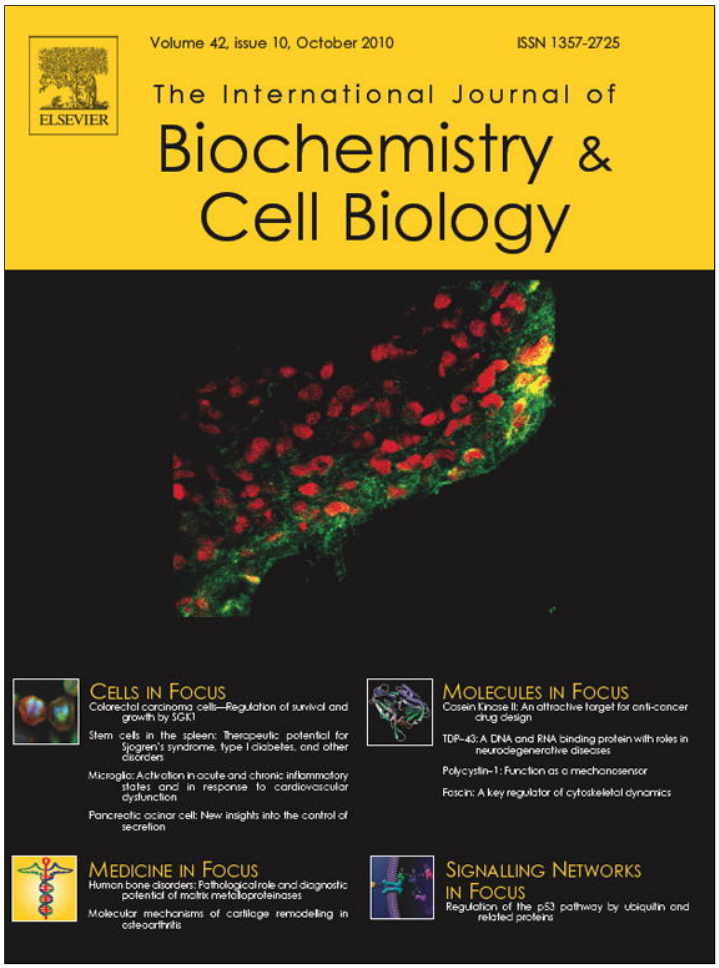

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Proteomics identifies multipotent and low oncogenic risk stem cells of the spleen

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ABSTRACT

The adult spleen harbors a population of naturally occurring multipotent stem cells of non-lymphoid lineage (CD45−). In animal models, these splenic stem cells can directly or indirectly contribute to regeneration of bone, inner ear, cranial nerves, islets, hearts and salivary glands. Here we characterize the CD45− stem cell proteome to determine its potential broader multipotency versus its protection from malignant transformation. Using state-of-the-art proteomics and *in vivo* testing, we performed functional analyses of unique proteins of CD45− (non-lymphoid) splenic stem cells, as compared with CD45+ (lymphoid) cells. CD45− stem cell-specific proteins were identical to those in iPS, including OCT3/4, SOX2, KLF4, c-MYC and NANOG. They also expressed Hox11, Gli3, Wnt2, and Adam12, the benchmark transcription factors of embryonic stem cells. These transcription factors were functional because their mRNA was upregulated in the spleen in association with ongoing damage to the pancreas and salivary glands, organs to which they normally contribute stem cells. We also show low likelihood of malignant transformation. Our proteomic and functional analyses reveals that naturally occurring CD45− stem cells of the spleen are the first-ever candidates for naturally occurring population of embryonic and iPS cells with low oncogenic risk. Given their presence in normal humans and mice, splenic stem cells are poised for translational research.

1. Introduction

The spleen of several adult species, including humans, contains a population of naturally occurring stem cells with multi-lineage capacities (Macias et al., 2001; Dieguez-Acuna et al., 2005, 2007; Khaldoyanidi et al., 2003; Kodama et al., 2003; Kodama et al., 2005a; Yin et al., 2006; Tran et al., 2007; Lonyai et al., 2008; Robertson et al., 2008; Swirski et al., 2009; Park et al., 2009). These stem cells are non-lymphoid (CD45−) cells, contrary to the spleen's predominant cell type, CD45+ lymphoid cells. The CD45− stem cells not only regenerate the structure or function of several other tissues and portions of organs, but they do so without *in vivo* or *ex vivo* manipulation (Dieguez-Acuna et al., 2007; Kodama et al., 2003, 2005a; Lonyai et al., 2008). Our next step was to characterize in mice the proteomes of the non-lymphoid and lymphoid populations with new instrumentation, namely state-of-the-art mass spectrometry (LTQ-FT) followed by shotgun and subtractive proteomics. Using the newly validated mass spectrometry methods and then matching proteins with the SEQUEST dataset, we identified 809 proteins unique to the spleen's CD45− stem cell population relative to its CD45+ cells (Dieguez-Acuna et al., 2005). We chose these two splenic cell types for comparison because they are the most similar differentiated cell populations in the same animals: both are from the same organ, but possess different regenerative abilities. Of the 809 stem cell-specific proteins, 98 bore developmental functions, according to our analysis of bioinformatics using gene ontology terms.

Here we perform a targeted and detailed analysis of stem cell functions by utilizing the datasets obtained from this new instrument, a state-of-the-art mass spectrometry (LTQ-FT), with a variety of other techniques. The overall goal was to understand the regulated stem cell biology of a novel splenic stem cell with multilineage commitments and identify the protein differences between splenic stem cells and cancer cells of closely related lineages. More specifically, our goal was to determine the lineage commitment of the spleen's CD45− stem cells, with emphasis on their multipotency and possible pluripotency. The more versatile the lineage commitment, the broader the functional implications for regenerative medicine, considering that CD45− stem cells are abundantly expressed in a nonessential organ, the human spleen, and they

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can be harvested without serious health consequences. Given the established link between other pluripotent stem cells and cancer, we also compare CD45− stem cell proteins, such as the developmental protein HOX11, with signature genes from a rare human leukemia also expressing the same HOX11 protein. Overall, our analyses were designed to identify proteins used for regeneration and those for preventing or facilitating oncogenic transformation, based on their relationship to signature protein products in HOX11 leukemia.

Two broad categories of prior evidence underlie the hypothesis that splenic CD45− stem cells are multipotent, as defined by expression of several well-known developmental transcription factors. First, the spleen's CD45− cells, in contrast to its CD45+ cells, normally express from embryogenesis into adulthood a well-established marker of an embryonic phenotype, the HOX11 protein (Kodama et al., 2005a). The *Hox11* gene encodes a highly conserved transcription factor found in many invertebrate and vertebrate species. It is among a family of genes contributing to embryonic development and to control of spatial patterning, cell fate, cell differentiation and/or regeneration (Dear et al., 1995; Raju et al., 1993).

The second line of evidence supporting broad multipotency draws on functional studies in animal models of several diseases. The normal target tissues for Hox11 stem cell migration and development include the pancreas, salivary glands, tongue, blood, certain cranial nerves, parts of the hindbrain and cochlea (Lonyai et al., 2008; Raju et al., 1993; Roberts et al., 1994). The evidence shows that administration of splenic stem cells, after disease or injury, leads to regeneration of the following tissues: pancreatic islets, osteoblast-like bone cells, T lymphocytes, salivary epithelial cells, and cranial neurons (Kodama et al., 2003; Lonyai et al., 2008; Tran et al., 2007; Macias et al., 2001; Khaldoyanidi et al., 2003; Park et al., 2009; Yin et al., 2006). Splenic stem cells have also been found to regenerate part of the inner ear of deaf mice and can restore hearing after complete deafness in some animals (Lonyai et al., 2008). Splenic stem cells harvested from birds can form insulin-secreting pancreatic islets *in vitro* (Robertson et al., 2008) similar to *in vivo* findings in mice with type 1 diabetes (Kodama et al., 2003) and most recently, the spleen serves as a reservoir for immature cells that function in repairing the heart after myocardial infarction (Swirski et al., 2009).

Multipotency as well as possible pluipotency however, represents a double-edged sword. Plasmid (DNA) transfection used to introduce genes into ES cells or induced pluripotent stem cells (iPS) is widely recognized to increase the risk of cancer. iPS cells are generated by reprogramming differentiated cells with a set of transcription factors during embryogenesis. iPS-induced tumors occur in germline transmitted mouse pups as they mature (Takahashi and Yamanaka, 2006; Aoi et al., 2008). Tumor formation is believed in part secondary to the retroviral vector necessary for integrating the transcription factors into the genome, as well as introduction of unregulated c-Myc transcription factor (Park et al., 2008; Kim et al., 2008; Okita et al., 2007). Naturally occurring stem cells also may initiate or contribute to perpetuation of certain cancers, according to the cancer stem cell hypothesis. That hypothesis holds that cancers arise from, and are sustained by, rare stem cells that become transformed into malignant cells (Dalerba et al., 2007). It has been proposed that CD45− splenic stem cells expressing HOX11+ are precursors of two rare spontaneous cancers that express HOX11, lymphoblastic leukemias of T cell lineage (T-ALL) and select pediatric brain tumors (Dieguez-Acuna et al., 2007). However, the rarity of these cancers implies that Hox11+ CD45− splenic stem cells are infrequently transformed to malignancies, most likely resulting from naturally occurring alterations in regulatory controls.

In this detailed characterization and analysis of proteomic data from splenic CD45− stem cells, we sought to answer several key questions surrounding the multi-lineage potency of these stem cells, as well as their likelihood of transformation to malignancies. (1) Does proteomics identify expressed proteins resembling those of embryonic stem and iPS cells? Pluripotency is defined by expression of transcription factors, signaling proteins, and developmental proteins similar to those in well-established pluripotent stem cells; (2) Do proteins expressed in splenic stem cells confirm the multi-lineage target tissues into which these stem cells already have been shown to differentiate? (3) In keeping with the evolutionary role of stem cells to replace injured or diseased cells, do splenic stem cells upregulate gene expression as a result of pathology in another organ? More specifically, in an animal model of type 1 diabetes, does pancreatic disease during the pre-diabetic phase trigger the spleen to upregulate stem cell proteins and/or the number of stem cells potentially available for migration and restoration of pancreatic function? (4) Given the carcinogenic risk of introducing stem cells harvested from embryos or iPS, do CD45− stem cells express proteins that overlap with those of the signature genes of HOX11+ T cell leukemias? Comparison between these two populations may reveal proteins necessary to target for prevention and treatment of this leukemia. Taken together, the proteins identified by this multi-pronged analysis will help to narrow the search for ways to harness naturally occurring splenic stem cells for multi-organ regeneration, therapeutics, or disease prevention, while protecting against malignant transformation.

2. Materials and methods

2.1. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis (Fig. 1)

LC–MS/MS was performed using an LCQ-Deca XP^{Plus} ion trap mass spectrometer and a LTQ-FT hybrid linear ion trap FTICR-MS (ThermoElectron, San Jose, CA). Samples were loaded (Famos autosampler, LC Packing, Sunnyvale, CA) to a 125 mm inner diameter fused silica C_{18} capillary column packed to 14 cm with Magic (Michrom BioResources) C_{18} resin (200 Å pore size, 5 m diameter) using an Agilent 1100 series binary pumps with an in-line flow splitter. Peptides were loaded onto the column for 15 min at 120 bar in buffer A (2.5% acetonitrile, 0.15% formic acid). Further details of these methods (Supplementary Materials and Methods).

2.2. Database searching and data analysis (Fig. 1)

Raw MS/MS data were searched against the mouse NCI non-redundant database with no enzyme constraint using SEQUEST (version 27) (Yates et al., 1995). Peptide identifications were performed with an estimated <1% false positive rate using a target/decoy database approach (Peng et al., 2003). Peptides identified from all LC–MS/MS runs for each gel slice were pooled to compare proteins unique to CD45− and CD45+ cell fractions, and total number of peptides is identified for each protein. The UNIPROT database was used to characterize proteins unique to CD45−, CD45+ or identified in both cell populations by gene name, protein name, and cell function. It provided the first list of known proteins in the splenic proteome and it provided each protein's function, if available. In addition, the functions of approximately 85% of all spleen proteins were matched to gene ontology classifications using GoMiner (Zeeberg et al., 2003) in an automated fashion. An Excel spreadsheet file is available (see Supplementary Tables 1–6) which contains all protein identifications and with UNIPROT and gene ontology characterizations for the entire dataset. For Supplementary Tables 1–6 all possible MS detected proteins were used with the raw MS/MS data from the CD45− and CD45+ LC–MS/MS runs. For the specific examination of the biological functions of the proteins from the two

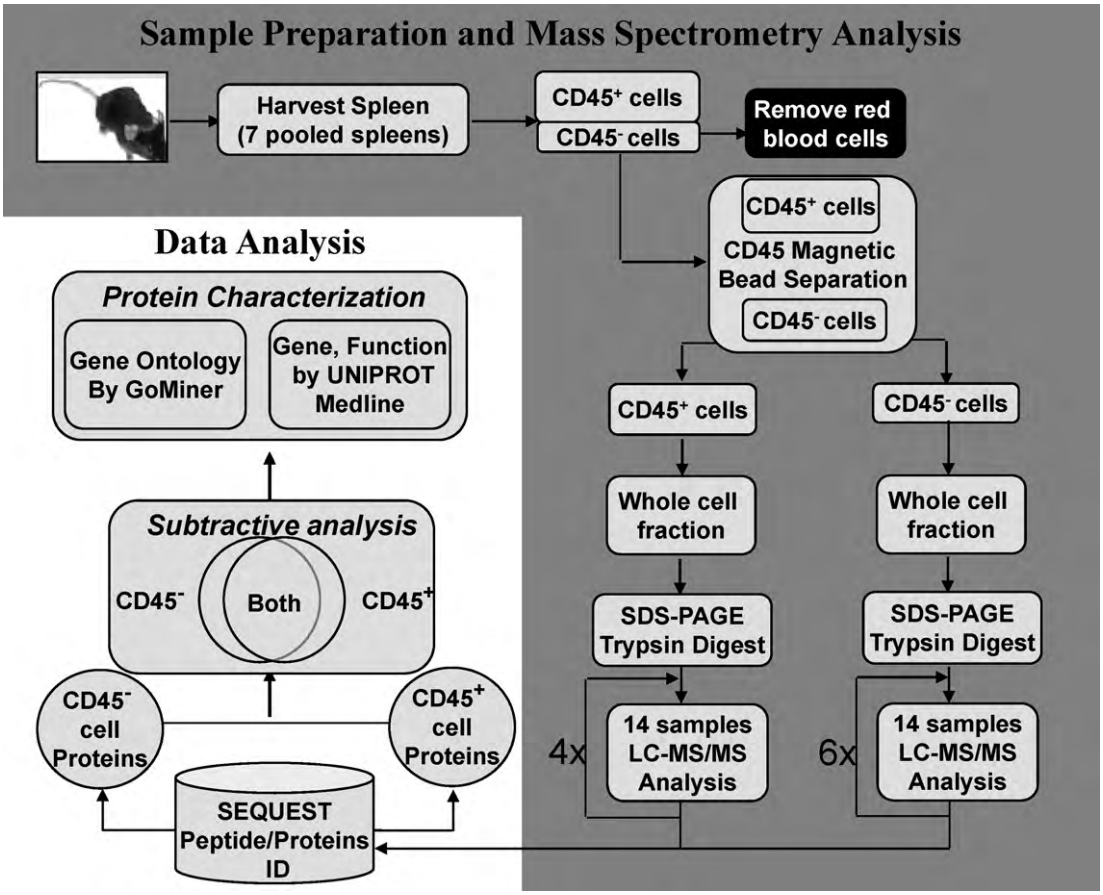

Fig. 1. Work-flow of proteomic characterization of non-lymphoid (CD45−) spleen stem cells versus CD45+ lymphoid cells. In a previous paper (Dieguez-Acuna et al., 2007), spleens were removed from $CByB6F_1$ mice and divided into CD45+ and CD45− populations by CD45 magnetic bead separations. Whole cell protein fractions were prepared and resolved by 1D SDS PAGE and 14 trypsin digests were prepared and analyzed by LC–MS/MS in 4–6 replicates. In this paper, stem cell-specific proteins identified by LC–MS/MS subtractive protein analysis of the CD45− and CD45+ cell populations and further studies using the SEQUEST algorithm on a Linux cluster analysis. Exclusive protein function of the CD45− stem cells was further characterized by UNIPROT and Medline, and by gene ontology assignment using the GoMiner application.

different cell populations, the MS data for each dataset were compared to UNIPROT database with exclusion of theoretical proteins (Fig. 4).

2.3. RT-PCR analysis and quantification

Whole spleens were harvested from C57BL/6 and NOD mice, and total RNA prepared using Qiagen RNeasy Midi kit or RNAquenous kit (Valencia, CA or Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The details of these methods are presented in the Supplementary Materials and Methods.

3. Results

3.1. The proteomics of splenic CD45− stem cells resemble embryonic stem cells and iPS cells

To determine the regenerative potential of CD45− stem cells, we undertook an analysis of proteins specific to CD45− stem cells. We had originally identified the developmental protein HOX11 exclusively in CD45− adult spleen cells by both RT-PCR and Western blot analysis (Kodama et al., 2005a). With the newly developed LC–MS/MS characterization, we identify a broad range of proteins exclusive to CD45− stem cells and expand our search for additional homeobox proteins exclusive to the spleen's CD45− stem cell population with these methods (see Supplementary Methods; Fig. 1). All HOX11-related, proteins identified in the CD45− splenic stem cells are presented as part of the Supplementary Tables 1–6 online compared to the lymphoid proteins of CD45+ cells.

We analyzed CD45− stem cell-specific proteins to determine whether they expressed transcription factors indicative of pluripotency. This targeted study was in part driven by LC–MS/MS data that revealed an excess of 17 embryonic stem (ES) cell-specific proteins, 57 embryo whole body proteins and 19 neonatal head fetal proteins, exclusive to the CD45− stem cells (see Supplementary Tables 1–6; Tables 1 and 2).

To further verify and extend our understanding of the multipotency of splenic stem cells identified by our proteomics effort, RT-PCR was performed on the spleen of two different adult mouse strains, one normal (C57BL/6) and the other spleen from a diseased murine model of type 1 diabetes, the NOD mouse. In both those murine strains, CD45− stem cells vividly expressed mRNA for the five key transcription factors—*Oct3/4*, *Sox2*, *c-Myc*, *Nanog* and *Klf4* or for closely related family members indicative of ES cell phenotype (Fig. 2). These are the same transcription factors that are actively expressed in ES cells or induced pluripotent stem (iPS) cells. Published studies have determined that these transcription factors, under select conditions, are sufficient to transform a somatic cell into a whole embryo (Okita et al., 2008).

Similarly, proteins known to be involved in signal transduction pathways during normal fetal development were also found among CD45− stem cell-specific proteins. Examples included WNT2, NOTCH3, and PTCH and GLI3, which are critical for the Wnt, Notch, and Sonic Hedgehog signaling pathways of development, respectively (Table 1).

Table 1
Splenic CD45− stem cell-specific proteins involved in signal transduction pathways related to stem cell control and development.

Gene	# of unique peptides	Protein name
Wnt signaling pathway		
Mark4	3	MAP/microtubule affinity-regulating kinase 4L
Wnt2	1	Wnt-2 protein precursor
Gsk3b	2	Glycogen synthase kinase-3 beta
Fgf8	2	Fibroblast growth factor-8 precursor
Csnk1A1	3	Casein kinase I, alpha
Csnk2A2	2	Casein kinase II, alpha
Phf2	3	PHD finger protein 2
Notch signaling pathway		
Notch3	6	Neurogenic locus notch homolog protein 3
Ctnnbl1	3	Beta-catenin-like protein 1
Adam10	1	A disintegrin and metalloproteinase domain 10
Psen1	2	Presenilin 1
Sel1l	2	Sel-1 homolog
Crb2	3	Crb2 protein
Wdr12	2	WD-repeat protein 12
Sonic Hedgehog signaling pathway		
Ptch	4	Patched protein homolog 1
Gli3	2	Zinc finger protein GLI3

3.2. Proteomics suggests CD45− stem cells differentiate into multiple tissues

Proteins exclusive to the CD45− cell population that are associated with organ development are also known to have regenerative functions in a wide variety of tissue types. We used gene ontology entries from GoMiner combined with the PubMed and UNIPROT databases to identify CD45− stem cell-specific proteins known to contribute specifically to regeneration of bone, blood, pancreas, and nerve development, the tissues known to be regenerated by the *in vivo* introduction of these stem cells. These same organ-specific proteins during fetal development were exclusively expressed in CD45− cells by LC–MS/MS analysis.

We identified many new proteins in CD45− stem cells involved in the development of bone, pancreas, cranial nerves, and blood (Table 2). These same tissues are reported *in vivo* to have cellular contributions to regeneration from splenic stem cells (Kodama et al., 2003; Lonyai et al., 2008; Tran et al., 2007; Swirski et al., 2009). The results indicate that many developmental proteins possibly required for regeneration of these tissues already are present in the naturally occurring CD45− spleen cells.

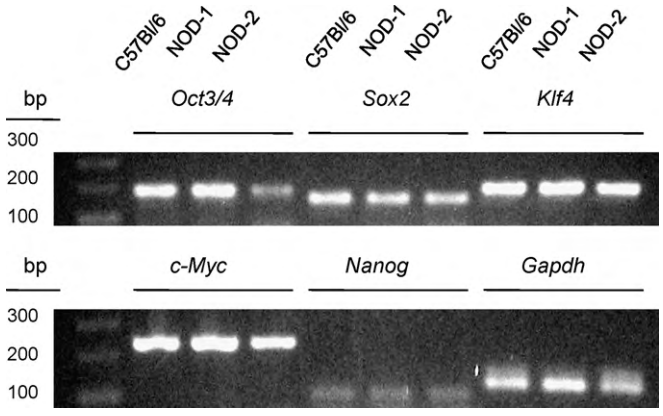

Fig. 2. RT-PCR of a normal C57BL/6 and two NOD mice (NOD-1, NOD-2) confirmed the database analysis regarding essential transcription factors for pluripotency. The data show active mRNA expression of five transcription factors of pluripotent stem cells. Primers to *Oct3/4*, *Sox2*, *Klf4*, *c-Myc*, *Nanog* and *Gapdh* made PCR fragments of 175 bp, 151 bp, 185 bp, 185 bp, 228 bp and 100 bp and 123 bp, respectively. The C57BL/6 and NOD-1 mice were 8 weeks of age, and the NOD-2 mouse was 12 weeks of age. The pre-diabetic NOD mice still had normoglycemia but with active pancreas disease, a time associated with compensatory islet regeneration in the pancreas.

LC–MS/MS analysis also revealed tissue-specific transcription factors of other organs such as skin ($n = 12$), heart ($n = 9$), eye ($n = 3$), testes ($n = 25$), thymus ($n = 33$), kidney ($n = 13$), liver ($n = 3$), lung ($n = 6$) and placenta ($n = 3$) as summarized in Table 2. Also abundant proteins of ES cells ($n = 17$), embryo whole body development ($n = 57$) and neonatal head development ($n = 19$) were detected in the proteome exclusive to CD45− stem cells (Table 2).

3.3. In situ activation of the spleen's CD45− stem cells occurs during pre-diabetic injury to the pancreas

We turned to *in vivo* studies of NOD mice during active pancreas disease (pre-diabetes) to determine whether pancreatic destruction resulted in upregulation of mRNA and CD45− stem cell-specific proteins of the spleen. Upregulation of stem cell-specific proteins in the same diseased mouse would confirm that ongoing pancreas damage is associated with an expansion or upregulation of the spleen's stem cell-specific transcription factors associated with regeneration and/or expansion of the stem cell population size. NOD mice regenerate islets after intravenous injection of CD45− stem cells harvested from normal, non-diabetic mice (Kodama et al., 2003).

The spleens of diseased NOD mice showed increased levels of mRNA for *Hox11*, *Gli3*, *Wnt2* and *Adam1*, four transcription factors associated with regeneration (Fig. 3a). The mRNA was only upregulated in the spleen during the active disease state, as opposed to the fully diabetic phase of severe hyperglycemia leading to death within days/weeks (data not shown). We interpreted the findings as signifying that, in advanced diabetes, regeneration of islets cannot keep pace with their active immune destruction and the severe metabolic state of uncontrolled hyperglycemia may lead to poor regenerative abilities. Pre-diabetic NOD mice, on the other hand, provide an opportunity to evaluate compensatory regeneration of several important developmental genes unique to CD45− spleen cells.

We also evaluated protein expression in the spleen of pre-diabetic NOD versus normal C57BL/6 mice (Fig. 3b). Using immunofluorescent antibodies to the stem cell proteins of interest, we examined the spleen of pre-diabetic NOD mice with active pancreatic disease. We found higher levels of staining for developmental proteins HOX11, GLI3 and ADAM12 in NOD spleens. Pre-diabetic mouse spleen also showed proliferative capacity by an increase in the total number of cells expressing these proteins. By contrast, CD45+ protein staining in spleen cells from both C57BL/6 and NOD mice showed similar levels of cells. Both CD45− and CD45+ cells were negative for expression of insulin protein.

3.4. Hox11 stem cells possess key differences and similarities with Hox11 leukemia cell database

Tumors expressing *Hox11*, although rare, have been the subject of gene expression profiles, from which signature genes have been identified. The most common Hox11+ tumor is a subtype of Acute T cell lymphoblastic leukemias (T-ALL). The *Hox11* T-ALL molecular phenotype is reported to have high expression of 20 signature genes, in addition to *Hox11* (Ferrando et al., 2002; Riz and Hawley, 2005).

Under the hypothesis that *Hox11*+ CD45− stem cells of the spleen might be precursor cells of this ALL leukemia subtype, we compared the 20 signature tumor genes with our 809 unique Hox11 stem cells proteins. Fig. 4 shows high overlap of 20 overexpressed signature tumor protein products (excluding *Hox11*) with

Table 2
Stem cell-specific proteins in CD45− stem cell fraction involved in organ, fetal or ES development.

Tissue/cell category	Proteins identified by LC–MS/MS analysis
Bone	A disintegrin and metalloproteinase domain 12 (ADAM12), a disintegrin and metalloproteinase domain 19 (ADAM19), collagen alpha 2(XI) chain (COL11A2), C lectin-related protein B (OCIL), guanine nucleotide-binding protein alpha subunit (GNAL), homeobox protein Hox-B4 (HOXB4), interleukin-5 (IL5), transforming growth factor beta 1 (TGFb1).
Nerve/CNS	A disintegrin and metalloproteinase domain 19 (ADAM19), homeobox protein Cux-2 (CUTL2), CADHERIN-7 (CDH7), multiple coagulation factor deficiency protein 2 (MCFD2), Rap guanine nucleotide exchange factor 5 (RAPGEF5), integrin alpha-V (ITGAV), sodium channel beta-3 subunit (SCN3B), neurofascin (NFASC), neurogenic locus notch homolog protein 3 (NOTCH3), Reelin (RELN), achaete-scute homolog 1 (ASCL1), Plexin 3 (PLXNA3), myosin Ib (MYO1B), zinc finger protein GLI3 (GLI3), inositol-1-monophosphatase1 (IMPA1), neuronal apoptosis inhibitory protein 5 (BIRC1E), MIR-interacting saponsin (TMEM4), neural proliferation protein (NPDC1), NADH-cytochrome B5 reductase (NQO3A2), protein phosphatase 1 regulatory subunit 11 (PPP1R11), glutaredoxin 1 (GLRX1), zinc-containing alcohol dehydrogenase like protein (ZADH1), adaptin-ear-binding coat associated protein 1 (1200016B17), RNA-binding protein Musashi homolog 2 (MSI2), olfactory receptor MOR283 (OLFR693), NPAT, retinoblastoma binding protein (RNF40), SUMO specific protease (SENP5), vigilin (HDLBP), BC024322, PECANEX1, TAF15, NCK2, ZFP291, similar to EPI64 (1110003P22), P20-CGGBP homolog (CGGBP1), ThiJ/Pfpl family (BC023835), serum deprivation response protein (SDRP).
Pancreas	Non-catalytic region of tyrosine kinase adaptor protein 2 (NCK2), Patched protein homolog 1 (PTC1), transforming growth factor beta 1 (TGFb1), transcription complex subunit NF-ATc4 (NFATC4), zinc finger protein GLI3 (GLI3), hepatocyte nuclear factor 6 (Hnf6), phosphatidylinositol 3-kinase regulatory alpha subunit (PIK3R1), DcoHprotein (DcoHm), anaphase promoting complex (NANPC7), Sid478p (ESD), Maf-like protein (1810009N02), HESB-like protein (HBLD2), hypothetical 22.2 kDa protein (LOH12CR1), transcription initiation factor IIE beta subunit (GTF2E2), gasdermin domain containing 1 (GSDMDC1), BOLA/YRBA family regulation DNA binding activator (1810037G04), oligoribonuclease mitochondrial homolog (SMFN), RNA helicase-related protein (DDX42), CGI-25 protein (Nosip), TRAM1, interferon inducible protein (IFITM3), MAP 17 homolog (MAP17), clone 1810038M14 protein, grancalcin (GCA), BolA-like protein (1810056020), hypothetical 23.7 kDa protein homolog (1500006O09RIK), ribosomal protein L27a (RPL27A), Gca protein similar to grancalcin (GCA), chemokine C–C ligand 17 (CCL17), synaptotagmin-like protein 4 (SYTL4), transcription initiation factor IIE, beta subunit (GTF2E2).
Skin	Keratin, type 1 (KRT16), gamma-carboxylase like protein (4633402N23), serpins containing protein (SERPINA12), S-adenosyl-L-methionine-dethytransferase (3300001M20), protein regulator of skin (CSTF3), zinc finger protein 101 (ZFP101), tetratricopeptide repeat protein (1810054D07), probably protein disulfide isomerase ER-60 (2810408E11), AA986860, 1110063F24, epithelial stem cell protein keratin, type 1 (KRT17).
Neonatal head	TLT-1 inhibitory receptor (TREML1), keratin complex 2 (KRT2-1), RAB (RAB22A), NADH-ubiquinone oxidoreductase subunit (NDUFA5), small nuclear ribonucleoprotein 70 kDa polypeptide A (SNRP70), mitochondrial ribosomal protein S24 (MRPS24), S-adenosyl-L-methionine-dependent methyltransferase (3300001M20), lipoptotien lipid site (8030451K01), squamous cell carcinoma antigen (SART3), PBEF1, SSR3, aldehyde dehydrogenase protein (2410004H02), CTD-binding SR-like protein RA4 (AA517739), threonyl-tRNA synthetase (2310044P18), Titin protein (TTN), dermatan sulfate 2-sulfotransferase (UST), Prunem1 (9230112005), aminotransferase class-I (C130053K05).
Embryo whole body	Coatomer protein complex (COPE), adaptin-ear-binding coat protein 2 (1110005F07), HSPC184 homolog (2610318K02), vacuolar sorting protein 28 homolog (VPS28), nuclear factor SBB122 homolog (1110020M19), protein AD-016 (2900091E11), prenyl group binding site protein (119003K14), nuclear distribution gene E-like (NDEL1), ARM repeat structure containing protein (2810037C14), ATP synthase delta chain (ATP5D), MUP11, MUP8 (2610016E04), prostrate cancer overexpressed gene 1 (SLC43A1), pseudouridine synthase 3 (PUS3), elongation protein 3 homolog (ELP3), cullin 4B protein (CUL4B), ovary-specific MOB-like protein (2700078K21), EAP30 subunit homolog (D11MOH34), tumor protein D54 homolog (TPD52L2), karyopherin beta 3 (KPNB3), Clast3 protein (TNFSF5lp1), hemogen (HEMGN), NIT protein 2 (NIY2), enoyl-CoA hydratase (ECHDC1), beta polypeptide (PCCB), heterogeneous nuclear ribonucleoprotein D-like (HNRPDL), mitochondrial ribosomal protein L16 (MRPL16), serine–arginine domain protein (SFRS2IP), DEAD/DEAH box helicase (DHX36), eukaryotic translation imitation factor 3 (EIF3S8), amidohydrolase (ACY-1), mitochondrial ribosomal protein 64 (MRPL51), polyamine modulated factor-1 (PMF1), Bcl2-associated athanogene 2 (BAG2), plyglutamine binding protein 1 (PQBP1), S-adenosyl-L-methionine methyltransferase (CIAPIN1), SMARCA1, PHAX, THOBTB1, PURB, six hairpin glycosytransferase (BC023151), MMRP19, vacuolar sorting protein 28 (VPS28), ubiquitin carboxyl terminal hydrolase (USP36), P63 protein (CKAP4), MCM10, ASH1L, TRIP12, VPS18, TPM4, BRE, SCAP2, MRPS2, SPAG7, D3ERTD194, glutamate rich WD-repeat protein (GRWD1), zinc finer protein GLI3 (GLI3), metaxin1 (MTX1).
ES cells	Perosismal 2-enolyl-CoA reductase (PECR), implantation-associated protein homolog (2610529C04), thyroid hormone receptor interactor 13 (TRIP13), aldehyde dehydrogenase family protein (2410004H02), hemoglobin beta adult major chain (HBA-A1), pyridine synthesis protein CAD homolog (CAD), ubiquitin fusion degradation 1 like protein (UFD1L), armadilo repeat protein (ARMC6), myeloid cell leukemia sequence 1 (MCL1), bifunctional aminoacyl-tran synthetase (EPRS), ribosomal protein L27a (RPL27A), helicase-related protein (DDX42), ribosomal protein S6kinase (RPS6KA6), arginine/serine-rich 2 homolog (2410002M20), splicing factor rich 7 (SFRS7), CG9578-like protein (1810011K17), grancalcin (GCA).
Heart	EPSP synthase (BC003479), RNI-like structure (E130107N23), PPP1R9B, spectrin beta chain (SPNB1), ARHGAP4, BLMH, CTPsH protein (CTPS2), NK13 serine protease inhibitor 12 (SERPINB6B).
Eye	Nucleoporin 210, FYVE/PHD zinc finger protein (E130113K22), eyes absent homolog 3 (EYA3).
Blood	Anamorsin (CIAPIN1), stromal interaction molecule 1 (STIM1), membrane-associated protein HEM-1 (HEM1), stem cell adaptor protein STAP-1 (STAP1).
Testes	Epididymal secretory protein (NPC2), XAP-5 protein (X5L), testis derived transcript 3 (TES3-PS), DAZ-associated protein 1 (DAZAP1), ribosomal protein L2 (MRPL37), wolffian duct like protein (1500034J01), tubulin alpha 7 (TUBA7), ribosomal protein L46 (MRPL46), testes expressed gene 9 (Tex9), actin-like protein (4921517D21), Atad1 protein (ATAD1), sperm-specific like protein SP-2 (4921536121F), secretory carrier-associated membrane protein 1 (SCAMP1), organic anion transporter polypeptide (OATP), B-box zinc finger domain (TRIM42), cytoplasmic 2 interacting protein (NFATC2IP), mitochondrial solute carrier protein (MSCP), HAD-like protein (NT5C2L1), kinesin protein 16A (KIF 16B), PML, BC027092, CDK4, CDADC1, steroidogenic factor 1 (NR5A1), aldose reductase-related protein 1 (AKR1B7).
Thymus	Thymus activation chemokine (CCL17), stem cell adaptor protein, STAP-1 (AI586015), thymus chemokine 1 (CXCL7), 3-phosphoserine phosphatase (PSPH), PNN, guanine exchange factor (ARHGEF6), SNF2 domain protein (4632409L19), TAN binding protein 2 (RANBP2), DNAJC8, LRCH4, DEF6, fusca protein homolog (1110032N12), PPP2R1A, host cell factor C1 (HCFC1), heterogeneous nuclear ribonucleoprotein U (HNRPU), mucin-like (MUC5B), STE20-like kinase homolog (A430105105), histone acetyltransferase type B catalytic subunit (HAT1), TNF binding protein 10 (RBM10), similar to DEC1 protein (C33023MO2), metallo-dependent hydrolases protein (TATDN1), glutaredoxin S-transferase C-terminus protein (PRGES2), bacterial acetolactate synthase (ILVBL), phospatidylethanolamine binding protein (MRPL38), NIPA, EPRS, APRIN, serine/threonine protein kinase (PAK2), steroid 5 alpha-reductase 2-like protein (SRD5A2L).

Table 2 (*Continued*)

Tissue/cell category	Proteins identified by LC–MS/MS analysis
Kidney	Endothelial differentiation-related factor (EDF1), kidney protein (0610007P06), carbonic anhydrase 2 (CAR2), cytochrome b-5 reductase (DIA1), Ethe1 protein (ETHE1), dienoyl-CoA reductase 1 (DECR1), interferon gamma induced GTPase (IGTP), p21-activated protein kinase-interacting protein 1 (PAK1IP1), glioma amplified sequence (GAS41), FNBP1, retinoid X receptor protein 110 (RXRIP110), beta-arrestin 1 (ARRB1).
Liver	Kell protein (Kell), ubiquitin-activating enzyme E1 (UBE1L), oligosccharyl transferase STT3 protein (130006C19).
Lung	Ribosomal protein S2 (RPS2), thymidylate kinase LPS inducible protein (TYKl), ATP-binding cassette protein (ABCB6), 46.9 kDa like protein (RBM22), PPR1R10.
Placenta	Prolactin-like protein C (PRLPC1), microtubule associated protein EMAP homolog (EML2), elastin microfibril interface-located protein 1 (EMILIN1).
Gut	Purine nucleoside phosphorylase (PNP), sporulation induced transcript 4 associated protein (D19ERTD70), actin related protein 2/3 complex (ARPC2), RNA-binding motif protein 7 (RBM7), trans-flycosidases protein (6530418L21).
Tongue	RNI-like structure protein (2310004L02), mitotic phosphoprotein 44 (NUP35), MDS025 homolog (2310015N07), casitas B lineage (CBLC), C3HC4 structure continuing protein (2310020H19), thioredoxin DNA binding domain protein (2310042M24), product E-1 enzyme homolog (2310057D15), SMC domain N terminal domain contain protein (SMC6L1), keratin complex 2 (KRT2-6B), metalloprotease 1 (PITRM1), glycoprotein 2 homolog (GP2), translation initiation factor 3 subunit (EIF3S8), nucleoside dephosphate linked moiety X (NUDT8), leucyl tran synthetase homolog (LARS), pyruvate dehydrogenase kinase, isoenyme 3 (PDK3).

our Hox11 stem cell-specific proteins. We found 10 of 20 (50%) exact matches. We also found 14 of 20 (70%) matches with closely related family members.

It should be remembered that the newly identified CD45− stem cell-specific proteins excluded the spleen's CD45+ lymphoid fraction. As is reported in this paper the starting material for the MS studies involving the physical separation of lymphoid (CD45+) cells from the non-lymphoid CD45− stem cell fraction and the stem cell-specific proteins were further defined by methods that involved multiple MS analysis runs with subtraction proteomics, thus "taking away" again any over lap with lymphoid cells. With this in mind, the discovery that CD45− stem cells overlap so closely with T-ALL cancer cells is amazing in light of the methods that virtually ruled out the possibility of lymphoid contamination of the preparations. Our data and methodology allowed us to concentrate on the 809 proteins unique to the CD45− stem cell fraction compared to cancer cells and thereby reinforces the possible similar lineages of these cells and the very unique and limited proteins that do not overlap. This reinforces the point that the strength of the observed overlap between the *Hox11*+ CD45− stem cells and *Hox11*+ cancer cells is unrelated to lymphocyte traits. The overlap between Hox11 CD45− stem cells and Hox11+ tumors (T-ALL) supports the hypothesis that splenic Hox11+ CD45− stem cells may be precursor cells of this form of cancer.

The largest overlapping groups of protein products expressed by both *Hox11*+ tumor cells and CD45− splenic stem cells consisted of those encoding DNA replication factors and DNA polymerases. Examples of the close overlap are the DNA replication licensing

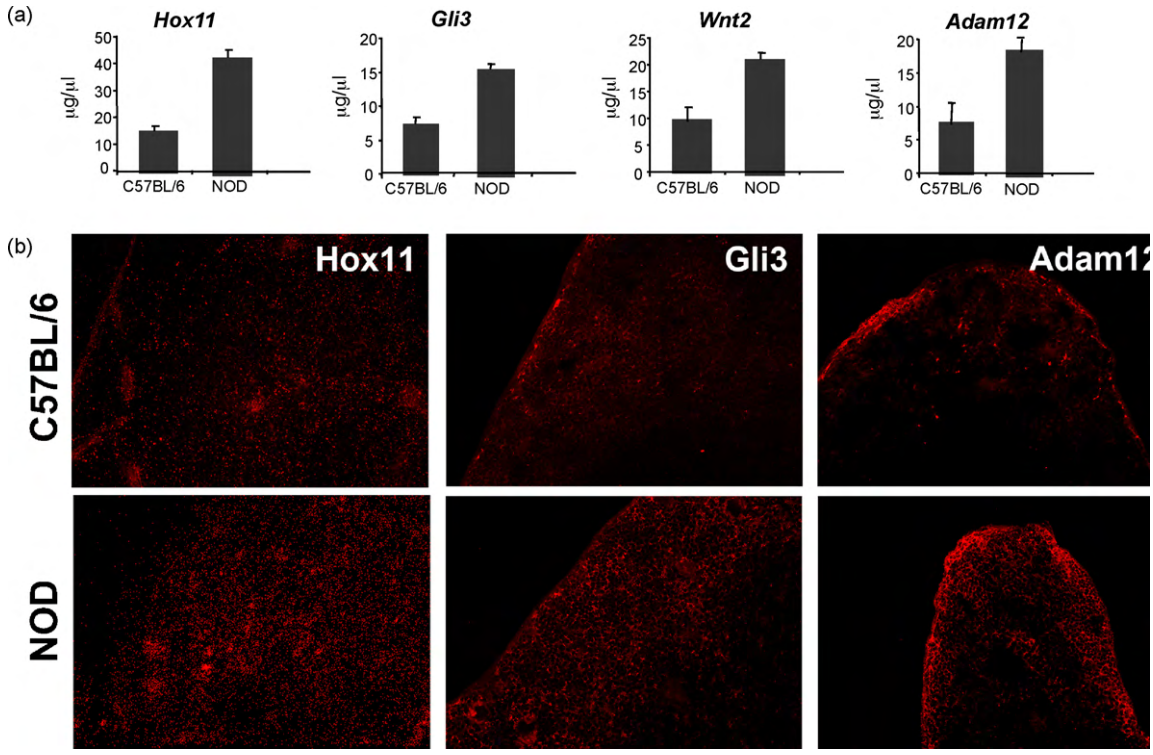

Fig. 3. Spleens from pre-diabetic adult NOD mice with active pancreatic islet disease show increased expression of genes and proteins involved in stem cell activity and replication. (a) NOD mice show increased expression of *Hox11*, *Gli3*, *Wnt2* and *Adam12* mRNA when compared to normal C57BL/6 mice. (b) Immunofluorescence staining of spleens shows increased expression and numbers of *Hox11*, *Gli3* and *Adam12* positive cells in NOD mice compared to C57BL/6 mice.

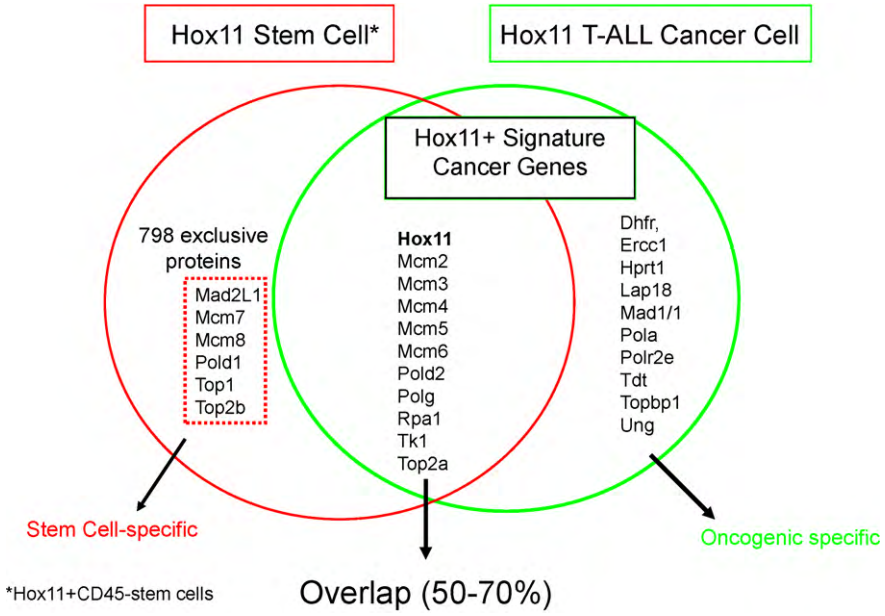

Fig. 4. Identical or close family member overlap (dashed red box) between Hox11 T-ALL signature cancer cells and stem cell-specific proteins of the spleen. Of the 798 proteins exclusive to the splenic stem cell, 6 close, but not identical, matches were also identified (red box). Those close stem cell family member matches to cancer cells were Mad2L1 similarity to Mad1/1, Mcm7 and Mcm8 with similarity to Mcm2, Mcm3, Mcm4, Mcm5 and Mcm6, Pold1 with similarity to Pola, Top1 and Taop2b with similarity to Top2a and Topbp1. The proteins used for splenic stem cells for this comparison represented matches to known proteins within the UNIPROT database. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

factors MCM family members, polymerase genes, replication A1 proteins and topoisomerase binding proteins.

Ten protein products of *Hox11+* tumor cells were found in neither the CD45− stem cell fraction nor in the CD45+ lymphoid cell fraction of the spleen (Fig. 4). Those tumor-specific genes are candidate oncogenes possibly controlling transformation of stem cells to cancer cells. Six of the tumor protein products are known markers of cancer. The six tumor-related genes lacking exact matches to the stem cell-specific proteins, or to closely related family members, were *Dhfr*, *Ercc1*, *Hprt1*, *Lap18*, *Tdt* and *Ung*. While some other genes, like *Top2b1*, also appeared to be unique to the cancer cells, they have many family members in common with splenic stem cells, but at low concentrations.

We also identified proteins unique to Hox11+ CD45− stem cells that were not expressed in Hox11+ tumor cells (Fig. 4). These proteins were MAD2Ll, MCM7, MCM8, POLD1, TOP1 and TOP2b. One of them, MCM7, which is also known as DNA replication licensing factor, may be indicative of regeneration, based on our analysis. Also unique is the expression of MAD2L1, a mitotic spindle assembly protein in stem cells. Lowered expression of this protein in cancer cells may be associated with chromosomal stability, functional spindles and rapid progression to anaphase.

4. Discussion

The regenerative potential of the spleen has long been underestimated (Kodama et al., 2005a,b; Jia and Pamer, 2009). A significant body of evidence in adults of several species, including humans, supports the spleen as holding a natural reservoir of non-lymphoid (CD45−) multi-lineage stem cells. Here we reanalyze and expand our previous proteomic analysis of these stem cells to explore their breadth of applications for regenerative medicine. We also evaluate their likelihood of oncogenic transformation, in keeping with research showing that oncogenesis is associated with introduction of embryonic stem cells and induced pluripotent stem cells.

We identified 98 CD45− stem cell proteins with developmental functions by combining results from our previous proteomic analysis and the results in this paper generated by examining the LC–MS analysis. Those proteins included the well-established developmental transcription factor HOX11. Using RT-PCR analysis, we found that splenic stem cells naturally express transcription factors Oct3/4, Sox2, Klf4, cMyc, and Nanog, all of which are characteristic and obligatory of iPS cells. These proteins, used in a large body of research, are sufficient to induce differentiated cells to become pluripotent stem cells, most commonly by using viral vectors and these cells in turn having multi-lineage potential (Okita et al., 2007; Chang et al., 2009; Senju et al., 2009; Karumbayaram et al., 2009). But the introduction of these genes carries risk of carcinogenesis. The naturally occurring stem cells of the spleen, on the other hand, possess these developmental proteins, but do not require manipulation of any type, a prospect that should reduce the possibility of malignant transformation. The concept of using naturally occurring stem cells in various states of embryonic development is also substantiated by recent publications in the adult neural stem cell literature. Neural derived adult stem cells, like splenic stem cells, naturally express Sox2 and c-Myc such that only introduction of Oct4 and Klf4 is sufficient to generate iPS cells (Kim et al., 2008).

In this paper, we identify within splenic stem cells dozens of proteins participating in signaling pathways necessary for regeneration. We also perform *in vivo* studies of autoimmune prone NOD mice to confirm stem cell functioning, namely by showing that regeneration in two organs the pancreas and salivary glands in young mice during active disease is associated with increased expression of stem cell-specific proteins in another organ, the spleen. Finally, we find that CD45− stem cells are unlikely to be carcinogenic, and instead may offer an explanation as to which proteins may prevent these stem cells from becoming transformed into Hox11+ tumor cells.

Our proteomic analysis of splenic stem cells identifies fetal tissue-specific developmental proteins that are expected based on the known direct and indirect regenerative ability of the spleen stem cells, i.e. pancreas, bone, cranial nerves/CNS, heart and blood. The proteomic analysis also identifies in the CD45− stem cells fetal

tissue-specific proteins that are indicative of a possible broader role in regeneration. There are proteins characteristic of the developing eye, testes, thymus, kidney, liver, lung, placenta, gut, tongue and skin. In fact, during the preparation of this manuscript was the discovery that a similarly located cell population in the mouse spleen contributes to repair of damaged heart tissue adding the list of possible candidate organs that can benefit from the direct or indirect contribution of splenic cells to regeneration (Swirski et al., 2009; Jia and Pamer, 2009). Neonatal head, embryo whole body and ES-specific proteins are also abundantly identified within the CD45− stem cells of the spleen. The exclusive identification of diverse developmental proteins in the CD45− cell population provides further evidence that a naturally occurring stem cell of the spleen may *in vivo* have broader regenerative potential beyond the literature to date.

Splenic stem cells naturally express key transcription factors, e.g., Oct4, Sox2, c-Myc, and Klf4, which are used to induce somatic cells to produce pluripotent stem cells (iPS). Some approaches attempt to turn somatic cells into stem cells, a process known as induced pluripotent stem cells (iPS), by introducing key transcription factors by retroviral vector or other vectors. Our discovery of HOX11+ CD45− cells in the spleen that naturally express these critical transcription factors for multi-lineage potential avoids many of the pitfalls associated with these methods of oncogenic transformations. By naturally expressing many of the proteins that other approaches seek to introduce is a possible solution to tumor transformation. There are likely to be fewer risks using a naturally occurring stem cell population with the advantage that its stem cells are found in abundance, even in humans, thereby precluding the need for *ex vivo* expansion. Finally, CD45− stem cells already have been shown to restore structure and function of injured or diseased cells of their normal lineage and for the normal lifespan of the animals.

Among the CD45− stem cell-specific developmental proteins identified there are members of the WNT, NOTCH, and SHH signal transduction pathways. These pathways repeatedly have been shown to contribute to normal embryonic development, stem cell function, and, when dysregulated, cancer cell progression (Blank et al., 2008; Reya and Cleavers, 2005). Some of the most significant proteins identified were NOTCH3, WNT2, PTC1, and GLI3. NOTCH proteins are cell surface proteins involved in the control of normal embryonic development and organ regeneration (Wilson and Radtke, 2006). NOTCH3 plays a dominant role in brain development, normal T cell differentiation, and leukemia. NOTCH3 is not unique to the spleen, but appears to be abundantly expressed in CD45− spleen cells and may interact with other proteins to increase the spleen's regenerative potential. In fact, NOTCH3 overexpression was shown to protect mice from pancreatic degradation and streptozotocin-induced autoimmunity (Anastasi et al., 2003). WNT proteins are known to interact with NOTCH (Duncan et al., 2005) and HOX (Maloof et al., 1999) proteins and can function as stem cell growth factors (Willert et al., 2003). During embryonic development, WNT proteins are expressed in substantial amounts in limb buds and in the central nervous system (Christiansen et al., 1995; Gavin et al., 1990; Parr et al., 1993). The WNT2 protein has not been previously shown to be expressed in the adult spleen, but has been identified in adult mesenchymal stem cells prepared from various human donors (Etheridge et al., 2004). WNT2 has been implicated in heart development, placenta vascularization, and various human cancers and appears to function as an anti-apoptotic factor PTC1. The SHH signal transduction pathway is critical for early organ development, specifically for bone (Mo et al., 1997), pancreas (Kawahira et al., 2005), cerebral cortex (Theil, 2005) and lung (Li et al., 2004). GLI3 has been shown to function as a repressor of SHH signaling and thus to prevent the terminal differentiation of cells during development (Koziel et al., 2005). To date, few experiments have characterized the role of GLI3 in normal adult tissue.

Our finding that most, but not all, proteins in splenic stem cells overlap with 20 signature genes of T-ALL tumors is to be expected based on a large body of research showing that many of the same signaling pathways, which are key to embryonic development, are also key to regulating self-renewing tissues. This finding is also consistent with the hypothesis that CD45− splenic stem cells are the precursor stem cell for rare forms of oncogenic transformation into Hox11+ expressing cancers. Transformation is more likely to occur when normal pathways are deregulated. Despite the high overlap, it must be underscored that malignant transformation is highly unlikely, considering that *Hox11*-expressing tumors in humans are rare. Several tumor-related proteins were only found in T-ALL, and their very identification, as well as few numbers, may enable these proteins to become past and future targets for cancer therapies. Those proteins have control over the cell cycle. The largest overlap of proteins between stem cells-specific proteins and signature proteins of T-ALL are for controlling DNA replication by DNA polymerase. Signature genes of T-ALL show unique expression of cancer related genes or closely related family members. Dihydroflate reductase (Dhfr) and hypoxanthine phosphoribosyltransferase 1 or hypoxanthine phosphoribosy transferase (Hprt1) are known targets for cancer drugs that inhibit *de novo* purine synthesis and indeed these divergent proteins were identified as exclusive to the cancer cells not the splenic stem cells.

Dhfr, dihydroflate reductase, and *Hprt1*, hypoxanthine phosporibosyltransferase, are both involved in *de novo* synthesis of purines and have known associations with cancer. Chemotherapeutic agents like methotrexate, raltitrexed and pemetrexed act to prevent *de novo* purine synthesis by inhibition of these genes. *Ercc1*, another unique cancer cell gene, participates in excision repair cross-complementing protein. This protein is also reported as a protein marker for cancers with poor prognoses (Matsubara et al., 2008). *Lap18* is known as oncoprotein 18 and has been found overexpressed in carcinomas and carcinomas with poor prognosis (Chen et al., 2003) and expression was exclusive to Hox11 cancer cells.

To prevent transformation to malignancy, several prominent proteins unique to the CD45− fraction may be involved. The proteins are known to be responsible for control over cell replication and transformation. They are *Mad2Ll*, *Mcm7*, *Mcm8*, *Pold1*, *Top1* and *Top2b*. One of them, *Mcm7*, which is also known as DNA replication licensing factor, may be indicative of regeneration, not oncogenic potential, based on this analysis. Also unique is the expression of *Mad2L1*, a mitotic spindle assembly protein considered a checkpoint in only splenic stem cells. Lowered expression of this protein in cancer may prevent chromosomal instability, no functional spindle and rapid progression to anaphase.

Should it be a surprise that the spleen of many species contains a subpopulation of multi-lineage stem cells with a protein signature of possible pluripotency? One possible reason may trace to developmental biology where the spleen forms from a region of the mammalian embryo known as the aorta–gonad–mesohephros (AGM). This is the first region of embryonic hematopoisis (Cumano et al., 2001; Medvinsky and Dzierzak, 1996). The AGM cells early in fetal life can be labeled and transplanted into neonatal mice. These AGM cells show pluripotency and form many organs and tissues of the adult mouse (Medvinsky et al., 1993). In the human literature the spleen's removal in certain conditions unrelated to diabetes and heart disease can eventually lead to the onset of insulin dependent diabetes, despite patients having intact pancreas or lead to heart disease (Lee et al., 1985; Bannerman et al., 1967). In humans, diabetes occurs years after removal of both the spleen and left side of the pancreas to treat chronic pancreatitis, whereas diabetes does

not occur after the removal of only the right side of the pancreas with intact spleen (Hutchins et al., 2002; Govil and Imrie, 1999). A long-term follow-up of 740 American servicemen splenectomized because of trauma during the 1939–1945 war showed a significant excess mortality from ischemic heart disease from poor cardiac health (Robinette and Fraumeni, 1977). This suggests a possible life long homeostatic role for the spleen in maintaining pancreas and cardiac health in humans.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biocel.2009.12.001.

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