-HSCs (Goodell et al., 1997). In the mouse, the SP is largely lin−/lo Sca1+ and about 1000-fold enriched for LT-HSCs (Goodell et al., 1996). Dye toxicity prevents clinical use (Durand and Olive, 1982).</td>
</tr>
<tr>
<td>Tip-SP CD34−LSK</td>
<td>Cells with strongest dye efflux activity, further selected for a CD34−LSK phenotype. These are practically pure LT-HSCs in the mouse (Matsuzaki et al., 2004). The fraction represents only 0.001-0.01% of marrow mononuclear cells, and possibly not all murine LT-HSCs are confined to this very rare subset.</td>
</tr>
<tr>
<td>CD150+CD48−</td>
<td>A recently published simple isolation strategy for murine HSCs, based on the expression pattern of various SLAM family cell surface receptors (Kiel et al., 2005). Allows enrichment efficiency comparable to LSK. In combination with CD41− selection, almost half of the population is capable of long-term reconstitution as single cells.</td>
</tr>
</tbody>
</table>

The hematopoietic microenvironment of the bone marrow is only beginning to be understood. HSCs are thought to lodge primarily in two specific marrow niches. The most dormant stem cells are located on endosteal surfaces (Gong, 1978; Zhang et al., 2003; Arai et al., 2004). They are anchored via N-cadherin to osteoblasts, which regulate the number and function of HSCs (Zhang et al., 2003; Arai et al., 2004; Calvi et
Somatic stem cells

HSCs are also found in association with the marrow sinusoidal endothelium (Kiel et al., 2005), where they proliferate more actively (Wilson and Trumpp, 2006). Small numbers of HSCs are normally present in the circulation, quickly migrating through blood and exiting within minutes (Goodman and Hodgson, 1962; Wright et al., 2001). This may serve to maintain hematopoietic homeostasis by distributing HSCs to unoccupied niches, but could also be a means to delete unwanted stem cells (Abkowitz et al., 2003). Rare HSCs can also be found in most other organs (Asakura and Rudnicki, 2002; Kotton et al., 2005; McKinney-Freeman et al., 2002).

Most HSCs in adult bone marrow are quiescent at any one time (Cheshier et al., 1999; Kiel et al., 2005). Yet, they are regularly recruited to cycle and can be rapidly activated and mobilized in response to stress or injury (Wilson and Trumpp, 2006). The hematopoietic cytokine CSF3 is commonly used in the clinic to mobilize HSCs to blood for transplantation; interestingly, the process appears to be regulated by the sympathetic nervous system (Katayama et al., 2006). Membrane-bound kit ligand expressed by osteoblasts is essential for long-term maintenance of HSC activity (Lyman and Jacobsen, 1998). Osteoblasts maintain stem cell quiescence through Tek/angiopoietin-1 signalling (Arai et al., 2004).

HSCs have been shown to generate differentiating progeny by asymmetric cell division (Suda et al., 1984; Takano et al., 2004; Giebel et al., 2006), but the mechanisms inducing the asymmetry are not understood (Wilson and Trumpp, 2006). Notch and Wnt signalling are thought to promote self-renewal and inhibit differentiation in an integrated manner (Duncan et al., 2005; Reya and Clevers, 2005; Maillard et al., 2003). Also the transcription factors HoxB4 and c-myc and the tumour suppressor PTEN have been implicated as important regulators of HSC renewal and differentiation (Antonchuk et al., 2002; Wilson et al., 2004; Zhang et al., 2006b).

4.1.3. Mesenchymal stem cells

Bone marrow also contains a non-hematopoietic stem cell system, the mesenchymal stem cells (MSCs; Prockop, 1997; Väänänen, 2005). Cells generating clones of mesenchymal lineages (fibroblasts, osteoblasts, chondroblasts and adipocytes) can be enriched by simple plastic adhesion from the marrow and several other tissues (Friedenstein et al., 1970; Friedenstein et al., 1987; Kuznetsov et al., 2001; Zuk et al.,
These cells are highly expandable in culture, growing up to 50 population doublings, and multipotent on a single-cell basis (Friedenstein et al., 1987; Colter et al., 2000; Pittenger et al., 1999; de Bari et al., 2006). They are thus regarded as stem cells, although their stem-like properties have not been assayed as rigorously as those of HSCs (Javazon et al., 2004). MSCs are poorly characterized and little is known of their in vivo biology; they are of nevertheless of considerable clinical interest, due to the favourable in vitro properties (Väänänen, 2005).

MSCs differ from HSCs in several ways. Turnover of mesenchymal tissues is very slow compared with blood; thus, the production of differentiated cells is limited, and also the self-renewal capacity of MSCs in vivo is poorly elucidated (Dennis and Charbord, 2002). MSC lineages appear to be less strictly defined than hematopoietic lineages. The commitment to a specific blood cell lineage is thought to be irreversible under normal conditions (Akashi et al., 2000). Fully differentiated osteoblasts, adipocytes and chondrocytes, by contrast, may transdifferentiate to other mesenchymal lineages (Song and Tuan, 2004). On the other hand, MSCs and HSCs may be functionally connected. MSCs produce hematopoiesis-supporting stroma (Dennis and Charbord, 2002) and colocalize with HSCs throughout ontogeny (Mendes et al., 2005). MSCs are found in the fetal circulation, suggesting that they might migrate through blood like HSCs (Campagnoli et al., 2001).

No specific markers for MSCs are known, although they are generally negative for hematopoietic and endothelial markers, and can be enriched by integrin α1 positivity or using the monoclonal antibody STRO-1 (Deschaseaux and Charbord, 2000; Simmons and Torok-Storb, 1991; Stewart et al., 2003; Gronthos et al., 2003; Javazon et al., 2004). Therefore, MSCs are primarily identified by their in vitro differentiation capacity, and all MSC populations investigated are heterogeneous (Javazon et al., 2004). MSC cultures can be fractionated by FACS scatter analysis to small rapidly renewing cells, which are highly clonogenic, and larger, granular, slowly proliferating cells (Colter et al., 2000; Smith et al., 2004).

Recently, progenitor cells capable of generating mesenchymal and other lineages have been isolated from bone marrow and other tissues (Reyes and Verfaillie, 2001; Jiang et al., 2002a; Jiang et al., 2002b). These multipotent adult progenitors (MAPCs) were obtained by prolonged culture of cells depleted for hematopoietic markers, in a medium supplemented by epidermal growth factor, platelet-derived growth factor BB, and for the murine cells, leukemia inhibitory factor (Jiang et al., 2002a). MAPCs may
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represent a rare subset of MSCs, but could also be a product of the culture conditions used to isolate them.

4.1.4. Epithelial stem cells

Epithelial stem cells have been primarily studied in the rapidly renewing epithelia of the skin and the intestine (Rizvi and Wong, 2005). In contrast to the HSC niches, the microanatomy of these epithelial stem cell systems is well defined, but less is known of their cell biology. The stem cells are typically identified as retaining a label in the chromosomal DNA, introduced by modified nucleotides or histone proteins (Tumbar et al., 2004). In non-stem cells, the label is gradually diluted by DNA replication or is lost by cell death, while stem cells retain the label because of their relative quiescence or, possibly, due to selective chromatin segregation (Potten et al., 2002).

The epidermis is continuously renewed every one to three weeks, depending on species and location (Potten, 1975a; Potten, 1975b). Cells proliferate at the basal layer next to the basement membrane, then move towards the surface, gradually differentiating into dying proteinaceous sacs, which are finally sloughed off (Alonso and Fuchs, 2003). In normal epidermal homeostasis, the tissue is replenished by basal layer stem cells, which probably comprise about 10% of all basal cells, and their progeny, the rapidly proliferating transit amplifying cells (Levy et al., 2005; Ito et al., 2005; Mackenzie, 1997). Still, the most multipotent epidermal stem cells are found in the hair follicles (Fig. 2a; Moore and Lemischka, 2006). The follicle is a complex epidermal appendage, consisting of a permanent upper portion and a cycling lower portion which generates the hair (Alonso and Fuchs, 2003). The bulge region of the outer root sheath, below the sebaceous gland and near the attachment to the arrector pili muscle, is considered to be the follicular stem cell niche (Cotsarelis et al., 1990; Alonso and Fuchs, 2003). A single cultured bulge stem cell is able to generate all epithelial lineages of the hair follicle and sebaceous gland and also transiently contributes to damaged interfollicular epidermis (Blanpain et al., 2004; Claudinot et al., 2005; Levy et al., 2005; Ito et al., 2005). The stem cells are self-renewing. They can be cultivated for more than 120 doublings, theoretically producing far more epithelium than necessary to cover every human being (Rochat et al., 1994). The progeny of a single cultured cell can generate thousands of hair follicles, even in serial transplantation to a secondary recipient (Claudinot et al., 2005; Blanpain et al., 2004).
Figure 2. Epithelial stem cell niches.

a) Multipotent epidermal stem cells in the hair follicle. Based on Reya and Clevers (2005) and Ross (2003).


No specific markers for epidermal stem cells are available, but they can be enriched by α6 and β1 integrin and CD34 (Li et al., 1998; Jones et al., 1995; Trempus et al., 2003). In addition, the bulge area is positive for cytokeratin 15 (Lyle et al., 1998). Highly purified DNA label-retaining cells have been isolated from the basal layer as the Hoechst dye effluxing side population (Dunnwald et al., 2001).

The intestinal epithelium is replaced in a few days by stem cells residing in the intestinal crypts, in a spatially well-defined process (Potten, 1998). An estimated four to six multipotent stem cells, which may be originally monoclonal, are located at a specific position near the base of each crypt (Fig. 2b; Rizvi and Wong, 2005). They generate all four differentiated cell types of the epithelium (Gordon et al., 1992). Progeny of the stem cells migrate up towards the intestinal lumen. They first become
Somatic stem cells

Transit amplifying cells at the upper part of the crypt, then differentiate and stop proliferating, and finally are exfoliated to the lumen (Potten, 1998). Paneth cells in the small intestine follow an opposite course, settling at the crypt bases (Rizvi and Wong, 2005). The villi of the small intestine are covered by sectors of epithelium originating from several neighbouring crypts (Bjerknes and Cheng, 1999).

Studies of the intestinal epithelium have been hindered by the lack of markers. Recently, the RNA binding protein Musashi-1 was reported to label stem and early progenitor cells in the intestine (Potten et al., 2003; Kayahara et al., 2003).

The Wnt signalling cascade is essential in the regulation of epithelial proliferation and differentiation in both the skin and the intestine (Reya and Clevers, 2005). It is required for the maintenance of intestinal crypt progenitors (Korinek et al., 1998), and persistent activation of the pathway is an initiating event in colorectal cancer (Reya and Clevers, 2005). On the other hand, Wnt signals induce the differentiation of Paneth cells (van Es et al., 2005). Through the EphB/ephrin-B system, the Wnt pathway also affects the sorting and positioning of intestinal epithelial cells (Batlle et al., 2002). In the epidermis, Wnt signalling is thought to promote proliferation and hair follicle development (Reya and Clevers, 2005). The bone morphogenetic protein (BMP) signalling pathway is integrated with Wnt to negatively regulate stem cell proliferation and stimulate differentiation (Moore and Lemischka, 2006). Notch signalling is also implicated in the regulation of differentiation in both the epidermis and the intestinal epithelium (Rizvi and Wong, 2005).

4.1.5. Neuronal stem cells

New neurons are continuously produced in the brains of adult vertebrates, contrary to the long-held dogma (Gross, 2000; Garcia-Verdugo et al., 2002). Adult neurogenesis was originally suggested by DNA labelling experiments in the rodent hippocampus (Altman and Das, 1965), and then shown to be associated with vocal learning in songbirds (for a historical review, see Nottebohm, 2004). However, this theory was not generally accepted until the 1990s (Ming and Song, 2005). Multipotent neural stem cells are now known to exist in the hippocampus and in the walls of the lateral ventricles of adult rodents and humans (Fig. 3; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Palmer et al., 1997; Eriksson et al., 1998; Kukekov et al., 1999; Ahn and Joyner, 2005). They are capable of self-renewal and clonal generation of neurons and glia in vitro and
in vivo. In culture, the stem cells typically form floating aggregates of cells called neurospheres (Reynolds and Weiss, 1992). In the hippocampus, new neurons are generated in the subgranular zone of the dentate gyrus and then differentiate into granular neurons (Altman and Das, 1965; Kaplan and Bell, 1984; Bayer, 1982; Stanfield and Trice, 1988). The stem cells in the ventricular walls are located at the subventricular zone, below the ependymal layer (Lois and Alvarez-Buylla, 1993; Chiasson et al., 1999). In rodents, their progeny migrates to the olfactory bulb, along the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Sanai et al., 2004). In other areas of the central nervous system, the generation of new neurons is controversial and species-dependent (Falk and Frisen, 2005; Garcia-Verdugo et al., 2002). The cerebral cortex of healthy adult humans showed no neuronal turnover in an ingenious study applying $^{14}$C dating to cellular DNA (Spalding et al., 2005).

**Figure 3. Neuronal stem cell niches in the murine brain.**
Schematic sagittal view. Based on Uchida (2000) and Sidman et al. (1971).

In both germinal regions, neuronal stem cells are astroglia-like cells expressing glial fibrillary acidic protein (GFAP), a traditional marker of mature astroglia (Doetsch et al., 1999a; Seri et al., 2001; Garcia et al., 2004). They also express nestin (Lendahl et al., 1990) and prominin-1 (Uchida et al., 2000). Yet, labelling of dividing cells remains the principal method for reliably identifying them and their progeny (Ming and Song, 2005).

The neuronal stem cell niches appear to be associated with blood vessels (Doetsch, 2003). Hippocampal neurogenesis occurs around capillaries in proliferative clusters
also containing endothelial precursors (Palmer et al., 2000). In the subventricular zone, the proliferating cells are in contact with specialized extensions of the vascular basal lamina (Mercier et al., 2002), and the stem cells occasionally also extend to the ventricle (Doetsch et al., 1999b).

Neuronal stem cells are thought to generate several stages of transit-amplifying cells, which then differentiate into postmitotic neurons (Alvarez-Buylla et al., 2001; Kempermann et al., 2004). They acquire a neuronal phenotype, express neuron-specific receptor proteins, establish synaptic connections, display typical electrophysiological properties and respond to stimuli (Carlén et al., 2002; van Praag et al., 2002; Song et al., 2002; Belluzzi et al., 2003; Carleton et al., 2003). The new neurons are thought to differentiate in an activity-dependent manner, possibly sensing activities of the local neuronal network even before receiving synaptic innervation (Carleton et al., 2003). The process resembles the neuronal development in the embryo (Esposito et al., 2005).

The functional significance of adult neurogenesis is still unclear, largely due to the extreme complexity of the central nervous system. It seems to be associated with learning and memory, although the process is too slow for acute benefits (Kempermann et al., 2004). Neurogenesis is also stimulated by physical exercise or injury, and possibly reduced in stress and depression (Ming and Song, 2005).

Major developmental signalling pathways regulate stem cells also in the central nervous system (Alvarez-Buylla and Lim, 2004). Wnt signalling is required for neurogenesis, at least in the hippocampus (Lie et al., 2005), whereas Sonic hedgehog and Notch are thought to maintain both stem cell populations (Alvarez-Buylla and Lim, 2004). The tumour suppressors PTEN and p53 inhibit stem cell self-renewal (Groszer et al., 2006; Meletis et al., 2006).

### 4.1.6. Somatic stem cell plasticity

Somatic stem cells are traditionally thought to be restricted to particular fates, producing only cell types found in the tissue of their origin (Lakshmipathy and Verfaillie, 2005; Wagers and Weissman, 2004). This dogma has been challenged by reports of stem cell plasticity, claiming that somatic stem cells are also capable of generating cells of nonrelated tissues. Transplanted bone marrow cells have been reported to produce in vivo skeletal and cardiac muscle (Ferrari et al., 1998; LaBarge and Blau, 2002; Orlic et al., 2001), liver (Petersen et al., 1999; Theise et al., 2000),

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endothelium (Asahara et al., 1999), epithelia (Okamoto et al., 2002; Körbling et al., 2002; Kale et al., 2003), neurons (Mezey et al., 2000; Brazelton et al., 2000; Mezey et al., 2003; Prillier et al., 2001) and even oocytes and male germ cells (Johnson et al., 2005; Nayernia et al., 2006). Of the various bone marrow lineages, purified HSCs (typically defined as the LSK fraction) also seem to contribute to muscle (Corbel et al., 2003; Camargo et al., 2003), liver (Lagasse et al., 2000), endothelium (Grant et al., 2002; Bailey et al., 2004), various epithelia (Krause et al., 2001) as well as astrocytes and oligodendrocytes (Koshizuka et al., 2004). Similarly, MSCs were reported to differentiate into cardiomyocytes (Toma et al., 2002) and astroglia (Kopen et al., 1999), besides the various mesenchymal lineages. In addition to these bone marrow-derived stem cells, MAPCs isolated from several tissues generate hematopoietic cells, various epithelia, and skeletal muscle (Jiang et al., 2002a; Jiang et al., 2002b; Muguruma et al., 2003); skeletal muscle appears to produce hematopoietic cells (Jackson et al., 1999); and neuronal stem cells may give rise to blood (Bjornson et al., 1999), muscle (Galli et al., 2000) and, indeed, in chimeric embryos, to all germ layers (Clarke et al., 2000).

Plasticity of somatic stem cells could revolutionize our understanding of tissue development and maintenance, and a multitude of clinical applications may be envisioned (Herzog et al., 2003). Unfortunately, it has often been problematic to reproduce the reported findings. Bone marrow transplants, purified HSCs and parabiosis partners have repeatedly failed to yield neuronal cells in an intact or injured brain, although their contribution to microglia is remarkable (Castro et al., 2002; Roybon et al., 2006; Massengale et al., 2005). Instead, some of the bone marrow hematopoietic populations were confusingly found to express proteins regarded as neuronal markers. In another study, recipients of single HSCs showed no contribution to most tissues, and only a single donor-derived neuron was detected in more than 13 million total cells examined, despite a robust hematopoietic engraftment (Wagers et al., 2002). HSCs also failed to generate cardiomyocytes (Murry et al., 2004), and no oocytes were derived from bone marrow transplants or long-term parabiosis partners (Eggan et al., 2006). Generally, reported frequencies of bone marrow-derived engraftment vary several orders of magnitude, and are usually low, around 0.5-2% of non-hematopoietic cells in various tissues and experiments (Goodell, 2003). Concerning neuronal stem cells, hematopoietic capacity was undetected in almost $130 \cdot 10^6$ neurosphere cells transplanted to more than 100 recipient mice (Morshead et al., 2002). Hence, the
plasticity of somatic stem cells is clearly a controversial issue (Goodell, 2003; Raff, 2003; Wagers and Weissman, 2004).

The outcomes of the experiments obviously vary depending on the type and status of the transplant recipient. Tissue damage generally promotes engraftment (Petersen et al., 1999; LaBarge and Blau, 2002; Okamoto et al., 2002); this is in many studies intentional, but poorly defined damage is also induced by common transplantation procedures (Prise et al., 2005). The transplanted cells are to a variable degree affected by the manipulation involved in their isolation and possible cultivation (Morshead et al., 2002).

Some of the findings may even be due to detection artefacts; the risk is increased in the analysis of exceedingly rare events. The transplanted cells can be traced in recipient tissues by labels such as the Y chromosome, a detectable genetic polymorphism, or a transgene, typically expressing green fluorescent protein (GFP) or β-galactosidase. Colocalization of the label with various phenotypic markers, most commonly using immunostaining, then reveals the fates of donor-derived cells (Herzog et al., 2003). In ordinary light microscopy, superimposed cells may produce an illusion of marker colocalization (Goodell, 2003). Any labelling method used to track stem cells and their progeny is subject to potential false positives: in situ hybridization to genomic markers could be unspecific, autofluorescence may confuse interpretation of GFP signals and endogenous β-galactosidase activity occurs in some conditions (Herzog et al., 2003). Regarding derivatives of human bone marrow transplants, false positives may be caused by male cells existing in female tissues in the absence of transplantation (Stevens et al., 2004; Khosrotehrani et al., 2004). These may be of fetal origin, but are found quite often even in women who have not given birth to a son (Yan et al., 2005). False negatives may be caused by unstable or low-level transgene expression (Torensma and Figdor, 2004). In addition, antibodies (or even antigens!) used to assay the phenotypes of labelled cells are often not completely specific (Goodell, 2003).

Still, detection artefacts cannot account for all of the observations suggestive of somatic cell plasticity. Confocal microscopy with 3D reconstruction and double staining with hematopoietic markers have been used to resolve potential cell overlays (LaBarge and Blau, 2002; Jiang et al., 2002a). Specificity of labels can be ensured with careful controls or by the verification of results with several different labelling systems (Bailey et al., 2004). Unambiguous morphological properties of cells or functional analysis support the immunostaining data in some works (Priller et al., 2001; Lagasse et
al., 2000). A stepwise progression of bone marrow cells to muscle, in response to various stimuli, has also been documented (LaBarge and Blau, 2002).

Several biological explanations may account for the apparent plasticity, depending on the experimental setup (Fig. 4). These have been directly addressed in few studies (Raff, 2003; Wagers and Weissman, 2004; Lakshmipathy and Verfaillie, 2005).

True plasticity would involve transdifferentiation (Fig. 4a) or dedifferentiation (Fig. 4b) of somatic cells. Transdifferentiation refers to a direct lineage change to a completely different cell type, while dedifferentiation implies a conversion of a tissue-specific cell to a more primitive stem cell, followed by redifferentiation along a new pathway (Wagers and Weissman, 2004). These processes have not been unambiguously documented in mammals (Wagers and Weissman, 2004). Transit-amplifying precursors may be converted to stem cells in the brain (Doetsch et al., 2002) and intestinal epithelium (Potten, 1998); also, HSCs express a multitude of genes related to non-hematopoietic tissues (Akashi et al., 2003). Theoretically, plasticity clearly should be possible. Nuclei of even extremely specialized cells like lymphocytes can sometimes be reprogrammed to enable cloning of an animal (Hochedlinger and Jaenisch, 2002), and regeneration involving plasticity of differentiated cells occurs readily in urodele amphibians (Brockes and Kumar, 2002).

Unfortunately, in many of the cases presented above, the transplanted cell populations are far from homogeneous. Unfractioned bone marrow preparations are likely to contain several kinds of stem and progenitors cells, instead of a single highly plastic type (Fig. 4c; Wagers and Weissman, 2004). Yet, in several studies, single purified cells have been transplanted, indicating true multipotency and attributing this capacity to actual stem cells at least indirectly (Krause et al., 2001; Jiang et al., 2002a; Bailey et al., 2004).

Alternatively, rare multipotent stem cells may contaminate the isolated tissue-specific stem cells (Fig. 4d; Wagers and Weissman, 2004). Contaminating HSCs seem to account for the reported hematopoietic capacity of skeletal muscle (McKinney-Freeman et al., 2002). In addition, as presented in section 4.1.3., highly potent MAPCs have been derived from many tissues, although their actual existence in vivo is unclear (Jiang et al., 2002b). In transplantations of single stringently purified LSK HSCs, multilineage differentiation obviously indicates actual plasticity of HSCs defined by the currently recognized criteria; even this population is, however, known to be heterogeneous (Osawa et al., 1996).
Figure 4. Potential mechanisms for apparent somatic stem cell plasticity.

(a) Transdifferentiation.
(b) Dedifferentiation.
(c) Multiple stem cells (a heterogeneous population isolated).
(d) A rare multipotent stem cell among tissue-specific stem cells.
(e) Cell fusion.

Based on Herzog et al. (2003) and Wagers and Weissman (2004).

Finally, bone marrow-derived cells have been shown to contribute to several tissues by cell fusion (Fig. 4e). In culture, bone marrow cells fuse to embryonic stem cells, adopting their phenotype (Terada et al., 2002). Under selective pressure, bone marrow-derived hepatocytes \textit{in vivo} arise primarily by fusion (Wang et al., 2003). In another studies, bone marrow cells fused to liver, Purkinje cells and cardiac muscle in irradiated recipients, with no actual transdifferentiation events detected (Alvarez-Dolado et al., 2003), and to intestinal stem cells (Rizvi et al., 2006). Fusion to Purkinje cells was shown to produce stable heterokaryons, where the bone marrow cell nucleus is reprogrammed (Weimann et al., 2003). Cell fusion is a well-known property of macrophages (Vignery, 2005), and indeed, HSCs appear to contribute to skeletal muscle through myeloid intermediates rather than directly (Camargo et al., 2003). On the other hand, fusion events are rare in the absence of selection (Wagers and Weissman, 2004). Observations of HSC and neuronal stem cell plasticity have also been reported in the absence of cell fusion (Jang et al., 2004; Bailey et al., 2004; Wurms et al., 2004).

Taken together, the significance of somatic stem cell plasticity is still unclear, and understanding the often apparently contradictory observations requires further research. To convincingly demonstrate an actual transdifferentiation event, several stringent criteria are proposed: the investigated cells should be minimally manipulated and cultured; clonal analysis should be performed; the transdifferentiated cells must be
unambiguously identified, phenotyped and shown to be functionally engrafted to tissue; nuclear reprogramming should be assessed, and cell fusion must be excluded (Wagers and Weissman, 2004). However, clinical applications may be feasible, even if transdifferentiation is not a physiologically relevant process.

4.1.7. What makes a stem cell?

The basis of “stemness” is not understood, despite intensive research (Mikkers and Frisen, 2005). The commonly used definitions of a stem cell are ambiguous: some differentiated cells are able to self-renew (Luckey et al., 2006), and some stem cells, such as those of the germline, are unipotent, generating a single type of progeny (de Rooij and Grootegoed, 1998).

Stem cells do share some characteristics. Several signalling pathways, including Wnt and Notch, are common to multiple stem cell types, as presented above. In addition, specific small RNAs are emerging as important stem cell regulators (Zhang et al., 2006a). Stem cells in various tissues have been enriched by their dye efflux ability, as the side population. The enhanced membrane transport may be involved in protection of long-lived stem cells from cytotoxic substances (Challen and Little, 2006). Stem cells also seem to carefully protect their genomic DNA. Telomerase is commonly expressed by stem and progenitor cells, preserving chromosome ends from degradation (Harrington, 2004). Evidence is also accumulating for the immortal strand hypothesis; asymmetrically dividing stem cells apparently retain the original template strands, passing the newly synthesized chromatids to the committed daughters (Cairns, 1975; Merok et al., 2002; Potten et al., 2002; Smith, 2005; Karpowicz et al., 2005; Shinin et al., 2006). In contrast, some forms of DNA repair may actually be downregulated; potentially mutated stem cells are preferentially replaced, in agreement with a strict control of genomic stability (Cairns, 2002).

Despite such general features, no unambiguous molecular markers of stem cells are available, let alone markers common to all stem cell types (Zipori, 2004). In search of a stem cell signature, transcriptomes of embryonic, hematopoietic, and neuronal stem cells have been compared by three independent groups using DNA array technology (Ivanova et al., 2002; Ramalho-Santos et al., 2002; Fortunel et al., 2003). All groups found more than 200 genes upregulated in all stem cell types, thus representing putative “stemness” genes. These gene sets were, however, strikingly different; only a
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A single gene, integrin α6, was identified in all three studies, although a multitude of genes enriched for embryonic or neuronal stem cells were reported in good agreement (Fortunel et al., 2003). While variations in stem cell purification methods and shortcomings in the current DNA arrays partially explain the discrepancies, it appears that specific stemness genes are expressed at a low level or only transiently, or may not exist at all (Fortunel et al., 2003; Zipori, 2004). Instead, stemness may be established by subtle differences in the expression of many genes, or different stem cells may use distinct genetic programs for self-renewal and differentiation (Fortunel et al., 2003).

Figure 5. Stem cell: a blank state (a) or priming for multipotentiality (b)?
Adapted from Zipori (2004) with permission.

HSCs show a highly promiscuous gene expression pattern, expressing multiple genes active in specific hematopoietic lineages, and even genes characteristic of non-hematopoietic cells (Akashi et al., 2003). As the cells differentiate to various committed progenitors, the expression pattern is progressively restricted. HSCs actively express genes associated with energy metabolism and translation (Hüttmann et al., 2006). They thus appear to be quiescent only with regard to proliferation, otherwise prepared to rapidly respond to environmental stimuli. Stem cells may in fact be characterized by a wide-open chromatin structure (Mikkers and Frisen, 2005). Rather than representing a blank state that gradually obtains properties of differentiated cells (Fig. 5a), stem cells could be primed for multipotentiality by expression of many genes, which are then selectively repressed or enhanced during differentiation (Fig. 5b; Zipori, 2004). The importance of chromatin modification in the specification of stemness is illustrated by the requirement of Bmi1 in self-renewal of hematopoietic and neuronal stem cells, as well as many murine and human cancers (Valk-Lingbeek et al., 2004). This polycomb group member is involved in epigenetic regulation.
Difficulties in defining a stem cell, heterogeneity of isolated stem cell populations, and observed plasticity have led some authors to suggest that stemness is a regulatable state rather than a discrete entity (Zipori, 2004; Blau et al., 2001; Fig. 6). Perhaps any cell is able to function as a stem cell, given the correct environment (although differentiated cells are less likely to do so!)

Figure 6. Route stem cell.

A stem cell landscape inspired by the initial reports of plasticity. Stem cells are envisioned here as travelling in the bloodstream, entering diverse tissues and generating appropriate cell types in response to local cues. Reproduced from Blau et al. (2001) with permission. Original artwork by N. Gewertz and B. Colyear.

Indeed, the niche appears essential in stem cell biology, as exemplified above for various stem cell types. The niche is a specialized microenvironment sheltering the stem cells, consisting of neighbouring cells and extracellular substance (Moore and Lemischka, 2006; Fuchs et al., 2004; Spradling et al., 2001). By interactive signalling, the niche maintains a balance of stem cell quiescence and activity, regulating self-renewal and differentiation. Best characterized in the Drosophila ovary, asymmetry and
subsequent differentiation may be commonly achieved by directional cell division, where one daughter remains attached to the niche, and the other is separated, as schematically shown in Fig. 7 (Spradling et al., 2001). Little is known, though, of the strategies actually used by mammalian somatic stem cells (Morrison and Kimble, 2006).

**Figure 7.** Symmetric and asymmetric division in the niche.

Symmetric divisions expand the stem cell population; asymmetric divisions generate differentiating daughter cells and renew the stem cells. Based on Spradling et al. (2001).

The state and history of the cell are still likely to affect the interpretation of environmental signals (Mikkers and Frisen, 2005). This is a central principle in developmental biology (Gilbert, 2006). As previously presented, intestinal stem cells and Paneth cells reside in intimate proximity, yet certain signals seem to affect them in almost opposite ways. Stem cells may be described as being halted in the progression towards differentiation, an apparently inherent tendency of mammalian cells (Fig. 8; Mikkers and Frisen, 2005; Clarke and Fuller, 2006). This status is maintained by complex interactions between the stem cell and the niche (Mikkers and Frisen, 2005).
4.2. The freemartin

Cattle provide a unique large animal model for stem cell research: most bovine twins are naturally chimeric as a result of conjoined fetal circulations. It is thus unnecessary to apply the typical transplantation procedures, where poorly defined radiation damage affects the results (Prise et al., 2005). Isolation and *in vitro* cultivation of transplanted cells are also avoided, minimizing alterations caused by manipulation (Morshead et al., 2002).

Vascular anastomosis occurs in about 92% of cattle twins, due to fused chorions (Fig. 9; Marcum, 1974; Lillie, 1917). The anastomoses form early in the development, from the 10- to 15-mm crown-rump stage (Lillie, 1917; Bissonnette, 1924; Ohno and Gropp, 1965). This corresponds to about 30-35 days post-coitum or Carnegie stages 15-18; the limb buds and forelimb plates are visible, many internal organs develop rapidly, and the genital ridges appear (Ohno and Gropp, 1965; Rüsse, 1991; Butler and Juurlink, 1987; Bissonnette, 1924). The blood is thus effectively mixed between the fetuses for most of the 280-day gestation. Postnatally, the twins permanently share identical
composite blood types, indicating that HSCs are exchanged and successfully engraft in the recipient (Owen, 1945). The proportions of donor-derived nucleated cells in blood and bone marrow in different twins are reported to be randomly distributed between less than 5% and more than 95% (Wilkes et al., 1981). The twins are tolerant to tissue allografts from each other (Anderson et al., 1951; Billingham et al., 1952). This serendipitous discovery led to further research in mice and chickens, unravelling the basis of acquired immunological tolerance (Billingham et al., 1953); the findings earned Peter Medawar a Nobel prize in 1960 (Brent, 1997).

In other species, chimerism caused by vascular anastomosis is rare, although it has been reported in at least sheep, goats and pigs (Marcum, 1974). Low-level microchimerism appears to be surprisingly common in humans (Boklage, 2006). However, this may be primarily caused by cell exchange between temporarily fused early embryos, rather than through vascular connections between fetuses or between a fetus and the mother.

Donor-derived cells can be readily identified in a female animal born as a twin to a male, as only bull cells contain a Y chromosome. In cattle, a female calf born as a twin to a bull is usually infertile due to disturbances in the development of the reproductive organs. These are probably caused by the anti-Müllerian hormone produced by the male fetus (Jost, 1970; Vigier et al., 1977; Vigier et al., 1987); the bovine twins were a key research model in the early days of reproductive endocrinology (Lillie, 1917; Capel and Coveney, 2004). Otherwise, the animals develop normally (Padula, 2005).

The infertile heifers are called freemartins. The “free” may derive from the Scottish farrow, for a cow not giving milk or without a calf, or from the Anglo-Saxon fearr, a bull (Forbes, 1946). The “martin” possibly originates from the Gaelic and Irish mart, meaning cow, heifer, ox, cow for slaughter, or beef. The term was often applied to cattle killed at the time of the Martinmas festival in November, to preserve the meat for winter; the non-productive freemartins were probably selected for slaughter. In the scientific literature, freemartins were first described more than two hundred years ago by John Hunter (1779):

“It is a known fact, and, I believe, is understood to be universal, that when a cow brings forth two calves, and that one of them is a bull-calf, and the other a cow to appearance, the cow-calf is unfit for propagation; but the bull-calf becomes a very proper bull. They are known not to breed: they do not even shew the least inclination for the bull, nor does the bull ever take the least notice of them.”
Diagnostic methods for freemartinism range from clinical examination to Y-chromosome-specific PCR (Padula, 2005). Composition of freemartin tissues has been analysed by cytogenetic techniques (Wilkes et al., 1981). Still, due to limitations of these methods, little is known of the contribution of bull-derived cells to the non-hematopoietic tissues.

![Cattle twins.](image)

**Figure 9.** *Cattle twins.*

1: arterial anastomosis; 2: cotyledon with venous connection with both sides; 3: opened amniotic sacs; 4: clitoris of the freemartin. Reproduced from Lillie (1917) with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Copyright © 1917 John Wiley & Sons, Inc.

4.3. Generation of B lymphocytes in ruminants

Our current understanding of the immune system is mostly based on mouse research, and to some extent on the human system. However, it turns out that these species are not representative of all mammals (Reynolds, 1997). A striking lesson is provided by the ontogeny of the antibody-producing B lymphocytes in ruminants. In this part of the review, I discuss the strategies ruminants use for the generation of their antibody repertoire, and the major role of the ileal Peyer's patch in this process.
4.3.1. Production of the ruminant antibody repertoire

Antibodies are able to specifically recognize almost any substance due to tremendous variation in their antigen-binding sites (Janeway et al., 2001). Every immunoglobulin (Ig) variant cannot be directly encoded in the genome because they are far more numerous than all of our genes. Instead, vertebrates use various alternative strategies to generate the necessary diversity (Ratcliffe, 2002).

The variable regions of Igs are assembled from several gene segments by rearrangements of genomic DNA in developing B lymphocytes (Fig. 10). In the mouse and human, multiple slightly different copies of each gene segment are available, allowing a high number of possible combinations. Additional diversity is generated in the joints between gene segments by addition and removal of nucleotides (Janeway et al., 2001). This preimmune repertoire is produced in the absence of antigenic stimulation (Griebel and Hein, 1996). In postnatal mice and humans, the process occurs in bone marrow, continuing more or less throughout life (Nunez et al., 1996; Min et al., 2006). Since B cells arise there from uncommitted progenitors (see Fig. 1), the bone marrow is regarded as a primary or central lymphoid tissue (Janeway et al., 2001). Igs are fine-tuned later, as B cells are exposed to antigens, to create the immune repertoire. In the process of somatic hypermutation, point mutations are introduced at a high rate into the variable region genes. This takes place in secondary or peripheral lymphoid tissues, such as the lymph nodes (Janeway et al., 2001).

In chicken and in several mammalian species, gut-associated lymphoid tissues (GALT) are essential for the generation of B lymphocytes (Reynolds, 1997; Weill and Reynaud, 1998). Actually, B cells were first identified as bursa-dependent lymphocytes in the chicken, produced in the cloaca-associated bursa of Fabricius (Glick et al., 1956). Lymphoid follicles in the ileal Peyer’s patch appear to function analogously in ruminants, the best-characterized of which are sheep (Yasuda et al., 2006). Cattle are speculated to be similar.

The GALT species seem to rely less on the combinatorial diversity than the mouse and human. Instead, their preimmune Ig repertoire is generated largely in GALT by antigen-independent somatic hypermutation or gene conversion, where pseudogene sequences replace parts of the functional Ig genes (Ratcliffe, 2002).

Sheep were originally reported to have a very limited selection of Ig variable region gene segments, even fewer of which are actually used (Reynaud et al., 1991). Igs are subsequently diversified by somatic hypermutation, where point mutations are
introduced to the variable regions at an extremely high rate. The process starts antigen-independently in the sterile fetus and continues after birth (Reynaud et al., 1995). However, more Ig gene segments have later been identified in the ovine genome, suggesting that the combinatorial mechanisms contribute more greatly to Ig diversity than previously appreciated (Jenne et al., 2003). While somatic hypermutation does play a role, reassessment of the original data suggests that it occurs mostly after birth, when the animal is exposed to foreign antigens.

**Figure 10. Construction of an immunoglobulin heavy chain.**

A simplified view; the actual number of available gene segments is larger and species-dependent. V: variable segment, D: diversity segment, J: joining segment, C: constant segment specifying the antibody isotype. Based on Janeway et al. (2001) and Tizard (2004).

Cattle are thought to generate their antibody repertoire using strategies similar to sheep, although available data are even more limited (Zhao et al., 2006; Kaushik et al.,
A small number of utilized Ig gene segments have been detected (Sinclair et al., 1997; Saini et al., 1997; Berens et al., 1997). Both somatic hypermutation and gene conversion have been proposed to be involved in the Ig diversification (Parng et al., 1996; Berens et al., 1997; Lucier et al., 1998). CDR3, one of the highly variable complementarity-determining regions of Igs, shows marked length heterogeneity in cattle Ig heavy chains (Berens et al., 1997; Sinclair et al., 1997; Saini et al., 1997; Saini and Kaushik, 2002). In a substantial proportion of bovine antibodies, the CDR3 is longer than found in any other species (Saini et al., 1999), at least partially due to exceptionally long diversity (D) segments in the germline genome (Shojai et al., 2003). This is thought to provide additional material for somatic hypermutation in the diversification of bovine immunoglobulins (Kaushik et al., 2002).

4.3.2. The ileal Peyer’s patch

Lymphoid organs called Peyer’s patches (PPs) exist in the walls of the mammalian small intestine (Yasuda et al., 2006; Griebel and Hein, 1996). They are accumulations of lymphoid follicles, with considerable variation in structure and distribution between species. In ruminants, pigs, horses, dogs and humans, most of the follicles are contained in a single ileal PP, while in jejunum multiple discrete accumulations are found (Griebel and Hein, 1996). PPs are generally responsible for mucosal immunity. In the mammalian GALT species, however, the large ileal PP is important for the entire peripheral B cell pool (Reynolds, 1997).

The lymphoid follicles in the PPs extend from the lamina propria to the submucosa of the intestine (Fig. 11). In the ileal PP, they are larger and more cylindrical than in the jejunum (Reynolds and Morris, 1983b). A dome covered by a specialized follicle-associated epithelium protrudes towards the mucosa; this is more prominent in the follicles of jejunal PPs. Each follicle is encapsulated by connective tissue and surrounded by lymphatic sinuses draining from the intestinal villi (Reynolds and Morris, 1983b; Lowden and Heath, 1992). The ileal follicles contain mostly B cells expressing surface IgM, supported by a rich network of stromal cells (Hein et al., 1989; Griebel and Hein, 1996; Reynolds, 1997).

The ileal PP develops relatively late in the ruminant fetus, as compared with jejunal PPs and other lymphoid organs. In sheep, it forms by day 110 of the 150-day gestation (Reynolds and Morris, 1983b). In cattle, the first ileal lymphocyte clusters are seen at
day 150, and the follicular structures can be distinguished by 180-185 days’ gestation (Ishino et al., 1991; Beyaz and Asti, 2004; Doughri et al., 1972). The PP is seeded by B cells, which have already rearranged one Ig light chain allele and express surface IgM (Jenne et al., 2006; Reynaud et al., 1991; Griebel et al., 1992). The B cells are thought to originate at least partially from the spleen, although they can also be produced in other locations (Lucier et al., 1998; Press et al., 2001). The essential population appears to exist already at day 63, when an injection of anti-IgM antibody is able to perturb later PP development (Press et al., 1996).

By observing Ig light chain rearrangement patterns, sheep ileal PP follicles were discovered to be oligoclonal, indicating that each follicle is founded by a small number of B cells (Reynaud et al., 1995). Igs are then diversified by somatic hypermutation. Based on sequence genealogies, diversification of the light chain variable regions has been shown to occur in the ileal PP also in cattle; some of the diversity is, however, already present in the fetal spleen (Lucier et al., 1998).

Growing until 6-8 weeks after birth, the ileal PP is up to 2.5 meters long in young lambs and contains about $10^5$ lymphoid follicles; it accounts for up to 1.2% of body weight, more than the T cell-producing thymus (Reynolds and Morris, 1983b). The ovine ileal PP starts to involute at about 3 months postnatally and is mostly replaced by connective tissue by 18 months of age (Reynolds and Morris, 1983b). The jejunal PPs remain even in old animals.

The follicular B cells in the ileal PP proliferate rapidly already in the fetus. About 5% of them enter metaphase each hour, equalling the production of $3.6 \times 10^9$ new cells; this is approximately ten times the mitotic rate in the thymus (Reynolds, 1986; Reynolds, 1987). Uncontrolled, the mass of such an expanding population would exceed that of the whole animal within a week (Reynolds, 1997). About 5% of the B cells leave the PP and are distributed to all lymphoid tissues, with no preference for the mucosal sites (Pabst and Reynolds, 1987; Reynolds et al., 1991). Most of the rest are removed by apoptosis (Pabst and Reynolds, 1986; Pabst and Reynolds, 1986; Motyka and Reynolds, 1991). A poorly understood selection process may be involved in the deletion of B cells (Yasuda et al., 2006).

Bovine ileal PP follicles were recently characterized by serial analysis of gene expression (Neill et al., 2006). Conforming to earlier studies in sheep, the data indicate active protein synthesis, IgM-dominant expression of Ig genes and apoptotic activity.
Figure 11. *The ileal Peyer’s patch.*

A longitudinal section from cattle ileum, showing follicles of the ileal PP. The lumen is at the top of the image. MU = mucosa, SM = submucosa, ME = muscularis externa. F = a single outlined follicle, D = dome area.

Removal of the ileal PP in neonatal lambs causes a prolonged, although not fully permanent B cell deficiency, suggesting an important role for the PP in ovine lymphopoiesis (Gerber et al., 1986; Reynolds, 1997). The sheep bone marrow contains few lymphocytes compared with that in rodents or humans (Tizard, 2004). As the PP involutes at puberty, adult sheep are thought to depend on the peripheral B cell pool originally created in the young animal (Reynolds, 1997).

The ovine ileal PP is sometimes referred to as a primary lymphoid organ, since the preimmune antibody repertoire is largely produced there. However, new B cells do not arise from uncommitted precursors in the PP, and rearrangements of Ig genes do not occur (Reynolds, 1997). In addition, antigen exposure is necessary for the postnatal function of the ileal PP, as it develops normally in isolated, sterile intestinal loops until birth, but then involutes prematurely (Reynolds and Morris, 1983a; Reynolds and Morris, 1984; Reynaud et al., 1995; Yasuda et al., 2006). The primary and secondary functions are thus not clearly distinct and separated. The traditional classification arises from the mouse and human systems and appears largely artificial with regard to GALT species (Griebel and Hein, 1996).
5. Objectives of the study

The present work aimed to characterize the differentiation potential of somatic stem cells in a large, unmanipulated mammal to complement the data obtained from mouse and human transplantation experiments. We studied naturally chimeric twin cattle, where a substantial proportion of hematopoietic stem cells originates from the sibling. The donor contribution to the immune system was also examined to investigate the role of the ileal Peyer’s patch in the generation and maintenance of bovine B lymphocytes. The following specific objectives were set:

1. To develop methods for detecting bull-derived cells in the tissues of female twin siblings (freemartins), while preserving tissue architecture (III).

2. To select and develop tools for identifying the phenotypes of bull-derived cells in freemartin tissues (I, V).

3. To use these methods to characterize the distribution and phenotypes of bull-derived cells in freemartin tissues (II).

4. To analyse the distribution of bull-derived cells in various leukocyte populations in freemartins to better understand the function of the ileal Peyer’s patch in the generation and maintenance of the B cell pool in cattle (III, IV).
6. Materials and methods

Detailed descriptions of methods, including primer and probe sequences, are available in the original publications.

6.1. Animals, tissues and cells (I-V)

Heterosexual cattle twins were located by veterinarian contacts or the cattle database maintained by the Finnish Animal Breeding Association. The chimerism was quantitated by Y chromosome-specific in situ hybridization to blood samples, as described below. Blood samples were acquired from farms. Tissues were collected from chimeric and normal cattle, which were either anaesthetized and exsanguinated at the facilities of the Department of Basic Veterinary Sciences or slaughtered routinely at an abattoir. Altogether 12 freemartins aged 20 days to 20 months were studied.

To obtain newly generated granulation tissue, block-shaped CellSpon cellulose viscose sponges (Cellomeda, Finland; Viljanto and Kivikoski, 1962) were implanted subcutaneously in the prescapular region of a 70-day-old freemartin calf. Five sponges were implanted at one-week intervals. Operations were performed under surgical anaesthesia, by A. Iivanainen. The calf was sacrificed and implants recovered one week after the last operation.

Tissue samples were processed for paraffin sections or cryosections. For paraffin sections, they were fixed either in 4% phosphate-buffered paraformaldehyde (PFA) for 24 h at +4°C or in 100% ethanol for 2 h at +4°C followed by 120 h at -20°C, embedded in paraffin and cut to 2- to 4-μm sections on Superfrost Plus slides (Menzel-Gläser, Germany). For cryosections, the samples were frozen in liquid nitrogen, stored at -80°C and cut to 10-μm sections.

Leukocytes were collected from heparinized blood samples and bone marrow suspensions by Ficoll gradient centrifugation. Erythrocytes were lysed where necessary. Bone marrow was obtained from sternal pieces from slaughtered animals or as aspirates from the wing of the ilium, taken under surgical anaesthesia prior to exsanguination. For isolation of cells from the spleen, lymph nodes and thymus, tissue samples were cut into small pieces in medium, and the released cells filtered through a Cell Strainer (Becton Dickinson Labware, NJ) and centrifuged on a Ficoll gradient. Lymphocytes from the ileal mucosa were isolated as described previously (Parsons et al., 1996).
Cells were generally processed for cytocentrifuge preparations at 10^5 cells per slide, fixed with ethanol or acetone, air-dried and stored at -80°C. Smears were prepared from bone marrow aspirates.

6.1.1. Animal welfare (I-IV)

The use of animals was approved by the local authorities. Care was taken to minimize any pain and suffering, and mostly post-mortem material was used.

6.2. In situ hybridization

6.2.1. Genomic in situ hybridization (II-IV)

Optimized in situ hybridization (ISH) protocols were developed for the detection of the Y chromosome in bovine tissue sections and cytocentrifuge preparations. For tissue material, paraffin sections were used. The sections were deparaffinized and then permeabilized by detergent treatment, microwave heating in 2×SSC pH 6, and protease P6911 (for PFA-fixed material; Sigma-Aldrich, MO) or detergent and protease only (ethanol-fixed material). Protease concentration (5-250 μg/ml) was optimized for each tissue. The sections were then quickly fixed in PFA, dehydrated in graded ethanol, and air-dried. Genomic DNA was denatured by heating slides at +85°C after applying hybridization solution and coverslips. Hybridization was carried out for 40 h in humidified chambers at room temperature. The hybridization solution contained 7.5-15 nM digoxigenin (DIG)-labelled oligonucleotide probe (see below), 50% formamide, 4×SSPE pH 7.4, 1 mg/ml ssDNA and 5×Denhardt's solution. The hybridized sections were washed in 50% formamide, 4×SSPE and in 1.5×SSPE, at room temperature. DIG-labelled probe was detected by alkaline phosphatase-conjugated anti-DIG-F_{ab} fragments (Roche, Germany) and visualized using the NBT/BCIP chromogen (Roche). Sections were lightly counterstained with Mayer's haematoxylin and mounted, usually with Faramount (Dako Cytomation, Denmark).

In most cases, the slides were assembled in Shandon Coverplates (ThermoElectron), with the exception of the hybridization steps. Normal bull and cow tissues and cells were used as positive and negative controls in the analysis of freemartin material. Cytocentrifuge preparations and bone marrow smears were processed essentially similarly, with optimized pretreatments and an overnight hybridization.
6.2.2. Tyramide amplified mRNA in situ hybridization (V)

To analyse the expression of the CD34 gene in bovine tissues, ISH to mRNA was performed. Fresh cryosections were used for this purpose. The sections were heated at +60°C for 1 min and air dried. They were fixed in ice-cold 4% PFA pH 7.4 for 1 hour, acetylated, incubated in 0.2 M HCl for 6 min, treated with 0.5 – 1 µg/ml proteinase K for 30 min at room temperature, dehydrated, and air dried. The probes were diluted at 200-400 pg/µl in a hybridization buffer containing 50% formamide, 4×SSPE pH 7.4, 1 mg/ml ssDNA, and 5×Denhardt’s, denatured, and hybridized to the tissue sections at +52°C overnight. The slides were washed in 4×SSPE; 4×SSPE, 50% formamide; and 1.5×SSPE, at +52°C. They were then assembled with Shandon Coverplates. The sections were incubated in a blocking reagent and anti-DIG-peroxidase conjugate (both Roche). Tyramide amplification was performed using the TSA Plus DNP alkaline phosphatase system (Perkin Elmer, MA), with the NBT/BCIP substrate (Roche). The sections were then counterstained with haematoxylin and mounted.

6.2.3. Probes (II-V)

The Y chromosome probe, designed by A. livanainen, was a degenerate 28mer oligonucleotide [5'-TT(A/C/T) TCA GCC CTG TGC C(C/T)T GG(A/C/G/T) (A/G)A(C/T) TGT G-3'] corresponding to the repetitive bovine btDYZ locus (Perret et al., 1990). As a positive control, a previously described oligonucleotide probe (Modi et al., 1993) for the bovine autosomal 1.709 satellite sequence (Skowronski et al., 1984) was applied. Both probes contained a single DIG label and were synthesized by Proligo (France).

DIG-labelled antisense riboprobes and sense control probes were prepared by in vitro transcription from plasmids containing the respective cDNA sequences, using T7 and SP6 polymerases and DIG-11-UTP (Roche, Germany). The CD34 probe consisted of the sequence for the extracellular part (bases 57-859). The DNA template was removed by RQ1 DNase (Promega, WI), and the probes purified by ethanol-LiCl precipitation. Products were checked by agarose gel electrophoresis and by a UV absorption spectrum analysis using a Nanodrop spectrophotometer (Nanodrop Technologies, DL).
6.3. Immunostaining

6.3.1. Antibodies and antigen retrieval (I-V)

We evaluated the usefulness of 39 antibodies and lectins for the identification of major cell types in paraffin-embedded cattle tissues. Antibodies raised against bovine antigens or known to be bovine cross-reactive were used where possible. If no information on bovine reactivity was available, antibodies with the broadest species cross-reactivity were selected. A comprehensive list of these markers can be found in open-access publication I. Some additional anti-bovine leukocyte antibodies were used in publication IV, as described in Results. An anti-bovine CD34 antibody was generated as a part of the work (V); all other antibodies and lectins are commercially available.

Immunostaining with each antibody was optimized with regard to tissue fixation (PFA or ethanol) and antigen retrieval methods, as described in publication I. Each antibody was tested with a standard test battery of retrieval procedures. This included microwave heating in an acid, neutral or alkaline buffer (for PFA-fixed material) and protease treatments (for PFA- and ethanol-fixed material). The specificity and sensitivity of the staining in various tissues were evaluated, and the fixation and antigen retrieval methods yielding the best results selected for subsequent use.

6.3.2. Immunohistochemistry (I-V)

Immunohistochemistry (IH) was performed using either the avidin biotin complex (ABC) method or tyramide amplification, in most cases applying Shandon Coverplates. Paraffin-embedded sections were dewaxed, rehydrated, subjected to an optimized antigen retrieval procedure and permeabilized with Tween-20 in PBS. The sections were then blocked for endogenous biotin, when necessary, and for non-specific binding with goat serum in PBS. They were incubated in the primary antibody overnight at +4°C, in PBS containing 1% BSA, washed and incubated with goat biotinylated anti-mouse or anti-rabbit secondary antibody (Dako, Denmark) for 2 h at room temperature. The ABC detection was performed using the Vectastain Elite ABC kit and the diaminobenzidine substrate kit (both Vector Laboratories, CA) according to manufacturer’s instructions. For tyramide amplification, sections were incubated in avidin D conjugated peroxidase (Vector), in biotinylated tyramide (Hopman et al.,
1998), again in avidin-peroxidase and in diaminobenzidine substrate. The sections were counterstained with Mayer’s haematoxylin and embedded with Faramount.

6.3.3. Immunofluorescence (V)

Immunofluorescence staining was performed essentially as described above for IH. Secondary antibodies conjugated with Alexa Fluor dyes (Invitrogen, OR) were used. Autofluorescence was suppressed where necessary by incubation in 0.1% Sudan Black B (Merck, Germany) in 70% ethanol. For cytocentrifuge and smear preparations, no antigen retrieval was performed, and antibody incubations were shortened. The slides were embedded using Mowiol 40-88 (Aldrich, Germany).

6.4. Combined ISH and IH double staining (II)

Combining ISH and IH was necessary to reliably analyse the phenotypes of bull-derived cells. This was problematic, as the ISH protocol destroyed many of the antigens, and the IH reaction products caused false negatives in ISH. As a solution, tyramide amplification was utilized in IH, depositing a covalently bound label to the target before the colour reaction. Thus, the immunodetection could be started first. After the tyramide reaction, ISH was performed, and thereafter the IH protocol was finished. Diaminobenzidine (Vector Laboratories) was used as the peroxidase substrate. Sections were counterstained with Mayer’s haematoxylin and mounted with Faramount (Dako).

For lectin histochemistry, the ISH protocol was beneficial. In combined genomic ISH and lectin histochemistry, the lectin staining was therefore performed after ISH.

6.5. Microscopy and photomicrography

The stained preparations were viewed and documented using an Olympus BH2 microscope (Olympus Life and Material Science Europa, Germany) equipped with a ColorView 12 digital camera and AnalySIS 3.0 image analysis software (both Soft Imaging System, Münster, Germany), or a Leica DM4000 microscope (Leica Microsystems, Germany) with an Olympus DP70 camera and Cell^P software (both Olympus).
6.6. Cell counting and statistics (II, IV)

To determine the frequency of Y chromosome-containing (Y+) cells, several tissue sections or cytocentrifuge preparates were hybridized, and the samples with the highest Y+ cell frequencies were selected. They provided the most reliable data, as no false positives were detected in any of the control female samples (see Results).

6.6.1. Counting of donor-derived non-hematopoietic cells in tissues (II)

Y+ and Y- nuclei were counted in digitally photographed tissue sections doubly stained for the Y chromosome and the hematopoietic marker CD45. The touch count tool of AnalySIS 3.0 image analysis software was used. Nuclei in all cells negative for hematopoietic markers in a field of 230 μm × 185 μm were counted. The process was repeated for additional fields until the cumulative percentage of Y+ nuclei no longer fluctuated more than 0.2 percentage units. If no non-leukocyte Y+ cells were found, the whole section was viewed, and the total number of screened nuclei was estimated by measuring the area viewed. It should be noted that for tissues with multinucleated cells, such as the skeletal muscle, the Y+ count represents the proportion of donor-derived nuclei, rather than entire cells.

Numbers were corrected with counts obtained from similar bull tissues. All statistical analyses were performed in SPSS 10.0 for Windows. Mann-Whitney U test was used for estimating statistical significance of differences in cell frequencies. Similarity of the implanted animal to the other freemartins was evaluated by comparing scatterplots of cell counting results from different animals.

6.6.2. Counting of donor-derived cells in leukocyte fractions (IV)

Y+ and Y- cells were counted in cytocentrifuge preparates processed for genomic ISH. A total of 300-500 cells were counted from each slide until the variation in the cumulative percentage was less than 2 percentage units. Accuracy of counting was controlled by analysing leukocytes from normal cows and bulls mixed in known proportions.
6.7. PCR-based IgH analysis (III)

Genomic PCR was used to analyse the size of complementarity-determining region 3 (CDR3) within the IgH locus, in individual ileal Peyer’s patch (PP) follicles. CDR3 size can be used to mark a single B cell clone (Arnold et al., 1983). The assay is especially feasible in cattle due to the large variation in the CDR3 size (Saini et al., 1999).

6.7.1. Laser capture microdissection

Individual ileal Peyer's patch follicles were laser microdissected from membrane-mounted, light green-stained, PFA-fixed paraffin sections of freemartin ileum using a PALM laser microscope system (P.A.L.M. Microlaser Technologies, Germany), essentially as described previously (Bohm et al., 1997).

6.7.2. Hybridoma culture

For controls, two bovine B cell – mouse myeloma hybridoma lines (αBL5C2.870005 and αBL5C2.870009) were obtained from ATCC. These were cultured in Dulbecco's modified Eagle's medium with 4.5 g/l glucose, supplemented with 10% horse serum.

6.7.3. Preparation of genomic DNA templates

The microdissected follicles were digested with proteinase K at +56°C for 5-7 days. The enzyme was inactivated by heating. The crude digest was then used as PCR template. For controls, genomic DNA was isolated from bovine peripheral blood leukocytes and from the two B cell hybridoma lines.

6.7.4. PCR amplification and analysis of products

A region containing the hypervariable CDR3 segment of the rearranged bovine IgH locus was PCR amplified. Primer cocktails were designed based on bovine IgH sequences in GenBank. The 5’ primer was specific to the framework 3 region of the IgH gene, and the 3’ primer was specific to the framework 4.

The PCR products were analysed by agarose gel electrophoresis. The specificity of products was confirmed by Southern blotting using a DIG-labelled internal framework 3 probe. Alkaline phosphatase-conjugated anti-DIG-F_ab fragments (Roche) and the CDP-
Star substrate (Roche) were used for detection. Results were visualized using the Molecular Imager phosphor imager (Bio-Rad, Hercules, CA).

6.8. Leukocyte fractionation (II, V)

Magnetic-activated cell sorting (MACS) was used to isolate various lymphocyte fractions from Ficoll-isolated mononuclear cells and to deplete bone marrow cells for lineage markers. MACS columns (Miltenyi Biotec Inc., Germany) were used according to the manufacturer’s instructions for indirect separation. The efficiency of separation and depletion was controlled by FACS after incubation of the cells with a fluorescently labelled secondary antibody.

Granulocytes were isolated using Ficoll gradient centrifugation. They were obtained from the cell fraction under Ficoll by lysis of the red blood cells.

Cell fractionations were performed by T. Pessa-Morikawa.

6.9. Generation of a polyclonal anti-bovine CD34 antibody (V)

6.9.1. Cloning of the full-length CD34 cDNA

Full-length CD34 cDNA was produced by RT-PCR from total RNA of bone marrow mononuclear cells. The total RNA was isolated by the single-step guanidium-isothiocyanate method (Kingston et al., 1996).

The first strand was synthesized using M-MuLV reverse transcriptase (Finnzymes, Finland) and a primer specific for the 3’-UTR (Zhou et al., 2001a). The product was amplified in semi-nested PCR using DynaZyme EXT polymerase (Finnzymes) and primers specific for the 5’-UTR and 3’-UTR of bovine CD34.

The cDNA was ligated into a SacII/SalI digested pBluescript II SK plasmid (Stratagene, CA), and the recombinant plasmid was used for transforming Escherichia coli. Five clones were sequenced at the DNA sequencing laboratory of the Viikki campus. A full-length sequence conforming to previously published data (Zhou et al., 2001a) was then assembled from these clones.

6.9.2. Expression and purification of polypeptide fragments

The following three fragments of the CD34 polypeptide were selected for antigen expression based on structure analysis with several prediction software programs: the
predicted extracellular domain, a part of the extracellular domain without most of the predicted glycosylation sites and the predicted cytoplasmic domain.

The corresponding DNA sequences were PCR amplified from the cDNA plasmid and ligated into a Ncol/XhoI digested pET-22b expression plasmid (Novagen, WI) containing the T7lac promoter and sequences for the pelB periplasmic localization signal and C-terminal 6×His-tag. The plasmids were then sequenced to exclude potential PCR errors.

Protein expression was performed using *E. coli* strain BL21(DE3) (Novagen). The partial extracellular fragment and the cytoplasmic fragment were selected for large-scale production, as the complete extracellular domain was found insoluble. Bacterial cells were cultured in Luria-Bertani medium with 100 μg/ml ampicillin, and protein expression induced with 1 mM isopropyl-β-d-galactoside. The cytoplasmic fragment was collected from the supernatant, and the extracellular fragment from the supernatant and the periplasmic space. Both fractions were dialysed to remove free histidine and histidine-containing oligopeptides.

The His-tagged polypeptides were purified by metal affinity chromatography, using the Ni-NTA Superflow nickel-nitriloacetic acid resin (Qiagen, MD). The polypeptides were bound to the resin, washed with buffer containing 1-20 mM imidazole and eluted with 250 mM imidazole.

The fractions were quantitated with the BCA assay and analysed using SDS-PAGE electrophoresis. Identities of the polypeptides were confirmed by N-terminal sequencing and mass spectrometry from reversed-phase high-performance liquid chromatography fractions, at the protein chemistry core facility of Viikki campus.

### 6.9.3. Antibody production

Rabbit polyclonal antibodies were raised against the CD34 polypeptide fragments. The antibodies were produced by Sigma Genosys (Cambridge, UK) according to a standard custom protocol. They were then purified by affinity chromatography on antigen coupled to NHS-activated Sepharose (Amersham, Sweden).

### 6.10. Western blot (V)

Samples of homogenized bovine tissues were run on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes and probed with the CD34 antibody. To test
specificity, free polypeptide antigen was included in some experiments. After incubation with a peroxidase-conjugated goat anti-rabbit antibody (Dako Cytomation), the blots were developed by enhanced chemiluminescence substrates (ECL, Amersham) and visualized using a Fuji LAS3000 camera (Fuji Photo Co., Ltd., Japan). T. Pessa-Morikawa was responsible for the Western blots.

6.11. Analysis of the bovine CD34 gene and mRNA expression (V)

6.11.1. Expression of CD34 mRNA

The possible alternative splicing of the CD34 mRNA in cattle was first examined by bioinformatics. The tentative intron 7 splice sites in the bovine CD34 gene were predicted by a CLUSTAL W (Thompson et al., 1994) alignment of the human and cattle genomic sequences (Genbank accessions NT_021877 and NW_929130). This part of the work was performed by A. Iivanainen.

The expression of CD34 mRNA in various bovine tissues was analysed using RT-PCR by R. Ra. To detect putative splice variants, a strategy published previously for murine CD34 was used (Suda et al., 1992). Total RNA was reverse-transcribed using the M-MuLV reverse transcriptase (Fermentas), and the products were PCR amplified with the Dynazyme EXT DNA polymerase (Finnzymes). Primers specific for exons 7 and 8 of the bovine CD34 gene, enclosing the possible exon 7 extension, were used in these reactions. Products were then analysed by agarose gel electrophoresis.

In addition, the expression pattern of CD34 mRNA was analysed by in situ hybridization, as described above.

6.11.2. Assaying single nucleotide polymorphism

A potential single nucleotide polymorphism (SNP) in the bovine CD34 gene was suggested by a difference in our sequence to the previously published data. The presence of the SNP was assayed by restriction fragment length in bovine genomic DNA samples (9 different breeds, 49 animals in total). DNA was extracted from blood samples using the Wizard genomic DNA purification kit (Promega, WI) or obtained from the Nordic Gene Bank Farm Animals (the Japanese black cattle sample from Professor Masahiro Yasuda, University of Miyazaki). The genomic DNA was then PCR amplified using primers corresponding to bovine CD34 exons 4 and 6. The products were digested using BshTI and analysed by agarose gel electrophoresis.
7. Results

7.1. Detection of bull cells (II-IV)

The optimized Y chromosome in situ hybridization (ISH) protocol was shown to be both sensitive and highly specific (III / Fig. 1). In bull tissues, approximately 40-70% of cells were strongly labelled, depending on tissue type, even in double staining combined with immunohistochemistry (II). In the 2- to 4-μm tissue sections, the Y chromosomes were unavoidably excluded from some nuclei. No false-positive signals were seen in any of the female control samples (more than 1700 hybridizations using 20 different tissue types). In the counting of bull-derived cells on cytocentrifuge preparations, the results of control experiments with known proportions of bull and cow cells varied less than 3 percentage units from the actual values (IV / Fig. 2). For paraformaldehyde-fixed paraffin sections, effective hybridization required an enhanced pre-treatment with both microwave heating and protease.

7.2. Selection of phenotypic markers (I)

To facilitate the analysis of the phenotypes of the bull-derived cells, various markers were evaluated for immunohistochemistry (IHI) and lectin histochemistry. A panel of 31 useful markers was selected. These allowed the identification of all major differentiated cell types in cattle tissue sections, including epithelia, connective tissue, muscle and nervous tissue, as well as cell proliferation and apoptosis (I). With paraformaldehyde-fixed material, heat-induced antigen retrieval at a specific pH was beneficial for most antibodies. By selecting a suitable retrieval protocol, most of these markers could be successfully applied to this type of material.

7.3. Bull-derived cells in freemartin tissues (II, III)

To characterize the distribution of bull-derived cells in freemartin tissues, a wide Y chromosome ISH screening was first performed. Samples from 20-74 different body sites per animal were analysed from 12 freemartins. In total, more than 1650 freemartin tissue sections were successfully hybridized. Y chromosome-positive (Y⁺) bull-derived cells were detected in all of the animals in all tissues examined. The frequency of Y⁺
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cells was highest in blood, bone marrow and lymphoid tissues, with considerable variation present between individual animals. In blood mononuclear cells, the proportion of Y$^+$ cells varied from 10% to 90%. The variation was less pronounced in other organs.

The distribution of the bull-derived cells and double stainings with cell type-specific immunohistochemistry (primarily neuronal markers; unpublished observations) suggested that a significant proportion of the Y$^+$ cells were infiltrating leukocytes. These are often difficult to distinguish from parenchymal cells in a standard histological staining. To reliably label the leukocytes residing in the tissues, Y chromosome ISH was combined with immunohistochemistry for the pan-leukocyte marker CD45 (Thomas, 1989), as described in Methods. In the analysis of the central nervous system, mistletoe lectin I (ML-I) histochemistry was used to label bovine microglial cells (Hewicker-Trautwein et al., 1996).

Using double staining, 14 different tissues were examined, representing all major tissue types and derivatives of all embryonic layers. A total of 472 successfully double-stained freemartin tissue sections were viewed. CD45/ML-I Y$^+$ cells were identified in most tissues, but in 11 of the 12 calves they were mostly sporadic. Thus, the Y$^+$ cells were overwhelmingly of the hematopoietic lineage (II / Fig. 1A-G). One of the calves (designated FM10) was a striking exception, with high numbers of CD45/ML-I Y$^+$ cells in several tissues, as discussed below.

The proportion of Y chromosome-positive CD45/ML-I cells among all CD45/ML-I cells was determined for 4-5 calves per tissue. With the exception of FM10, the calves did not differ markedly in the relative frequency of CD45/ML-I Y$^+$ cells, which constituted less than 1% of all CD45/ML-I cells in most tissues investigated (II / Fig. 2). No obvious correlation was observed in the frequency of Y$^+$ cells between blood and non-hematopoietic tissues, or between various non-hematopoietic tissues in a single animal.

In 11 of the 12 freemartins, the highest numbers of CD45/ML-I Y$^+$ cells were detected in the mammary gland connective tissue (0.94±0.67%), liver (0.85±0.41%, mostly not hepatocytes), and skeletal muscle (0.83±0.26% of nuclei). The average frequency of CD45 Y$^+$ cells was 0.1% or less in cardiac and smooth muscle, renal epithelia, and the epidermis. Bull-derived non-hematopoietic cells were markedly less frequent in the ectoderm-derived epidermis and the central nervous system. In the brain, ML-I Y$^+$ cells were extremely rare, despite a robust contribution to the microglia
Bull-derived cells in freemartin tissues

In the cerebral cortex and the underlying white matter, 0.0020±0.0023% of ML-I- cells were Y+. No ML-I-Y+ cells were detected in the hippocampus or the olfactory bulb, among the approximately 2-10⁵ cells screened per tissue. No Y+ cells showing unambiguous neuronal morphology were seen in more than 700 successfully hybridized sections of the freemartin central nervous system. These included samples from the cerebral cortex, cerebellum, brainstem, corpus callosum, hippocampus, olfactory bulb, thalamus, ventricular walls, hypophysis and spinal cord, representing approximately 28-10⁶ cells in total.

In FM10, the distribution of CD45/ML-I- Y+ cells differed substantially from the other calves. Higher numbers of CD45-Y+ cells were detected in the epithelium of small intestine (19%, II / Fig. 1H), in the connective tissue of mammary gland (15%), and in liver (4.3%), epithelium of mammary gland (4.0%), skeletal muscle (2.3%), cardiac muscle (1.0%) and smooth muscle (0.5%). In the brain and renal epithelia, the frequency did not differ notably from the other calves. Continuous strands of CD45 Y+ cells were seen in the villi of the intestinal mucosa (II / Fig. 1H). In the hematopoietic system, the bull contribution to B cells was clearly weaker than to the total leukocyte pool (data not shown).

Newly formed granulation tissue in subcutaneously implanted cellulose viscose sponges contained relatively high numbers of CD45 Y+ cells (II / Fig. 1I and Fig. 2). The frequency increased within an incubation period of 4 weeks. At 4 weeks, the frequency of CD45 Y+ cells in the newly forming tissue was significantly higher than in most other non-hematopoietic tissues of the same animal (P=0.12 for liver, P<0.02 for any other tissue investigated). However, no obvious Y+ cell clusters indicating local proliferation were seen. In the other tissues of the sponge recipient, the frequencies of CD45/ML-I- Y+ cells did not differ markedly from those in the 10 other calves of the majority type.

7.4. Bull-derived cells in freemartin lymphocyte populations (III, IV)

In the ileal Peyer's patch (PP) of freemartin cattle, a striking variation in the frequency of Y+ cells was observed between individual follicles (Fig. 12 and III / Fig. 2A, B). Some follicles were almost exclusively of the male genotype, while others were female. The variation appeared discontinuous, with 2 or 3 discrete intermediate groups of follicles identifiable based on the frequency of bull-derived cells. The mosaic pattern was

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specific to the ileal PP follicles. In the jejunal PPs (III / Fig. 2C, D), the interfollicular variation in the frequency of Y⁺ cells was very modest.

Figure 12. Y chromosome-specific in situ hybridization to the ileal Peyer's patch of a freemartin calf.

A composite image assembled from several photomicrographs. The intestinal lumen is at the top of the image. For a description of the structure, see Fig. 11.

To directly estimate the number of founder clones per follicle, the size distribution of the CDR3 regions of the rearranged IgH genes in B cells isolated from individual follicles were analysed. A small number of different PCR products were generated from the ileal follicles, in comparison with reactions from blood cells (III / Fig. 3) or from the jejunal or splenic follicles (data not shown).

To investigate the significance of the ileal PP for the bovine peripheral B lymphocyte pool, we compared the frequencies of bull-derived cells in freemartin B cells, T cells and other leukocytes isolated from blood and various lymphoid tissues. In 2 freemartins (designated as FmC and FmD) of the 16 analysed, a striking difference in the bull contribution was observed between blood B and T cells. The proportion of Y⁺ cells was only 8% and 9% among B lymphocytes, but as much as 90% and 63% in T lymphocytes (IV / Fig. 1). Slightly less pronounced yet substantial differences were also seen in two other calves. In the first two calves, FmC and FmD, the values remained stable for a follow-up period of 9 months (IV / Fig. 3). They were also essentially the same in lymphocytes isolated from the lymph nodes and spleen, and in T cells from the thymus (IV / Fig. 5). This was reflected by Y chromosome ISH staining of lymphoid tissue sections, where high numbers of Y⁺ cells were seen in T cell-rich areas, while B cell follicles were mostly devoid of them (IV). In the ileal PP of these animals, the great majority of follicles were dominated by female cells. The bull contribution to granulocytes and monocytes closely matched that of T cells (Fig. IV / Fig. 4). The twin
brother of one of these freemartins was also examined, and similar proportions of Y+ cells were found in the various leukocyte fractions and tissues (IV / Fig. 4).

7.5. Generation of a polyclonal anti-bovine CD34 antibody (V)

To facilitate a direct analysis of the bull contribution to various stem cell populations in freemartins, we set out to generate antibodies against the bovine CD34, one of the most widely used markers for hematopoietic stem and progenitor cells in other species (Krause et al., 1996).

7.5.1. Cloning of bovine CD34 cDNA

RT-PCR of bone marrow mononuclear cell total RNA yielded cDNA clones with a 1149-bp open reading frame. A sequence identical to the previously published bovine CD34 cDNA (Zhou et al., 2001a) was assembled, except for a difference of one nucleotide (T781 → C), changing one amino acid in the polypeptide (Trp261 → Arg). This position was identical in all of our clones. A C781 was identified in all samples analysed from 49 animals, representing 9 different cattle breeds (Finnish Ayrshire, Finnish Holstein-Friesian, Eastern Finncattle, Northern Finncattle, Western Finncattle, Icelandic cattle, Doela cattle, Danish Red, and Japanese black cattle). This also is in agreement with the cattle genomic data in GenBank (accession NW_929130).

7.5.2. Expression of CD34 polypeptide fragments

The partial extracellular domain and the cytoplasmic domain of the CD34 polypeptide were successfully expressed in E. coli, while the complete extracellular domain was insoluble. The His-tagged polypeptides were adequately purified by metal affinity chromatography, as shown by SDS-PAGE (for the partial extracellular domain, see Fig. 13). Mass spectrometry indicated the expected fragment sizes, and N-terminal sequencing confirmed that the periplasmic signal peptide introduced by the expression vector was correctly cleaved.

7.5.3. Production and characterization of a polyclonal antibody

A useful rabbit polyclonal antibody against the bovine CD34 was obtained with the partial extracellular domain of the polypeptide. The antibody was affinity-purified and
tested in Western blots and immunostaining of cytocentrifuge preparates and tissue sections.

The antibody, designated as boCD34ecf, recognized a protein of predicted size in Western blots, showing broad and hazy bands characteristic of heavily glycosylated membrane proteins (V / Fig. 4). An expected staining pattern, with positively labelled blood vessels, was also seen in immunostained paraformaldehyde-fixed paraffin sections (V / Fig. 5); a heat-induced antigen retrieval in an acid buffer and a mild protease treatment were necessary. Some unspecific staining was observed, most intensely in erythrocytes and the epidermis. The antibody did not label living cells. Thus, it could not be used in fluorescence activated cell sorting.

**Figure 13.** Purification of the His-tagged extracellular fragment of the CD34 polypeptide by metal affinity chromatography, as seen in SDS-PAGE.

Crude culture medium (med), unbound fraction (ub) and fractions eluted with 10, 20 and 250 mM imidazole are shown. STD: size standard (band sizes shown in kDa).

7.6. Expression of CD34 mRNA and protein in bovine tissues (V)

We characterized the expression of CD34 mRNA in bovine tissues, and used the boCD34ecf antibody to investigate the expression at the protein level.

CD34 mRNA was detected by RT-PCR in all tissues analysed, including blood leukocytes (V). Two products of expected sizes were obtained, confirming the existence of two splice variants as predicted by analysis of genomic data. Tyramide amplified in situ hybridization showed CD34 mRNA in vascular endothelia (V / Fig. 5O) and fibroblast-like connective tissue cells.

In Western blots, the approximate molecular weight of the bovine CD34 was 105-115 kDa in the lung and lymph nodes, and 125-132 kDa in lysates of the brain, muscle, spleen and bone marrow cells enriched for lineage marker negative cells (V / Fig. 4). In
the testis, two bands were present, with sizes of around 118 and 150 kDa. The expression was weak in lymph nodes and the spleen. All of these bands were blocked by free antigen (shown in V).

To further investigate the distribution of the CD34 protein in bovine tissues, double immunofluorescence staining using boCD34ecf and the endothelial marker lectin GSL I-B4 was applied. Vascular endothelia were widely labelled by the antibody (V / Fig. 5). The staining was generally strongest in blood capillary endothelium, which was CD34 positive in all tissues examined. The endothelium of hepatic sinuses was negative (V).

In larger blood vessels, the expression of CD34 was variable and generally weaker. The endocardial endothelium was also weakly boCD34ecf positive. In contrast, all histologically identifiable lymphatic vessels were negative for CD34 (V / Fig. 5D, E).

In cytocentrifuge preparates of bone marrow mononuclear cells, around 7.5% of the cells were positive for boCD34ecf immunofluorescence (V). The proportion of labelled cells was increased by enrichment for lineage negative cells (using antibodies against CD3, IgM, CD14, CD41/61, IgG, granulocytes and red blood cells).

In addition to endothelia and primitive hematopoietic cells, certain other cell populations were stained by the antibody. Smooth muscle in most vessel walls and other tissues was strongly positive in the immunofluorescent staining with boCD34ecf. Large numbers of fibroblast-like cells were labelled in connective tissues (V / Fig. 5B, D, I). In the brain, cytoplasmic staining was seen in subpopulations of neurons, such as the cerebellar Purkinje cells and rare neurons in the thalamus and olfactory bulb. Thymic epithelioreticular cells were stained (shown in V). In addition, immunofluorescence labelling was associated with spermatids in the seminiferous tubules, but not with mature spermatozoa or earlier phases of spermatogenesis (V / Fig. 5I, L). In all of these tissues, the staining was blocked with the antigenic polypeptide. For maximal specificity, the tissues were also stained using tyramide amplified immunohistochemistry, which allows very dilute solutions of antibodies. By this method, endothelia and fibroblasts were strongly labelled, but most smooth muscle was negative or weakly stained, and no boCD34ecf staining could be seen in neurons (V / Fig. 5A), epithelioreticularocytes and spermatids.

In bovine fetuses of the second trimester (110-180 days of gestation), vascular endothelia were generally intensely stained in immunofluorescence. Liver sinusoids were negative, as in adult tissues. Large numbers of individual cells were labelled in fetal liver (V / Fig. 5H).
8. Discussion

8.1. Differentiation potential of somatic cells in adult cattle

At the time that this work was started, transplanted bone marrow was reported to generate non-hematopoietic tissues in mice (Ferrari et al., 1998; Petersen et al., 1999). We set out to investigate somatic stem cell differentiation potential in an unmanipulated large mammal. Chimeric cattle twins are a unique model for this purpose; placental anastomosis allows mixing of blood for most of the fetal period, resulting in high-level hematopoietic chimerism (Lillie, 1917; Owen, 1945; II-IV). The permanent nature of the chimerism proves the exchange of hematopoietic stem cells (HSCs) by the strictest criteria applied in any model system, the long-term repopulation, although no specific markers for cattle HSCs are available. In addition, other stem cell populations circulating in the fetal blood are likely to be exchanged, such as the mesenchymal stem cells (MSCs; Campagnoli et al., 2001); this was not directly assayed in the present work. The sibling-derived cells observed in twin cattle thus are the progeny of HSCs and any other cells received during the fetal period.

Due to the probable heterogeneous nature of this originating population, the twins cannot be used to confirm true plasticity of any somatic stem cell type. Rather, they may provide information on the differentiation potential of circulating somatic stem cells at a general level, or potentially reject the plasticity of any stem cell type. This may seem a drawback compared with the commonly used mouse models. However, the isolation of absolutely pure and homogenous stem cell populations is uncertain, a matter of methodological definition, even in mice (Osawa et al., 1996; Wagers and Weissman, 2004). Processes of tissue maintenance and regeneration in small short-lived rodents may differ from those in large animals, and manipulations necessary for the induction of artificial chimerism may affect the results. Thus, naturally chimeric cattle twins valuably complement the data obtained in mouse experiments and clinical human transplantations.

8.1.1. Evaluation of methods

Y chromosome-specific in situ hybridization (ISH) was shown to be a powerful method for tracing the bull-derived cells in freemartin tissues. In contrast to cytogenetic
techniques previously used (Wilkes et al., 1981). ISH is not restricted to dividing cells, and tissue structure is preserved. The method was demonstrated to be highly specific and sufficiently sensitive (II, III). The high signal intensity allowed rapid screening of large numbers of cells. Furthermore, quantification of bull-derived cells on cytocentrifuge preparations was highly accurate (IV).

Identification of the phenotypes of donor-derived cells is complicated by the narrow range of markers available for cattle research, as compared with mouse or human tools. In the present work, we were able to assemble a panel of antibodies and lectins allowing identification of all major differentiated cell types in paraffin sections of bovine tissues (I). While only some of them were fully exploited in the context of this work, they provide a basis for further investigations. Few bovine stem cell markers are available. Hence, we generated the first anti-bovine CD34 antibodies, as discussed below.

Ordinary transmitted light microscopy was used to analyse the localization of the genetic and phenotypic markers. Serial sectioning or confocal microscopy is generally regarded as necessary for unambiguous marker colocalization. In this work, however, the double staining for the Y chromosome and hematopoietic markers was used as negative selection, to identify the non-hematopoietic bull-derived cells (II). Possible false positives thus did not affect the results essentially.

Cell fusion has recently been implicated as a major cause of apparent plasticity (Wagers and Weissman, 2004). Multinucleated foreign body giant cells with both Y chromosome-positive and -negative nuclei were seen in the granulation tissue (II). However, our attempts of assaying nuclear fusion events between bull-derived cells and female cells were unsuccessful due to technical limitations in the labelling of other chromosomes (unpublished observations). Chromosomal probes suitable for such experiments should be developed in the future.

8.1.2. Implications of bull-derived cells in freemartin tissues

An abundance of bull-derived (Y⁺) cells was observed in most freemartin tissues, including the intestinal mucosa, liver, and brain (II-IV). However, double staining with hematopoietic markers indicated that a vast majority of them were infiltrating leukocytes (II). In most cases, clearly less than 1% of all non-hematopoietic (CD45/ML-1 Y⁺) cells in a tissue were of bull origin. The lack of donor contribution was especially
striking in the central nervous system, although a significant proportion of microglia were bull-derived. This contrasts with several reports of bone marrow cells generating neurons (Mezey et al., 2000; Brazelton et al., 2000; Priller et al., 2001). Less than 0.005% of all non-hematopoietic cells were $Y^+$ in the cerebral cortex. The hippocampus and the olfactory bulb are involved in the generation of new neurons in adult mammals (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994); not a single bull-derived non-hematopoietic cell was detected in these areas. No groups of non-hematopoietic $Y^+$ cells indicating local clonal expansion were seen in any tissues in the majority of calves.

One of the freemartins (FM10) showed an exceptionally high frequency of donor-derived cells in several tissues, especially in the intestinal mucosa and the mammary gland, but not in the brain or the renal epithelia (II). In the intestinal villi, strands of CD45$^-$ $Y^+$ cells were observed, suggesting that some of the crypt stem cells were bull-derived. This was surprising, as the intestinal epithelium is thought to be derived from the primitive gut endoderm (Trier and Moxey, 1979; Ponder et al., 1985). A similar engraftment has, however, been reported with intravenously injected multipotent adult progenitor cells (MAPCs) in immunodeficient mice (Jiang et al., 2002a). On the other hand, the mammary gland is a highly plastic organ, theoretically a candidate target for circulating stem cells. In the infertile freemartins, however, the glands obviously are not engaged in the cyclic expansion and involution associated with pregnancy and lactation.

The reasons for the remarkable donor contribution in the single freemartin (1 of 12) are unknown. It was of the same breed as the majority of the other freemartins. While it was the oldest animal examined (20 months), the difference in age to the closest cases was modest (only 4 and 6 months). The animal was apparently healthy (unpublished observations), but an earlier insult on the intestinal epithelium or other tissues could have gone undetected. Genetic deficiencies in the freemartin cells could provide donor cells with a competitive advantage in some tissues; in absence of such data, this remains speculative. A very early formation of vascular anastomoses in the fetus might result in an exchange of more multipotent primitive cells than available later, but this is unlikely to occur, as discussed in study II. Instead, a rare fusion of early embryos could result in substantial chimerism in random tissues. The $Y^+$ cells in FM10 could originate from either the surviving twin brother, or a third embryo lost before birth. In the first case, the embryonic event would be impossible to retrospectively distinguish from the effects of placental anastomosis, whereas triplets would be detectable by careful genetic
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Intriguingly, spontaneous chimerism resulting from embryonic cell exchange was recently proposed to have a 10% incidence in humans (Boklage, 2006). The high bull contribution in FM10 may thus be explained by factors other than placental anastomoses.

Our results indicate that HSCs (and any other somatic cell type circulating in the fetal blood) generally do not produce significant numbers of non-hematopoietic cells in the development, growth or turnover of bovine tissues. HSC plasticity does not appear as a major physiological phenomenon, and the same is probably true for MSCs. It is possible that a stronger contribution of donor-derived cells would be seen in aged animals. However, this is unlikely, as no obvious correlation was observed between age and the level of chimerism in calves from 20 days to 20 months old (II).

In contrast, the progeny of circulating cells may be important in tissue repair and regeneration. To begin to investigate such processes in cattle, we induced the formation of granulation tissue in subcutaneous cellulose viscose implants (II). The proportion of non-hematopoietic Y+ cells was markedly high in the newly forming granulation tissue, and increased over time. Apparently, cells are recruited from the circulation or adjacent tissues, from a population with a higher frequency of Y+ cells than in the intact subcutaneous tissue. They probably do not proliferate extensively, as no clusters of bull-derived cells were seen. The origin of cells in granulation tissue is controversial, with evidence for both local (Ross et al., 1970; Rangan, 1967) and systemic (Petrakis et al., 1961) sources. Peripheral blood cells positive for vimentin, fibronectin, collagen, CD45 and CD34 have been implicated in tissue repair (Abe et al., 2001). They could be derived from the HSCs, a major proportion of which must be bull-derived in freemartins. In the present study, though, the leukocyte lineages were excluded due to the CD45 expression, unless it is downregulated after extravasation. MSCs would be another candidate source, although the frequency of Y+ cells in this population has not been determined.

Notably, the relatively short follow-up time of 4 weeks does not allow us to conclude that the donor-derived cells stay in the repaired tissue permanently. Instead, they may be accessory cells transiently promoting the repair process. Compatible with this hypothesis, the frequency of Y+ cells around the fully healed ear tag holes was comparable with that of the surrounding tissues (unpublished observations).

No comprehensive analysis with various cell type-specific markers was carried out in this work due to the generally low frequencies of donor-derived cells. Thus, the exact
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phenotypes of the CD45/ML-1-Y\(^+\) cells in freemartin tissues were not determined. The morphology and spatial organization of donor-derived non-hematopoietic cells suggest, however, that they are primarily fibroblast-like cells, pericytes, possibly myocytes and/or associated satellite cells, and occasional epithelial cells (II). No cells with an unambiguously endothelial phenotype were found. A majority of the donor-derived non-hematopoietic cells may represent interstitial cell populations, and integration into parenchymal populations appears rare. The phenotypes of Y\(^+\) cells in the granulation tissue will be characterized in the future.

The results from the present work are in agreement with several recent reports questioning the plasticity of HSCs and other bone marrow cells (Castro et al., 2002; Massengale et al., 2005; Roybon et al., 2006; Wagers et al., 2002; Murry et al., 2004). In other studies, markedly higher contribution of transplants to non-hematopoietic tissues was observed, as discussed in the literature review. Especially in the central nervous system, engraftment rates in our material were strikingly lower than those reported in some earlier experiments. These disparities may be explained by the effects of transplantation procedures, such as radiation damage to the blood-brain barrier, variable criteria for defining the donor-derived cells or species differences. The MAPCs, convincingly documented as highly multipotent (Jiang et al., 2002a), may not have been exchanged through fetal blood, although this would be expected due to the probable MSC circulation. Alternatively, they could indeed be non-existent \textit{in vivo}, arising through the culture methods used to isolate them.

Our results do, however, support the findings that injury stimulates the contribution of bone marrow to non-hematopoietic tissues, at least in the short term (Petersen et al., 1999; LaBarge and Blau, 2002; Okamoto et al., 2002). Due to the very restricted sample size in this work, these processes in freemartins warrant further investigation.

The low level of chimerism in non-hematopoietic tissues of freemartins should enable the transplantation and follow-up of such cells or tissues from their male twins, naturally tolerated by the recipient. The cattle twins may thus be used as a large animal model for investigations of non-hematopoietic stem cell systems.
8.2. Role of the ileal Peyer’s patch in generation of the B cell pool in cattle

In study III, we report that the bovine ileal Peyer’s patch follicles are oligoclonal. This was clearly shown by the discontinuous variation in the proportion of Y+ cells in the ileal Peyer’s patch follicles, together with the small number of different PCR products of the rearranged IgH loci obtained from single follicles. Apparently, each follicle is founded by a few B cells only, and later entry is prevented, as the oldest animals in the study were about 1.5 years of age. Theoretically, a restricted CDR3 length polymorphism could be produced by an intense selection process, but this would probably not affect the distribution of bull-derived cells in the observed manner. In contrast, oligoclonality of lymphoid follicles was not observed in the jejunal PPs or other lymphoid organs.

Surprisingly, a very uneven bull contribution to B and T lymphocytes was found in some freemartins (IV). In the two animals studied in detail, the proportion of Y+ cells in B cells was drastically lower than in T cells and other leukocyte populations. The difference was uniform in all lymphocyte compartments and stable for at least 9 months.

The mechanisms establishing and maintaining the disparity are unclear. The common dominance of either bull or cow cells throughout the hematopoietic system of freemartins may be explained by subtle differences in the speed of early fetal development. Fetal liver is colonized by HSCs around days 27-32 of gestation (Rüsse, 1991). This coincides with the earliest vascular anastomoses detected at about day 30, immediately accompanied by chimerism in the liver (Ohno and Gropp, 1965). Cells from a slightly more advanced fetus may thus have the opportunity to colonize the whole conjoined system. However, the timing alone does not readily explain the minor donor contribution specifically to the B cell pool. The bull-derived hematopoietic progenitors may have had a weaker tendency to commit to the B lineage, or they could have been less effective in the population of the PP follicles. Alternatively, they could have been inferior in their capacity for rapid proliferation or for the survival of the selection processes. Testing these hypotheses would require the isolation of uncommitted progenitors in this type of freemartins and their brothers and a comparison of their B cell generation potential in vitro or in transplantation models.

The observations are also compatible with a self-sufficient pool of cells committed to the B lineage. The stability of the differences between lymphocyte populations may
indicate that few B cells are continuously generated from HSCs or common lymphoid progenitors in young cattle (see Fig. 1). As the majority of all other leukocytes investigated were bull-derived, the donor contribution to HSCs was probably substantial. This would be expected also for *de novo* produced B cells, unless the B lymphopoietic potency of the donor-derived progenitors is persistently severely inferior.

Our results indicate a special role for the ileal PP in the production of B lymphocytes in cattle, and suggest that it may be responsible for the generation and maintenance of the bovine peripheral B cell pool in juvenile animals. Moreover, the limited number of IgH rearrangements in the PP follicles is compatible with the view that cattle rely on post-rearrangement mechanisms in the generation of antibody diversity. Thus, cattle appear largely similar to sheep with regard to strategies for B lymphopoiesis. For a detailed description of bovine B lymphocyte ontogeny, further studies are necessary. The sites where ruminant B cells are originally generated from uncommitted precursors remain unknown. The production of B cells in aged cattle has also not yet been investigated.

**8.3. Generation of an anti-bovine CD34 antibody (V)**

Direct investigation of various stem cell populations requires specific markers, which are scarcely available for cattle. To expand the tool kit, the first antibodies against bovine CD34 were generated in the present work. This sialomucin is commonly used as a marker for purification of human hematopoietic HSCs (Krause et al., 1996). CD34-enriched cells have also been reported to contribute to several non-hematopoietic tissues (Krause et al., 2001). Although CD34 is hardly a perfect stem cell marker, as discussed in the literature review, it certainly is one of the best known. Thus, it is a reasonable starting point for stem cell investigations in cattle and provides a basis for comparative studies. In addition, CD34 is useful as a vascular marker, being expressed in endothelial cells from the earliest stages of development to adulthood (Young et al., 1995).

Polypeptide fragments produced in *E. coli* were used as immunogens, although bacteria are unable to produce the heavy glycosylation of the native CD34 protein (Krause et al., 1996). The glycosylation is cell type-specific (Shailubhai et al., 1997), but antibodies directed against the polypeptide backbone may recognize the protein in any
cell. The extracellular polypeptide fragment yielded a polyclonal antibody, designated boCD34ecf, which was found useful in Western blots, cytocentrifuge preparations, and paraffin sections. However, it did not label living cells. While this is a major shortcoming, such a reagent is still valuable for veterinary research and for the use of cattle as a large animal model in stem cell and cardiovascular biology. In addition, the polyclonal antibody will be helpful in the generation of monoclonal antibodies against the glycosylated CD34 protein.

8.4. Expression of CD34 mRNA and protein in bovine tissues (V)

The expression of bovine CD34 was characterized at the mRNA level. The boCD34ecf antibody was then used to investigate the previously unknown distribution of the CD34 protein in cattle tissues.

RT-PCR analysis of the CD34 mRNA expression confirmed that two splice variants are expressed in cattle, as predicted by the genomic data. The cytoplasmic domain is truncated in the alternatively spliced variant by an exon extension introducing a stop codon. This is the case also in human (Nakamura et al., 1993) and mice (Suda et al., 1992), but has not been demonstrated previously for cattle (Zhou et al., 2001a). Both isoforms were detected in all tissues investigated. The method applied did not provide reliable quantitative information. However, any tissue-level analysis would nevertheless primarily reflect vascular densities; based on in situ hybridization, the majority of CD34 mRNA is produced by endothelial cells. Studying the expression patterns of the two splice variants specifically in situ will be interesting. They may be produced by different cell types or in different cellular states, thus reflecting the poorly known physiological roles of CD34.

In Western blots, several forms of the bovine CD34 protein with different apparent molecular weights (105-150 kDa) were detected, depending on the tissue. These probably represent both alternatively spliced and variably glycosylated forms of the protein.

Immunofluorescence staining showed the tissue distribution of the bovine CD34 to be comparable with the other species investigated. The endothelium was positive in most blood vessels, generally most intensely in the capillaries, arterioles and venules. Hepatic sinusoids and lymphatic vessels were negative. BoCD34ecf staining was enriched by lineage depletion of bone marrow mononuclear cells, suggesting CD34 as a
marker of primitive hematopoietic cells also in cattle. Assaying CD34 expression in the actual HSCs obviously requires the isolation of live cells and testing of their differentiation and self-renewal capacity, impossible with the current antibody.

The boCD34ecf antibody also stained fibroblast subpopulations, thymic epithelioreticulocytes, some types of neurons and smooth muscle. These have previously been reported to express the CD34 protein in mice and humans (Lin et al., 1995; Miettinen et al., 1994). Only the fibroblast staining could be reproduced in tyramide amplified immunohistochemistry, where a more specific staining is expected due to highly diluted antibodies. We also were unable to detect CD34 mRNA in the other cells and tissues by tyramide amplified in situ hybridization. Although the CD34 protein is known to be more long-lived than the mRNA (Beauchamp et al., 2000), these immunofluorescence results must be interpreted with caution. The antibody may recognize a related, highly immunogenic epitope on another protein in the more permissive conditions of non-amplified immunofluorescence stainings.

BoCD34ecf immunofluorescence was associated with late spermatids in the walls of seminiferous tubules. The spermatid tails were apparently labelled, although it is not possible to distinguish these from the surrounding Sertoli cells at the level of resolution provided by standard epifluorescence microscopy. Mature spermatozoa in the lumen were negative, as were the sections of the tubules without spermatids. The CD34 ligand L-selectin (Baumheter et al., 1993) has been reported in rat Sertoli cells (Freeman et al., 2002). It is tempting to speculate that CD34 may have a role in cell adhesion and control of differentiation at the late stages of spermatogenesis, as it does in hematopoietic progenitors and muscle satellite cells (Majdic et al., 1994; Healy et al., 1995; Hu and Chien, 1998; Gordon et al., 2000; Beauchamp et al., 2000). However, the findings could not be reproduced by tyramide amplified immunohistochemistry or confirmed by in situ hybridization. Further studies are thus needed to validate the immunofluorescence findings.
9. Conclusions

a) The chimeric freemartin cattle are a useful large animal model for somatic stem cell research.

b) Y chromosome in situ hybridization, combined with immunohistochemistry, is a powerful method for analysing the contribution of bull-derived cells to freemartin tissues.

c) Hematopoietic stem cells, and the progeny of other cell types circulating in the fetal blood, do not generate significant numbers of non-hematopoietic cells in the development, growth and physiological turnover of bovine tissues. However, they may be important in tissue repair and regeneration.

d) The ileal Peyer's patch has a dominant role in the generation and maintenance of the bovine B lymphocyte pool, as it is thought to have in sheep. Peripheral B cells are probably produced within the ileal Peyer's patch, rather than generated de novo from uncommitted bone marrow precursors, at least in young animals. The oligoclonal nature of the Peyer's patch follicles suggests that cattle rely largely on post-rearrangement strategies in the diversification of immunoglobulins.

e) The first anti-bovine CD34 antibody was produced in this work. The polyclonal antibody can be used to study cattle hematopoietic and cardiovascular systems in tissue sections, cytocentrifuge preparations and Western blot. The bovine CD34 mRNA is alternatively spliced, and both splice variants are widely expressed. The CD34 protein was detected in most blood vessel endothelia, primitive hematopoietic cells and also in some non-hematopoietic cells.
10. References


References


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11. Original publications