The Promise of Hox11+ Stem Cells of the Spleen for Treating Autoimmune Diseases

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Key words
- spleen
- regeneration
- Hox11
- Sjogren's syndrome
- type 1 diabetes
- hearing loss

Abstract

The spleen of human adults uniquely possesses a reservoir of multilineage adult stem cells that express the developmental transcription factor *Hox11*. In contrast to hematopoietic stem cells, *Hox11+* stem cells hold potentially broader therapeutic applications because they are less lineage restricted. *Hox11/Tlx1* is part of a homeodomain gene family essential for organogenesis of the spleen and for contributions to development of hindbrain, cochlea, pancreas, salivary glands, among other organs and tissues. While *Hox11/Tlx1* displays widespread patterns of expression during embryogenesis, its expression was thought to cease after birth. Recent findings in human post-mortem tissue have shattered this dogma, finding that *Hox11/Tlx1* stem cells are uniquely and abundantly expressed throughout adulthood in the human spleen. While their role in humans is not yet understood, *Hox11/Tlx1* stem cells from the spleen of normal mice have been harvested to assist in both the treatment and cure at least two autoimmune diseases: type 1 diabetes, Sjogren's syndrome, and possibly their comorbid hearing loss. The splenic stem cells are infused, with an immune therapy, into diseased NOD mice, where they can home to the diseased organ, differentiate into the appropriate cell type, and assume normal functioning with the endogenous regeneration of the animal due to disease removal. This review covers *Hox11/Tlx1+* stem cells' success in an animal model and their potential for treating autoimmune diseases in organs that mirror their extensive expression patterns during embryogenesis.

received 26.09.2007
accepted 26.10.2007

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DOI 10.1055/s-2007-1022560
Horm Metab Res 2008;
40: 137–146

ISSN 0018-5043

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Introduction

Research from our laboratory has disclosed that the spleen of normal adults is the only site in the body harboring a reservoir of multi-lineage stem cells that express the highly conserved nuclear transcription factor *Hox11/Tlx1* [1, 2]. *Hox11/Tlx1+* cells possess key stem cell characteristics by virtue of their capacity for self-renewal and capacity to differentiate into cells of multiple lineages [2–7]. During embryogenesis, *Hox11/Tlx1* is indispensable for development of the spleen, and it contributes to development of the pancreas and portions of the nervous system, among a plethora of other tissues and organs of both vertebrates and invertebrates [8–10]. *Hox11/Tlx1* stem cells in this previously unknown splenic reservoir do not express the hematopoietic marker CD45+, which suggests that they may have broader multilineage potential than do hematopoietic stem cells (HSCs) with the more restrictive CD45+ marker.

Identifying a reservoir in the spleen of adult stem cells in humans is an outgrowth of our previous findings in mice [3]. We have reported that adult mice contain a reservoir of *Hox11/Tlx1*-expressing stem cells in their spleen, from which cells were harvested to contribute to the permanent reversal of type 1 diabetes by pancreas regeneration. After extracting *Hox11/Tlx1* stem cells from the spleens of a healthy donor mice and injecting them into NOD mice, the infused cells homed to the host pancreas. There the stem cells differentiated into fully functional β cells with insulin and contributed to normoglycemia, as long as the host animals were also given an immune therapy to eradicate the underlying autoimmunity. The observed regeneration in the pancreas was a combination of regeneration assisted with the spleen cells as well as endogenous regeneration of the treated host [3]. Splenic stem cells have also been found in animal or *in vitro* models to differentiate into functional cells for treating Sjogren's disease and possibly some of the

comorbidities of autoimmunity, among other conditions [6,7]. Although *Hox11/Tlx1* expression may not have been evaluated in each of these particular experiments, the findings point to the multilineage potential of CD45 stem cells culled from the spleen.

This review begins with the historical origins of *Hox11/Tlx1* (also known as *Tlx1*) and its patterns of expression during embryogenesis. These patterns are essential, for the evidence presented in this review suggests that *Hox11/Tlx1* stem cells hold autoimmune treatment potential in target tissues that recapitulate the lineages seen in development. The review proceeds to discussing the identification of a *Hox11/Tlx1* stem cell reservoir in the human spleen. This discovery is a platform for illuminating the promise of *Hox11/Tlx1* expressing stem cells as cellular therapies for type 1 diabetes, other autoimmune diseases, and comorbidities, such as hearing loss. Finally, the review turns to the safety posed by *Hox11/Tlx1*-expressing stem cells and how any heretofore unknown risks of *Hox11/Tlx1* stem cells could be minimized by splenectomy, considering that the spleen is an expendable organ. Because the focus of this review is on autoimmune diseases, we have purposefully omitted discussion of two important studies showing that splenic stem cells can differentiate into partially functional osteoblast-like cells [6,7]. Nevertheless, these studies reinforce the multilineage potential of splenic stem cells.

Historical Overview of *Hox11*

Hox11 first identified in human leukemia cells

Oncology researchers are often the first to identify seemingly deleterious proteins expressed in tumors that eventually turn out to possess beneficial applications to human health and disease treatment. A decade before the vital role of *Hox11/Tlx1* cells was identified in normal development, first it was discovered as a proto-oncogene in certain human T cell acute lymphoblastic leukemia (ALL) [11,12] and some CNS tumors of childhood [13]. The etiologies of ALL are diverse, but one type exhibits dysregulation of *Hox11/Tlx1* proto-oncogenes. Because cells with dysregulation of *Hox11/Tlx1* proto-oncogenes frequently co-expressed chromosomal translocations on chromosome 10 [11], the translocations were thought to remove regulatory elements upstream of the *Hox11* gene. That, in turn, led to overexpression of *Hox11* and subsequent tumor genesis. In support of this hypothesis, transfection of *Hox11* was found to immortalize hematopoietic precursor cells [14]. Therefore, prior to the work on using *Hox11/Tlx1* splenic murine stem cells in regeneration of select organs, *Hox11* was clearly established as an oncogene and an immortalization factor for embryonic stem cells with induced overexpression.

Normal developmental expression of *Hox11* in animal models

Hox11/Tlx1 is a part of a group of highly conserved homeobox genes. The protein it encodes is a highly conserved transcription factor that activates a constellation of genes controlling cell fate and differentiation [9,15]. The *Hox* gene family is found over a wide range of species, such as zebrafish, chicks, Xenopus, and mammals [16–20]. In these species, *Hox11/Tlx1* is expressed in organs and tissues similar to those found in detailed studies of mouse embryos.

Table 1 Profile of Hox11 expression in developing mouse embryo*

Organ or Tissues	Mouse embryonic day
brachial arches and presumptive pharynx	
first branchial arch	8.5
mandibular component	8.5
surface ectoderm	10.5
thyroid rudiment	8.5
pharynx	8.5
tongue	
branchial arches	11.5
lingual surface epithelium	12.5–15.5
presumptive muscle	12.5
intrinsic and extrinsic muscle tracts	13.5
papillae	10
mandible	
anterior surface epithelium	12.5
tooth primordia	12.5
muscle tracts	13.5–15.5
ear	
internal margin of pinna	13.5
lining of external auditory meatus	13.5
salivary gland	
salivary glands (parotid, submandibular, and sublingual)	14.5
different glands distinguishable	16
submandibular gland	14.5–16.5
hindbrain, spinal cord, and cranial ganglia	
trigeminal, facioacoustic, and glossopharyngeal ganglia	10.5–13.5
cochlear complex and inner ear	13.5–15.5
spinal chord	10.5–15.5
pons and media	12.5–15.5
spleen	11.5–13.5
splanchnic mesoderm	11.5
Splenic primordium	12.5
cellular organization and mesenchymal cell condensation	12.5
pancreas	
pancreatic primordium	12–15.5

*Sources: Raju et al. [21]; Roberts et al. [22]; Dear et al. [23]

In mouse embryos, *Hox11/Tlx1* is widely expressed in organs and tissues that transcend ectodermal, mesodermal, and endodermal germ cell layers (**Table 1**). It is essential for organogenesis of the spleen, which will be discussed later. Understanding the widespread patterns of *Hox11/Tlx1* expression is essential for providing clues about the range of future applications for treating autoimmune disease. In other words, the developmental patterns of expression suggest that organs and tissues, if later diseased, might benefit from *Hox11/Tlx1*-stem cell therapies. Our laboratory thus far has reported therapeutic benefit from *Hox11/Tlx1* stem cells for three conditions: type 1 diabetes, Sjogren's syndrome, and comorbid hearing loss. But it is clear from embryology studies that *Hox11/Tlx1* expression is upregulated in a much wider array of organs and tissues (**Table 1**).

The most detailed study of the patterns of *Hox11/Tlx1* expression in embryogenesis was undertaken on *Tlx1,* the murine homologue of *Hox11* [21–23]. *Tlx1* transcripts were identified as early as mouse embryonic day 8.5 (E8.5) on the surface of the ectoderm and central mesenchyme of the first brachial arch. From that day until E15, the expression of *Tlx1* distributes to multiple sites in the body, exhibiting complex patterns in these organs or tissues: the pancreas, cranial ganglia, spinal cord, tongue, man-

dible, ear, and salivary gland, among others (**Table 1**). Subsequent studies found that *Hox11/Tlx1*-expressing cells also condense to form the spleen as early as day E11.5, and, by the next day, *Hox11/Tlx1*-expressing cells become apparent in the splenic primordium. *Hox11/Tlx1* expression persists for another day, but then ceases [23]. When the *Hox11/Tlx1* gene is knocked out (*Hox11*–/–), the spleen is completely missing [22,23]. In knockouts, the spleen begins to develop on the appropriate embryonic day, but then is rapidly and completely resorbed by day E13.5 [22,23]. The spleen is the only missing organ in knockouts, whereas the other organs in which *Hox11/Tlx1* is expressed are grossly normal in appearance although microscopic studies have not been conducted. Given the widespread embryological distribution of *Hox11/Tlx1*, the limited impact of *Hox11/Tlx1* knockouts is perplexing. The investigators postulated that the limited consequences of knocking out *Hox11/Tlx1* may be attributable to functional redundancy of related *Hox* gene family members – *Hox11L1* and *Hox11L2* – which are also found in mouse embryos [23,24].

Further research has established the complex molecular cascade that controls the actions of *Hox11/Tlx1* in the spleen's ontogeny. Using several types of knockout mice, two other transcription factors upstream of *Hox11* – *Bapx1* and *Pbx1* – have been identified to regulate *Hox11* transcriptional activity [25,26].

How does *Hox11/Tlx1* exert its developmental functions, especially the formation of the spleen? The answer is not fully known, although there are clues. From knockout experiments, it can be inferred that *Hox11/Tlx1* normally regulates splenic development most likely by increased proliferation of mesenchymal cells or, possibly by decreased apoptosis [26]. A role of *Hox11/Tlx1* in proliferation is consistent with cancer literature report indicating that *Hox11*+ human acute lymphoblastic leukemia is caused by a translocation on chromosome 10. This translation breaks-off *Hox11* from its upstream regulatory elements, yielding uncontrolled proliferation of *Hox11* target genes [11,12,24,27]. Taken together, these findings point to *Hox11* as having a proliferative role. Still, the genes on which *Hox11* acts to induce proliferation or reduce apoptosis have not fully been identified. It is known that *Hox11* does act on several genes, including *Aldh1*, which it represses [28] and Wilm's tumor gene (Wt1), which it also regulates [29]. There may be many other genes on which *Hox11* acts, and they are likely to vary, considering *Hox11* contributes to development of wide-ranging organs and tissues. The full repertoire of diverse genes the *Hox11* regulates to allow multilineage cells and regeneration are unknown, but the oncology literature has defined 20 signature genes that are commonly expressed in all *Hox11* tumors and are most commonly genes related to cell proliferation and the cell cycle [30,31].

Role of Spleen In Human Development and Adulthood

The spleen's main roles in adulthood are to filter blood and to contribute to immune surveillance and response. During adulthood, the spleen's macrophages filter blood to remove senescent, dysfunctional, or antibody-coated RBCs (red blood cells), senescent platelets, and apoptotic cells [26]. The spleen also stores a small fraction of the body's RBCs. These functions are performed in the spleen's red pulp, which occupies about one half of the spleen. The white pulp, which is the spleen's lymphoid compartment, produces antibodies to attack invading pathogens. It also releases platelets and neutrophils to counter bleeding or infection. Finally, the white pulp also stores up to one third of the body's platelets [26,32].

One of the adult spleen's most underappreciated functions in the white pulp is the capacity for extramedullary hematopoiesis when the body's bone marrow is overwhelmed by trauma or disease. For that purpose, the spleen contains stem cells with the capacity to differentiate into any type of hematopoietic cell [33]. Because this capacity has only been studied in mice, it is not known whether the human spleen, under stress or trauma, has more pluripotent capacity or relies on more lineage-restricted HSCs.

The spleen's capacity for hematopoiesis is not limited to adulthood. In fact hematopoiesis is a primary function of the spleen during several months of embryogenesis. The fetal liver is the first site of hematopoiesis, after the yolk sack. At six weeks of gestation, the spleen takes over from the liver and remains the main site of hematopoiesis through the fifth gestational month. It is only then that the bone marrow becomes responsible for hematopoiesis [33].

Hox11-Expressing Stem Cells Found in Human Spleen of Adults

Recent research has found that the human spleen is the only bodily organ that houses a reservoir of *Hox11* expressing stem cells [1]. The stem cells are abundantly expressed throughout adulthood, with no differences in expression by gender or age. Further, these stem cells display autonomous proliferation in tissue culture. They are readily maintained in culture for at least two months, displaying persistent expression of *Hox11* [1].

These findings were obtained by analysis of samples from 30 normal human postmortem spleens using RT-PCR and DNA quantitative analysis. The spleens had been procured during harvesting procedures for organ donation. *Hox11* expression was found to be abundant in the human spleen relative to negligible expression in the following organs and tissues of lymphoid and nonlymphoid origin: bone marrow, kidney, liver, and tonsil. Abundant expression is especially useful for transplantation because it means that the stem cells need not be expanded in culture prior to transplantation. In contrast to mice, the stem cells in humans are distributed throughout the organ, rather than being concentrated in the spleen's capsule. Because they do not display the marker CD45+, splenic stem cells are less restricted to only become hematopoietic stem cells. Interestingly, postmortem samples from three patients with type 2 diabetes tended to express the highest levels of *Hox11*. This finding is consistent with that from our murine model of type 1 diabetes, in which pancreatic beta islet cells originating from inject spleen cells are harvested from normal donors. These spleen cells can home to the damaged pancrease where they contribute to the autoimmune destruction of the pancreatic cells. Similarly, the examination of the spleen of NOD (nonobese diabetic) animals with ongoing autoimmune destruction reveals a marked expanion of *Hox11/Tlx1* stem cells in the spleen leading one to believe this is the spontaneous protective response to contribute to promote endogenous regeneration (unpublished data). Furthermore, a recent report also observes an indirect contribution of splenic cells to pancreas regeneration suggesting perhaps a secreted factor from the spleen can promote pancreatic islet regrowth after damage as well as a direct migration of stem cells to the pancreas [34].

The findings are striking in light of the prevailing dogma among developmental biologists that *Hox11* ceases to be expressed after birth. This dogma held sway because of the robust role that *Hox11* plays during embryogenesis in controlling cell fate and

differentiation across a wide range of developing organs and tissues [21,23]. Also the vivid *Hox11* expression in tumors led all to believe that postnatal expression was a sign of malignancy. Most embryologists expect *Hox11* expression to end before birth, most likely because of the body of evidence that *Hox11* is so essential as a developmental transcription factor that its absence, when *Hox11*–/– mice were created, leaves animals without a spleen [23,35]. Perhaps, these are the reasons for scanty systematic examination of *Hox11* expression in the human or animal spleen mice after birth.

The findings from human adults raise important questions. The first is why is a *Hox11* stem cell reservoir sequestered only in the spleen, considering the wide array of organs and tissues in which it is expressed during development? And why does *Hox11* continue to be expressed in the spleen throughout human adulthood? These questions are as yet unanswerable, but, yet again, there are clues from developmental biology. The spleen plays a prominent role in embryogenesis as one the first sites of hematopoiesis during gestation, as discussed in the previous section. The fetal spleen generates the full range of hematopoietic cells as early as six weeks' gestation, long before the bone marrow assumes that capacity. Thereafter, production in the spleen decreases as production in bone marrow increases [32]. It bears repeating, from the previous section, that after birth and throughout adulthood, the spleen takes over hematopoiesis when the bone marrow cannot satisfy the body's requirements during times of stress and disease [32]. It is worth speculating that the reservoir of *Hox11* expressing cells in the spleen is there to assume that residual capacity, as well as to be summoned for a broader range of lineages, if the need arises, especially with the need for cell repair.

If an abundant supply of stem cells persists into older adulthood, are these cells functional, and do they respond to signals emanating from damaged tissues? It has been presumed that the reason for incomplete or poor regeneration with aging is due to a shortage of stem cells. Yet, the spleen shows an abundant expression of *Hox11* positive cells into adulthood. Further, our research reveals preliminarily that *Hox11* expression may increase as a result of disease both by more vivid *Hox11* expression per stem cell but also the expansion of *Hox11* stem cells in both the mouse as well as the human [1]. The findings in adult mice, to which we now turn, suggest that *Hox11* expressing stem cells of the human spleen may possess multilineage capacity that could be marshaled to treat several autoimmune diseases.

Potential Benefits of Stem Cell Therapies

This section explores the potential benefits of stem cell therapies for two autoimmune diseases – type 1 diabetes and Sjogren's syndrome – and a condition that is comorbid with both, hearing loss. The evidence thus far suggests that this seemingly unusual combination of autoimmune disorders and one comorbid condition may stand to benefit from *Hox11/Tlx1* stem cell therapies.

We propose that the three organs in which these conditions are manifest – the pancreas, salivary glands, and cochlea – may be linked pathophysiologically by their common lineage tracing back to *Hox11/Tlx1* expression in embryology [21]. The pathophysiological linkage is reinforced by epidemiological evidence of high comorbidity between all three autoimmune diseases [16,17,36]. Up to 55% of individuals with type 1 diabetes also experience Sjogren's syndrome or symptoms. Further, both autoimmune conditions are found in conjunction with hearing loss that, in some cases, is a primary manifestation, rather than a secondary effect of autoimmunity. The overlap of all three is even more apparent in NOD mice, an animal model of both type 1 diabetes and Sjogren's syndrome. The comorbidity of the two diseases in NOD mice stands at 80–90% [36]. And, as already described, we have recently have found that 100% of NOD mice are deaf – at early stages of development prior to the onset of either type 1 diabetes or Sjogren's syndrome.

Type 1 Diabetes

Type 1 diabetes has been the subject of most advances in splenic stem cell therapies. Affecting millions worldwide, type 1 diabetes is responsible for significant morbidity and mortality. Although current treatments and better glucose management are helping to reduce morbidity and mortality in the US [37], treatment shortcomings still abound, with serious repercussions [38]. Among them are: (1) tight glycemic control is difficult to maintain with exogenous insulin; (2) high rates of certain serious complications (e.g., heart disease, stroke, kidney failure, amputations, blindness, etc.); (3) long-term immunosuppression appears to carry previously unrecognized adverse affects; and (4) there is a paucity of human donor pancreata if therapy fails [39,40].

Cellular therapies would be a landmark contribution because they could replace beta islet cells, the targets of autoimmune attack in type 1 diabetes. But, to be effective, cellular therapies must be combined with an immunotherapy to avert the disease's fundamental defect, namely the destruction of beta islets or other targets by autoreactive T cells. Otherwise, new islet cells would become fresh targets for immune destruction. Thus, many in the field view cellular therapies combined with an immunotherapy as an ideal combination. While this review covers immunotherapies to some degree, its primary focus is on the enormous potential of cellular therapies.

The focus of our laboratory has been expressly on adult stem cell therapies. Adult stem cell therapies from the spleen have several advantages over other types of adult cellular therapies: (1) their capacity for self-renewal is robust, only few cells need be harvested or subsequently expanded in tissue culture; (2) because the spleen is an expendable organ, there is much less likelihood of a scarcity of donors; (3) harvesting splenic cells from adults, rather than from an embryo, obviates the ethical controversies of using embryonic stem cells. It is worth reiterating, however, that splenic stem cell therapies, no matter how successful by themselves, still encounter the same obstacle in treating autoimmunity as does any other type of cellular therapy – attack by autoreactive T cells. Stem cell therapies cannot be administered alone: they must be combined with some form of immunotherapy that effectively destroys autoreactive T cells, or the cellular therapies face destruction.

Until recently, it was believed that beta islet cells of the pancreas, once fully differentiated, had no capacity for self-renewal. That view propelled intense research interest, using murine models, in exogenous cell therapies, at first from humans and pigs and more recently from stem cells. In one of the first studies of its kind, embryonic stem cells cultured under conditions that coaxed them to express insulin were found, after injection into diabetic mice, to successfully replace beta islet cells [41], but other groups were not able to replicate the findings. Embryonic stem cells also raise concerns about instability in their differentiated state and risk of transformation to tumor cells [42,43].

For these reasons, adult stem cells from adult bone marrow or peripheral blood became attractive. Indeed, adult hematopoietic

stem cells were found to facilitate islet cell replacement in mice with streptozotocin-induced pancreatic damage [43]. But hematopoietic stem cells only may be acting indirectly, namely by release of growth factors that stimulate regeneration of beta cells that are endogenous to the pancreas. Bone marrow derived endothelial cells, which were transplanted to the pancreas, may have succeeded in recovering beta islets by inducing neovascularization [44]. In a separate study, bone marrow transplants were used in an unusual way to combine with pancreatic cells to reduce autoimmunity and permit natural methods of endogenous islets to replenish themselves [45]. Finally, one study found that hematopoietic stem cells were capable of transdifferentiating into insulin-expressing cells, but at rates too low (1.7–3.0%) to be clinically effective [46].

The concept that the pancreatic islets could briskly regenerate was at first doubted. Indeed in our own work, we were not allowed to use the work regeneration in the title of our first paper showing massive islet regeneration in the pancreas [47] and could only speculate that the reappearance of the large islets might be regeneration. Gradual acceptance that the pancreas could regenerate has come and published data now shows the pancreas can regenerate in multiple ways. Mechanisms included replication of progenitor cells in the pancreas followed by their differentiation into islets, cells into pancreatic cells, or proliferation of differentiated beta cells into new beta cells. Using lineage tracing techniques, mitosis of already differentiated beta islet cells was found to be the route of islet cell turn over, at least in normal mice [48]. A cautionary note to these important studies is that the mechanisms of replication found in normal animals may not apply to diabetic animals. Key questions are the extent to which islet cell regeneration from endogenous differentiated cells may vary by host, underlying disease or injury, or administered treatments. Still, the underlying issue remained: how to eradicate autoimmune attack on beta cells?

Several years earlier, our laboratory tackled two questions expressly for type 1 diabetes: could we eradicate the autoreactive T cells completely, and could we then replenish the beta islet cells with stem cells from outside the pancreas, under the assumption that pancreatic cells could not replenish themselves? We succeeded on both counts. We found that end-stage diabetic mice could be permanently cured of diabetes and have their islet cells replenished [3,47]. The treatment strategy was two-pronged: an immunotherapy to kill autoreactive T cells, followed by stem cell therapy to replenish beta islet cells. The immunotherapy consisted of inducing TNF production and infusing MHC (major histocompatability complex) class I and self-peptide complexes either as isolated complexes or on harvested lymphoid cells form normal MHC matched cells. Autoreactive T cells are vulnerable to TNF-α as a result of errors in intracellular signaling by transcription factor NF-κB [49,50]. In another study, we revealed that islet cell regeneration were due to: 1) spontaneous regeneration from endogenous cellular sources within the pancreas; and 2) infusion of adult stem cells from the spleen of normal animals. According to two labeling techniques, we found that the infused cells homed to the pancreas where they differentiated into islet cells [3]. By comparison to our control groups, we found that the spleen cells hastened the speed of cure from 120 days to 40 days, but did not affect the overall rate of cure, which remained unchanged.

The findings were startling in three ways: firstly, diabetes in the NOD model of spontaneous autoimmunity had never before been permanently cured, especially with a nontoxic brief limited intervention. Secondly, spontaneous regeneration in the pancreas allowed long-term normoglycemia that has never been reported. Lastly, a unique adult stem cell in the spleen had not yet been previously characterized.

During the next 5 years there was an international effort to duplicate our findings. In 2006, four laboratories confirmed that the two limb treatment of brief treatment of TNF induction and MHC class I and self-peptide cured end stage mice [51–54]. The unresolved question was whether splenic stem cell infusions were necessary [55]. Three of the papers cured the end stage diabetic mice to varying degrees and confirmed our observed regeneration, but only observed our previously reported endogenous regeneration and not the time boosted regeneration with the spleen cell. We had originally shown that the regenerative potential of the diabetic mouse pancreas was intact, even in late stage diabetes, and could recover without live stem cells albeit at slowed kinetics. We contended that methodological differences precluded each team from finding a splenic contribution [55]. Subsequent independent data now confirms that the end stage diabetic mouse pancreas can regenerate both from endogenous mechanisms as well as from a splenic stem cell contribution [4,55].

A subsequently published study from investigators at the NIH observed the splenic cells did have the capacity to regenerate portions of the insulin secreting cells in the pancreas [55]. Like past data, the splenic assisted regeneration was not the only means for functional pancreatic restoration but contributed in a manner that tracked with the degree of underlying disease. For instance, if the underlying autoimmunity in the pancreas was advanced, exogenous splenic stem cells contributed to larger portions of the regenerated islets so that the minor forms of splenic cell engraftment are in animals with only early signs of pancreatic islet destruction [55]. It also found that splenic stem cells succeeded in regenerating salivary epithelial cells, a topic discussed in the next section [4,55]. More recently, another laboratory has also found a beneficial role of the spleen in islet cell regeneration when they implanted splenic cells below the kidney capsule. The authors inferred that the spleen cells secreted factors that facilitated endogenous islet regeneration [34]. With the escalating interest in stem cells facilitating endogenous regeneration and the interest in stem cell growth factors facilitating regeneration, the novel ability to restore blood sugar to normal through pancreas regeneration seems forthcoming and confirmed by world wide efforts, at least in end stage mice.

Sjogren's Syndrome

Like autoimmune humans, nonobese diabetic (NOD) mice exhibit autoimmune diabetes and Sjögren's syndrome. Since *Hox11/Tlx1* stem cells during fetal life contribute to the regeneration of the salivary glands it was logical that additional studies would extend the adult splenic regeneration studies to another *Hox11/Tlx1* lineage gland.

Using the same immune intervention to remove diabetes in end stage NOD mice, the NOD mice were also studied for removal of salivary disease. All NOD mice receiving the immune therapy had a complete recovery of salivary flow and were protected from diabetes [4]. Salivary gland recovery resulted from a combination of rescue and regeneration of the gland, as confirmed with immunohistochemistry. All untreated NOD mice showed a continuous decline in salivary flow, followed by hyperglycemia and death. This study establishes that a brief intervention into NOD mice with Sjögren's syndrome can reverse salivary gland

malfunction, in part from regeneration from endogenous and exogenously assisted mechanisms, that is, introduced splenic stem cells.

It should not be too surprising that an intervention that thwarts one form of autoimmunity might have an impact on a co-existing form of autoimmunity. The Tran et al. data indicates that an immune intervention that thwarts autoimmune diabetes in the NOD mouse is also effective in reversing established Sjögren's syndrome in the same animal model. The efficiency of therapies that protect NOD mice from Sjögren's syndrome and autoimmune diabetes may largely depend on the timing of the treatment to show different forms of regeneration in the respective organs. In our work in diabetes, we tested the efficacy of a therapy that reverses end-stage diabetes, typically in mice over 22 weeks of age. The Tran et al. study examined NOD mice at an early stage in their diabetes but a late stage of their autoimmune salivary gland function, that is, the NOD mice were 14 weeks of age with advanced Sjogren's syndrome. This treatment timing showed 100 % protection for progression to diabetes and restoration of complete salivary flow through salivary tissue regeneration. This percentage diabetes cure is higher than the 85 % success rate reported by our group in end-stage diabetic NOD at 22- to 40-week-old, suggesting the importance of early intervention [3,47]. Lineage tracking methods confirmed that although both islet rescue from the recipient and islet regeneration from donor injected splenocytes occurred, in earlier stage animals (14-week-old NOD used in this study) the islet rescue (or host-derived regeneration) dominated. Tran et al. demonstrated that salivary gland function, as measured by salivary flow rates was completely restored. A small number of donor splenocytes colonized the salivary gland and differentiated into salivary epithelial cells. At this stage of salivary disease, though the chimerism occurs, it is unlikely to account for the full functional regeneration since endogenous regeneration and rescue are more dominant.

This study demonstrates that a two-limb intervention can stably reverse two forms of established autoimmune disease, that is, diabetes and Sjögren's syndrome. The results support the notion that disease removal is of central importance to promote diverse forms of parenchymal regeneration and/or rescue.

Comorbid Hearing Loss

Hearing loss is commonly viewed as a secondary effect of both type 1 diabetes and Sjogren's syndrome. But epidemiology studies revealing its high comorbidity with type 1 diabetes and Sjogren's syndrome led us to look for a physiological explanation, considering that *Hox11/Tlx1* is expressed in all three organs and tissues. One of the intriguing findings was that hearing loss and Sjogren's syndrome is independent of disease duration. What has been observed is that patients with new onset of Sjogren's Syndrome can already have moderate to advanced hearing loss. This might suggest that hearing loss is a primary effect. With regard to type 1 diabetes, the evidence is certainly strong that hearing loss is often a secondary effect of vascular damage. But, in some cases, a recent study found that cochlear changes may be a primary effect of the disease. On the basis of both the epidemiological evidence and the linkage to *Hox11/Tlx1* patterns in embryogenesis, we sought to tease apart whether development contributes to autoimmunity. We found that development does indeed contribute to autoimmunity by predicting which organs may be targeted in autoimmunity. This finding challenges conventional wisdom that autoimmunity is exclusively caused by a defective immune system.

Table 2 Functional recovery of glucose levels and hearing in NOD mice with autoimmune treatment*

	NOD treated	NOD untreated	C56BL/7
glucose levels	100 % (8) corrected	0 % (5) uncorrected	no change
hearing improvement			
permanent, complete	25 % (2)	–	no change
partial or temporary	25 % (2)	–	no change
none	50 % (4)	100 % (5)	no change

*NOD mice were given autoimmune therapy at 15 weeks of age and were examined up to 20 weeks post-treatment. Of the eight NOD-treated mice, all successfully corrected their glucose levels by 33 weeks of age. Twenty-five percent (n = 2) had permanent and complete functional recovery of hearing, and 25 % (n = 2) had partial or temporary recovery of hearing by 33 weeks of age. The other 50 % (n = 5) of treated animals showed no recovery. The control mice C56BL/7 (n = 10) showed normal hearing throughout the study period for an aged mouse

In the course of our investigation, we hypothesized that hearing loss had a physiological relationship to comorbid autoimmune disorders. By examining the anatomy and physiology of the cochlea, we recently reported that NOD animals are almost completely deaf (Lonyai et al., submitted). This finding is not surprising considering that *Hox11/Tlx1* embryonic expression is firmly linked to the eighth cranial nerve and the cochlea [21]. Nonobese diabetic animals were found to have profound structural abnormalities in the cochlea, and these abnormalities were detected along before the onset of immune-mediated disease (either type 1 diabetes or Sjogren's syndrome). When we compared very young but post-natal NOD animals with NOD*scid* (NOD mice without an immune system), we saw no difference in structural deformities. This suggests that the defect was driven more by development rather than driven by the immune system.

Despite our conclusion that deafness in NOD mice is more of a developmental rather than solely an autoimmune defect, we sought to determine whether *Hox11/Tlx1*+ stem cells from normal mice could ameliorate deafness in NOD mice. In a preliminary study, we compared cochlear structure and function in three groups of mice: NOD-treated animals (n = 8) given both *Hox11/Tlx1*+ stem cells from normal animals plus the autoimmune removal therapy (TNF in the form of CFA) or examined NOD-untreated mice (n = 5) given neither limb of therapy, and normal control mice (CF7BL/6) (n = 10). There was no need to give another control group of NOD mice only one of the two limbs of therapy because we previously demonstrated in large numbers of animals that only one limb of the therapy produces partial efficacy for autoimmune disease reversal [47]. We introduced the 2-limbed therapy by a biweekly splenic stem cell infusion at 15 weeks and then evaluated the structure and function of the inner ear, especially the cochlea at various times thereafter, up to 35 weeks of age.

Of the treated group, 8 of 8 NOD animals were cured of type 1 diabetes, whereas all 5 of 5 untreated NOD animals did not show any spontaneous recovery in glucose levels (**Table 2**). Of the NOD-treated mice, two showed complete and permanent recovery in hearing function, while two others showed slight increases, as measured by lower thresholds in the auditory brainstem response (ABR) test (**Fig. 1**). Lower thresholds reflect better

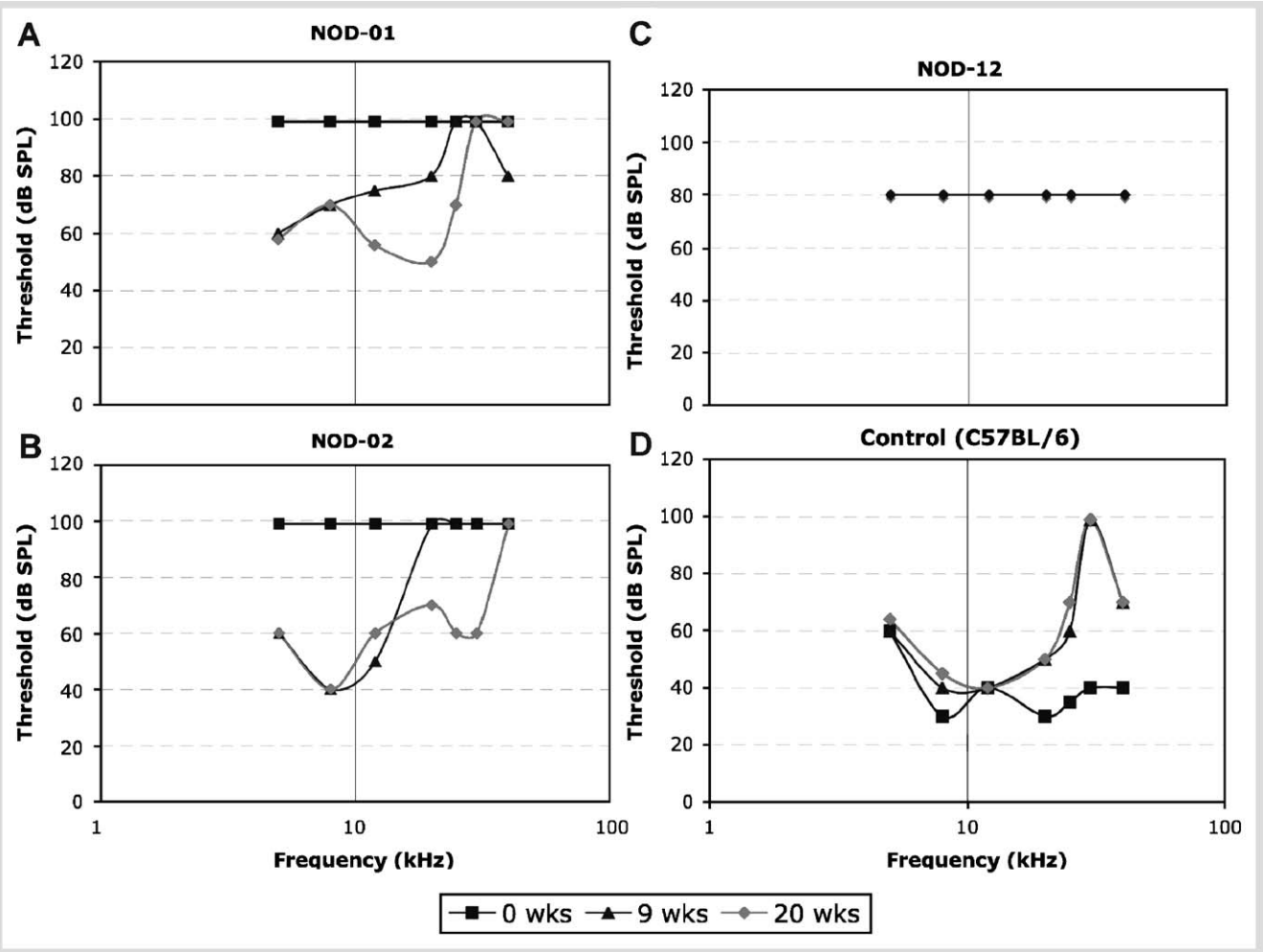

Fig. 1 Audiograms of hearing thresholds for three NOD mice over time-course of autoimmune reversing treatment. Shown in this figure are two NOD animals (NOD-01 and NOD-02) who received the autoimmune treatment, which included the injection of *Hox11* adult stem cells from normal donors, and one that did not (NOD-12). This long term therapy prevented diabetes in the two treated NOD mice, thus "curing" diabetes in those animals. Treatment was started at 15 weeks of age and successfully reversed the hearing loss in two of the treated NOD mice. The characteristic audiogram is shown for the C57BL/6 control with an increase in hearing thresholds with age, especially at the lowest and highest frequencies (**D**). Arrows indicate that thresholds above 100 dB could not be measured. All three NOD animals had no measurable hearing at 15 weeks of age as indicated by thresholds above 100 dB (**A–C**). NOD-01 and NOD-02 showed a return of low and mid-frequency hearing as indicated by a lower threshold at frequencies from 7 to 25 kHz over the course of treatment (**A**,**B**). NOD-12 had a high threshold (80 dB) at 15 weeks of age and died before the next measurement (**C**).

hearing. Half of the treated animals showed no improvement in hearing function.

The function of the cochlea by audio brainstem responses (ABRs) is depicted in **Fig. 2**. In the normal control, the characteristic pattern is somewhat of a U-shaped curve. By 35 weeks (or 20 weeks post-treatment for the treated animals), frequency thresholds are lowest at 10 kHz, but rise again at higher frequencies. In the only two successfully treated NOD mice with full restoration of hearing function and full glucose restoration (NOD-001, NOD-002), the normal pattern begins to resemble the normal curve by 10–12 weeks post-treatment. In another treated NOD mouse, the glucose was restored but unsuccessful hearing improvement (NOD-012). The functional data are compared to a similarly aged C57BL/6 control (**Fig. 1**). Individual audiograms reveal that two successfully treated NOD mice, NOD-01 and NOD-02, showed a return of most low and mid-frequency hearing as early as 9 weeks post-treatment, while the unsuccessfully treated NOD mouse, NOD-012, exhibited no measurable hearing recovery throughout the period being studied (**Fig. 2a–d**).

In order to determine whether treatment and recovery from type 1 diabetes had an effect on cochlear structure, the same three NOD animals were also compared histologically. As seen from **Fig. 1** for the two successfully treated NOD mice, the histology reveals what appears to be a normal spiral ligament with a full, or nearly full, population of cells in the lower third turn (**Fig. 2a, c**) and lower second turn (**Fig. 2b,d**) of the cochlea. Also, the organ of Corti was restored in both, with a full population of cells, including hair cells, and apparent restoration of the tunnel of Corti in the lower second and third turns (**Fig. 2a–d**). The unsuccessfully treated NOD mouse, NOD-12, showed severe atrophy of cells in the spiral ligament of the lower third turn and lower second turn. This is depicted by a depopulation of cells in the area that corresponds to the spiral ligament in **Fig. 2e–f**. This animal did have some apparent improvement in the organ of Corti, that is, the lower second turn contained a few cells, especially inner and outer hair cells (**Fig. 2f**). The organ of

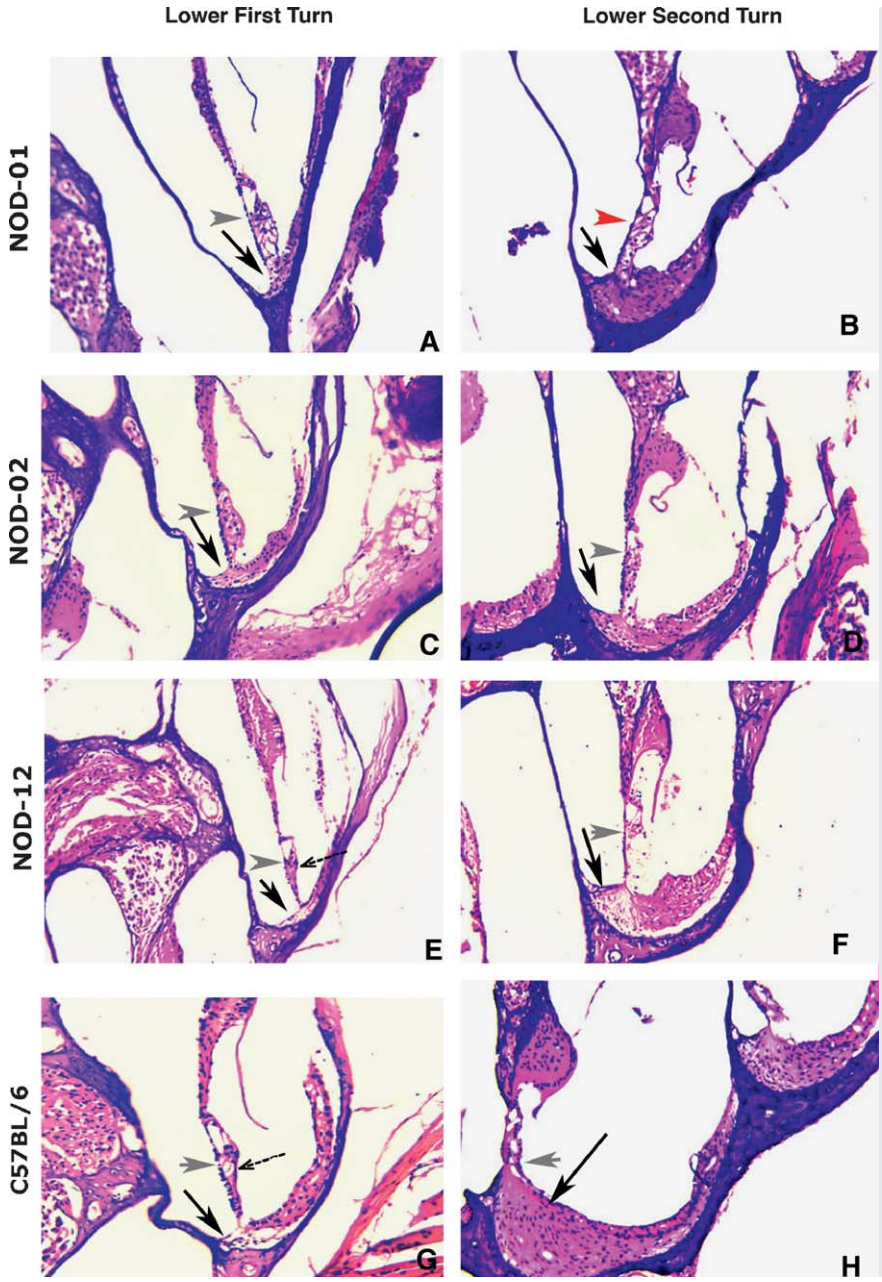

Fig. 2 Cochlear morphology of two treated NOD mice with partial restoration of hearing. NOD-01 and NOD-02 received CFA treatment to restore their long-term insulin levels, thereby "curing" diabetes in the animals, while NOD-12 was not treated. The treatment successfully restored insulin levels in NOD-01 and NOD-02, while insulin levels in NOD-12 remained unchanged. The spiral ligament (black arrow) and organ of Corti (gray arrowhead) appear to be normal with a full cell population including inner hair cells and normal morphology in the lower first and second turns in NOD-01 (**A**,**B**) and NOD-02 (**C**,**D**). The spiral ligament appears depopulated in both the lower first and second turns in the NOD-12 (**E**,**F**). Also, organ of Corti appears to have fewer cells including hair cells in the lower second turn (**F**) and appears to have not developed the tunnel of Corti in the lower first turn. A dotted arrow points to where the tunnel of Corti is located in the C57BL/6 control and where it is missing in the lower first turn of NOD-12. C57BL/6 controls are shown in **G**,**H**. All images taken at 20× magnification.

Corti of NOD-012 of the lower third turn had a slightly less severe loss of cells in the lower second turn compared to an untreated NOD mouse, but had persistently poor development of the tunnel of Corti (▶ **Fig. 2e**) as compared with the normal C57BL/6 control (▶ **Fig. 2g**). This preliminary study reveals that *Hox11/Tlx1* stem cells, combined with immune therapy, have therapeutic potential to reverse or ameliorate hearing loss, but further study is warranted.

Safety Considerations

Multi-lineage stem cells, no matter how promising in animal models, always raise safety risks for humans. One of the foremost issues facing stem cell research of any kind is to weigh the enormous promise for regeneration and cure against the possible risks of stem cell transformation into cancer cells. Safety concerns are especially applicable to the *Hox11/Tlx1* gene, considering that it was originally identified, more than 15 years ago, as a proto-oncogene in human T cell acute lymphoblastic leukemia [11, 12]. Nonetheless, the findings regarding *Hox11*-expressing ALL indicated that tumorigenesis resulted from translocation of the *Hox11* gene from its upstream regulatory genes. Consequently, potential donors of *Hox11* stem cell therapies could be genotyped for chromosomal translocations to reduce the possibility of tumorigenesis.

On the other hand, there is a paradoxical implication of finding a stem cell reservoir in the spleen, all this is unrelated to treatment of autoimmune disease. What began with finding an isolated reservoir of stem cells potentially *worth* harvesting as a cellular therapy for type 1 diabetes may turn out to be a reservoir potentially *worth* removing for treating *Hox11*-expressing acute lymphoblastic leukemia. According to the stem cell hypothesis of cancer, rare stem cells are considered to be obligatory to the onset, perpetuation, and recurrences of human cancers. The hypothesis is an outgrowth of a body of literature finding that most cancer cells, except cancer stem cells, cannot be expanded in culture. The hypothesis calls for removal of the dysregulated or mutated stem cells rather than the tumor itself because they induce and perpetuate several forms of cancer [4].

Based on our finding of a reservoir of *Hox11* stem cells in the spleen, we have proposed splenectomy as a low risk procedure for adult and pediatric patients with few treatment options and a poor prognosis [1]. Paradoxes never cease on the frontiers of stem cell research.

Conclusions

The main conclusion of this article is that removal of the underlying autoimmune disease is essential to see the regeneration of the pancreas, salivary gland, or even portions of the inner ear. The data clearly shows, at least in the NOD mouse, that disease removal permits both endogenous regeneration as well hastened regeneration facilitated by the introduction of an adult *Hox11* stem cell from the spleen. *Hox11* stem cells are no longer a paradox of the mouse, but similarly identified in humans of all ages and appear to be stimulated to expand *in vivo* in humans with diseases of the pancreases where they promote regeneration. The enormous potential of *Hox11*- expressing splenic stem cells for treatments for autoimmune diseases affecting organs in that *Hox11* is expressed during embryogenesis in areas such as the salivary glands, pancreas, and ear. Future research will need to be aimed at identifying whether additional organs at the *Hox11* splenic stem cells could provide cells of regeneration, and of course to see if *Hox11* stem cells in humans can have additional and beneficial roles.

Acknowledgments

This work was supported by The Iacocca Foundation, The Zwanziger Foundation, and the Sjogren's Syndrome Foundation. We appreciate the secretarial assistance of Ms Lynne Murphy.

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