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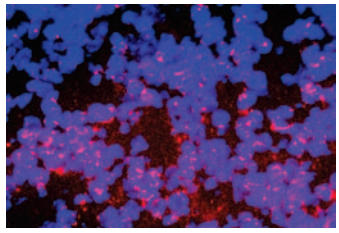

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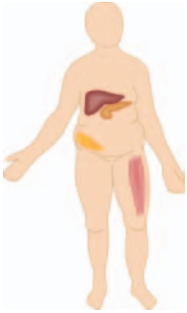

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Diabetes and Stem Cell Researchers Turn to the Lowly Spleen

Shohta Kodama, Miriam Davis and Denise L. Faustman
Shohta Kodama and Denise L. Faustman are at Harvard Medical School and Massachusetts General Hospital-East, Boston, MA 02192, USA. Miriam Davis is at the School of Public Health and Health Services, George Washington University, Washington, DC 20052, USA. E-mail: faustman@helix.mgh.harvard.edu (D.L.F.)

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Introduction

The spleen, an organ that filters blood and contributes to the immune response, has long occupied the bottom rung of the medical pecking order. It is one of the only organs to be removed and discarded in times of trouble. Splenectomy is routine when treating certain traumatic injuries, leukemias, and hematological diseases. But what if the seemingly superfluous spleen possesses a range of stem cells found in no other organ? The spleen might just be the fountain of youth for diseases of aging.

For many years, the spleen has been known to contain a reserve of hematopoietic stem cells (HSCs) with the capacity, during periods of severe stress or disease, to supplement the cells produced by the bone marrow, the major site of hematopoiesis (see Fuller Perspective). But extramedullary hematopoiesis (that is, hematopoiesis occurring outside the bone marrow) hardly makes the spleen unique, considering that other sites like the lymph nodes, thymus, and liver also have this capacity.

The spleen's recent ascension is fueled by findings that this organ harbors populations of newly identified stem cells that can differentiate into fully functional insulin-producing islet cells of the pancreas (1, 2) or partially functional osteoblast-like cells (3, 4). The spleen importance is further reinforced by the finding (see below) that one population of splenic stem cells expresses a key embryonic transcription factor, Hox11, that regulates organogenesis and participates in the development of the nervous system. These tantalizing findings, when taken together, suggest that the spleen might become a source of stem cells to replace cells jeopardized by diseases of aging, such as diabetes [the prevalence of which is highest in those over 60 years of age (5)], bone diseases, and central nervous system diseases such as Parkinson's and Alzheimer's.

This Perspective strives to elevate the importance of the lowly spleen. It draws on a range of research describing the normal physiology of the spleen, its role in development and disease, and its potential application as a source of stem cells for treating diabetes and other diseases of aging.

The Spleen's Undervalued Role in Development and Disease

The easily maligned spleen's best-known role is as a multitasking housekeeper. Situated at the intersection of the immune and circulatory systems, this organ is loosely divided into histological areas of red and white pulp. The red pulp possesses macrophages, whose normal role is to filter and remove from the blood senescent and dysfunctional red blood cells (RBCs), as well as antibody-coated bacteria or RBCs. The higher concentration of RBCs gives the red pulp its name. The white pulp, a lymphoid compartment, plays a pivotal role in immune surveillance and response. The white pulp pumps out antibodies to counter invading pathogens and releases platelets and neutrophils to counteract bleeding or infection. The spleen also has a storage function, because it houses up to one-third of the body's platelets (6). The human spleen stores a small percentage of the body's RBCs, but the spleen's storage capacity in other mammals, such as seals and horses, is far greater (7).

Another established function of the mature spleen is to undergo extramedullary hematopoiesis in times of high demand related to trauma or disease. The spleen holds a population of HSCs that can be summoned, when the need arises, to multiply and differentiate into any type of hematopoietic cell (6). The capacity for hematopoiesis in human adulthood is a replay of the spleen's role during gestation: After the fetal liver, the spleen is the next likely fetal site of hematopoiesis, several months before the bone marrow takes over. Starting with the sixth week of human gestation, the spleen churns out hematopoietic cells from stem cells, although it is not clear whether the cells are circulating or resident cells (6). Later, during the fifth month of gestation, the spleen's role diminishes as the bone marrow assumes center stage in hematopoiesis. Although the spleen's role has been studied in mouse development (8), less is known about extramedullary hematopoiesis in human embryogenesis and adulthood; for example, it is not known (i) whether splenic stem cells are pluripotent or more lineage-restricted HSCs or (ii) whether the pool of cells undergoing hematopoiesis has been stored in the spleen since embryogenesis or has more recently migrated from the bone marrow (or vice versa). Nevertheless, what is clear is that the spleen, beyond its normal blood filtration and immune functions, holds HSCs that, during times of stress or disease, undergo hematopoiesis as a backup to hematopoiesis in the bone marrow.

The Newly Discovered Job of the Spleen: A Stem Cell Reservoir for Diabetic Mice

Types 1 and 2 diabetes are highly disabling diseases (9) that are growing in incidence and prevalence (5, 10). Self-reported prevalence of diabetes (largely type 2) in a nationally representative U.S. sample is highest among those over 60 years of age. For a variety of reasons, including weight gain, diabetes prevalence in this age group has increased over the past decade by 10 to 17% (5) (see Mizuno Review and "Greasing Aging's Downward Slide"). In the more common type 2 diabetes (in which cells no longer respond to insulin), islet cells are eventually lost, perhaps because of excessive insulin secretory requirements; whereas in the less common type 1 diabetes (in which the pancreas does not produce insulin), islet cells are the direct

target of autoimmune attack.

The possibility of using stem cells to replace islet cells of the pancreas is seen as a potential milestone in diabetes therapy. Current treatments, which include insulin and oral hypoglycemic agents, are far from ideal: (i) tight glycemic control is difficult to achieve; (ii) there are complications with exogenous insulin or oral therapies; and (iii) for alternative therapies for type 1 diabetes, there is a limited supply of human donor pancreata or isolated insulin-secreting islets and a growing recognition of the adverse effects of long-term immunosuppression, which accompanies any transplant (11, 12).

Stem cells, with their capacity for self-renewal and differentiation to a variety of specialized cells, are advantageous for replacing islets. The first advantage is that few cells would need to be harvested, because their number could be readily expanded in vivo or ex vivo, given their potent proliferative capacity. The second is that autologous stem cells (stem cells from the patient's own body) could be used, thereby eliminating the need for long-term immunosuppressive therapy. The third is that autologous (or heterologous) cells can be harvested from an adult, thereby avoiding the more ethically controversial destruction of an embryo. The potential use of stem cells specifically for type 1 diabetes, however, could succeed only if the underlying autoimmune defect were reversed, because any replacement islets would become fresh targets for immune attack.

Before our identification of islet stem cells in the spleen, researchers focused on attempting to find appropriate stem cells in the pancreas and other sources. One team of researchers found that embryonic stem cells could be used to form insulin-secreting cells (13); however, the results proved controversial when other groups were not able to replicate them. A further concern was that embryonic stem cells are inherently more unstable in their differentiated state than are adult stem cells, and they pose a greater risk of transformation to tumor cells (14, 15). Adult stem cells for islets have been identified thus far in the bone marrow or peripheral blood (16-19), liver (20), and pancreas. Although the pancreas is the most immediate source, there has been much disagreement about which cell types participate, how restricted their lineage is, and by what mechanisms they differentiate into islets: for example, via the proliferation of differentiated β cells, stem cell differentiation, or transdifferentiation (see below) (21-23). Dor and colleagues concluded, after applying lineage tracing techniques, that self-duplication of differentiated β cells is the major route of islet cell replacement in normal animals (24). The mechanisms of regeneration in normal animals, however, may not be the same as those in diseased animals. Indeed, the mature spleen only undergoes extramedullary hematopoiesis in times of high demand related to trauma or disease; the multilineage stem cells of the spleen may need similar prompting. It is possible that islet cell regeneration may vary depending on the host, the underlying disease, and the administered treatments.

Our laboratory, while searching for immunotherapy for type 1 diabetes, discovered that the spleen contained a population of islet stem cells that could effectively treat diabetes in a mouse model (1, 2). Our first set of experiments revealed that splenic cells, after being harvested from donor mice and coinjected into a diabetic host with a drug that induces tumor necrosis factor-α (for the selective killing of pathogenic T cells), migrated to the pancreas and differentiated into fully functional β cells of the islets that normalized blood sugar concentrations. The response in the host was rapid, durable, and robust. The stem cells did not need to be expanded in culture before injection, and they remained fully functional for >120 days in 92% of mice (2).

We used two lineage tracking techniques to demonstrate that splenic stem cells differentiated into islet cells without fusing with host cells (2). Ruling out fusion has become essential in the stem cell field. Early claims that stem cells had transdifferentiated--switched from one tissue lineage to another--have been undermined by the demonstration that donor cells had actually fused with host cells rather than switched their lineages (25). Although our experiments excluded fusion, the splenocytes, by virtue of their location in the adult spleen, could have originated from lymphoid cells or lymphoid precursors. Instead we found a new and separate population of nonlymphoid cells in the spleen. We also have shown that our functional islets in formerly diabetic mice were from the spleen's nonlymphoid cells (which lack a cell surface marker protein called CD45) rather than lymphoid cells (which express CD45) (2). Bone marrow-derived stem cells fail or contribute poorly to direct and robust islet regeneration by direct lineage differentiation.

Support for the Spleen as a Source of Stem Cells

Disparate lines of evidence support our findings that the spleen holds a reservoir of stem cells for generating islets, at least in times of stress. The spleen's removal in certain diseases unrelated to diabetes can eventually lead to the onset of insulin-dependent diabetes, despite patients having intact pancreata. For example, children with severe thalassemia, a hereditary form of anemia, develop diabetes after removal of their spleen (if it becomes enlarged), whereas children who retain their spleen are less likely to develop diabetes (26, 27). Diabetes also occurs years after removal of both the spleen and the left side of the pancreas to treat chronic pancreatitis, whereas diabetes does not occur after the removal only of the right side of the pancreas (28, 29). Because of shared vasculature, left hemipancreatectomy involves removal of the spleen, but right hemipancreatectomy leaves the spleen intact. Knockout mice that lack the gene for a transcription factor (PTF1-p48) that appears to control formation of the exocrine pancreas (which secretes digestive enzymes) and spatial organization of the endocrine pancreas (which secretes insulin and other hormones) are born normoglycemic (30). Although these mice lack a pancreas, their islets are found to develop in their spleen, yet not in ectopic locations along the digestive tract. One possible explanation for this finding in knockout animals is that the islets formed from differentiating stem cells in the spleen.

If the spleen is a natural harbor for certain stem cells, then it follows that certain heterologous cells would preferentially migrate to the spleen and remain there, instead of to other bodily sites. Human pancreatic stem cells, after having been implanted into immunocompetent mice, take up long-term residence in the mouse spleen, in preference to mouse bone marrow or peripheral blood. The human cells remain in the mouse's spleen for at least 60 days (31), a period far longer than would be expected if the spleen were merely the site of passive blood filtration. More striking is the fact that, decades after pregnancy, human fetal cells have been found in the mother's spleen. Their number was orders of magnitude higher in the spleen than in the maternal lymph nodes and eight other maternal sites (32). The fact that cells of a different species in the first study, and fetal cells in the latter study, escape immune rejection and preferentially remain in the spleen up to decades later implies that the spleen offers an environment for stem cell immortalization, especially of early embryonic cells.

Spleen and Pancreas Dance Together During Development

The spleen as a harbor of pancreatic stem cells is consistent with the interrelationships between the two organs during embryonic development. Decades of descriptive embryology in a variety of vertebrate species have revealed that the spleen and pancreas are formed at the same time and in close proximity during embryogenesis (33-35). The spleen and an embryonic portion of the pancreas (the dorsal pancreatic mesoderm) share mesodermal lineage. Although the main body of the pancreas is formed from budding epithelial tissue of the endoderm and the spleen is formed from mesoderm, the two tissues interact. Classic embryological experiments from the 1960s showed that the development of the endodermally derived pancreas hinges on trophic factors released from the dorsal pancreatic mesoderm (34, 36). Subsequent research revealed that the release of at least four mesenchymal factors is vital for development of the pancreas (22). Close interrelationships between spleen and pancreas during development are also consistent with findings from the knockout study noted earlier, in which mice lacking an exocrine pancreas formed fully functional islets in the spleen instead of in the missing pancreas (30). The converse is true when knockout mice lack a spleen: Their spleen anlage (the embryonic tissue destined to become spleen) relocates during gestation to the mesenchyme of the pancreas, without colonization by hematopoietic cells (37).

Spleen Stem Cells with Developmental Moxie (through Hox-i-ness)

What makes mobilized and injected splenic stem cells so effective and potent? They become functional pancreatic islet cells for months--at the least--without the need for expansion in culture. To learn more about their stem cell prowess, we investigated their expression of a key embryonic transcription factor, Hox11. We hypothesized that splenic stem cells might, through sustained Hox11 expression, retain certain very early fetal capacities through adulthood.

Hox11 (also known as Tlx1 or TCL-3) was originally identified in T cell acute lymphoblastic leukemia (38). Yet the next decade's worth of research showed that Hox11 plays a fundamental role in development: It is a transcription factor encoded by a highly conserved homeobox gene that activates a cascade of genes controlling cell fate and cell differentiation (39-42). Furthermore, it plays a striking role in regeneration, because persistent up-regulation of a *Hox11* -like gene in newts contributes to the regeneration of entire limbs and tail (43, 44). *Hox11* expression in vertebrate embryos is essential for development of the spleen, because the spleen in *Hox11* $^{-/-}$ mice is missing (45).

We investigated *Hox11* expression in the spleen of adult and embryonic mice, using a combination of reverse transcription polymerase chain reaction (RT-PCR) with primers specific for *Hox11* , as well as studies of intranuclear protein expression. Previous research had shown *Hox11* expression in multiple embryonic tissues to be time-limited during an 8-day window in embryonic mice [embryonic day 8.5 (E8.5) through E16] (46). Our findings indicate that the spleen of adult mice contains a putative mesenchymal stem cell with these three characteristics: (i) expression of *Hox11* (in four strains of mice) (Fig. 1A); (ii) lack of expression of *Pdx* , the early pancreatic lineage marker of islet commitment (Fig. 1A); and (iii) nonlymphoid ($CD45^-$) rather than lymphoid ($CD45^+$) origin (Fig. 1B), as confirmed from *Hox11* expression in one mouse strain devoid of most lymphoid cells (NOD SCID) (Fig. 1A). Through immunofluorescence analysis, we also localized *Hox11* protein largely to the nucleus of cells in the subcapsular region of the adult spleen (Fig. 1C). That region of the spleen is where $CD45^-$ cells are predominately situated during adulthood (47). *Hox11* was expressed at similar levels in the spleen of adult mice and embryos at E15. But it was not detectable in adult mouse peripheral blood lymphocytes (PBLs) or in bone marrow cells (Fig. 1D). These findings suggest that the capacity of $CD45^-$ splenic cells to participate in regenerating pancreatic islets is specific to this cell type and is not a characteristic of Hox11-negative cells of bone marrow lineage. Our mRNA and protein findings regarding the pattern of *Hox11* expression in the spleen suggest that these cells are early stem cells; that is, not yet lineage-committed to form only islets.

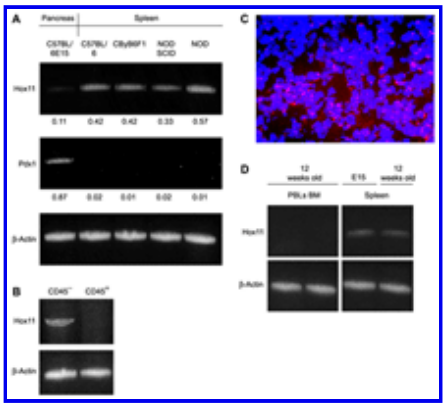

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Fig. 1. Expression of *Hox11* in the spleen of adult mice. (**A**) Polyadenylated RNA isolated from the pancreas of a C57BL/6 mouse embryo at E15 or from the spleen of 12-week-old C57BL/6, CByB6F1, NOD SCID, or NOD mice was subjected to RT-PCR analysis with primers specific for *Hox11* , *Pdx1* , or the β-actin gene. The amounts of PCR products derived from *Hox11* and *Pdx1* mRNAs were determined by densitometry and normalized by the corresponding amount of that derived from β-actin mRNA; the normalized values are shown below each lane. (**B**) Expression of *Hox11* in $CD45^-$ splenocytes. Polyadenylated RNA isolated from $CD45^-$ or $CD45^+$ splenocytes of 12-week-old C57BL/6 mice was analyzed by RT-PCR with primers specific for *Hox11* or the β-actin gene. (**C**) Immunofluorescence analysis of the spleen from a 12-week-old C57BL/6 mouse stained with antibodies to Hox11 (red) and with 4',6-diamidino-2-phenylindole (blue nuclei). (**D**) Polyadenylated RNA isolated from PBLs, bone marrow cells (BM), and the spleen of 12-week-old C57BL/6 mice and from the spleen of a C57BL/6 embryo at E15 was subjected to RT-PCR analysis with primers specific for *Hox11* or the β-actin gene.

Hox11 and other homeobox genes are conserved through evolution. They encode transcription factors that act as developmental switches in controlling spatial patterning, cell fate, and cell differentiation. Up-regulated expression of a *Hox11* -like gene in sponges and newts coincides with the proliferation of progenitor cells, whereas down-regulation occurs during cell differentiation (48). Overexpression of *Hox11* triggers immortalization of certain embryonic precursor cells (49). The splenic stem cell population that we identified highly expresses *Hox11* and has the capacity to contribute to the regeneration of pancreatic β and duct cells in diabetic mice. The expression of Hox11 without expression of tissue-specific or lineage-committed transcription factors suggests that the splenic stem cell has some pluripotency/multipotency. Furthermore, the persistent expression of Hox11, an embryonically early transcription factor, distinguishes these cells from the more differentiated bone marrow- or tissue-specific stem cells of adult mammals.

HSCs Versus Splenic Stem Cells: Different Origins and States of Fetalness?

Most stem cell biologists have placed HSCs, since their isolation 15 years ago (50), on something of a pedestal. Their allure comes from their ability to give rise to robust and reproducible hematopoietic cell types of most lineages, their accessibility in the blood and bone marrow, and their robustness, given the need for perpetual replenishment of blood cells. The possibility that HSCs are plastic--that they might jump from one lineage to another--is another draw, even if rarely demonstrated (25). But how effective are HSCs at replacing pancreatic islets or other adult organs?

HSCs help to regenerate islets, according to several experimental studies in mice, but the effect is most likely indirect. After transplantation into a host, HSCs do not themselves robustly differentiate into islets, but they stimulate regeneration by endogenous cells, possibly by the release of growth factors (16). Another study, using transplanted bone marrow-derived endothelial cells, found that HSCs do not become insulin-expressing cells, but they do lead to the recovery of endogenous islets, most likely by neovascularization (19). Bone marrow transplants, in a separate study (18), were used in a novel way to combat the underlying autoimmune defect in type 1 diabetes. The idea was to use the transplants to induce chimerism (the coexistence of donor and host cells) to dampen the host's autoimmune response against its own islets. The approach successfully reduced autoimmune attack and allowed natural mechanisms of endogenous islet regeneration to occur. Another study (17) demonstrated with lineage tracing that HSCs transdifferentiated into insulin-expressing cells, but only with a low frequency of 1.7 to 3.0%. Our experiments, on the other hand, showed rapid and robust differentiation of nonlymphoid ($CD45^-$) but not lymphoid ($CD45^+$) splenic stem cells into functional islet cells (2). Bone marrow cells fail to express *Hox11* in adults, whereas splenic stem cells express it into adulthood (Fig. 1D). The evidence, taken as a whole, suggests that splenic stem cells appear to be less

lineage-committed. Perhaps they are frozen in an earlier embryonic state that allows more diverse lineage commitment to bone, islets, ducts, etc., at least in the mouse, under set experimental conditions.

Versatility of Splenic Stem Cells May Trace to Embryogenesis

Why does the spleen hold a population of stem cells with apparent multilineage potential? The answer may partly trace to embryogenesis. The spleen is formed in mammals from a region of embryonic mesoderm known as the aorta-gonad-mesonephros (AGM), which appears at 10 to 12 days post coitum (dpc). This intraembryonic region, which includes the splanchnic mesoderm, surrounds the endoderm of the developing gut and the endothelium of arteries at day 8.5 to 10 dpc. The AGM region forms from the paraaortic splanchnopleura (P-Sp) (51, 52). This process represents the formation of the coelomic cavity that subdivides the intraembryonic mesoderm into two regions--that is, the mesoderm close to the ectoderm and the mesoderm close to the endoderm. It is this latter region that is the spanchnopleura. The aorta then develops from the splanchnopleura and the region becomes the P-Sp. In the past few decades, the AGM has gained recognition along with the yolk sac as two prime locations for generating independent pockets of multipotent hematopoietic precursors before the establishment of circulation (8).

Before the 1970s, the embryonic yolk sac was seen as the first and only site giving rise to hematopoietic cells. A series of studies in several vertebrate species, including mouse embryos (53, 54), determined that the yolk sac and the AGM region are both original sites of hematopoiesis, although their relative contributions are still debated. Later, cells migrate from the yolk sac or AGM and colonize the liver, which is the first embryological organ to produce hematopoietic cells. In the mouse, the sequence proceeds from the yolk sac or AGM to the liver, then the spleen, and lastly to the bone marrow, which remains the major site of hematopoiesis throughout life (8). Precursor P-Sp cells, but not yolk sac hematopoietic precursors, isolated before embryonic circulation, provide long-term multilineage and robust adult hematopoietic reconstitution (52). This finding suggests the yolk sac cells have a more restricted or more differentiated state, even in early development, whereas P-Sp cells have broader and multilineage, at least hematopoietic, potential (51, 52).

Our finding in the mouse spleen of a stem cell population with possibly broad lineage potential may be explained by early patterns of cell migration from the AGM region. Some AGM cells, after migration, may remain in the spleen in an undifferentiated state. A recent study has found that AGM cells, in particular $CD45^-$ cells, harvested from mouse embryos not only have the capacity to differentiate into cells in the blood, bone marrow, and spleen, but the $CD45^-$ AGM cells also differentiate into nonhematopoietic cells (stroma-like cells and fibroblasts) found in the adult liver, kidney, lung, small intestine, and uterus (55). The investigators studied the fate of AGM cells by transplanting them from transgenic mouse embryos (expressing enhanced green fluorescent protein) into the liver of neonatal mice and then tracking the pattern of labeled cells once the animals had developed into adults. This suggests that the AGM cells, even in nondiseased animals, can play a homeostatic role for these target organs. The developmental fate of AGM cells in animal models of disease, such as spontaneous diabetes, has not yet been established.

Summary and Applications to Aging

This Perspective has largely focused on the potential value of stem cells from the spleen for treating diabetes, but other potential applications loom large. The most immediate applications hinge on finding *Hox11* expression in introduced splenic stem cells that regenerate pancreatic islet cells. Hox11 is a pivotal and early player in the embryonic development of vertebrates and invertebrates. Not only is this transcription factor involved in organogenesis of the spleen and other mesoderm-derived structures, but it also is involved in development and patterning of the nervous system. *Hox11* or its ortholog is transiently expressed in certain neuron populations of mice (46, 56, 57) and chicks (58). Its expression patterns in zebrafish coincide with a critical time of axon outgrowth, suggesting that Hox11 plays a role in neuron specification and patterning (39). Members of the Hox family of proteins also are found in developing spinal motoneurons, where sequential waves of expression are thought to be responsible for motoneurons' identity and regional organization (59, 60). Given the breadth of Hox's role during nervous system development, splenic stem cells might be used to replace neurons lost to diseases of aging, such as Alzheimer's, Parkinson's, and stroke. One potential advantage is that Hox11 may confer the capacity to form appropriate connection patterns. This potential capacity is reinforced by Hox11's role in regenerating entire limbs and tails of newts (43, 44).

The adult spleen possesses some populations of stem cells that play a role in diverse adult forms of regeneration. Splenic stem cells can assist in hematopoiesis, islet regeneration (1, 2), and the formation of osteoblast-like cells (3, 4). The expression of Hox11 in the spleen's insulin-producing stem cells raises the possibility that this population is multipotent: a possibility that may trace to embryological migration patterns. If splenic stem cells have the capacity to form neurons and other cell types, they may be vital for replacing cells destroyed by diabetes and other diseases of aging. Given the range of possibilities, it is not far-fetched to consider the spleen for one-stop stem cell shopping.

January 19, 2005

Comment on Article

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