

Regenerative medicine: a radical reappraisal of the spleen

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The spleen has long been considered a dispensable organ. Recent research, however, has found that the spleen of adult mice holds a reservoir of stem cells that can rapidly and robustly differentiate into functional cells of diverse lineages. Splenic stem cells express *Hox11*, a key embryonic transcription factor that regulates organogenesis. The presence of multi-lineage stem cells in the spleen might represent lifelong persistence of cells from a primitive embryonic region called the aorta–gonad–mesonephros. By bringing together findings from diverse disciplines, we propose that the adult spleen is an important source of multi-lineage stem cells for future cellular therapies for diabetes and other diseases.

The spleen is undervalued

Conventional medical wisdom holds that the spleen is a dispensable organ. Its removal is standard procedure with certain types of trauma and diseases of the blood or pancreas. But newly characterized stem-cell (see Glossary) populations found in the spleen are beginning to challenge conventional wisdom. Recent studies in animal models have identified populations of splenic stem cells that are capable of differentiating into cells of other organs, including pancreatic β -islet cells [1,2] or new bone matrix from spleen stem cells with osteoblast potential [3,4]. Furthermore, it has long been established that the spleen contains a reserve population of hematopoietic stem cells that is tapped when the bone marrow cannot fully meet the body's demand. These lines of evidence point to the adult spleen as a potential reservoir of stem cells to harvest as cellular therapies for diabetes and other diseases. Adult stem cells are advantageous because they can be harvested from the same individual in which they are to be used, thereby avoiding the need for immunosuppressive drugs, and because they avoid the more ethically controversial use of embryonic stem cells. Drawing on studies on the physiology of the spleen, formation during development and use in animal models of disease, we propose the spleen as a source of stem cells for future cellular therapies.

The spleen supports hematopoiesis in development and disease

At the interface of the circulatory and immune systems, the spleen has dual roles in maintenance and adaptation to stress or disease [5]. The red pulp of the spleen holds macrophages that normally filter and remove senescent or defective red blood cells (RBCs) and antibody-coated bacteria or RBCs from the circulation. The white pulp of the spleen – its lymphoid compartment – is crucial for immune surveillance and response. It synthesizes antibodies against invading pathogens and releases platelets and neutrophils in response to bleeding or infection. The spleen is a storage site for up to one-third of the body's platelets [5] but only a small fraction of RBCs [6].

A lesser-known but well-established function of the spleen is in extramedullary hematopoiesis. When the bone marrow cannot fulfill the body's demand in times of stress and disease, the spleen can serve as a backup by generating all types of cellular components of the blood [5]. The capacity of the mature spleen for hematopoiesis is not surprising, considering that it is among the first sites of hematopoiesis during gestation – even before the bone marrow. As early as six weeks' gestation, the fetal spleen produces the full range of hematopoietic cells. By the fifth gestational month, production in the spleen trails off as bone-marrow hematopoiesis starts [5]. It is unknown whether the pool of splenic stem cells undergoing extramedullary hematopoiesis in adulthood are more-pluripotent stem cells versus more-restricted hematopoietic stem cells, and whether the pool has been sequestered there

Glossary

AGM (aorta–gonad–mesonephros): Region of the mammalian embryo that gives rise to cells of the aorta, mesonephros (the functional kidneys of the embryo) and genital ridges or gonads. Recent data also show AGM cells form the liver, lung, small intestine and uterus.

Extramedullary hematopoiesis: The production of hematopoietic cells outside the bone marrow during times of stress or disease.

Hox11: Highly conserved transcription factor regulating growth and development in vertebrates and invertebrates.

Multi-lineage stem cells: stem cells from one organ system or tissue with the capacity to differentiate into cells of at least one additional organ system or tissue (e.g. spleen stem cells that form mature cells of the pancreas).

Stem cells: undifferentiated cells with the capacity not only to replace themselves (through mitosis) but also to form multiple types of specialized cells.

Transdifferentiation: cells of one specialized type switching to another specialized cell type.

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since embryogenesis or has migrated there more recently from the bone marrow. The main point, however, is that one normal function of the adult spleen is to house hematopoietic stem cells and initiate hematopoiesis in times of stress or disease.

Spleen as reservoir of stem cells for diabetes

The search for islet stem cells is a high priority for the 150 million patients worldwide with diabetes. By virtue of their capacity for self-renewal and differentiation into specialized cell types, stem cells are seen as a potential therapy to replace insulin-secreting β -islet cells of the pancreas. Pancreatic β -islet cells are the target of an autoimmune attack in type 1 diabetes. In type 2 diabetes, β -islet cells produce insufficient insulin to overcome insulin resistance in the target tissues (e.g. muscle), in which insulin receptor signaling is altered, and eventually die. The impetus behind the search for insulin-secreting stem cells is the difficulty of normalizing metabolic control with exogenous insulin, the high rate of complications with existing insulin therapy and the limited supply of human donor pancreata as alternative therapy [7]. Stem cells offer a potential way to meet the demand, because only a small quantity need be harvested or expanded *in vitro* before implantation. Furthermore, the use of autologous stem cells would surmount the need for lifelong immunosuppression. Nevertheless, whether from autologous or heterologous sources, stem cells can only be a viable cell-replacement strategy for type 1 diabetes if the underlying autoimmune defect is eliminated. Without disease elimination, replacement islets would be subject to autoimmune destruction.

Although the consensus view is that islet stem cells should reside in the pancreas, bone marrow or progenitor tissues, the search for islet stem cells in these sites has not uncovered a definitive source. One group reported that embryos possessed stem cells that could form insulin-secreting cells [8], but other groups could not replicate the finding [9]. Amid concerns about instability and tumorigenic risk of embryonic stem cells [10,11], most attention has shifted to adult stem cells. Adult stem cells have the advantage of greater stability for maintaining a differentiated state and lower likelihood of transformation to tumor cells. These advantages are important considering that any cell-based therapy for diabetes would need to remain functional over the long term and, if for type 1 diabetes, would need to be introduced at a young age. Adult stem cells for potential islet regeneration have been isolated from bone marrow or peripheral blood [12–15] and liver [16]. Another source is the pancreas itself. But there is lack of consensus on which cell types are responsible for islet regeneration, how restricted is their lineage and by what mechanisms regeneration occurs (e.g. proliferation of differentiated β -islet cells, stem-cell differentiation or transdifferentiation) [9,17]. There is evidence for stem or progenitor cells residing in islets, ducts or acinar cells. Most studies, however, were histological in nature and did not examine *in vivo* the functional capacity of the cell to restore normal glycemic control in the setting of spontaneous disease, such as type 1 diabetes. The exception is a study of normal animals using lineage tracing [18]. The

study found that the predominant route of islet-cell replacement is by self-duplication of differentiated β -islet cells rather than by stem-cell recruitment and differentiation. Although the importance of β -islet-cell replication cannot be underestimated, it might be more applicable to normal than disease states. The mechanism of islet regeneration might depend on numerous factors, including the host, the underlying disease and the administered treatments.

In the mouse, a subpopulation of islet stem cells exists in the spleen which, upon infusion into a diseased host, can migrate to the pancreas and become functional islets that restore blood sugar levels to normal [1,2]. Two lineage-tracking techniques were used to show that donor spleen cells had, after intravenous transfer, homed to the host's pancreas where they differentiated, without fusing with host cells, into β -islet cells. The donor cells remained fully functional for >120 days in 92% of mice [2]. Only nonlymphoid ($CD45^-$) cells of the spleen, as opposed to $CD45^+$ cells, directly contributed to newly formed islets. Bone-marrow-derived stem cells, in a separate experiment, did not contribute to adequate or robust islet regeneration, assessed by the restoration of blood sugars [19]. The results suggest that the splenic $CD45^-$ cells, once in the pancreas, had differentiated rather than transdifferentiated across tissue lineages, because they bore markers of early development instead of the pancreatic lineage. The long-term restoration of glycemic control by the injection of small numbers of fresh $CD45^-$ stem cells, which did not need expansion in tissue culture, thus meets the criteria for stem-cell efficacy and robustness, durability and function [20].

Other lines of evidence point to the spleen as a source of β -islet stem cells that can be recruited throughout adulthood in diabetes or pancreatic disease. For example, insulin-dependent diabetes is the eventual outcome for individuals with chronic pancreatitis who, years earlier, had left-hemipancreatectomy, but not right-hemipancreatectomy [21,22]. Left-hemipancreatectomy requires removal of the spleen along with the pancreas owing to their shared vasculature, whereas right hemi-pancreatectomy does not involve spleen removal. Similarly, spleen removal in children with severe thalassemias leads to the eventual development of insulin-dependent diabetes [23,24]. An experimental study by Krapp and co-authors [25] found that knockout mice lacking a pancreas (due to ablation of PTF1-p48, which is a transcription factor controlling exocrine pancreas development) were born normoglycemic; their endocrine-derived β -islet cells, which were not directly affected by the gene knockout, had developed in the spleen instead of their usual location in the pancreas, which was missing. The islets in the spleen were functional insofar as they controlled blood sugar levels. The islets did not appear at ectopic locations along the digestive tract. Although the investigators had not designed the study to test the lineage, development and migration patterns of islets, our hypothesis is that the islets formed in the spleen from differentiating splenic stem cells. An alternative explanation is that a yet to be identified pancreatic islet precursor cells migrated to the spleen.

If the spleen is a natural harbor for adult stem cells for the body to draw upon in disease, it follows that the spleen is a preferential site for stem cells to home. Abraham and colleagues [26] implanted human pancreatic stem cells into immunocompetent mice and found that human stem cells took up residence in the host's spleen, in preference to its bone marrow or peripheral blood, and remained there for at least 60 days. Remarkably, in humans, fetal cells were found – decades after pregnancy – in far greater numbers in the maternal spleen compared with the pancreas and nine other maternal organs, including lymph nodes [27]. The persistence of fetal cells in maternal tissues has been reported for 20 years, but the functional implications for the mother are not fully understood [28].

Although the apparent preference of fetal cells or stem cells for the spleen might reflect the more passive blood-filtration function of the spleen, the duration of their residence in the spleen, for months to decades, suggests that the spleen is a specific harbor that facilitates stem-cell immortality into adulthood.

Spleen stem cells express markers for early embryonic development

A series of experiments on adult mice examined whether their splenic stem cells express a marker of early development, *Hox11*. The hypothesis driving the experiments was that the capacity of splenic stem cells for regenerating islet cells might come from retention of fetal capacities into adulthood. *Hox11* (also known as Tlx1 or TCL-3) was first found in adult mammals in association with cancers, such as T-cell acute lymphoblastic leukemia [29]. The protein is encoded by a highly conserved homeobox gene and is an important transcription factor regulating early development in both vertebrates and invertebrates [30–33]. In the newt, persistent and marked upregulation of a *Hox11*-like gene has a role in the regeneration of entire limbs and the tail [34,35]. In vertebrate embryos, *Hox11* expression is obligatory for spleen development, because *Hox11*^{-/-} mice lack a spleen [36].

Using reverse transcription (RT) and polymerase chain reaction (PCR) with primers specific for *Hox11*, Kodama *et al.* [19] found that the spleen of adult mice contains a putative mesenchymal stem cell which (i) expresses *Hox11* (in embryos and adults of four different mouse species); (ii) does not express *Pdx*, the early pancreatic lineage marker of islet commitment; and (iii) is of nonlymphoid (CD45⁻) origin. Using immunofluorescence, *Hox11* protein was localized predominantly in the nucleus of cells in the subcapsular region of the adult spleen. Finally, the study failed to find *Hox11* expression in peripheral blood lymphocytes or bone-marrow cells of adult mice. This finding suggests that the ability of CD45⁻ cells of the spleen to contribute to the regeneration of pancreatic islets is specific to this cell type and is not mimicked by *Hox11*-negative cells of the bone-marrow lineage.

Homeobox genes such as *Hox11* are evolutionarily conserved and encode transcription factors that act as developmental switches in the orchestrated control of cell fate. The expression of a *Hox11*-like gene in sponges and newts is associated with the proliferation of progenitor

cells, with expression downregulated during cell differentiation [37]. The artificial overexpression of *Hox11* also induces immortalization of embryonic precursors derived from embryonic stem cells [38].

The spleen and pancreas interact during development

The possibility of the spleen being a reservoir of islet stem cells is supported by close interrelationships between the spleen and pancreas during development. The two organs form simultaneously and in close proximity during the early stages of organogenesis, according to decades of descriptive embryology in mammals, birds, reptiles and amphibians [39–41]. In zebrafish, the pancreas specification also depends on overlapping factors, such as retinoic acid, bone morphogenetic protein or hedgehog signaling; the overexpression of bone morphogenetic protein causes expanded or ectopic pancreas development – a finding that links bone-formation transcription factors to pancreas development [42].

The spleen forms from the mesoderm, and the main corpus of the pancreas forms from two patches of epithelium that bud from the gut epithelium. The growing pancreas epithelium is surrounded by mesenchymal cells, which are obligatory for growth and exocrine differentiation. The spleen either forms directly from the dorsal pancreatic mesenchyme (DPM) or the two form from the same pool of mesodermal stem cells. The shared mesodermal lineage of the DPM and spleen has been confirmed by gene-knockout experiments. The DPM and spleen are intact after the knockout of *Pdx1* [43] and *Hlxb9* [44,45], which are key transcription factors for the development of the pancreas. Knocking out these genes eliminates the tissue destined to become the exocrine and endocrine pancreas. The endocrine pancreas normally forms by the fusion of dorsal and ventral pancreatic buds and is apparent even as the pancreas begins to bud from the gut [41]. Conversely, the development of the spleen and DPM were disrupted in an experiment that induced the ectopic expression of the regulatory protein Shh [46].

Close developmental interrelationships between the spleen and pancreas are also supported by the knockout study in which mice that are genetically deficient for the development of the exocrine pancreas had formed fully functional and abundant islets located in the spleen, in lieu of the pancreas [25]. Conversely, *Hox11*^{-/-} mice lack a spleen, but the spleen anlage (i.e. forerunner) relocates during development to the mesenchyme of the pancreas without hematopoietic cell colonization [47]. These observations support coordinated embryonic development of the pancreas and spleen.

Classic embryological experiments from the 1960s found that interactions between mesenchymal and budding epithelial tissue are necessary for the development of the pancreas. Removal of the DPM, which surrounds the dorsal pancreatic bud, arrests the development of the pancreas, whereas the reintroduction of mesenchymal tissue leads to regrowth [40,48]. The precise mechanisms of signaling between the DPM and the pancreas have begun to be elucidated through gene-knockout experiments. At least four mesenchymal factors are essential for the development of the pancreas [9].

Spleen stem cells are distinct from bone-marrow stem cells

During the past decade, much of the focus of the stem-cell field has been on hematopoietic stem cells and their potential plasticity (transdifferentiation) to become specialized cells of nonhematopoietic lineages. Early enthusiasm concerning evidence of plasticity has been tempered, however, by findings that stem-cell plasticity is a rare event and instead might be accounted for by other forms of regeneration, such as fusion or the release of trophic factors that stimulate the differentiation of tissue-specific host cells [49]. Several studies have examined the potential of hematopoietic stem cells (HSCs) to regenerate islet cells. The findings thus far indicate that HSCs facilitate islet regeneration, but predominantly by indirect means. Hess and colleagues [12] found that transplanted HSCs, which contain endothelial progenitor cells, do not develop into functional islet cells but instead stimulate regeneration by endogenous cells, presumably through the release of growth factors. Similarly, another study found that bone-marrow-derived endothelial cells induce the recovery of endogenous islets, probably by neovascularization [15]. Another study used bone-marrow transplants in a novel way to address the underlying autoimmune defect in type 1 diabetes; the transplanted marrow induces chimerism in the host, thereby tempering its autoimmune response and enabling natural mechanisms of endogenous islet regeneration to occur [14]. Finally, one study did establish, through lineage tracing, that HSCs could transdifferentiate to express insulin, but the frequency of transdifferentiated cells in islets was only 1.7–3% [13].

A study with nonlymphoid (CD45⁻) stem cells of the spleen showed them to be responsible for the rapid and robust differentiation into functional islet cells when transplanted into NOD mice (a murine model of type 1 diabetes with spontaneous hyperglycemia) [2]. The study also found that lymphoid cells of the spleen (CD45⁺) did not contribute to the formation of functional islets. In a later experiment, splenic CD45⁻ stem cells were compared with bone-marrow cells, which contain HSCs. Bone-marrow cells did not express *Hox11* in adulthood, in contrast to splenic stem cells, which expressed this early developmental regulator throughout adulthood. Taken together, the evidence suggests that splenic cells are distinctive in their capacity for regeneration.

Developmental origins of splenic stem cells

Stem cells of the spleen appear to have multi-lineage potential on the basis of their *Hox11* expression and capacity to differentiate into functional islet cells or osteoblast-like cells [1–4]. Why are these multi-lineage stem cells found in the spleen? One possibility traces their developmental origin to a region of the mammalian embryo known as the aorta–gonad–mesonephros (AGM).

The AGM is formed at embryonic day 10 in mice, from mesodermal tissue found in close contact with endodermal tissue near the embryonic aorta – an embryonic area known as para-aortic splanchnopleura (P-Sp) (Figure 1) [50,51]. Because the AGM is formed directly from the P-Sp, it is often referred to as the P-Sp/AGM. The AGM is

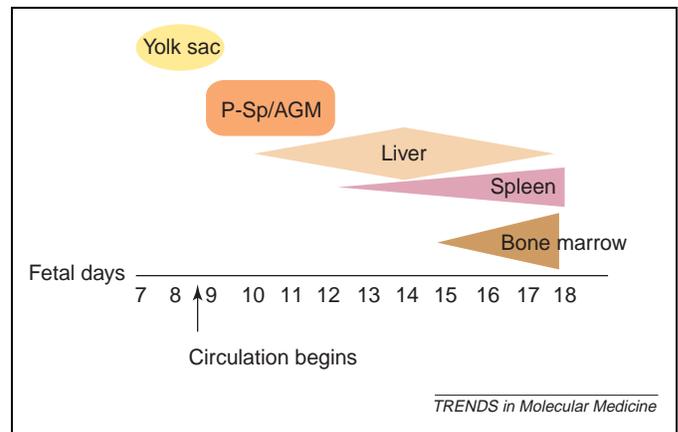


Figure 1. The approximate time of appearance of hematopoietic activity in different fetal tissues of the mouse. Normal gestation is for 21 days. The y-axis represents days of fetal mouse development. Redrawn from [53]. Abbreviations: AGM, aorta–gonad–mesonephros; P-Sp, para-aortic splanchnopleural.

a band of embryonic tissue that, as the name implies, forms the aorta, mesonephros (the functional kidneys of the embryo) and genital ridges or gonads. But before those tissues form, the AGM region (at embryonic day 10) has another highly significant function: it is the first intra-embryonic site of hematopoiesis [50,52]. The only other site of hematopoiesis around this time is the yolk sac, which is extra-embryonic. Both sites temporarily generate hematopoietic cells. As the mouse embryo matures, hematopoietic stem cells migrate from these two sites to the embryonic liver and later to the spleen, where they also produce hematopoietic cells – again only temporarily. By embryonic day 15, hematopoietic stem cells have migrated to the bone marrow, which takes over as the central site of hematopoiesis for the remainder of life [53]. The formation of the AGM is probably independent of yolk-sac formation and is established before the circulatory system.

Although much of the research has focused on the production of hematopoietic cells by the AGM, a recent study suggests that AGM cells have even broader lineage potential. The study harvested CD45⁻ cells from the AGM region of mice expressing the marker green fluorescent protein. After being transplanted into the liver of neonatal mice, the labeled cells were found in adult hematopoietic tissues, including the spleen and bone marrow, in addition to nonhematopoietic tissues – liver, kidney, lung, small intestine and uterus – where they differentiated into vascular cells, stroma-like cells or fibroblasts [54]. The authors did not examine the pancreas, but for the parenchymal organs examined the expression was broad within the abdomen.

These disparate lines of evidence suggest the possibility that stem cells with multi-lineage potential are found in the spleen as a result of early migration patterns from the AGM. Once in the spleen, the stem cells can persist throughout the life of the organism in an undifferentiated state.

Concluding remarks

We view the adult spleen as a potential source of stem cells for treating disease. Research in experimental models

reveals that the spleen houses a variety of stem cells for restoring glycemic control in diabetes, producing bone matrix and hematopoiesis. Splenic stem cells already have an established role in hematopoiesis during development and adulthood. Their versatility in producing functional cells of several lineages might be attributable to the persistence of expression into adulthood of *Hox11*, a key transcription factor in many vertebrate species that regulates organogenesis and regeneration. To bring splenic stem cells to the clinic, more research is needed to characterize them in animal models of disease and understand their multi-lineage potential in disease states.

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