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SERPINB3 is associated with longer survival in transgenic mice

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The physiological roles of the protease inhibitor SERPINB3 (SB3) are still largely unknown. The study was addressed to assess the biological effects of this serpin *in vivo* using a SB3 transgenic mouse model. Two colonies of mice (123 transgenic for SB3 and 148 C57BL/6J controls) have been studied. Transgenic (TG) mice showed longer survival than controls and the difference was more remarkable in males than in females (18.5% vs 12.7% life span increase). In TG mice decreased IL-6 in serum and lower p66shc in the liver were observed. In addition, TG males showed higher expression of mTOR in the liver. Liver histology showed age-dependent increase of steatosis and decrease of glycogen storage in both groups and none of the animals developed neoplastic lesions. In conclusion, the gain in life span observed in SB3-transgenic mice could be determined by multiple mechanisms, including the decrease of circulating IL-6 and the modulation of ageing genes in the liver.

The serpins are a family of serine or cysteine protease inhibitors involved in multiple biological functions and cell homeostasis control[1]. They are characterized by unique common structural features, extra and intracellular localization and inhibitory mechanisms of specific proteases[2,3].

Serpins act as inhibitors of serine proteases, although some of them have evolved mechanisms into different proteases, such as SERPINB3 (SB3) (known as Squamous Cell Carcinoma Antigen 1 or SCCA1) which exerts inhibition versus cysteine proteases[4]. The divergent functions or the specificity of target depend mainly on the variety of the reactive-site loop which is involved in the initial interaction with the protease, its recognition and cut, resulting in its inhibition[5,6].

SERPINB3 (SB3) was originally purified from squamous cell carcinoma of the uterine cervix[7] and genomic sequencing revealed that it belongs to the ov-albumin serine protease inhibitor family (ov-serpins)[8]. The biological role of this serpin has not been yet completely defined. SB3 is physiologically expressed in squamous epithelia, in endothelial cells of the veins, in arteries walls[9] and in peripheral blood mononuclear cells[10]. It is also overexpressed in neoplastic cells of epithelial origin[11,12]. Normal liver does not contain detectable amounts of this serpin, while it is over-expressed in primary liver cancer and in preneoplastic lesions[13–15], where remarkable levels have been found also in serum as IgM-linked immunocomplex[16–18]. Several data indicate that SB3 induces cellular protection from apoptotic death caused by different kinds of stimuli and the suggested molecular target is located upstream to caspase-3[19,20], with supporting evidence of cytochrome c release inhibition by mithocondria[21]. This serpin induces cell proliferation and deregulation of adhesion processes, leading to epithelial-mesenchymal transition (EMT) with increased invasiveness potential[22]. In addition, it has been reported that it induces TGF-β expression[23,24] and promotes fibrogenesis in experimental models[25]. On the basis of these considerations, SB3 could play a role in the development of cancer phenotype. In agreement with these observations, in hepatocarcinoma, where SB3 has been found overexpressed, a significant inhibition of apoptotic cell death has been reported[26]. However, the actual knowledge on the biological functions of SB3 *in vivo* are still limited[27] and it has not been investigated yet if this serpin plays a role in the complex process of aging, which induces typical molecular and genetic events[28]. The determinants of the length of life are multifactorial and involve composite steps, most of which are yet to be understood. Interestingly, most of the intracellular factors involved in the aging process, such as sirtuins, foxhead box O group (FoxO), the target of rapamycin (mTOR) kinase, p53 and p66shc are part of interconnected pathways associated to both cellular metabolism and oncogenic processes.

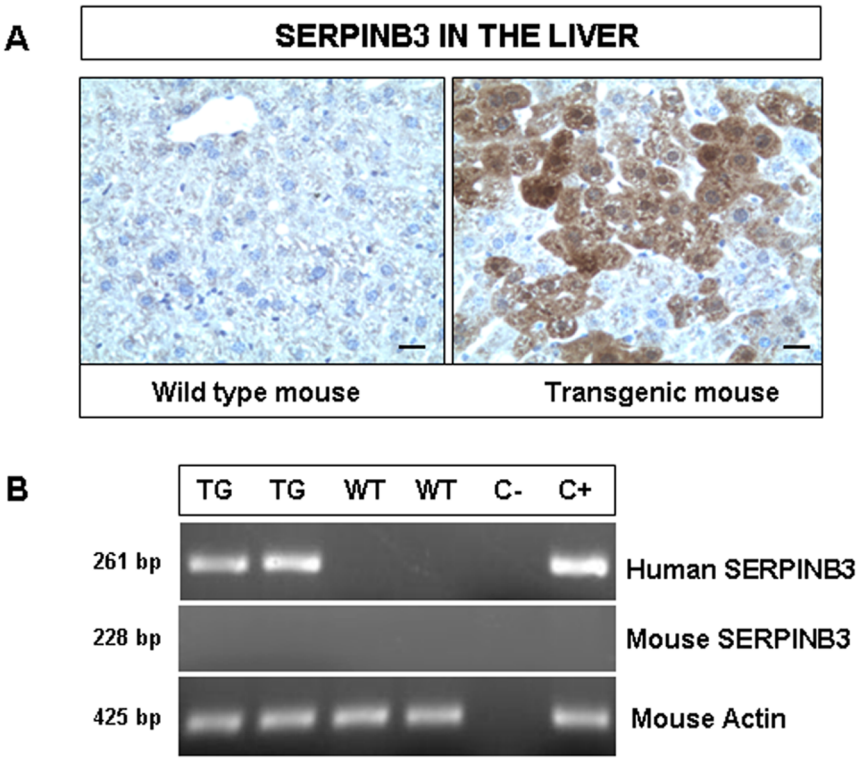

Figure 1 | **SB3 expression in mice.** (a) Immunohistochemistry for human SB3. TG mice showed strong immunostaining in hepatocytes, not detectable in wild type animals. (b) Example of agarose gel electrophoresis of real time PCR amplification products for human SB3, mouse SERPINB3b and mouse housekeeping gene actin in two transgenic mice (TG) and in two wild type mice (WT); C- refers to the negative control and C+ refers to the positive control. Bar = 1 μm.

The aim of the present study was to explore biological effects of SB3 *in vivo*, with a particular focus on survival and aging genes expression using a transgenic mouse model.

Results

The groups of transgenic (TG) and control wild type (WT) mice were homogeneous for sex distribution (M/F ratio: TG 69/54 vs WT 78/70, p = 0.7003). Human SB3 was detectable by immunohistochemistry in liver hepatocytes of TG animals (Figure 1, upper panel) and mRNA levels were similar in the liver of males and females (mean ± SD: 3.05 ± 3.6 pg/μg RNA vs 3.54 ± 4.37 pg/μg RNA, p = 0.8303), but were not detectable in the WT genotype. The mouse-homologous SB3 was not found in the liver of neither TG nor control WT mice (Figure 1, lower panel).

Length of survival. The observational follow up revealed that in the 123 TG mice the age range of death was 2 to 29 months, and 50% survival was reached at month 13. In the 148 WT mice the age range

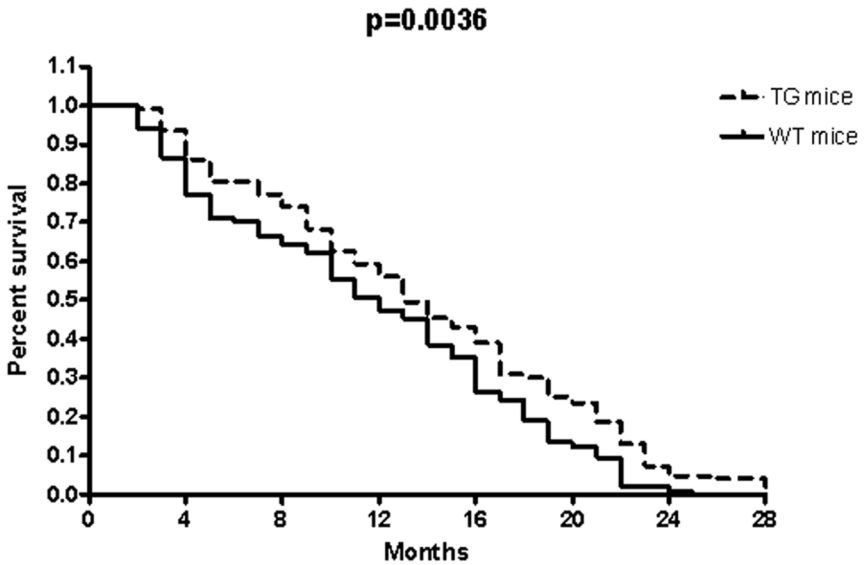

Figure 2 | **Survival curves in mice populations.** Survival curves in mice transgenic for SB3 (TG = 123) and in control wild type mice (WT = 148). A significant gain in life span is detectable in TG mice. The curves were developed using the Kaplan-Meier method (Logrank Test).

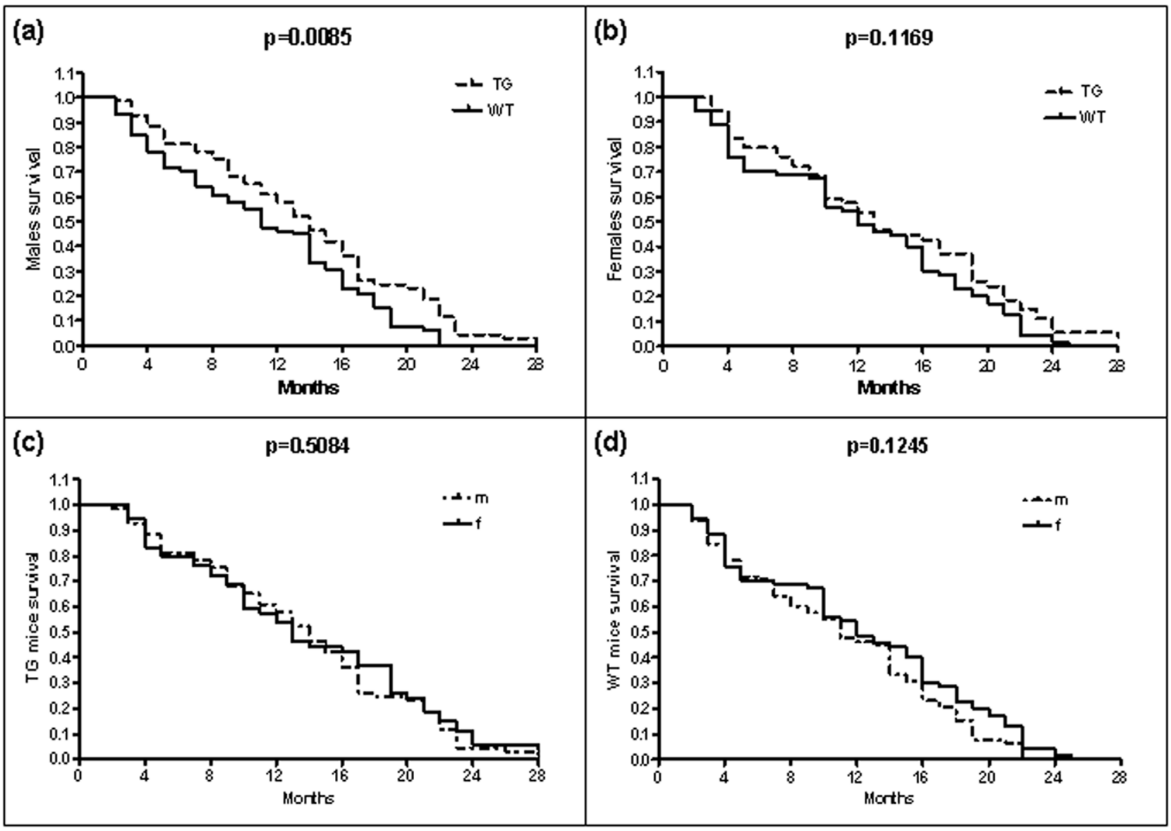

Figure 3 | **Survival curves in relation to sex.** (a–b) Comparison of survival between transgenic (TG) and wild type mice (WT). TG males show significantly higher survival time than WT males (A: $p = 0.0085$); (c–d) Comparison of survival between females and males in TG mice (c) and in WT mice (d). No significant gender differences were observed within each group. The curves were developed using the Kaplan-Meier method (Logrank Test).

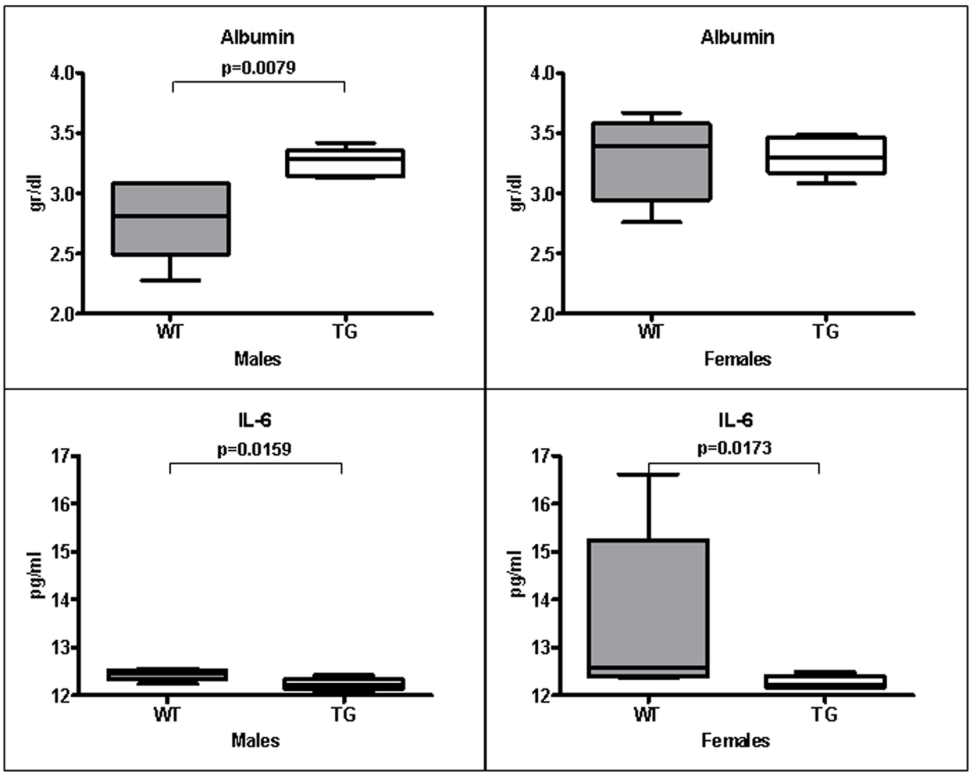

Figure 4 | **Albumin and IL-6 levels in relation to gender.** Albumin and IL-6 levels serum of transgenic (TG) and wild type (WT) mice in relation to gender. Box indicate the lower and the upper quartile and the middle lines indicate the median value; bars indicate the range of value distribution (Mann-Whitney Test).

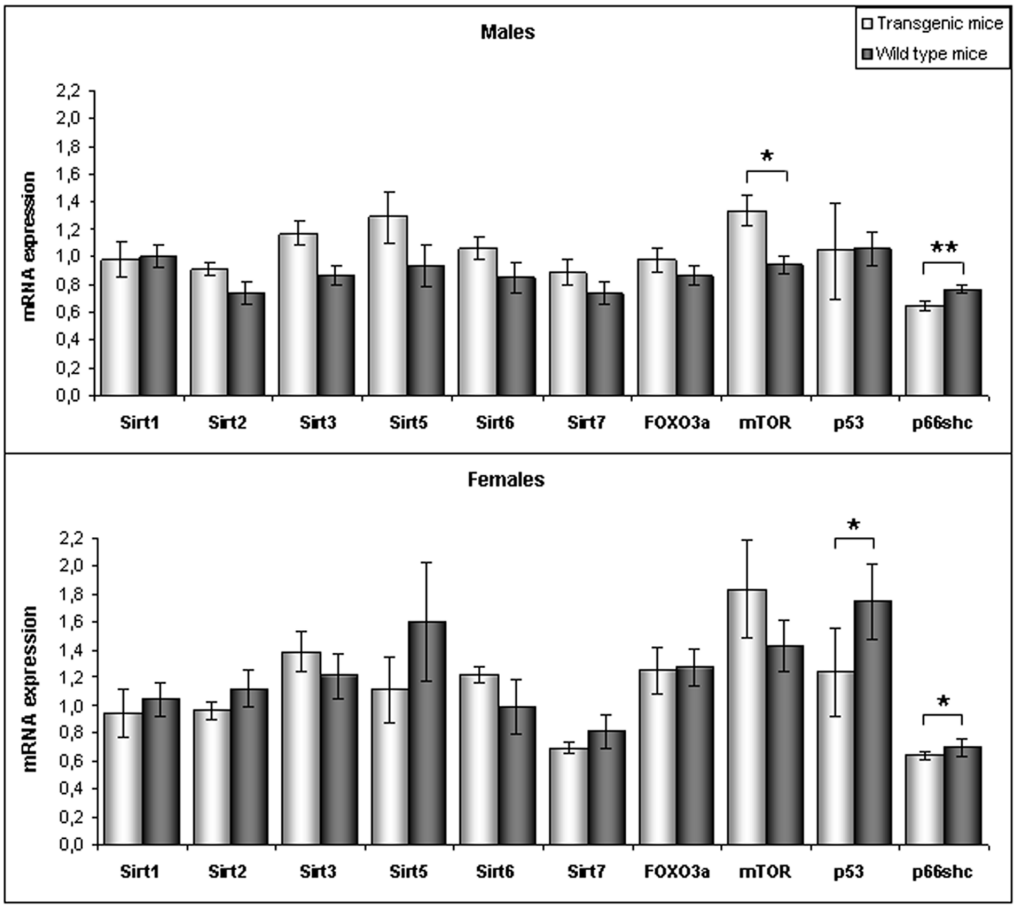

Figure 5 | Longevity genes mRNA expression in males and females. Mean values of longevity genes in liver tissue of TG (n = 21) and WT (n = 24) mice, detected in males (upper panel) and females (lower panel). Vertical bars represent standard deviation (SD). Significant gender differences were observed for mTOR in males, for p53 in females and for p66shc in both. The analysis was independently performed three times for each gene (Mann-Whitney Test). * $p < 0.05$; ** $p < 0.01$.

of death was 2 to 25 months, and 50% survival was reached at month 11. The survey of the overall mice population documented that the TG mice maintained a survival time significantly higher than WT mice (mean ± SD: 13.83 ± 0.65 months vs 11.73 ± 0.55 months, $p = 0.0036$), as shown in Figure 2. This feature was more prominent in males (mean ± SD, TG: 13.72 ± 0.83 months vs WT: 11.19 ± 0.71 months, $p = 0.0085$), while females survival was not significantly different in the two genotypes (mean ± SD, TG: 13.96 ± 1.03 months vs WT: 12.33 ± 0.84 months, $p = 0.1169$) as shown in Figure 3 (A, B). Within each genotype, however, no significant gender-related differences in survival were observed (Figure 3, C, D).

Serological parameters. Liver function tests as aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and fasting plasma glucose were similar in TG and WT mice. These parameters were further analysed in males and females separately and this approach revealed that albumin concentration in males was higher in TG mice than in control WT mice (mean ± SD: 3.26 ± 0.12 gr/dl vs 2.79 ± 0.33 gr/dl, $p = 0.0079$), while similar levels were observed in females of both genotypes (mean ± SD: 3.31 ± 0.15 g/dL vs 3.29 ± 0.35 g/dL, $p = 0.7922$) (Figure 4).

Overall IL-6 levels were significantly higher in WT mice than in TG animals (mean ± SD: 13.00 ± 1.35 pg/ml vs 12.25 ± 0.12 pg/ml, $p = 0.0020$) and the difference was maintained also when the results were analysed in relation to gender (Figure 4). It should be noted that in WT animals higher levels were detected in females than in males ($p < 0.005$), while in TG animals both groups showed very low IL-6 values.

Expression of longevity genes. Different profiles of longevity gene expression, including Sirtuins, Foxo3a, mTOR, p53 and p66shc, were detected in the liver of WT and TG mice. In TG mice a significant decrease of p66shc expression, compared to control WT mice ($p = 0.0005$), was observed. In addition, when gene expression analysis was carried out in relation to gender, in TG mice mTOR become significantly higher in males ($p = 0.0273$) and p53 lower in females ($p = 0.0318$), compared to the corresponding WT animals (Figure 5).

In order to assess whether these differences were due to a direct effect of sex hormones on liver parenchymal cells in relation to their SB3 content, HepG2 cell stably expressing SB3 or control HepG2 cells, stably transfected with the plasmid vector alone, were treated with sex hormones, including testosterone, progesterone and estrogens. In these experimental conditions, no significant modifications of mTOR, p66shc, p53 and sirtuins were observed, while testosterone induced significantly higher levels of FOXO3a in control HepG2 cells, but not in cells expressing SB3 (Figure 6).

Western blot analysis. Western blot analysis of liver lysates from TG and WT mice was carried out in order to confirm transcriptional results. Densitometric determination showed lower expression of p66shc in TG animals, compared to WT mice. In TG males increased expression of mTOR, compared to controls, was also detected, while trivial levels of p53 protein were observed, especially in females, not allowing consistent comparison (Figure 7).

Histological findings. At macroscopic level no hepatic nodules were detected in the liver of neither TG nor WT mice and these findings

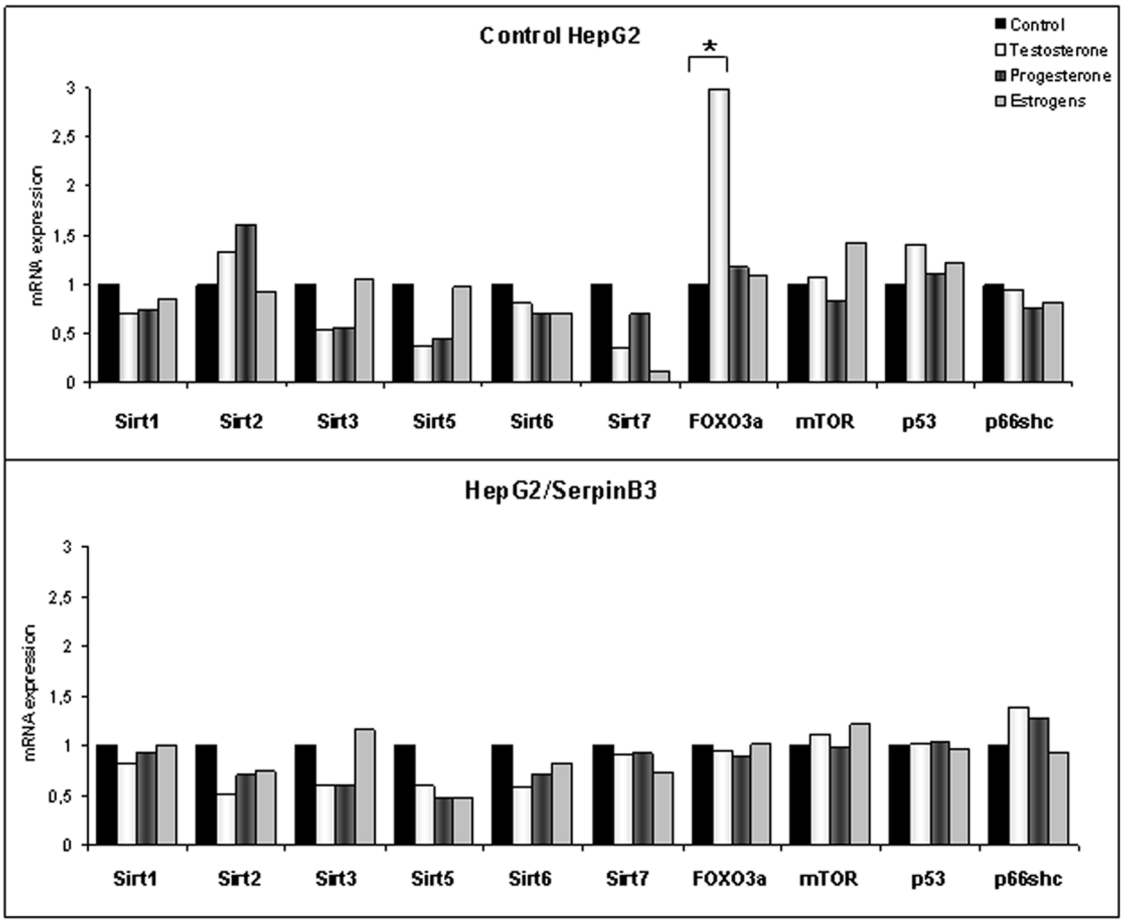

Figure 6 | Effect of sex hormones on longevity genes. Effect of sex hormones on longevity genes in HepG2 cells stably expressing SB3. Median values of longevity genes in HepG2 cells (upper panel) and in HepG2 cells expressing SB3 (lower panel) incubated with sex hormones, including testosterone, progesterone and β-estradiol (Estrogens). The analysis was independently performed three times (Mann-Whitney Test). * $p < 0.05$.

were confirmed at microscopic level, where no hepatic foci of neoplastic transformation were observed. Histological parameters, including inflammation, steatosis, glycogen storage and hepatocyte dysplasia were evaluated. At univariate analysis, as shown in Table 1, in TG mice higher degree of steatosis and lower glycogen storage, compared to WT mice (Figure 8) were detected. However, multivariate stepwise analysis adjusted for age and sex, revealed that only age, affected by SB3 in TG mice, was identified as a significant parameter associated to steatosis accumulation and lower glycogen deposition in the liver (Table 2).

Discussion

The biological role of the cysteine protease inhibitor SB3 is still largely unknown. While this serpin is physiologically expressed in squamous epithelia, it is not detectable in normal liver, where it can be induced by chronic liver damage and it is over-expressed in pre-neoplastic and neoplastic liver lesions[13–15]. In our study we have evaluated the effect of SB3 over-expression in the life span of a large colony of TG mice, expressing this protein in the liver and in the inflammatory cells[25,29].

The results indicate that this serpin induces a gain of about 15% of survival length in TG mice compared to WT controls and this effect was more remarkable in males than in females. These features were associated with lower expression of the ageing gene p66shc in the livers. In addition, mTOR was increased in the liver of TG males, while the difference was not significant in TG females. These findings can support the life span extension observed in TG animals, with a more prominent effect on TG males. The increased albumin synthesis detected in TG males, reflecting a gender-related metabolic modification, was in keeping with the observed results. In TG females lower p53 mRNA levels were detected, compared to WT females, however, protein expression was barely detectable in both groups, impeding proper comparison. The gender-dependent effect of the serpin on aging genes modulation suggests a possible role of sexual hormones that requires to be further explored. The "in vitro" model of hepatoma cells expressing SB3, used in the present study, demonstrated that the observed transcriptional modifications observed in TG mice were unlikely due to a direct effect of sex hormones on liver cells, but rather reflect more complex interactions with the micro-environment and/or immune system components.

At serological level, TG animals showed lower levels of IL-6 cytokine, compared to WT mice, with a more prominent drop in females than in males. Interleukin-6 is a pleiotropic cytokine that has a differential effect on tissue growth, repair and regeneration[30]. It may be essential for tissue regeneration in the acute setting, but under certain conditions, prolonged exposure to IL-6 may lead to activation of apoptosis, cell death and lower the threshold for liver injury[31]. In agreement with these findings, recent data have documented an association between serum IL-6 concentrations and mortality due to cardiovascular disease, cancer, and liver diseases in older adults[32].

The multiple biological effects of ageing genes are still under investigation. Beside metabolic control and cell survival, potential oncogenic properties have been described recently. Among ageing genes, p66shc, found down-regulated in TG mice, is classically known as a signalling protein implicated in receptor tyrosine kinase signal transduction[33]. More recently p66shc has also been identified

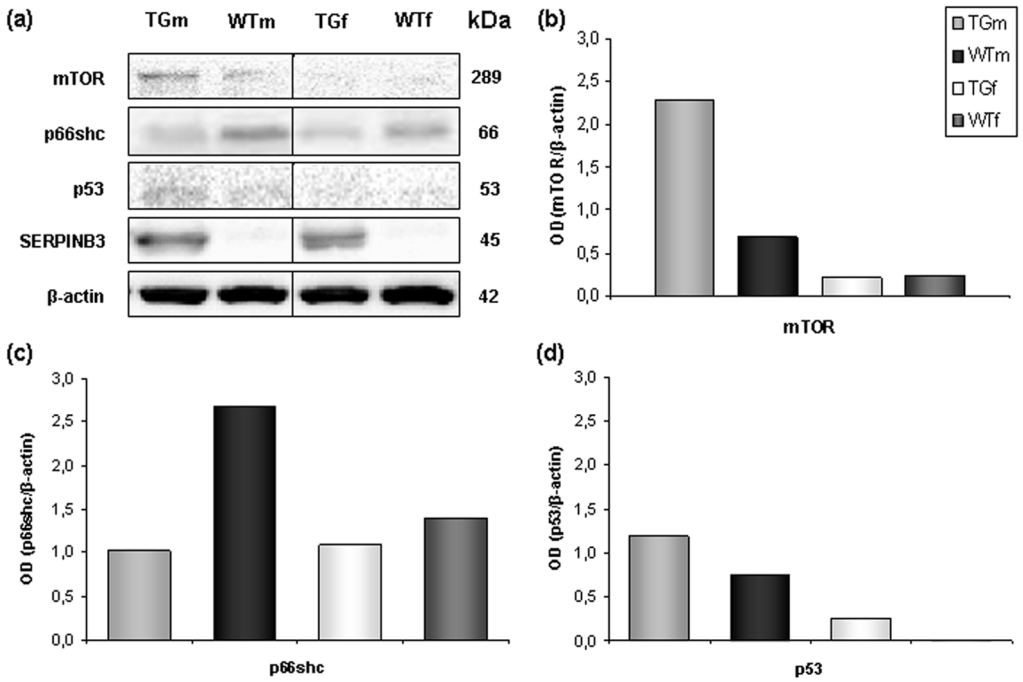

Figure 7 | Western blot analysis. Example of Western blot analysis obtained in four mice (one TG male, one WT male, one TG female and one WT female. (a) Cropped blots showing protein expression levels of mTOR, p66shc, p53 and human SB3 in mouse liver homogenates. Bar graphs show the corresponding densitometric analysis, normalised to β-actin, of mTOR protein (b), of p66shc protein (c) and of p53 protein (d). These results were representative of those obtained in the subgroup of mice (21 TG and 24 WT mice) sacrificed at 12 months of age and evaluated for aging gene expression. TGm: transgenic males; WTm: wild type males; TGf: transgenic females; WTf: wild type females.

as a sensor of oxidative stress-induced apoptosis and as a protein affecting longevity in mammals[34]. FOXO and sirtuin family members have been reported as key regulators of mammalian vascular development and disease, providing new perspectives on disease mechanisms of aging[35]. In our study these genes were slightly higher in TG mice, especially in males, without reaching significant differences, compared to control WT animals. As reported above, significantly higher levels of mTOR were found in the liver of male TG animals. As part of the mTORC1 and mTORC2 complexes, mTOR has key roles in several pathways, including regulation of cell growth by modulating protein synthesis, ribosome biogenesis and autophagy[36]. The activation of mTOR via PI3K/Akt pathway has been identified as one of the most commonly altered pathways in human tumors[37]. Its gender-related modulation by SB3 observed in the liver of TG mice, with higher values in TG males, might explain, at least in part, the higher risk of liver tumor development detected in males[38]. Hepatocellular carcinoma develops indeed in the majority of the cases in cirrhotic livers[39] where SB3 is expressed frequently, while it is not detectable in normal liver parenchymal cells[15].

Beside the observed modulator effects determined by SB3 in the present experimental model, the gain in life span observed in TG animals could be determined, at least in part, by the direct anti-apoptotic effect of this serpin[40], already described also in the TG mice after partial hepatectomy[29]. This serpin did not induce neoplastic transformation by itself, as documented by the absence of neoplastic transformation foci and of increased displastic hepatocytes in the liver of TG mice. SB3 can be therefore considered a protective factor which enhances survival mechanisms increasing cellular life span. Recent findings have revealed that this serpin is also able to determine epithelial-mesenchymal transition (EMT) and to increase cell invasiveness by autocrine and paracrine ways[22]. The close interconnection of the pathways associated to both cellular protection/survival and oncogenic processes is further supported by the fact that chronically damaged hepatocytes start to synthesize SB3, while the highest levels are achieved in preneoplastic and neoplastic liver lesions[15,16]. This defence mechanism to oxidative stress can determine initially a higher resistance to apoptotic cell death, but also a higher oncogenic potential over time. Indeed, the newly acquired cellular characteristics determined by chronic liver inflammation could explain, at least in part, the higher risk of neoplastic transformation found in liver cirrhosis, which can be considered the end stage of a chronic inflammatory process.

Table 1 | Histological parameters evaluated in SB3-transgenic mice (TG) and in control wild type mice (WT)

	Group	(No. of tested animals)	Mean	Std Dev	P*
Inflammation	TG	42	0.6579	0.7807	0.1245
	WT	98	0.5143	0.7425	
Steatosis	TG	42	**0.6053**	0.8865	**0.0332**
	WT	98	**0.2**	0.6325	
Glycogen storage	TG	42	**1.973**	0.9276	**0.0183**
	WT	98	**2.4839**	0.8112	
Hepatocyte dysplasia	TG	42	0.4211	0.5517	0.2376
	WT	98	0.5143	0.7425	

*Mann-Whitney Test.

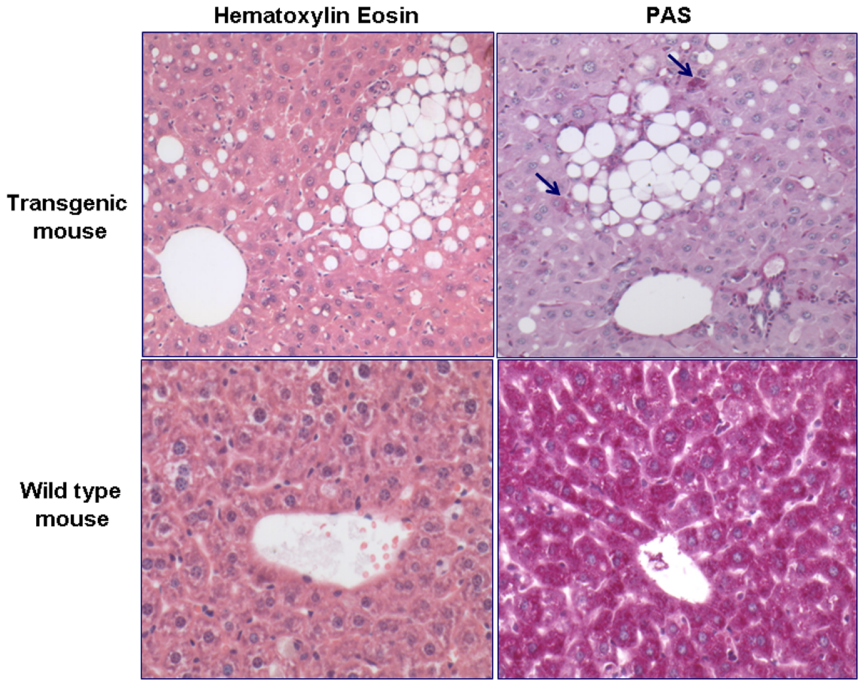

Figure 8 | Example of the degree of steatosis and of glycogen storage in transgenic and wild type mice. Hematoxylin eosin staining has been used to assess the degree of steatosis and PAS staining has been employed to evaluate glycogen storage. In the transgenic mouse hematoxylin eosin shows grade 2 hepatocyte steatosis and sporadic (grade 1) PAS positive hepatocytes (arrows). In wild type mouse hematoxylin eosin shows no steatosis (grade 0) and diffuse and strong PAS positive (grade 3) hepatocytes. Original magnification 40×.

In conclusion, the gain in life span observed in SB3-TG animals could be determined by multiple mechanisms, including the decrease of IL-6 cytokine and the modification of liver ageing genes that was in part modulated differently by sex hormones.

Methods

SB3 transgenic mice. The study was carried out in C57BL/6J mice transgenic for human SB3 and the insert was located under the α1-antitripsin (α1AT) promoter[29]. C57BL/6J WT mice (Charles River Italia S.p.A, Calco, Lecco) were used as control. By immunohistochemistry the serpin was detected in transgenic (TG) animals in parenchymal cells in the liver and in inflammatory cells/macrophages in the lung[25]. Mice were maintained under a 12 hours light/dark cycle in temperature-controlled cages (22 ± 3°C) and humidity (50 ± 5%), with free access to food and water.

The study was carried out in accordance with the guidelines for the care and use of laboratory animals and mice were bred at the Animal Care Facility of the Experimental Surgery Division of the University of Padua. The experimental protocol was approved by the Animal Investigation Committee of the Italian Ministry of Health.

Overall, 123 mice transgenic (TG) for SB3 and 148 control WT mice have been followed up till natural death. Soon after death the liver was removed and fixed with 4% paraformaldehyde in PBS, followed by paraffin embedding for histological analysis (hematoxylin-eosin and PAS stains). Blood samples were obtained in 48 TG and 55 WT alive mice (at 6 months of age) by tail venipuncture and serum was stored at −80°C for further analysis.

An additional subgroup of 45 mice (21 TG and 24 WT mice) were sacrificed at 12 months of age and the livers were frozen immediately in liquid nitrogen and stored at −80°C for molecular studies.

SB3-expressing cell cultures. In order to better assess the possible modulating effect of sex hormones on ageing genes of liver parenchymal cells in relation to SB3, HepG2 cells stably transfected with SB3 or with the plasmid vector alone as control, were used[22] and they were free from mycoplasma infections.

Cell cultures were treated with culture medium supplemented with 20 nM testosterone, or 2 nM progesterone, or 0.2 nM β-estradiol (Sigma-Aldrich, St. Louis, MO). After four days incubation at 37°C in a 5% CO_2 incubator, cells were trypsinized and the cellular pellets were maintained at −80°C in RNAse free tubes until use.

Serological parameters. Liver function was monitored by measuring ALT, AST and bilirubin concentrations in serum by laboratory routine assays. Albumin was measured in serum by a colorimetric assay (Eurokit S.r.l., Gorizia, Italy), according to manufacturer's instructions. Albumin concentration in each sample was expressed in

Table 2 | Multivariate stepwise analysis of the histological parameters considered in liver tissue in SB3-transgenic mice (TG) and in control wild type mice (WT) in relation to gender and age

Variable	Factor	Odds ratio	Confidence interval		P*
Inflammation	Females vs Males	2.036	0.716	5.792	0.1825
	TG vs WT	1.400	0.486	4.034	0.5331
	Age	1.095	0.971	1.234	0.1407
Steatosis	Females vs Males	0.269	0.053	1.358	0.1120
	TG vs WT	2.044	0.431	9.683	0.3678
	Age	**1.535**	1.183	1.992	**0.0012**
Glycogen storage	Females vs Males	0.778	0.209	2.895	0.7080
	TG vs WT	0.336	0.084	1.340	0.1224
	Age	**0.826**	0.698	0.978	**0.0265**
Hepatocyte dysplasia	Females vs Males	0.700	0.250	1.964	0.4985
	TG vs WT	0.985	0.345	2.815	0.9773
	Age	0.971	0.863	1.092	0.6204

*Wald Chi-Square.

g/dL and calculated using a calibration curve obtained by serial dilutions of the reference standard, ranging from 4 to 0.5 g/dL. IL-6 was determined using a mouse IL-6 enzyme-linked immunosorbent assay (ELISA) quantification kit (BioSource, Nivelles, Belgium), according to manufacturer's specifications.

Quantitative PCR analysis. *SerpinB3 detection.* The levels of SB3 mRNA were measured in liver tissue by SYBR green assay. Briefly, total RNA was extracted from frozen tissue using Trizol Reagent (Invitrogen, Carlsbad, CA) and 2 μg of each tissue sample were reverse transcribed using Superscript II reverse transcriptase (Invitrogen).

The SYBR Green real-time PCR was performed with a Light Cycler Apparatus (Roche, Monza, Italy), using a FastStart DNA MasterPLUS SYBR Green Kit™ (Roche, Monza, Italy) in glass capillaries. After an initial denaturation step at 95°C for 10 min, 45 cycles of amplification were carried out and included the following conditions, denaturation at 94°C for 1 sec, annealing at 58°C for 10 sec and extension at 72°C for 10 sec. Amplification of specific transcripts was confirmed by melting curve profiles at the end of each PCR cycle, using the specific routine built-up in the Light Cycler instrument. SB3 mRNA quantification (pg/μg RNA) was established using serial 10-fold dilutions of the target SB3 gene contained in a pUC18 plasmid vector[41] as standard curve.

The human SB3 mRNA (GenBank accession no. NM006919) and the mouse-homologous to human SB3 mRNA (mouse SERPINB3b)[42,43] were determined using the sets of primers as follows: human SB3 (sense: 5′-GCAAATGCTCCAGAAG-AAAG-3′ and antisense: 5′-CGAGGCAAAATGAAAAGATG-3′), mouse SB3 (sense: 5′-TTTGGCTGAACAAGAACACA-3′ and antisense: 5′-AAGTCAGCCT-TCTGTGGATCA-3′).

Longevity genes detection. A panel of hepatic genes implicated in longevity, including Sirtuins, Foxo3a, mTOR, p53 and p66shc, was evaluated in the livers of TG and WT mice.

Quantitative PCR (Q-PCR) assay was performed in a Thermal Cycler CFX96 Real Time-PCR detection system (Bio-Rad, Hercules, CA, USA). The PCR reaction was performed in a 25 μl final reaction volume containing 200 nmol of each primer and 5× EVA Green SuperMix (Bio-Rad, Hercules, CA, USA). All the reactions were performed in 96-well plates, in triplicate.

A negative control containing all reagents but no cDNA template was included in all runs. Primers were designed from sequences derived from the GenBank database using Primer 3 (Whitehead Institute, Massachusetts, USA) and Operon's Oligo software (Operon, California, USA) and were purchased from Eurofins MWG (Ebersberg, Germany). The primers used were as follows: sirtuine1 (sense: 5′-CAG-TAGCACTAATTCCAAGTTCTA-3′ and antisense: 5′-TTGGCATATTCACCA-CCTAGC-3′), sirtuine2 (sense: 5′-CAGAGTGTGGTAAAGCCTGATA-3′ and antisense: 5′-TAGTGGTGCCTTGCTGATGA-3′), sirtuine3 (sense: 5′-CATC-CCGGACTTCAGATCC-3′ and antisense: 5′-TGAGTGACATTGGGCCTGTA-3′), sirtuine5 (sense: 5′-GGAAATCCACGGAACCTTATT-3′ and antisense: 5′-TGTC-CACCTCCTCCAGAA-3′), sirtuine6 (sense: 5′-CAACCCACAAAACATGACCG-3′ and antisense: 5′-TCAGCCTTGAGTGCTACTG-3′), sirtuine7 (sense: 5′-TCC-AGCTTGAAGGTACTAAAGAA-3′ and antisense: 5′-AATCTCCAGGCCC-AGTTCAT-3′), Foxo3a (sense: 5′-TCACCCATGCAGACTATCCA-3′ and anti-sense: 5′-GGAGTCACTCAAGCCCATGT-3′), p66shc (sense: 5′-TGACAGG-ATGGCTGGCTT-3′ and antisense: 5′-ACGGACTTCATGGTCTCC-3′), mTOR (sense: 5′-TCTCCATCAAGCTGTTAGCC-3′ and antisense: 5′-TCGCACGAGG-ACTTTATTCAC-3′), p53 (sense: 5′-TTCTCCGAAGACTGGATGACT-3′ and antisense: 5′-CAGGTGGAAGCCATAGTTGC-3′) and β-Actin (sense: 5′-AGAG-CTACGAGCTGCCTGAC-3′ and antisense: 5′-GGATGCCACAGGACTCCA-3′).

Validation of specificity of Q-PCR assay was performed by melt-curve analysis and by agarose gel analysis. β-actin was used as reference gene. For each target gene, a calibration curve was generated with threshold cycle values from serial dilutions of cDNA (from 10^6 to 10 copies/reaction) to determine reaction efficiencies, linearity, detection and quantification limits.

Data analysis was performed with the CFX Manager software (Bio-Rad, Hercules, CA, USA). The comparative cycle threshold method ($2^{-\Delta\Delta Ct}$)[44], which compares the between groups difference in cycle threshold values, was used to obtain the relative fold change of gene expression.

Western blot analysis. The protein expression of genes showing significant differences by real time-PCR, including mTOR, p53 and p66shc, was assessed by Western blot analysis using cellular extracts of liver tissue. SB3 protein was used as control and β-actin as housekeeping. Liver homogenates were obtained by lysis buffer and centrifugation, as described previously[29].

The expression of each protein was detected using the following primary antibodies: polyclonal anti-mTOR (1 : 1000, Cell Signaling Technology, Danvers, MA, USA), polyclonal anti-p53 (1 : 1000, Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-Shc/p66 (mAb, 1 μg/ml, Calbiochem, Darmstadt, Germany), oligoclonal anti-SB3 (Hepa-Ab, 8 μg/ml, Xeptagen, Venice, Italy) and monoclonal anti-β actin (1 : 1000, Sigma-Aldrich, Milan, Italy). Anti-mouse IgG (1 : 1000, Amersham Bioscience, Arlington Height, IL, USA) and anti-rabbit IgG (1 : 2000, Sigma-Aldrich, Milan, Italy) were used as horseradish peroxidase conjugated secondary antibodies.

Antigenic detection was carried out by enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) and densitometric analysis was assessed using the VersaDoc Imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The quantitative densitometric value of each protein was normalized to β-actin and displayed in histograms.

Histological parameters. Liver specimens have been evaluated for the following parameters: inflammation, steatosis, glycogen storage, hepatocyte dysplasia and presence of neoplastic transformation foci.

In each specimen, the parameter expression was scored on a four-tiered scale (from 0 to 3), with score 0 denoting absence, while the other values meaning varied depending on the parameter: for inflammation, score 1 denoted mild, score 2 moderate and score 3 high grade; for steatosis and glycogen storage, score 1 indicated positivity in 1–30% of hepatocytes, score 2 in 31–50% and score 3 in >50%. For dysplasia score 1 was equivalent to mild, score 2 to moderate and score 3 to severe degree of displasia.

SERPINB3 Immunohistochemistry. The evaluation of SB3 expression was carried out on formalin-fixed paraffin-embedded liver sections of TG mice, rehydrated and processed for immunohistochemistry. Liver sections were labelled with a rabbit anti-SB3 antibody (Xeptagen, Marghera, VE, Italy); immunostaining was performed using the avidin-biotin-peroxidase complex technique and 3-3′ diaminobenzidine as chromogen (Vector Laboratories, Burlingame, CA, USA). The sections were then lightly counterstained with Mayer's hematoxylin. Parallel negative controls, obtained by replacing primary antibody with PBS, were run in all cases.

Statistical analysis. Demographic data and the animal histological findings were subjected to descriptive analysis, including mean, standard deviation, minimum and maximum value in case of continuous variables, and frequencies and percentages in case of qualitative variables. Statistical analysis was carried out using Sas System (SAS Institute Inc, North Carolina, USA) for Windows 9.01.

The following tests were used, when appropriate: the Student's t-test, the non-parametric Mann-Whitney test. Life table estimates were calculated according to the Kaplan–Meier method, and compared by the log-rank test. All tests were two-tailed and the significance was set as $p < 0.05$.

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Author contributions

G.V. designed and performed the experiments, analyzed data and drafted the manuscript, M.R., G.C., C.T., A.C. preformed molecular biology experiments, S.Q., A.B. performed cell culture and western blot experiments, F.C. performed histological analysis, N.T. handled the mice colony, C.M. carried out statistical analysis, A.A., A.G. were involved in the discussion and manuscript editing, P.P. was involved in the experimental design of the study, in the analysis of the data and manuscript editing. All authors reviewed the manuscript.

Additional information

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