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ORIGINAL PAPERS

SLUG in cancer development

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The *SNAIL*-related zinc-finger transcription factor, *SLUG* (*SNAI2*), is critical for the normal development of neural crest-derived cells and loss-of-function *SLUG* mutations have been proven to contribute to piebaldism and Waardenburg syndrome type 2 in a dose-dependent fashion. While aberrant induction of SLUG has been documented in cancer cells, relatively little is known about the consequences of *SLUG* overexpression in malignancy. To investigate the potential role of *SLUG* overexpression in development and in cancer, we generated mice carrying a tetracycline-repressible Slug transgene. These mice were morphologically normal at birth, and developed mesenchymal tumours (leukaemia and sarcomas) in almost all cases examined. Suppression of the *Slug* transgene did not rescue the malignant phenotype. Furthermore, the *BCR–ABL* oncogene, which induces Slug expression in leukaemic cells, did not induce leukaemia in Slug-deficient mice, implicating Slug in *BCR–ABL* leukaemogenesis *in vivo*. Overall, the findings indicate that while *Slug* overexpression is not sufficient to cause overt morphogenetic defects in mice, they demonstrate a specific and critical role for *Slug* in the pathogenesis of mesenchymal tumours.
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1. Introduction

Recent years have seen the identification of numerous genes that regulate embryonic development, and the discovery that mutation of such genes is implicated in genetic disorders in humans and mice, ranging from birth defects to cancer. *SLUG (SNAI2)*, a vertebrate-specific Snail-related gene that was first identified in the neural crest and developing mesoderm in the chick embryos (Nieto, 1994), is expressed by cells undergoing epithelial-to-mesenchymal-transition (EMT) in the developing chick embryo, where it is critical in formation of the primitive streak, endocardial cushions, decondensing somites and in closure of the palate. It has conserved and divergent roles in the chick and mouse embryo (Sefton *et al*., 1998), with evidence that the functional mouse homologue of cSlug is in fact *Snail* (Nieto, 2002), which is essential for gastrulation, EMT and cell survival in the mouse (Sefton *et al*., 1998; Sanchez-Martin *et al*., 2004).

In contrast, Slug-deficient mice are viable and have a white forehead blaze, patchy depigmentation of the ventral body, tail and feet and macrocytic anaemia and infertility, inferring an essential role for Slug in melanocytes, haematopoietic stem cells and germ cells (Perez-Losada *et al*., 2002). *Slug* is expressed in migratory but not premigratory neural crest cells (Jiang *et al*., 1998), where it is necessary for melanoblast migration and /or survival but not for neural crest formation (Sanchez-Martin *et al*., 2002), as well as being expressed in the craniofacial mesenchyme, developing bone, endocardial cushions and cardiac outflow tracts, and mesenchymal components of the lungs, kidneys and gut (Oram *et al*., 2003). The postnatal expression of *SLUG* (*SNAI2*) and the effects of *SLUG* deletion are similar in mouse and human (Cohen *et al*., 1998; Inukai *et al*., 1999; Perez-Losada *et al*., 2002; Sanchez-Martin *et al*., 2002; Oram *et al*., 2003). Heterozygous deletion of the *SNAI2* gene results in human piebaldism (Sanchez-Martin *et al*., 2003), while a homozygous deletion has been detected in two individuals with Waardenburg disease type 2 (Sanchez-Martin *et al*., 2002). In humans, SLUG maps to chromosome 8q11.21, comprises three exons and codes for a 268 aa (29 kDa) protein that contains five zinc-finger domains (Cohen *et al*., 1998).

Slug contributes to the function of the stem cell factor c-kit signalling pathway (Perez-Losada *et al*., 2002) and mediates the radioresistance biological function of the SCF/kit (Inoue *et al*., 2002; Perez-Losada *et al*., 2003). *SLUG* expression is found in t(17;19) leukaemic cells (Inukai *et al*., 1999) rhabdomyosarcoma cells expressing the translocation *PAX3-FKHR* (Khan *et al*., 1999), and in breast cancer, where it is strongly correlated with loss of E-cadherin (Hajra *et al*., 2002). These previous observations of aberrant *SLUG* expression in human

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cancer cells (Inukai *et al*., 1999; Khan *et al*., 1999; Hajra *et al*., 2002) lead us to investigate the role of *SLUG* overexpression in malignancy by generating and analysing Slug-overexpressing mice. These mice did not exhibit morphological defects at birth, but did develop mesenchymal cancers similar to those associated with *SLUG* expression in humans. The leukaemic phenotype seen in Slug-overexpressing mice could not be rescued by suppression of the Slug transgene. In contrast, leukaemia did not occur in compound heterozygous mice, expressing the *BCR–ABL* oncogene but deleted for both alleles of Slug (Slug−/−, *BCR–ABL*$^+$). Taken together, these findings indicate that alteration of *SLUG* expression plays a specific and critical role in the pathogenesis of cancer *in vivo*, notably in BCR–ABL-associated leukaemias.

Results

Derivation of CombitTA-Slug mice

In order to determine the effect of upregulation of *SLUG* expression in malignancy, we generated transgenic mice using the Combi-tTA system, in which the expression of *Slug* gene could be exogenously regulated. This system, which has the transactivator and the tet-operator minimal promoter driving the expression gene unit on a single plasmid (Schultze *et al*., 1996), ensures the integration of the transactivator and reporter gene units in equal copy numbers in a direct *cis*-configuration at the same chromosomal locus and prevents genetic segregation of the control elements during breeding. Insertion of the *mSlug* gene under the control of the tetO-minimal promoter yielded the plasmid CombitTA-Slug (Figure 1a). This was analysed in a cell system, using a murine haematopoietic precursor Ba/F3 cell line (Palacios and Steinmetz, 1985). In the absence of tetracycline, the tet-repressor protein (fused to the viral VP16 transactivator domain) binds to an engineered tet-operator minimal promoter and activates Slug transcription (CombitTA-Slug). In the presence of the tetracycline, binding is abolished and the promoter silenced (Figure 1a). CombitTA-Slug expression was determined in transfected Ba/F3 cells after culturing for 2 days in the presence or absence of tetracycline (Figure 1b). CombitTA-Slug was detected in Ba/F3 cells without tetracycline, but not in cells cultured with tetracycline (20 ng/ml). The physiological relevance of the CombitTA-Slug suppression was confirmed *in vitro* by assaying survival of Ba/F3 cells expressing CombitTA-Slug 24 h after IL-3 withdrawal. The effects of Slug expression on cell growth were evaluated by analysing internucleosomal DNA cleavage, leading to the formation of DNA ladders in agarose gels, which is a hallmark of apoptosis. Normally, Slug expression protects Ba/F3 cells from apoptosis following IL-3 withdrawal (Inukai *et al*., 1999) and, as we demonstrated, the level of CombitTA-Slug expression was sufficient in Ba/F3 cells to prevent cell death. A DNA cleavage ladder was observed in the Ba/F3 cells transfected with the empty plasmid undergoing apoptosis in response to IL-3 withdrawal, but not in Slug-expressing Ba/F3 cells. The sensitivity to IL-3 removal was restored by the addition of tetracycline (Figure 1c,d).

We generated two separate transgenic lines for CombitTA-mSlug (58A, 58B) (Figure 2a). In both lines, the CombitTA-Slug expression was detected in all tissues analysed (Figure 2b). Intercrossing of CombitTA-Slug mice and Slug-deficient mice rescued the null phenotype (discernable as a characteristic anaemia and depigmentation), confirming the efficacy of the CombitTA-Slug transgene *in vivo*. The CombitTA-Slug expression was the result of transactivation as the suppression of expression to undetectable values was confirmed when mice were supplied with tetracycline in their drinking water (4 mg/ml for 4 weeks) (Figure 2c).

CombitTA-Slug mice show no morphological abnormalities

Cohorts of Combi-tTA-Slug mice were generated to analyse the effect of the Slug expression *in vivo*. A total of 77 transgenic animals (48 mice corresponded to line 58A and 29 mice to line 58B) and 53 control animals were used in this study. Both transgenic lines were analysed in detail and similar phenotypic features were seen in both lines. Combi-Slug mice were born alive without overt morphological abnormalities, and were fully fertile with no differences apparent in the progeny. Autopsy of pups, including extensive histological analysis, revealed no abnormality of the kidneys, skin, brain or gastrointestinal tract of CombitTA-Slug mice, indicating that this level of overexpression of Slug does not perturb normal embryonic development. Approximately 20% of CombitTA-Slug mice died suddenly between 6 and 8 months of age with cardiomegaly and congestive heart failure on autopsy. Histological examination of CombitTA-Slug mice hearts showed no evidence of cardiac hypertrophy or fibrosis with similar cardiac myocyte cross-sectional areas to wild-type controls (Masson's trichrome staining, data not shown).

Cancer development in CombitTA-Slug mice

We next analysed whether the remaining CombitTA-Slug mice (62) develop cancer. All these CombitTA-Slug mice became unwell from approximately 9 months of age onwards (the age range for tumour development was from 9 to 14 months), with clinical manifestations that included decreased physical activity, tachypnea, piloerection, shivering and sustained weight loss, prior to killing. The cancers, which were all mesenchymal in origin, were acute leukaemia in 90% of CombitTA-Slug mice and soft-tissue sarcoma in the remaining 10% (Figure 3a,b). No epithelial tumours were seen in any of the CombitTA-Slug mice analysed, even though with ubiquitous expression of CombitTA-Slug. Macroscopic analysis of these animals showed splenomegaly (Figure 3a) or sarcomas (Figure 3b). Detailed analysis of the sarcoma tumour cells established the diagnosis as angiosarcoma (three cases), liposarcoma (two cases),

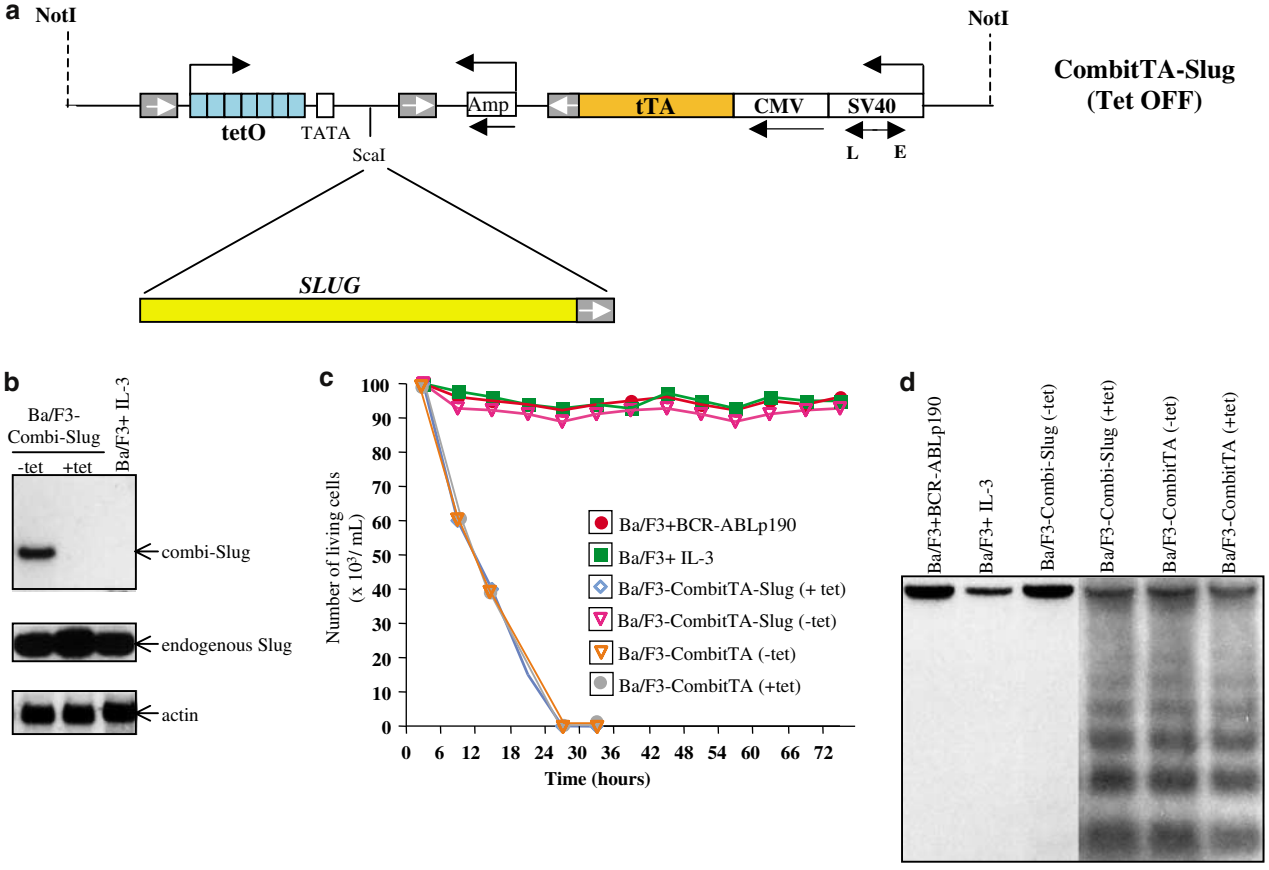

Figure 1 CombitTA-Slug: transgene construct, expression and effect of Slug on the survival of Ba/F3 cells deprived of growth factor. (**a**) Schematic representation of the CombitTA-Slug vector used in this study. (**b**) Analysis of tetracycline-dependent Slug expression by RT–PCR in Ba/F3 cells for Combi-Slug (−tet, +tet in the medium). The PCR products were transferred to a nylon membrane and analysed by hybridization with a specific probe for Slug. Actin was used to check cDNA integrity and loading. (**c**) Survival of Ba/F3 cells expressing Slug in the absence of IL-3. Cells growing exponentially in IL-3 supplemented media were adjusted to 5×10^5 cells/ml on day 0, and cultured after removal of IL-3. The cell number of viable cells is shown for BCR-ABLp190, Ba/F3 cells grown in the presence of IL-3, Slug-transfected Ba/F3 cells grown in the absence of IL-3 and Ba/F3 cells transfected with the empty plasmid (Ba/F3-CombitTA) grown in the absence of IL-3. *BCR–ABL* was used as a positive control of a gene that caused resistance to cell death. (**d**) Cell death is accompanied by nucleosome laddering after IL-3 deprivation. Low molecular weight DNA was isolated 24 h after IL-3 deprivation from Ba/F3 + BCR-ABLp190 (lane 1), Ba/F3 grown in the presence of IL-3 (lane 2), Ba/F3-Combi-Slug grown in the absence of IL-3 and doxycycline (−tet) (lane 3), Ba/F3-Combi-Slug grown in the absence of IL-3 and with doxycycline (+tet) (lane 4), Ba/F3-CombitTA grown in the absence of IL-3 and doxycycline (−tet) (lane 5), and Ba/F3-CombitTA grown in the absence of IL-3 and with doxycycline (+tet) (lane 6). The time of treatment with doxycycline was 48 h. DNA was end-labelled, resolved by electrophoresis in a 2% agarose gel and visualised by autoradiography

chondrosarcoma (one case), and fibrosarcoma (one case). We detected one type of sarcoma per animal although three of them also develop an acute leukaemia. Histological analysis revealed marked leukaemic cell infiltration of haematopoietic and nonhaematopoietic tissues. These leukaemic cells preferentially infiltrate the kidney, lung, WAT, heart and liver (Figure 3c). Peripheral blood mononuclear cells from leukaemic mice were identified by flow cytometry using a combination of specific antibodies. These studies defined the acute leukaemias as acute B-cell lymphoblastic leukaemia (60% of cases) or acute myeloid leukaemia (40% of cases) (Figure 3d). The thymi were normal.

To test the malignant potential of cells from the CombitTA-Slug mice, 1×10^6 peripheral blood blast cells from CombitTA-Slug leukaemias were injected subcutaneously into 16 40-day old nude mice. All 16 mice developed progressive tumours within 4–7 weeks of transplantation. The tumours in the nude mice were histologically identical to the original leukaemias and their clonal origin was confirmed by CombitTA-Slug PCR (data not shown). Overall, these data define that the leukaemias generated in the CombitTA-Slug mice were malignant mesenchymal cancers and indicate that Slug does not require tumour formation before dissemination can takeplace.

Irreversibility of alterations induced by Slug

The above results support the hypothesis that Slug expression facilitates cell migration (Sefton *et al*., 1998; Perez-Losada *et al*., 2002). Therefore, abolition of Slug overexpression might be expected to either halt or

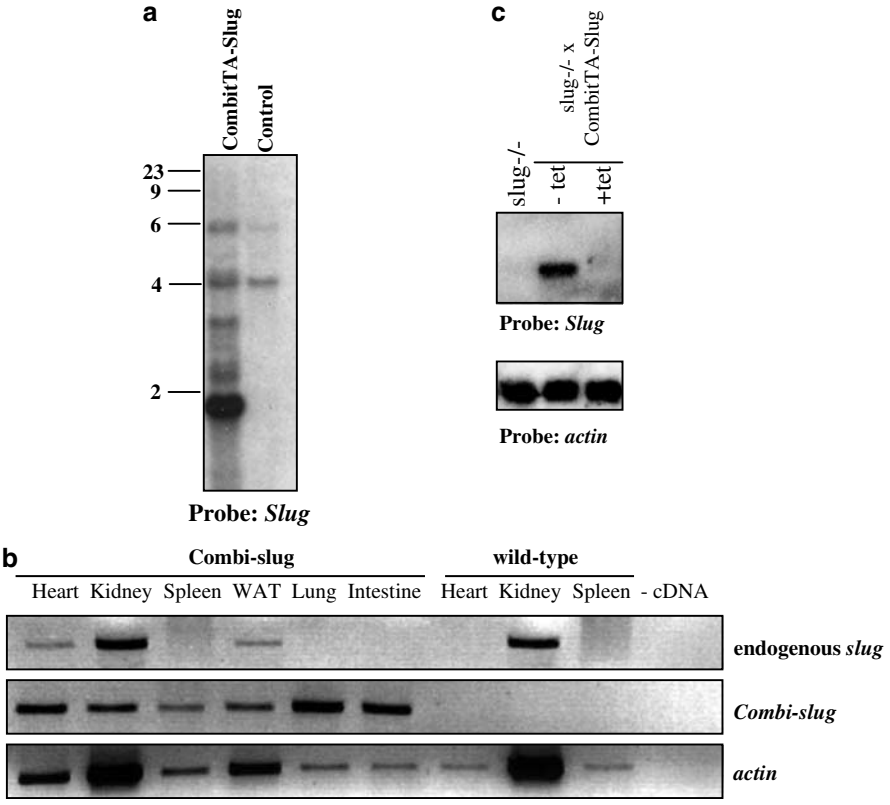

Figure 2 CombitTA-Slug mice: transgene expression. (**a**) Identification of transgenic mice by Southern analysis of tail snip DNA after *Eco*RI digestion. We used the cDNA for mouse *Slug* for detection of the transgene. (**b**) Expression of the transgene was demonstrated by RT–PCR. Expression of Combi-Slug and endogenous Slug was analysed by RT–PCR in tissues derived of Combi-Slug and control mice. The PCR products were transferred to a nylon membrane and analysed by hybridization with a specific probe. Actin was used to check cDNA integrity and loading. (**c**) Analysis of tetracycline-dependent Slug expression by RT–PCR in the bone marrow of Slug −/− mice for CombitTA-Slug (−tet, +tet in the medium). Tetracycline was given at 4 mg/ml for 4 weeks. When the Slug expression is suppressed, the phenotypic characteristics of Slug-deficient mice are evident again in 4 weeks

reduce the growth and/or spread of the Slug-expressing cells. To assess this, 10 leukaemic CombitTA-Slug mice were evaluated for disease progression by flow cytometry prior to and following administration of tetracycline (4 g/l in the drinking water for 2 weeks, a dose sufficient suppress exogenous Slug expression) (Figure 4a). None of the CombitTA-Slug mice exhibited amelioration of the leukaemic phenotype despite complete CombiTA-Slug suppression: flow cytometry analysis identified the persistence of leukemic cells in the peripheral blood (Figure 4b) with infiltration of nonhaematopoietic tissues evident on histology (Figure 4b). Autopsy of these animals identified cardiomegaly. Thus, these results show that the alterations, induced by Slug, become independent of Slug overexpression.

Suppression of Slug *blocks* in vivo *leukaemia development of BCR–ABL^{p190}*

A critical step in understanding how malignant transformation is mediated by cancer-associated oncogenes (such as *BCR–ABL*) is to identify target genes and pathways, which allow cells to grow outside their normal environment (Sanchez-Garcia, 1997). Although we have shown that *Slug* is sufficient to generate leukaemias and sarcomas and that the growth of these cells becomes independent of Slug expression, these results do not imply the specific requirement for Slug in the malignant transformation. In order to address this question, we used *BCR–ABL* oncogenesis (Shtivelman *et al*., 1985; Clark *et al*., 1987; Sanchez-Garcia and Grütz, 1995) as a model. *BCR–ABL* upregulates the expression of *Slug* in Ba/F3 cells, in blast cells of *BCR–ABL* transgenic mice (Figure 5a, b) and in blast cells from patients with Ph^1-positive human leukaemias, while *SLUG* mRNA is absent from normal human lymphocytes (Figure 5c, lane 1). *SLUG* expression was identified in t(9; 22)-positive cell lines derived from patients with CML and Ph^1-positive-ALL (K562, Nalm-1 and TOM-1), in cells from Ph^1-positive patients, and in other leukaemic cell lines lacking the t(9;22), including the 697 early B-lineage, the U937 myeloid, and the ALL-SIL and KOPTI-KI T-cell lines (Figure 5c). These and previous observations (10, 14) indicate that the aberrant expression of *SLUG* is a common finding in mesenchymal tumours.

To show whether Slug is essential for *BCR–ABL* leukaemogenesis, we generated BCR–ABL^{p190} transgenic

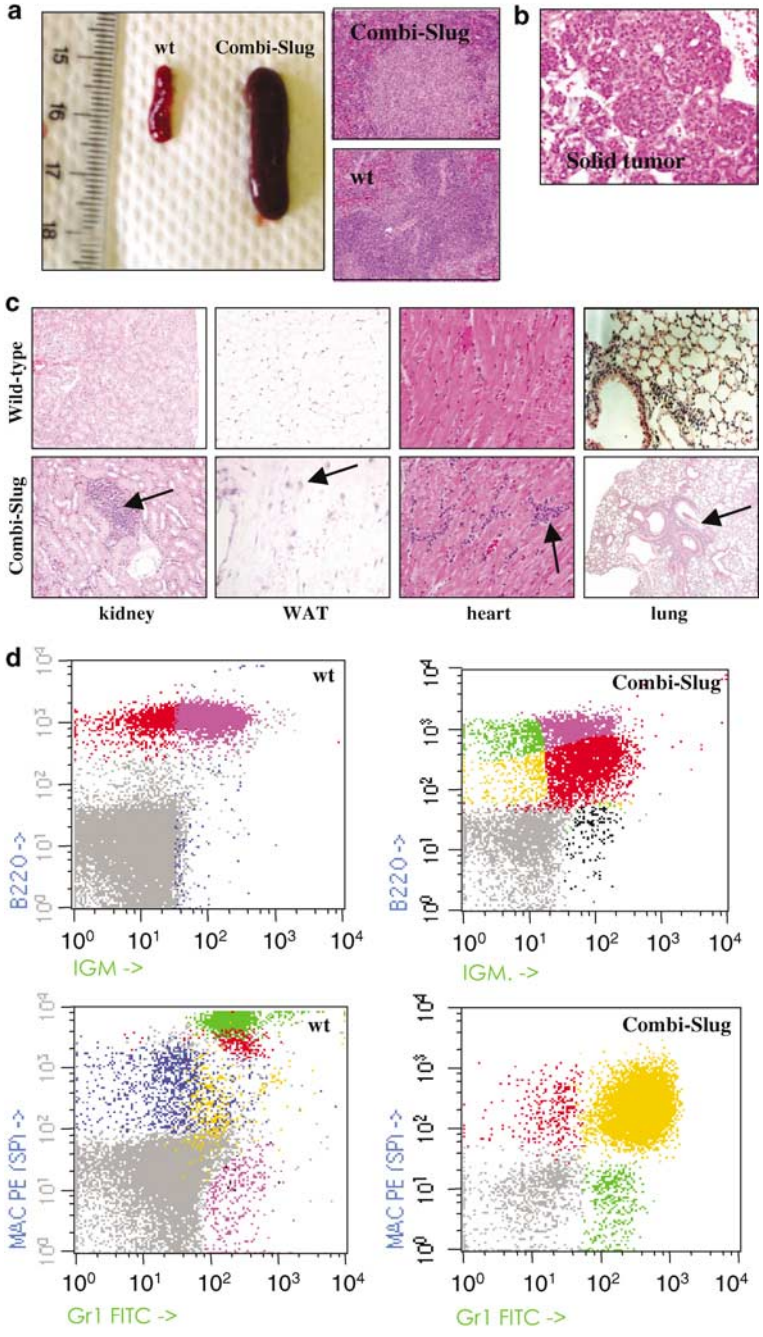

Figure 3 Mesenchymal cancer in CombiTA-Slug mice. (**a**) Macroscopic aspect and Haematoxylin/eosin-stained sections of the spleen of wild-type and CombiTA-Slug mice. The spleen from CombiTA-Slug mice shows the effacement of the normal spleen architecture. (**b**) Histologic appearance of a solid tumor (angiosarcoma) developed in CombiTA-Slug mice after Haematoxylin-eosin-staining. (**c**) Histologic appearance of tissues in CombitTA-Slug mice. Haematoxylin/eosin-stained sections of the kidney, WAT, heart and lung of wild-type and CombiTA-Slug mice. Slug-expressing cells disobey the social order of organ boundaries and migrate as individual cells giving metastasis to different regions. Metastases are indicated by arrowheads. (**d**) Phenotypic characteristics of leukaemias of CombiTA-Slug mice. Cells from peripheral blood of CombiTA-Slug mice were analysed by flow cytometry. Cells were identified with combinations of specific antibodies. Cells (10 000) were collected for each sample and dead cells were excluded from analysis by propidium iodide staining

mice (Perez-Caro *et al*, in preparation) and examined the leukaemogenic capacity of the *BCR–ABL*^{p190} oncogene in the presence and in the absence of Slug. Two founder lines were generated and backcrossed into the C57BL6 background to establish co-isogenic transgenic mice. Histological and phenotypic examination of 23 BCR–ABL^{p190} transgenic mice between 7 and 9 months of age confirmed the presence of blast cells in

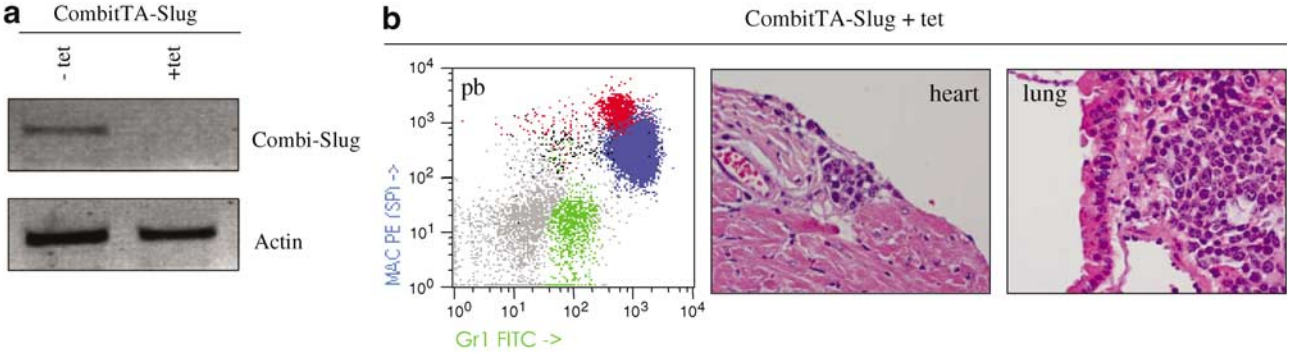

Figure 4 Phenotypes in CombiTA-Slug mice after suppression of Slug expression by tetracycline treatment. (**a**) Analysis of tetracycline-dependent Slug expression in peripheral blood of mice transgenic for CombiTA-Slug (−tet, +tet in water) by RT–PCR. β-Actin was used to check cDNA integrity and loading. (**b**) Flow cytometry phenotypic characteristics of cells from peripheral blood and Haematoxylin/eosin-stained sections of tissues in CombiTA-Slug mice after suppression of Slug expression by tetracycline treatment (4 g/l) for 4 weeks

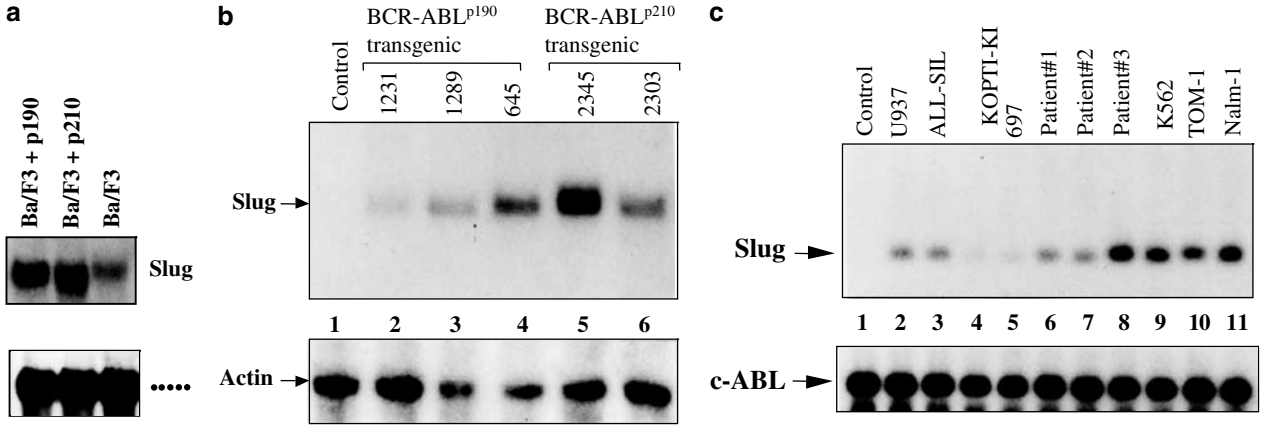

Figure 5 Expression of *SLUG* in BCR–ABL-expressing cells. Expression of both human and mouse *Slug* was analysed by RT-PCR. The PCR products were transferred to a nylon membrane and analysed by hybridization with a specific probe. Actin or ABL was used to check cDNA integrity and loading. **(a)** Analysis of *Slug* expression in the BCR–ABL-expressing Ba/F3 cells Ba/F3 + p190 (lane 1), and Ba/F3 + p210 (unpublished mice) (lane 2) and in Ba/F3 cells (lane 3). (**b**) Expression of *SLUG* in the peripheral blood of BCR–ABL^{p190} (human oncogene associated to B-cell ALL in humans) and BCR–ABL^{p210} (human oncogene associated to CML in humans) transgenic mice. Peripheral blood of control mice (lane 1), BCR–ABL^{p190} (lanes 2–4) and BCR–ABL^{p210} (lanes 5–6) transgenic mice. (**c**) *SLUG* is present in human leukaemic cell lines and in patient samples with t(9;22). Control human peripheral blood (lane 1), U937 myeloid leukaemic cell line (lane 2), ALL-SIL (lane 3) and KOPTI-KI (lane 4) T-cell leukaemic cells, the 697 pre-B leukaemic cell line (lane 5), peripheral blood from patient samples with t(9; 22) (lanes 6–8, where patients #1 and #3 had a Ph-B-ALL, and patient #2 a myeloid blast crisis), and K562 (lane 9), TOM-1 (lane 10), and Nalm-1 (lane 11) t(9; 22)-positive human leukaemic cell lines

haematopoietic and nonhaematopoietic tissues (Figure 6a). Murine blast cells co-expressed B-cell and myeloid markers (Figure 6b). Thus, these BCR–ABL^{p190} transgenic mice develop a disease similar to the human situation, where *BCR–ABL^{p190}* is associated with a B-cell acute leukaemia co-expressing myeloid markers (Tabernero *et al*., 2001). To study the biology of BCR–ABL^{p190}-B-ALL in the absence of Slug, we generated 10 BCR–ABL^{p190} transgenic mice homozygous for the targeted mutation of the *Slug* gene. No blast cells were detected on periodic analysis of peripheral blood samples from the BCR–ABL^{p190} × Slug−/− mice taken between 6 and 9 months of age. At 12 months of age, all 10 BCR–ABL^{p190} × Slug−/− mice were killed and no evidence of leukaemia was found at autopsy in any of the mice (Figure 6). These results show that *Slug* is an essential mediator of BCR–ABL^{p190} leukaemogenesis.

Discussion

Slug is a member of the Snail family of zinc-finger transcription factors that share an evolutionarily conserved role in mesoderm formation in invertebrates and vertebrates (Nieto, 1994, 2002; Sefton *et al*., 1998), In human and mouse, much of the knowledge regarding the function of SLUG has been derived from analysis of

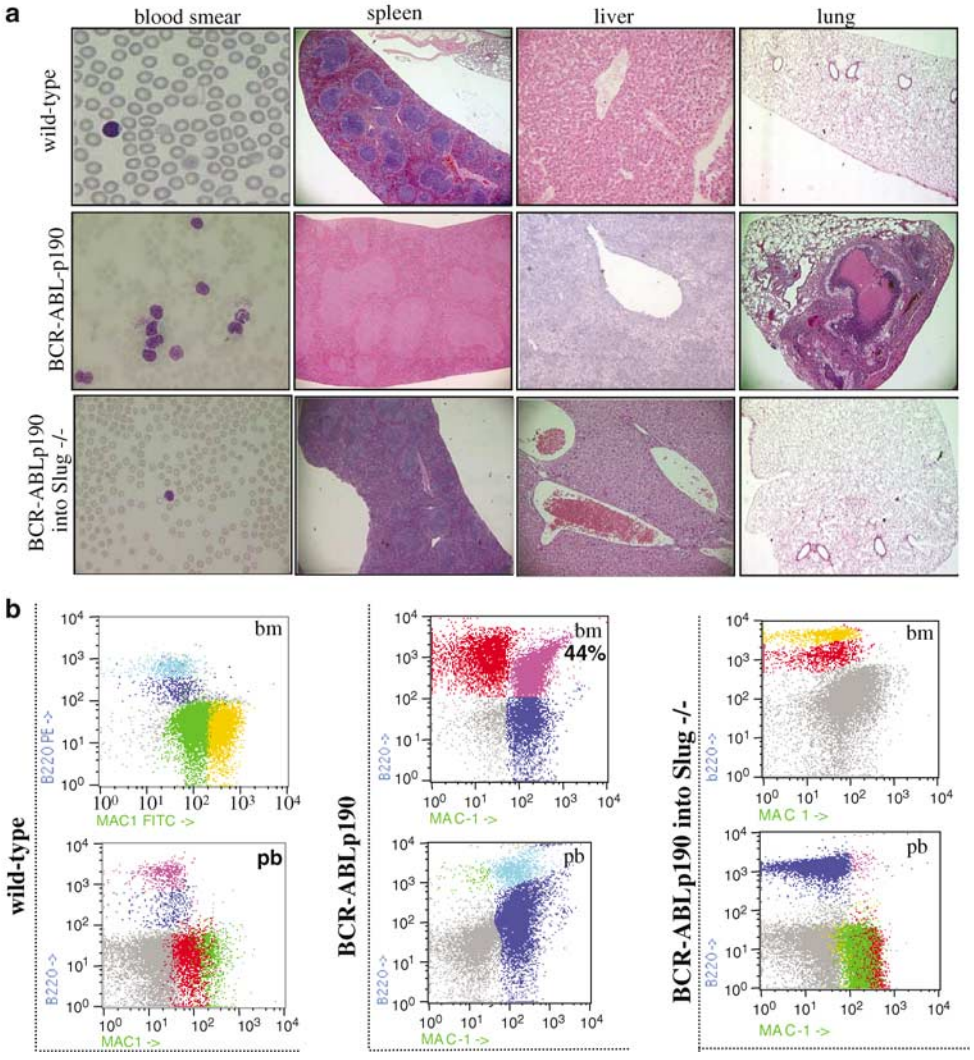

Figure 6 Requirement for Slug in tumorigenicity of BCR–ABL-expressing mice. (**a**) Histologic appearance of blood smears (Giemsa staining) and tissues (Haematoxylin/eosin stained sections) and (**b**) Flow cytometry analysis of bone marrow (bm) and peripheral blood (pb) cells with combinations of specific antibodies. Mice of the indicated genotypes were crossed and their offspring genotyped. Note that BCRABLp190 mice developed a B-ALL characterized by the presence of blast cells co-expressing Mac-1 and B220. This leukaemia is characterized by infiltration of spleen, liver and lung and the presence of lymphoblasts in blood smears. However, BCR–ABL^{p190} mice do not show leukaemia into *Slug* −/− background

loss-of-function mutations (Jiang *et al*., 1998; Perez-Losada *et al*., 2002; Sanchez-Martin *et al*., 2002, 2003), which have demonstrated that SLUG is critical for development of neural crest-derived cell lineages and from insights into the interaction of SLUG with specific oncogenes in human cancer, notably *BCR–ABL* (Inukai *et al*., 1999; Khan *et al*., 1999, and this manuscript). However, little is known as to how overexpression of *SLUG* might contribute to malignancy. We have utilized the single-plasmid system containing the regulating and expression elements of the original binary tetracycline system to allow high induction and tight control of gene expression by tetracycline in mice (Schultze *et al*., 1996) to try to understand the relevance of SLUG to human cancer development (Inukai *et al*., 1999; Khan *et al*., 1999, this manuscript). In the mouse *Slug* is not implicated in EMT (Sefton *et al*., 1998; Nieto, 2002), while the *Snail* gene has been shown to trigger EMT, an important pathway to acquisition of the invasive phenotype in epithelial solid tumours (Cano *et al*., 2000; Batlle *et al*., 2000). Our data support this observation, with neither epithelial alterations nor carcinomas in any of the CombitTA-Slug mice analysed. However, these results cannot exclude a role for Slug in carcinoma development in a context where murine epithelial cells show or accumulate previous tumour alterations.

The analysis of the Slug-expressing mice, with reference to known human phenotypes, identified that these mice develop mesenchymal cancers, mainly leukaemias. A significant percentage of mice develop cardiomyopathy, but the temporal relation to the cancer

favours the cardiomyopathy in the CombiTA–Slug mice being a manifestation of the incipient leukaemia rather than being a primary cardiomyopathy. In fact, endogenous Slug expression has not been identified in the ventricles either on embryonic wholemount LacZ staining (Sefton *et al*., 1998), immunohistochemistry (Oram *et al*., 2003) or by analysis of tissue extracts (this paper).

In the haematopoietic system, uncommitted progenitor cells differentiate to mature cells, at which time the expression of Slug is downregulated (Becker and Clarke, 2002; Inoue *et al*., 2002). When these normal uncommitted progenitor cells migrate, it is believed that Slug activation promotes their survival, permitting them to carry out their function or, in the absence of appropriate external stimuli, to undergo apoptosis (Metcalf, 1998). Thus, the mesenchymal cancers observed in the CombitTA-Slug mice represent an *in vivo* demonstration of the idea that transformation depends upon genetic changes that allow undifferentiated cells to grow outside their normal environment (Figure 3). Thus, these results provide evidence that Slug expression facilitates cell migration (Perez-Losada *et al*., 2002). However, the survival conferred by Slug, while reversible *in vitro* (Figure 1), can escape such control *in vivo*. The implications of these results in human stem cell-based therapies should be considered.

Our findings further define the biological function of Slug in cellular transformation by *BCR–ABL* and confirm that the *BCR–ABL* oncogene induces *SLUG* expression. *SLUG* expression is being increasingly recognized as an alteration in mesenchymal tumours (both leukaemias and sarcomas) (Inukai *et al*., 1999; Khan *et al*., 1999, and this paper), suggesting that SLUG may be a critical factor in tumour invasion not only of BCR–ABL-positive leukaemias but also potentially in other mesenchymal cancers. To show whether SLUG is required for BCR–ABL leukaemogenesis, we demonstrate that the leukaemogenic capacity of *BCR–ABLp190* oncogene is dependent on the presence of Slug, indicating that Slug has a fundamental role in the biology of BCR–ABL cancer cells. These results are consistent with a model in which tumour cells harbouring the BCR–ABL fusion protein constitutively express Slug, promoting both aberrant survival of the tumour cell and migration of the defective target cells into different environments (Figure 7). The finding that SLUG is commonly activated in mesenchymal tumour cells indicates that it may have a broader role in cancer biology than that specifically associated with BCR–ABL transformation. SLUG expression might therefore define a common pathway of cell invasion for other leukaemias and sarcomas. In conclusion, in the present work we have presented evidence that suggests that Slug might represent a potentially important mechanism of tumour invasion for mesenchymal tumours. As such, SLUG can be considered as both a marker of malignancy and an attractive target for therapeutic modulation of invasiveness in the treatment of human cancer.

Materials and methods

Generation of transgenic mice

The cDNA for mouse *Slug* was cloned into the Combi-tTA vector (16). Linear DNA fragments for microinjection were obtained by *Not*I digestion and injected into CBA × C57BL/6J fertilized eggs. We identified transgenic mice by Southern analysis of tail snip DNA after *Eco*RI digestion as described (Garcia-Hernandez *et al*., 1997). We used the cDNA for mouse *Slug* for detection of the transgene. Founder mice were crossed to the C57BL6 mice for five generations to establish co-isogenic transgenic mice. Similar phenotypic features were seen in all assays for both of the CombitTA-Slug transgenic lines generated.

Mice heterozygous and homozygotes for the $Slugh^{\Delta 1}$ mutation generated by removing the genomic sequences of the entire Slugh protein-coding region ($Slugh^{\Delta 1}$ mutant mice) have been previously described (Jiang *et al*., 1998). Heterozygous Slug +/− mice were bred to CombitTA-Slug and BCR–ABLp190 transgenic mice to generate compound heterozygotes. F1 animals were crossed to obtain null Slug −/− mice heterozygous for CombitTA-Slug and BCR–ABLp190 transgenic mice.

Histological analysis

Mice included in this study were subjected to standard necropsy. All major organs were closely examined under the dissecting microscope, and samples of each organ were processed into paraffin, sectioned and examined histologically. All tissue samples were taken from homogenous and viable portions of the resected sample by the pathologist and fixed within 2–5 min of excision. Haematoxylin- and eosin-stained sections of each tissue were reviewed by a single pathologist (TF). For comparative studies, age-matched mice were used (wild-type or Combi-Slug mice in the continous presence of tetracycline).

Cell culture

Cell lines used include Ba/F3 (Palacios and Steimetz, 1998) and Ba/F3 cells expressing the human proteins $BCR–ABL^{p190}$ (Ba/F3 + p190) and $BCR–ABL^{p210}$ (Ba/F3 + p210) (Sanchez-Garcia and Grütz, 1995). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). When required, 10% WEHI-3B-conditioned medium was added as a source of IL-3.

Cell transfection and cell survival assay

Ba/F3 cells were transfected by electroporation (960 μF, 220 V) with 20 μg of either CombitTA-Slug or empty vector (CombitTA). The neomycin-resistant pool of cells (Ba/F3 + CombitTA-Slug) was analysed by RT–PCR for Combit-TA-Slug expression in the presence and in the absence of tetracycline (20 ng/ml). These cells were resistant to IL-3 withdrawal during growth in the absence of tetracycline. Cells were screened for resistance to IL-3 withdrawal and cell viability was determined by trypan blue exclusion.

Low molecular weight DNA analysis

Low molecular weight DNA was isolated as follows. Cells were collected into 1.5 ml of culture medium and microcentrifuged for 1 min at 1500 r.p.m. (400 *g*), and the pellet was suspended in 300 μl of proteinase K buffer. After overnight

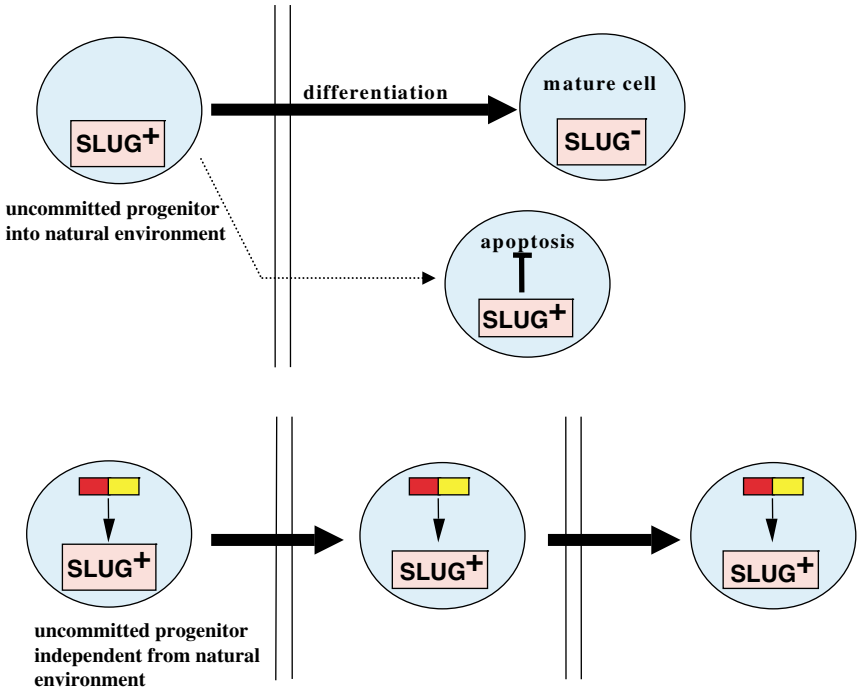

Figure 7 Model of the role of *Slug* in cancer development. (**a**) In the haematopoietic system, normal uncommitted progenitor cells differentiate to mature cells. During this transition, the expression of Slug is downregulated (Becker and Clarke, 2002; Inoue *et al*., 2002). These normal uncommitted progenitor cells are responsive to environmental cues, which regulate the number of mature cells produced and limit the self-renewal of immature cells. When in physiological situations, these normal uncommitted progenitor cells migrate, Slug would promote survival to allow them to carry out their function. If this is not achieved in a specific period of time, they would undergo apoptosis as they have been deprived of required external signals. (**b**) In cancer development, the differentiation capacity of the target cell is blocked, but inhibition of differentiation is not sufficient for transformation because survival and proliferation of target cells would be restricted to a particular microenvironment. Thus, other genetic changes that allow cells to grow outside their normal environment in addition to mutations that block differentiation must exist. Fusion oncogenes created as a result of chromosomal abnormalities associated with mesenchymal tumours (both leukaemias and solid tumours) block differentiation and have the capacity to activate target genes such as Slug, which promotes survival (with independence of the required external signals) and migration of the defective target cells into different environments

incubation at 55°C, DNA was ethanol-precipitated, suspended in 200 μl of TE buffer containing 50 μg/ml of RNase A, and incubated at 37°C for 2 h. DNA was extracted with phenol and chloroform and precipitated with ethanol. Aliquots of DNA (2 μg) were end-labelled with α32-dCTP and electrophoresed on 2% agarose gels. After electrophoresis, the gel was blotted onto Hybond-N (Amersham) and autoradiographied for 2 h at −70°C.

Reverse transcription-PCR (RT–PCR)

To analyse expression of CombitTA-*Slug* and endogenous Slug in mouse cell lines and mice, RT was performed according to the manufacturer's protocol in a 20-μl reaction containing 50 ng of random hexamers, 3 μg of total RNA, and 200 U of Superscript II RNase H$^-$ reverse transcriptase (GIBCO/BRL). The sequences of the specific primers were as follows: Combi-polyA-B1: 5′-TTGAGTGCATTCTAGTTGTG-3′; mSlugF: 5′-GTTTCAGTGCAATTTATGCAA-3′; mSlugB: 5′-TTAT ACATACTATTTGGTTG-3′. Amplification of *β-actin* RNA served as a control to assess the quality of each RNA sample. The PCR conditions used to amplify CombitTA-*Slug* and endogenous Slug were as follows: 30 cycles at 94°C for 1 min 56°C for 1 min, and 72°C for 2 min. The PCR products were confirmed by hybridization with specific internal probes. To analyse expression of *SLUG* in human cell lines and in peripheral blood samples of Ph1-positive patients, the thermocycling parameters for the PCR reactions and the sequences of the specific primers were as follows: SLUG, 30 cycles at 94°C for 1 min, 56°C for 1 min and 72°C for 2 min, sense primer 5'-GCCTCCAAAAAGCCAAACTA-3′ and antisense primer 5'-CACAGTGATGGGGCTGTATG-3′; c-ABL, 30 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, sense primer 5'-GTATCATCTGACTTTGAGCC-3′ and antisense primer 5'-GTACCAGGAGTGTTTCTCCA-3′. Amplification of *c-ABL* mRNA served as a control to assess the quality of each RNA sample.

Phenotype analysis

The following anti-mouse monoclonal antibodies from Pharmingen were used for cytometry staining: CD45R/B220, IgM, Mac1, Gr-1. Single-cell suspensions from the different tissue samples obtained by routine techniques were incubated with purified anti-mouse CD32/CD16 (Pharmingen) to block binding via Fc receptors and with an appropriate dilution of the different antibodies at room temperature or 4°C, respectively. The samples were washed twice with PBS and resuspended in PBS. Dead cells in samples were excluded by propidium iodide staining. The samples and the data were analysed in a FACScan using CellQuest software (Becton Dickinson).

Tumorigenicity assay

To test the tumorigenicity of the various cancers, 4- to 6-week-old athymic (nude) male mice were injected subcutaneously on both flanks with 10^6 cells resuspended in 200 μl of phosphate-buffered saline (PBS). The animals were examined for tumour formation for up to 3 weeks.

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