Sustained tissue -specific transgene expression from a vl30 retrotransposon-derived vector *in vivo*

Research Article

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Summary

Previous attempts to generate transgenic mice via retroviral transduction of pre-implantation embryos have usually not resulted in stable transgene expression. In these cases, inactivation of the retroviral LTR is associated with passage through the germ line. A subset of endogenous murine retrovirus-like retrotransposons (VL30's) are constitutively expressed in virtually all tissues, with no deleterious effects to the health of the animal. We surmised that these VL30s might be useful as vectors for stable lineage-specific transgene expression. A mouse VL30-derived retro-vector engineered to express a reporter gene (*Lac*Z) was used to generate transgenic mice via transduction of embryonic stem (ES) cells. A single copy of the vector was stably integrated into a unique site in the mouse genome. Sustained tissue specific expression was observed at both the mRNA and protein levels for several generations. Transgene expression was observed in distinct sub-populations of cells in both lung and spleen. In the lung, cells expressing the vector were identified as type II pneumocytes. These data illustrate for the first time that a VL30 LTR (NVL-3) is unique from its retroviral counterparts in that it can pass through the germ line repeatedly without undergoing transcriptional inactivation. Thus, VL30 vectors may be useful for both transgenesis and as alternatives to existing retroviral vectors for gene therapy.

I. Introduction

Currently, methods for deriving transgenic animals rely predominantly on the direct injection of DNA into the pronuclei of oocytes (Palmiter et al, 1982; Lacy et al 1983). This method results in the integration of random concatamers of the original construct with varying copy number. This characteristic frequently confounds the interpretation of the results, requiring the analysis of a large number of founders to ensure correct interpretation of the data (Si-Hoe et al, 1999).

An alternative approach was envisioned over two decades ago, involving the integration of a single copy retroviral vector into the germ line (Jaenisch, 1976). These studies, which employed Moloney murine leukemia virus (MoMLV), illustrated that these viruses failed to be expressed in the resulting animals. Infection of post-implantation embryos, however, does provide long-term expression of the transgene (Jahner et al, 1982). Failure of transgene expression in pre-implantation embryos is linked to lack of LTR enhancer activity (Brinster et al, 1981; Linney et al, 1984), binding of transcriptional repressors to the retrovirus tRNA primer binding site (Loh et al, 1987, 1988; Peterson et al, 1991), and possibly to DNA methylation of retroviral sequences as well as DNA flanking the integration site (Jahner et al, 1985; Barker et al, 1991; Hoeben et al, 1991).

Related to the murine type C retroviruses is a family of long terminal repeat- (LTR) bearing
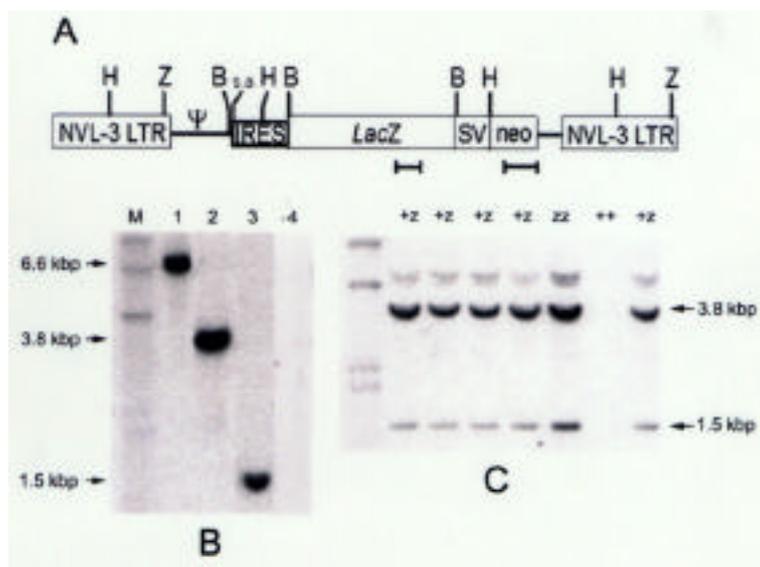
Figure 1. Production of a transgenic mouse line harboring the VLSAIBAG retrotransposon construct. The synthetic retrotransposon, VLSAIBAG, engineered for these studies is illustrated in panel A. Positions of the NVL-3 long terminal repeats (NVL-3 LTR), *Psi* packaging sequences ( ), splice acceptor site (s.a.), internal ribosome entry site (IRES), b-galactosidase expression cassette (*Lac-Z*), SV40 virus early region promoter (SV) and neomycin phosphotransferase expression cassette (neo) are shown. The positional locations of probes used are indicated by bars. Key restriction endonuclease recognition sites are indicated by letters: H= *Hind*III; Z= XhoI; B=*BamH*I. Panel B is a Southern blot illustrating single copy integration of an intact vector in the ES cell clone used to generate transgenic animals. Lanes: M, marker; l-*Hind*III; 1, *Xho*I digest hybridized with lacZ probe; 2, *Hind*III digest hybridized with lacZ probe; 3, *Hind*III digest hybridized with neo probe; 4, untransduced ES cell DNA; 5, EcoRV digest hybridized with *Lac-Z* probe. Panel C illustrates typical results for genotype analysis by Southern blot of a litter of pups derived by crossing two mice heterozygous for the transgene. Genotypes are listed above each lane. ++ = normal mouse; +Z = hemizygous for the transgene; ZZ = homozygous for the transgene.
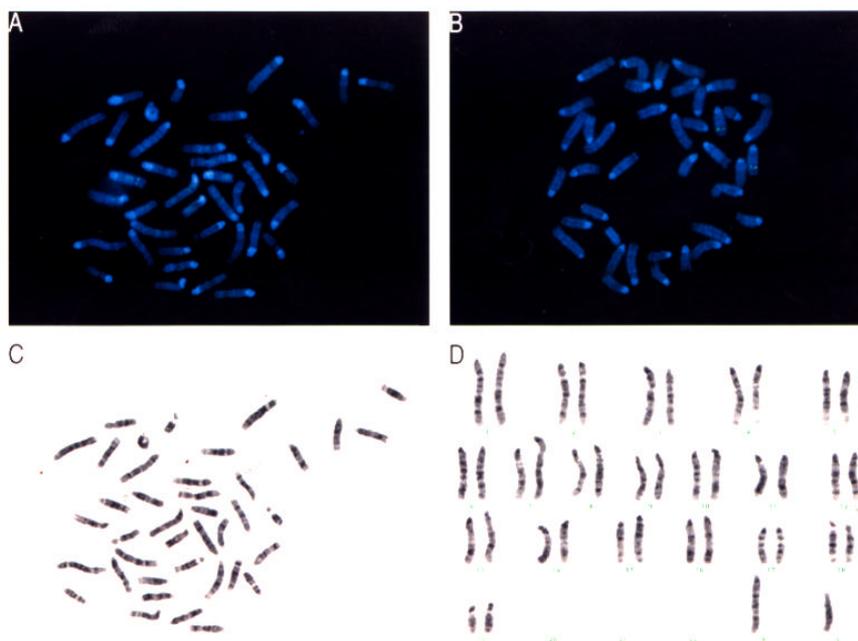

Figure 2. Cytogenetic analysis of VLSAIBAG integration site. (**A and B**) FISH signal showing vector integration site in two different metaphase spreads from splenocytes of homozygous transgenic mice. (**C**) G-banding analysis of same spread as in A. Chromosomes with fluorescent signal are indicated by arrows. (**D**) Typical mouse idiogram as reference for panel C.

retrotrotransposable elements (VL30's) present at 100 to 200 copies in the genomes of most species (Keshet et al, 1980; Courtney et al, 1982). Subsets of these endogenous elements are naturally expressed in many tissues (Norton et al, 1988; Nilsson and Bohm, 1994). During retroviral infections, mouse VL30 RNA is efficiently transmitted by pseudotyping (co-packaging) into retrovirus particles (Howk et al, 1978; Besmer et al, 1979; Scolnick et al, 1979). Vectors derived from murine VL30's are expressed in a variety of mammalian cell types, both primary and transformed (Chakraborty et al, 1993, 1995).

These characteristics of VL30 suggested that vectors derived from these elements might give sustained expression in vivo. To test this hypothesis, a construct expressing LacZ from a VL30 LTR was packaged using a standard retroviral packaging cell line, and the resulting virions were used to transduce murine embryonic stem cells. Selected transductants were used to derive a transgenic animal via injection of ES cells into 3.5 day blastocysts and surgical reimplantation. Sustained and tissue specific expression was observed in the resulting transgenic mouse line. These results illustrate the utility of VL30 derived retro-vectors for obtaining transgene expression in vivo.

II. Results

A. derivation of vector and construction of the transgenic mouse line

The vector used in these studies, which is referred to as VLSAIBAG (**Figure 1A**), was derived from the endogenous VL30 element NVL-3 (Carter et al, 1983). It contains a LacZ reporter gene expressed from the NVL-3 LTR. As VL30 RNA is poorly translated into protein (unpublished observations), an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) was cloned 5' of the LacZ open reading frame to ensure efficient translation of the reporter construct (Grunkemeyer et al, submitted). Embryonic stem cells were transduced using viral supernatants from PA317/VLSAIBAG vector producer cells (VPCs). A number of clonal cell lines were obtained by G418 drug selection. One such clone was selected for micro-injection into mouse blastocysts, resulting in the production of a single transgenic mouse line. The transgene was integrated as a single copy, without rearrangements, in both the ES cells (**Figure 1B**) and in the resulting transgenic animals (**Figure 1C**). Fluorescence in situ hybridization (FISH) analysis demonstrated that a single autosomal integrant was stable through several mouse generations (**Figure 2**).

B. Analysis of vector expression in vivo

Preliminary screening was carried out by reverse transcriptase polymerase chain reaction (RT-PCR) on total RNA from various embryonic and adult tissues. These analyses indicated expression in both spleen and lung (data not shown). RNase protection analysis was then performed using total RNA isolated from lung and spleen tissue from four and 15 week old animals. **Figure 3A** illustrates that a 194 bp protected fragment (corresponding to a portion of the lacZ structural gene) was present in both lung and spleen tissue. Significant amounts of vector RNA were observed in samples derived from both young (29 day old) and mature (99 day old) mice, illustrating that expression of the reporter gene is stable. To verify that the probe was detecting full-length transcripts of the appropriate molecular size, RNA blot analysis was performed using total RNA from lung tissue. **Figure 3B** demonstrates that the appropriate sized transcript (6.6 kb) was expressed in lung. Vector-derived transcript levels in the spleen were too low to be detected by this method.

Expression of vector-derived transcripts in RNA preparations from whole tissues might be derived from a specific cellular compartment within the tissue. To delineate transgene expression at the cellular level, we employed a histochemical staining technique for enzymatically active -gal protein. Results shown in **Figure 4** demonstrated significant transgene expression in a subset of cells in both lung (**Figure 4B, and D**) and spleen (**Figure 4F**). Neither the number of cells nor the intensity of staining appeared to vary significantly between lung tissue derived from animals of two-weeks (**Figure 4B**) versus 10-weeks (**Figure 4D**) of age. No staining was observed in these same tissues from age-matched non-transgenic littermates (**Figure 4A, C and E**).

Based upon the morphology, frequency, and localization of -gal positive staining cells of the lung, we surmised that they may be either macrophages or type II pneumocytes. To resolve this issue, tissues were first stained with X-gal, followed by staining of sections with antibodies specific for either macrophages or type II pneumocytes. The same fields were observed by both light and fluorescence microscopy to determine the identity of -gal positive cells. Analysis of **Figure 5A**, and **5B** clearly illustrates that cells staining positive for the macrophage marker are not positive for -galactosidase activity. Similar analysis was carried out using an antibody specific for the pro-peptide form of human surfactant protein C, which is an established marker for type II pneumocytes (Vorbroker et al, 1995). Comparison of **Figure 5C** with **Figure 5D** illustrates that all blue cells were also positive for surfactant protein C, identifying these -gal positive cells as type II pneumocytes. It should be noted, however, that not all antibody positive cells were blue, suggesting the transgene was expressed in a sub-population of type II pneumocytes in the lung.

Similar attempts to identify the Lac-Z positive cells in the spleen were inconclusive.

III. Discussion

The results described herein demonstrate that transgenic animals can be derived via transduction of mouse embryonic stem cells using retro-vectors engineered to express heterologous gene products from at

least one VL30 promoter (NVL-3). In the example described, a stably transduced ES cell clone was used to generate a transgenic mouse which expressed theVL30 vector, VLSAIBAG, in a tissue-specific manner. The retro-vector was integrated in single copy, and shown to pass through the germ-line several times (>5) without affecting either the expression levels or the cellular specificity of expression. Earlier attempts to derive transgenic mice from embryonic cells transduced with retroviral vectors resulted in inactivation of expression after passage through the germ line (Jahner and Jaenisch, 1985). Inactivation was attributed to methylation of both the retroviral genome and the DNA surrounding the site of integration, based on direct studies as well as experiments where the retroviral genome was activated by chemical demethylation of the DNA with 5-azacytidine (Jahner et al, 1982; Jahner and Jaenisch, 1985). Thus, this method for producing transgenic animals has been largely abandoned. VL30-derived vectors offer the opportunity to revisit this strategy.

The use of retroviral transduction of embryonic stem cells as a means of generating transgenic animals has obvious advantages over methods currently employed. The vector can be inserted in a single copy, avoiding complications due to concatamerization of the transgene. It allows for testing of gene therapy vectors *in vivo*, allowing quick assessment of tissue specificity and toxicity. There are more than 100 copies of VL30 in the mouse genome with wide ranging developmental and tissue specific expression patterns (Sanes et al*,* 1986; Norton and Hogan, 1988; Nilsson and Bohm, 1994), and inducibility (Rodland et al, 1986, 1988; Lenormand et al, 1992), suggesting that their use in transgenics might have broad applicability.

In an earlier report, Nilsson and Bohm, (1994) examined the endogenous expression patterns for a number of the known VL30 elements in mouse tissues. They demonstrated expression of the subclass of VL30 LTR's that includes NVL-3 are expressed in the lung and spleen. Our construct employed the NVL-3 LTR, and as reported herein, was expressed in both the lung and the spleen. Nilsson and Bohm did not resolve expression at the cellular level in these tissues. Our results suggest that the NVL-3 LTR is regulated in the appropriate developmental and tissue specific manner in the transgenic mouse line. However, because only one transgenic line was analyzed, it is not possible to say with certainty that the transgene functioned in a position-independent manner. Generation and analysis of more transgenic lines, derived from indepentdently-transduced ES cell clones, are necessary to resolve this issue. Indeed, expression of other retro-vectors have been shown to be sensitive to position effects (Hoeben et al, 1991). However, the fact that expression was observed in the same two tissues as observed previously by Nilsson and Bohm, (1994) strongly suggests that the LTRs are specific for these tissues.
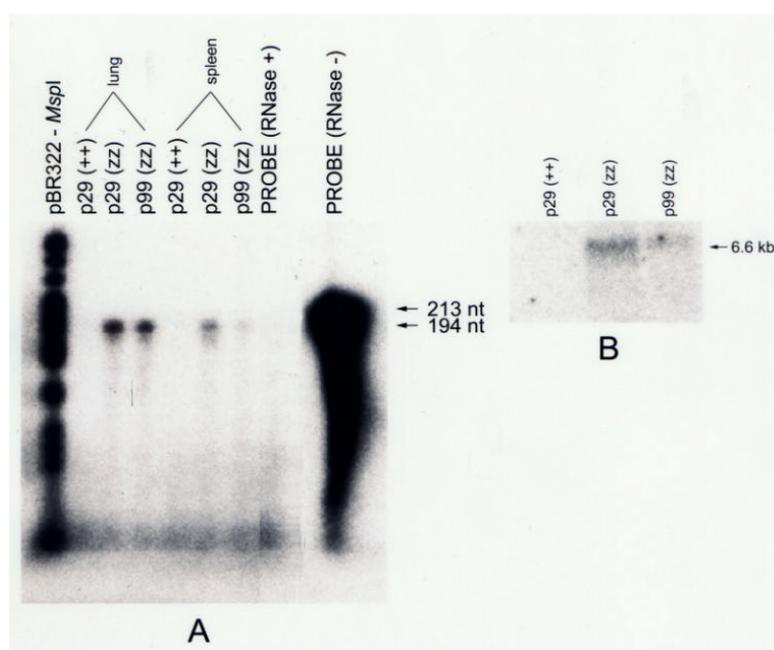

Figure 3. Expression of LTR-driven transcripts from lung and spleen in transgenic mice. Panel A is RNase protection analysis of total cellular RNA from both lung and spleen. Total RNA from either 29 day-old (p29) or 99 day-old (p99) mice were analyzed using a 213 base pair riboprobe. The expected size of the protected fragment was 194 base pairs. Probe (RNase +) is probe digested with ribonuclease; probe (RNase -) is undigested probe. (++) are wild type controls; (ZZ) are mice homozygous for the transgene. Panel B represents a northern blot of total lung RNA from either 29 day-old (p29) or 99 day-old (p99) transgenic mice hybridized with a probe derived from the *LacZ* structural gene (Figure 1A). The expected size band is visible at 6.6 kb.
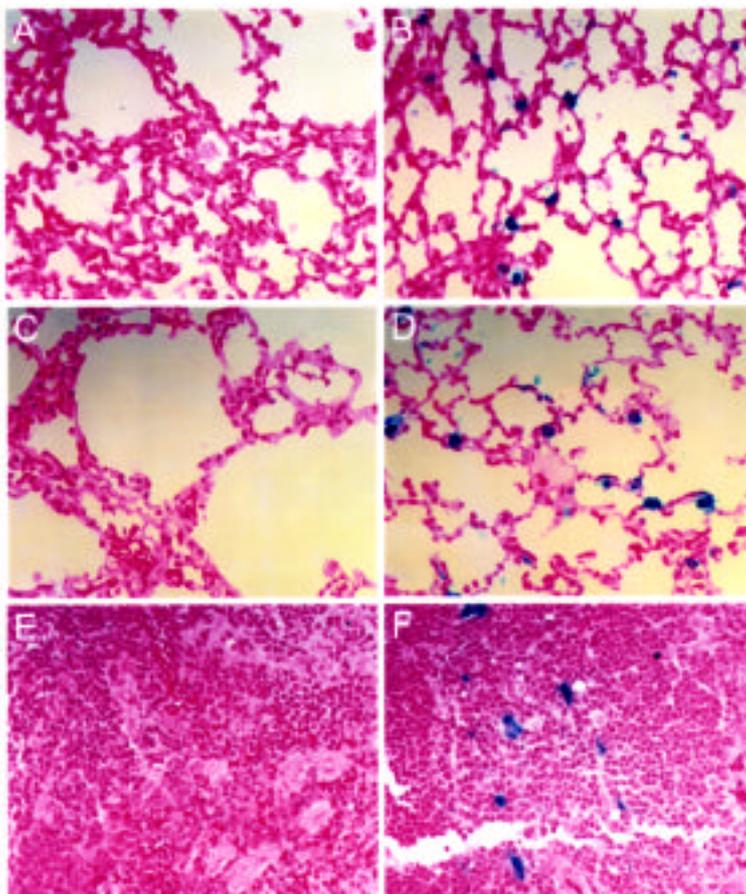
Figure 4. Expression of the VL30 transgene at the cellular level in lung and spleen. Tissues were stained histochemically for -galactosidase expression, then embedded in historesin plastic, and cut at 2.5 µM. Sections were counter-stained with eosin and hematoxylin and photographed at 200 X magnification. Panels A and B show lung from 15 day old normal (**A**) and homozygous transgenic (**B**) mouse. Panels C and D show lung from 71 day old non-transgenic (**C**) and homozygous transgenic (**D**) mice. Panels E and F show spleen from 71 day old normal (**E**) and homozygous transgenic (**F**) mice.
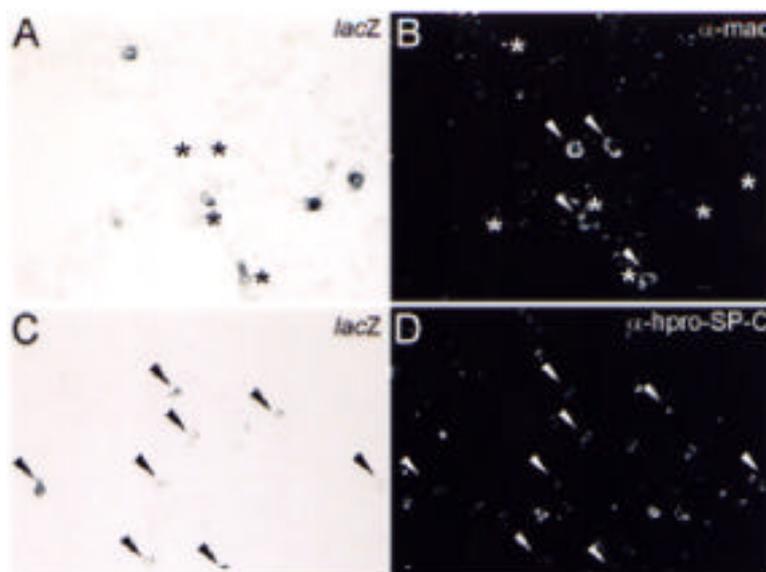

Figure 5. Identification of -galactosidase positive cells in the lung as type II pneumocytes. Lung tissue from a 71 day old homozygote was stained for -gal expression, embedded in aqueous mounting medium, and cryosections stained with either -Mac (a cell surface marker for macrophages, panel **B**), or anti-human surfactant protein C pro-peptide (-hpro-SP-C, a marker for type II pneumocytes, panel **D**). The same field was recorded by light microscopy (panels **A** and **C**) for -gal staining, and fluorescence microscopy (panels **B** and **D**) for the specific cell markers to allow identificaiton of dual positive cells as type II pneumocytes (indicated by arrows in panels **C** and **D**), and not macrophages (lack of dual positive cells indicated by non-overlapping asterisks and arrows in panel **B**).

This is the first time that tissue-specific expression of a transgene has been demonstrated using a VL30-derived vector. These findings point toward the use of VL30-derived vectors for the generation of transgenic animals expressing a heterologous gene in a tissue-specific or developmental stage-specific pattern. The large number of endogenous VL30 LTRs which have been shown to be expressed in distinct tissues at various times of development, may serve as a reservoir of promoters for transgenic constructs as well as potentially being useful for gene therapy.

Expression of the retro-vector in type II pneumocytes illustrates highly specific cellular expression of the NVL-3 LTR promoter. Indeed, only a subset of pro-SPC-positive type II pneumocytes were positive for -gal staining. Currently, pro-SPC is an accepted marker for the identification of type II pneumocytes (Vorbroker et al, 1995). Our data suggests that the capacity to express the retro-vector may delineate a subtype of type II pneumocyte or simply that vector expression in some type II pneumocytes is too low to be detected by the employed methods. Targeting expression to type II pneumocytes might provide therapeutic angles for diseases such as pulmonary emphysema, forms of which have been attributed to defects in matrix metalloproteinase expression (Ohnishi et al, 1998).

IV. Materials and Methods
A. Vector construction

All plasmids were constructed according to standard protocols (Ausubel et al, 1995). Plasmid pVLBAG was generated by cloning a blunted *lac*Z/simian virus-40 (SV40) early region transcriptional promoter/neomycin phosphotransferase resistance cassette from pDOL (Price et al, 1987) into the unique *Not*I site of plasmid pVLPP (Chakrablrty et al, 1993). Plasmid pVLPP is a synthetic VL30 vector containing the LTRs and *psi* packaging signal from the murine retrotransposon, NVL-3 Carter et al, 1983). Plasmid pVLIBAG was then constructed using pVLBAG as vector. A blunted 601 bp *Nco*I-*Sal*I fragment (from pG1IL2EN) (Treisman et al, 1995), containing the IRES sequence from encephalomyocarditis virus (Jang et al, 1988), was ligated into the unique *Pac*I site in pVLBAG immediately 5' of the -gal initiator AUG codon.This clone was used as vector for cloning pVLSAIBAG, the vector used in these studies. A 26 bp splice acceptor fragment was cloned immediately 5' of the IRES. The splice acceptor was found to be non-functional both *in vitro* and *in vivo* (unpublished observation). For RNase protection assays, plasmid pRIBOGAL was constructed by cloning 194 bp of the *lac*Z coding sequence into pBluescriptII (Stratagene, La Jolla, CA). All clones were verified by restriction and sequence analysis.

B. Viral transductions

The embryonic cell line RW4 (Genome Systems, St. Louis, MO) was cultured according to the method of Robertson, 1987. ES cells were transduced by supernatants according to standard protocols (Cepko et al, 1995). Viral supernatants were harvested from rapidly growing (just confluent) cultures of PA317s, filtered through 0.2 μ filter (Nalgene, Milwaukee, WI), and added to ES cell cultures which had been passed onto gelatinized plates. The following day, the cells were washed twice with PBS and allowed to grow for 48 hours. Transduced ES cells were selected in 175 μg/ml G418 for 10 days. G418 resistant clones were expanded, and DNA (Puregene kit; Gentra Systems, Inc., Research Triangle Park, NC) was isolated for determination of vector integrity (no major rearrangements or deletions) and copy number. Clonal cell lines with correct morphology and a single integrated copy of VLSAIBAG were expanded for injection into pre-implantation embryos.

C. Genetic typing

Hemizygote (designated +Z) males and females were maintained as breeders in the colony so that each litter had a likelihood of providing wild-type (++) and homozygous (ZZ) animals for experimentation. DNA was isolated from tail DNA according to the following method: an approximately 1 cm piece of tail was clipped with a sterile scalpel and incubated in 0.5 ml digestion buffer (50mM Tris-Cl, pH 7.5; 50 mM EDTA; 1% SDS and 10 μg/ml Proteinase K, Boehringer Mannheim) overnight at 37°C and purified as described previously (Hogan et al, 1994). Tail DNA was digested with *Hind*III, electrophoresed, and hybridized to ^{32}P-labelled probes for both the *lac*Z and *neo* coding sequences. After exposure of autoradiographic film, blots were exposed to a phospor screen to quantify the amount of signal in each lane and enable the distinction of hemi- and homozygous animals.

D. Fluorescence *in situ* hybridization analysis

Cells were prepared for FISH and G-banding (**Figure 2C**) analysis as previously described (Takashi et al, 1991). Briefly, splenocytes were cultured for 2-4 days in the presence of concanavalin A and lipopolysaccharide to stimulate cell division. Metaphase spreads were prepared by synchronizing the cells with 300 Ìg/ml thymidine, culturing in 5-bromo-deoxyuridine (BrdU) and arresting them at metaphase with colchicine. The spreads were subjected to FISH analysis as described.

The spreads were heat denatured and hybridized to a digoxigenin-labelled probe (dig-dUTP; Boehringer Mannheim), which was generated by nick translation of the *lac*Z probe (used for Southern and Northern analyses). After hybridization, slides were washed and incubated with a fluorescein-conjugated anti-digoxigenin antibody. The slides were viewed on an Olympus BH-2 microscope equipped for epi-illumination. DAPI (4,6 Diamidino-2-phenylindole) was used as a counterstain for the chromosomes (excitation = 367 nm, emission = 453 nm). FITC (Fluoroscein isothiocyanate) was used to label the probe (excitation = 497 nm, emission = 524 nm). Low light level fluorochrome signals were captured and enhanced using the Cytovision system (Applied Imaging Inc., Pittsburgh, PA).

E. Northern blot and RNase protection analyses

Isolation of RNA from tissues was done using TRIzol™ reagent (InVitrogen Corp, Gaithesburg, MD) according to the manufacturers instructions. Total RNA was fractionated by electrophoresis on agarose-formaldehyde gels (20 μg sample/lane). Blots were UV-crosslinked, air-dried and hybridized to a probe for the *lac*Z coding sequence. RNase protection assays were performed using the same total cellular RNA used for Northern analyses with a kit (RPA II kit) from Ambion, Inc. (Austin, TX) according to the manufacturer's instructions. Antisense riboprobes were synthesized *in vitro*

using T3 RNA polymerase (Boehringer Mannheim), labeled ^{32}P-UTP (Amersham), and *linearized* plasmid pRIBOGAL. The riboprobe contains extra vector sequences, so undigested probe (213 bases) can be differentiated from fully protected probe (194 bases) on a denaturing acrylamide gel.

F. Histochemical stain for -gal activity and visualization

Histochemical staining of tissues with X-gal was performed as previously described (Sanes et al, 1986). Tissues were fixed in 4% paraformaldehyde (PFA, Sigma Chemical Co.) for 1-2 hours at room temperature. After fixation, tissues were rinsed four times in PBS and incubated overnight at 30ºC in X-gal stain solution [5.0 mM $K_3Fe(CN)_6$; 5.0 mM $K_4Fe(CN)_6$; 1.5 mM $MgCl_2$; 0.02% NP-40; 0.01% sodium deoxycholate with 1.0 mg/ml X-gal (in DMSO) in PBS]. After staining, tissues were rinsed in PBS and embedded in Historesin using the methods described by the manufacturer (Leica; Heidelberg, Germany). Tissue blocks were sectioned at 2.5 μm with a Sorvall JB-4 microtome (Ivan Sorvall, Inc., Newton, CT) using a glass knife. To visualize cell morphology of cells stained blue (due to -gal activity) in the context of the surrounding cells, each section was stained with Harris' hematoxylin and counterstained in alcoholic eosin according to standard protocols (Allen et al, 1992). Sections were coverslipped with an organic mounting medium (Curtin Matheson Scientific, Inc., Houston, TX) and visualized on an Olympus BH2 oil immersion microscope fitted with a digital camera and imaging system (Cytovision, Applied Imaging, Pittsburgh, PA).

G. Immunohistochemical staining and visualization

Tissues stained with X-gal were also prepared for immunohistochemical analysis. After staining, tissues were rinsed in PBS and embedded in Tissue-Tek^{tm} (Miles Inc., Elkhart, IN) embedding medium. Tissues were frozen and stored at -150ºC and warmed to -25ºC prior to sectioning. Blocks were sectioned at 3 μ on a Microm HM 505 N cryostat (Carl Zeiss, Inc., Thornwood, NY)

Various antibodies/antisera were used to identify the specific cell types that express the vector VLSAIBAG *in vivo*. A polyclonal antisera against mouse macrophages (-mac; Accurate Chemical and Scientific Corp., Westbury, NY), and a polyclonal antibody against the pro-peptide of human surfactant protein C (-hpro-SP-C, Vorbroker et al, 1995) were used for immunohistochemical analysis on X-gal stained tissue sections. The basic method is as described (Watkins, 1989).

For the -mac antisera, normal goat immunoglobulin G (Rabbit IgG, Vector Laboratories, Inc., Burlingame, CA) was diluted to 12.5 μg/ml in 7% non-fat dry milk in PBS (milk/PBS) and incubated on the section for 10-30 minutes at room temp. Next, the primary antibody (polyclonal antibody/antisera) was diluted (1:300 dilution) in milk/PBS and placed on each section. Sections were incubated with the primary antibody overnight at 4ºC. After incubation with the primary antibody, the sections were washed three times in PBS before the FITC-conjugated (fluorescein isothiocyanate) secondary antibody was applied to the sections. The secondary antibody was diluted 1:100 in milk/PBS and incubated on each section for 2.5-3.5 hours at room temperature in the dark. Finally, the slides were washed three times in PBS and coverslipped with Vectashield^{tm} (Vector Laboratories, Inc., Burlingame, CA) mounting medium. The slides were documented with an Olympus BH2 oil immersion

microscope fitted with a digital camera and imaging system (Cytovision, Applied Imaging, Pittsburgh, PA).

For the -hpro-SP-C polyclonal, sections were blocked (2% normal goat serum; 0.2% Triton X-100 in PBS) for 90 minutes at room temperature before the addition of -hpro-SP-C (dilution of 1:1000 in 2% normal goat serum; 0.2% Triton X-100 in PBS). Primary antibody was incubated overnight at 4ºC. The next morning, the slides were washed with 0.2% Triton X-100 in PBS before addition of the FITC-conjugated secondary diluted 1:100 in PBS containing 0.2% Triton X-100. After 2.5 hours at room temperature in the dark, slides were washed with 0.2% Triton X-100 in PBS (1 wash for 5 minutes), PBS alone (2 washes for 5 minutes each) mounted with Vectashield, coverslipped and imaged.

Acknowledgements

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