Stem cell antigen 2: a new gene involved in the self-renewal of erythroid progenitors

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Abstract. *Objectives:* Stem cell antigen 2 (SCA2), also known as TSA1 and LY6E, is a glycosylphosphatidylinositol-anchored molecule that belongs to the Ly-6 family and whose function remains largely unknown. We have previously shown that *SCA2* is overexpressed in self-renewing avian erythroid progenitors (T2ECs) as opposed to differentiating T2ECs. The aim of this study was to define the role of SCA2 in the switch between self-renewal and differentiation of erythroid progenitors. *Materials and methods:* We have investigated the cellular processes controlled by SCA2 in T2ECs by RNA interference and overexpression approaches. Moreover, we have used a SAGE Querying and analysis tools developed in our laboratory, to investigate the expression level of *SCA2* gene in different human cell types. *Results:* We demonstrate the regulation of *SCA2* expression by TGF- β , a growth factor essential for self-renewal of T2ECs. We establish that *SCA2* knockdown by RNA interference reduced the proliferation and promoted the differentiation of T2ECs. In contrast, *SCA2* overexpression inhibited differentiation of T2ECs only. Furthermore, by using a bioinformatic approach, we found that *SCA2* is highly expressed in a variety of human cancer cells. We confirmed this result by quantitative PCR on human colon and kidney tissues. *Conclusions:* Altogether, these findings imply that SCA2 may function in a dose-dependent manner to support the self-renewal state and that its deregulation might contribute to the development of some human cancers.

INTRODUCTION

Stem cell antigen 2 (SCA2), also known as lymphocyte antigen 6 complex, locus E (LY6E) and thymic shared antigen 1 (TSA1), belongs to the Ly-6 family of cell surface proteins. Ly-6 molecules are low molecular weight glycoproteins with remarkable conservation of cysteine residues (Capone *et al.* 1996). The members of the family are anchored in the cell membrane by a C-terminal glycosylphosphatidylinositol (GPI) moiety. Although the biological functions of

these proteins are not well understood, there is evidence that they participate in signal transduction and cell activation (MacNeil *et al.* 1993; Fleming & Malek 1994).

Sequence homology of *SCA2* to members of the Ly-6 family is relatively low (20–30%) (Fleming & Malek 1994) and its exact function remains largely obscure; it has been shown to be expressed on various haematopoietic cells (Antica *et al.* 1997). It has been suggested that *SCA2* plays a key role in T-cell differentiation by participating in T-cell receptor/CD3-mediated apoptosis of immature thymocytes (Noda *et al.* 1996). Functional studies have also indicated that *SCA2* has an important regulatory function in the T-cell receptor signalling pathway of activated T cells (Saitoh *et al.* 1995).

In a previous study, we have shown that *SCA2* was up-regulated in self-renewing primary avian erythroid progenitors, called T2ECs [transforming growth factor (TGF)- α , TGF- β induced erythroid cells (Gandrillon *et al.* 1999)] in comparison with differentiated T2ECs (Damiola *et al.* 2004). T2ECs undergo long-term (>30 days) proliferation in the absence of overt differentiation, driven by a combination of TGF- α , TGF- β and dexamethasone. Once T2ECs cultures are established, the cells produce a sufficient amount of TGF- β to maintain a basal level of proliferation, through an autocrine loop, but exogenous TGF- β is required for long-term exponential proliferation (Gandrillon *et al.* 1999). At any time, T2ECs can be induced to terminally differentiate into fully mature erythrocytes by exposure to differentiation conditions such as to anaemic chicken serum (ACS) and insulin.

In the present work, we demonstrate that TGF- β increases expression of *SCA2* and that this regulation does not require *de novo* protein synthesis. To explore the functional role of *SCA2* in T2ECs, we used RNA interference and overexpression approaches. RNAi experiments revealed that down-regulating *SCA2* expression substantially reduced cell proliferation and increased differentiation of T2ECs. On the contrary, *SCA2* overexpression decreased differentiation of T2ECs, with no effect on proliferation.

Furthermore, using a new bioinformatic approach developed by our team, we observed that high levels of *SCA2* occur in a variety of cancer cell types. We confirmed this result by quantitative PCR, in two human tissues suggesting that *SCA2* may participate in tumorigenesis by perturbation of cell proliferation and differentiation processes.

MATERIALS AND METHODS

Reagents

Transforming growth factor- β 1 and TGF- α peptides were purchased from PeproTech (London, UK) as described previously (Gandrillon *et al.* 1999). The inhibitors of TGF- β receptor SB431542 and cycloheximide were purchased from Sigma (St. Louis, MO, USA).

Cell culture

T2ECs were generated from SPAFAS white leghorn chickens (PA12 line from INRA, Tours, France) as described previously (Gandrillon *et al.* 1999). These cells were expanded in LMI medium [α -MEM medium, 10% foetal bovine serum, 10^{-3} M HEPES, 10^{-4} M β -mercaptoethanol, 10^{-6} M dexamethasone, 5 ng/mL TGF- α , 1 ng/mL TGF- β 1, penicillin and streptomycin (100 U/mL)].

Plasmid constructs

An oligonucleotide cDNA insert encoding a short hairpin RNA (shRNA) specific for down-regulating *SCA2* mRNA was designed using the siDesign software (<http://www.dharmacon.com/>)

sidesign/default.aspx?source=0). The SCA2 shRNA coding sequence was constructed by annealing the following two primers: sense 5'-GCGGGCCCGCACCATGATCGCGTTTCTTTCAAGAGAA-GAAACGCCTTCATGGTGCTTTTTTGAATTCG-3' and antisense 5'-CGAATTCAAAAAAG-CACCATGAAGGCGTTTCTTCTCTTGAAAGAAACGCGATCATGGTGCGGGCCCCGC-3'. The two nucleotides mutated shRNA-SCA2 was constructed by annealing the following two primers: sense 5'-GCGGGCCCGCACCATGATCGCGTTTCTTTCAAGAGAAGAAACGCGATCATGGTGCTTTTTTGAATTCG-3' and antisense 5'-CGAATTCAAAAAAGCACCATGATCGCGTTTCTTCTCTTGAAAGAAACGCGATCATGGTGCGGGCCCCGC-3'. Then, these annealed oligonucleotides were cloned into *EcoRI* and *HindIII* sites in pSilencer 1.0-U6 (Ambion, Austin, TX, USA) to generate pSilencer 1.0-U6 shRNA constructs. The *SCA2* coding region was amplified from total RNA purified from T2ECs by RT-PCR using the upstream oligonucleotide 5'-GGAATTC-CACCATGAAGGCGTTTCTGTTCGCCGTGT-3' and downstream oligonucleotide 5'-GCTCTA-GAGCTTATCACTCACGAGCCCTGAGGACGT-3', and subcloned into *EcoRI* and *XbaI* sites of the pCRNCM (de la Pompa & Zeller 1993) plasmid to generate the pCRNCM-SCA2 vector.

A vector expressing a FLAG-tagged SCA2 was generated as follows. We inserted a FLAG-tag consisting of the eight-amino acid sequence Meth-Asp-Tyr-Lys-Asp-Asp-Asp-Lys at the N-terminal behind the signal peptide, between amino acids 14 and 15. A 120-bp fragment (with the FLAG-encoding sequence inserted) encoding the N-terminal part of SCA2 was synthesized (Invitrogen, Cergy Pontoise, France), introducing in *EcoRI* and *NsiI* restriction sites. This fragment was digested with *EcoRI* and *NsiI* and ligated *via* these restriction sites to the *SCA2* from pCRNCM-SCA2 plasmid resulting in pCRNCM-SCA2 FLAG.

Cell transfection

Transient transfections were performed using the nucleofection method (Amaxa, Cologne, Germany). For each nucleofection assay, cells were re-suspended in nucleofector buffer (Cell line Nucleofector kit V, reference VCA-1003; Amaxa) and were nucleofected with the T-16 programme. T2ECs were transfected with different expression vectors and selected based on the MACSelect™ – Transfected Cell Selection System (Miltenyi Biotec, Auburn, CA, USA). For that, the pMACS 4.1 vector encoding truncated CD4 surface marker is used for co-transfection in combination with these expression vectors. Sixteen hours after transfection, by co-expressing the CD4 marker on the cell surface, the transfected cells were magnetically isolated and separated from untransfected cells.

RT-PCR

RNA extraction and reverse transcription assays were performed as described previously (Gandrillon & Samarut 1998). Human normal and cancer tissue RNA were obtained from Ambion. Primer design was carried out using the Primer3 software system (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Real-time PCR was performed with a LightCycler system (Roche, Indianapolis, IN, USA) or MX3000P Stratagene (La Jolla, CA, USA) using the LightCycler FastStart DNA Master SYBR Green I (Roche) according to the manufacturer's instructions. We analysed expression of different invariant genes, namely, *T-Complex1* (Sigene accession number: BBSRC60295473F1.1.3.1), *hnRNP* (Sigene accession number: AB038230.1.3.153), *GAPDH* (GenBank accession number: K01458), *NADH deshydrogenase subunit 6* (Sigene accession number: BBSRC603019463F1.1.3.1), *eIF5* (GenBank accession number: NM_001006488.1) and *18 s ribosomal RNA* (GenBank accession number: NR_003286). The annealing temperature for all the primers used is 65 °C. In order to quantify gene expression, standard curves were generated for each pair of primers, by dilution series of a sample. The quantification for avian and human

SCA2 (GenBank accession number: NM_204775.1 and Z68179, respectively) was performed using a previously published mathematical method (Pfaffl 2001):

$$\text{Ratio} = (E_{\text{target}})^{\Delta\text{CP target (control - sample)}} / (E_{\text{reference}})^{\Delta\text{CP reference (control - sample)}}$$

The reference gene is a house-keeping gene transcript. For the target gene and the reference gene, E is the real-time PCR efficiency and ΔCP is the crossing point deviation of control versus sample. Real-time PCR efficiencies were calculated according to $E = 10^{(-1/\text{slope})}$. For information concerning the specific PCR conditions and primers sequences used, please contact the authors.

For semiquantitative RT-PCR, to determine *SCA2* and *hnRNP* (internal control) mRNA levels, the following PCR programme was performed: at 94 °C for 30 s, at 60 °C for 30 s, at 72 °C for 30 s, 30 cycles.

Cell population growth measurement

Cell proliferation was determined using the WST-1 assay (Roche) following the manufacturer's instructions at different times. Cells (10^5 cells/mL) were seeded in 96-well plates.

Differentiation assay

T2ECs were suspended at the concentration of 10^5 cells/mL in differentiation medium as described previously (Gandrillon *et al.* 1999). Preparation of ACS and level of differentiation determined by acidic benzidine staining, have been previously described (Gandrillon & Samarut 1998).

Cytospin

T2ECs were cytocentrifugated (55 g, 5 min) on glass slides and cells were fixed in benzidine solution (0.5% benzidine in ethanol 70%, with 2% H_2O_2). Cells were then stained with a solution containing 1% of Wright and 10% of Giemsa stains (Sigma).

Western blot analysis

Cells were lysed in FLB lysis buffer (25 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, pH 7.5) supplemented with 1% phosphatase inhibitor cocktail 2 (Sigma) and complete mini EDTA-free protease inhibitor cocktail (Roche). Protein concentration was determined by using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Protein samples, dissolved in Laemmli buffer, were analysed by SDS-PAGE (16% acrylamide) and were blotted on Protean BA85 nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). After blockage in 0.1% Tween-20 Tris-buffered saline (TBS) 5% non-fat milk for 1 h at room temperature, membranes were probed with primary antibodies (dilution 1/2000 in TBS–0.1% Tween–5% non-fat milk for anti-FLAG M2 antibody (Sigma) with incubation for 3 h at room temperature; dilution 1/30 000 in TBS–0.1% Tween–5% bovine serum albumin for GAPDH antibody (14C10, Cell Signalling, Danvers, MA, USA) with incubation of 4 h at room temperature). Membranes were washed twice in TBS–0.1% Tween before incubation for 1 h at room temperature with horseradish-peroxidase-coupled secondary antimouse antibody (dilution 1/5000 in TBS–0.1% Tween–5% non-fat milk) for FLAG analysis or with horseradish-peroxidase-coupled secondary antirabbit antibody (dilution 1/7000 in TBS–0.1% Tween–5% bovine serum albumin) for GAPDH analysis. Proteins were detected using the ECL plus Western blotting detection system (Amersham, Piscataway, NJ, USA).

Querying human gene expression databases

The SQUAT application has been developed in our group (Leyritz *et al.* submitted) and is available at <http://bsmc.insa-lyon.fr/squat/>. This database gives access to both raw SAGE data

and condensed information mined from those data, for three species (human, mouse and chicken). SQUAT is not only a SAGE data repository database but also is a user-friendly data mining platform, which is destined to bring the state-of-the-art mining tools to biologists. Raw data can be searched, such as expression level for a given gene, provided its corresponding tag has been identified in the course of a SAGE experiment. Raw data can be visualized using the 'Query on raw SAGE data/Expression submatrix creation' menu. This query allows the user to obtain an expression matrix containing a selection of tags and of libraries. Libraries are set in columns and tags in lines. The matrix contains, for each tag/library couple, the corresponding expression level. A zero means that the tag is not present in the library. Expression properties (like overexpression) in the matrix can be encoded in a Boolean matrix. Overexpression values (above the threshold) are highlighted in red in the matrix. When creating this submatrix, the programme automatically delineates normal, cancerous and cancer-associated situations, in human and murine cells. Furthermore, much more sophisticated queries can be performed, such as identification of sets of genes that are simultaneously overexpressed in a given set of biological situations. The L2L Microarray Analysis Tool (<http://departments.washington.edu/l2l/about.html>) can then be used for comparing our own list of genes, overexpressed in given SAGE libraries, with published lists of microarray results. One should note that SQUAT is a universal tool that can be used with any human, murine or chicken protein, provided the tag from the corresponding transcript has been identified in at least one SAGE experiment.

Statistical analysis

Student's *t*-test was used to determine statistical significance.

RESULTS

SCA2 gene is under the control of TGF- β in T2ECs

We investigated the expression level of *SCA2* gene, by kinetics experiments, in the absence of TGF- β . We observed that *SCA2* expression level is significantly reduced, 1.3-fold, in T2ECs cultured for 1 h in the absence of TGF- β in comparison with T2ECs maintained in LMI medium (Fig. 1a). From 24 h of culture in the absence of TGF- β , *SCA2* expression was strongly reduced (Fig. 1a,b). Inhibition of the effect of TGF- β , produced by the autocrine loop, with a TGF- β receptor inhibitor, reduced the level of *SCA2* gene expression to a greater extent (4.3-fold) than TGF- β deprivation (Fig. 1b). On the contrary, expression of *SCA2* was significantly induced when TGF- β was added, for 8 h, to cultures that had been grown in the absence of TGF- β for 48 h (Fig. 2a). When TGF- β stimulation was extended to 24 h, the level of *SCA2* continued to increase as far as 2.4-fold above the level detected in cells deprived of TGF- β . Increased expression of *SCA2* was also observed in response to TGF- β stimulation in the presence of cycloheximide, although to a lower extent (Fig. 2b). These results suggest that the *SCA2* expression level is strongly regulated by TGF- β and that this regulation does not require *de novo* protein synthesis.

Down-regulation of *SCA2* decreases the proliferation and promotes the differentiation of T2ECs

To evaluate the role of *SCA2* in avian erythroid progenitors, *SCA2* gene expression was knocked down in T2ECs by transiently transfecting cells with a plasmid expressing a shRNA directed specifically against *SCA2* mRNA.

Figure 1. Expression of *SCA2* in T2ECs following a TGF- β deprivation or a TGF- β receptor inhibition. *SCA2* expression was measured in T2ECs grown in LMI or in LMI without exogenous TGF- β (a, b) or in LMI without TGF- β supplemented with SB431542 (2 μ M) to inhibit the TGF- β receptor signalling (T β R) (b). RNA was extracted from T2ECs after 1–96 h (a), or after 48 h (b) of culture. The folds of variation are represented as LMI/TGF- β deprivation $\pm$ T β R inhibitor ratios. The bars correspond to the mean with standard deviations of three (a) to five (b) independent experiments. The ratio represents a mean calculated using two reference genes *T-Complex 1* and *hnRNP* (a) or three reference genes *T-Complex 1*, *NADH* and *GAPDH* (b). Ratios having *P* values less than 0.05 are considered statistically significant (*).

Expression of the shRNA-*SCA2* vector in T2ECs significantly reduced *SCA2* mRNA levels, from 1.3-fold to a peak of 1.8-fold 2 days after selection of these cells, compared to the empty vector control (Fig. 3a,b). Then, we observed the effect of this down-regulation on proliferation and differentiation of T2ECs. Proliferation was significantly slower, 1.3-fold after 2 days, in T2ECs expressing shRNA-*SCA2* compared to the empty vector control (Fig. 3c). Differentiation was analysed by staining haemoglobin with acidic benzidine. The percentage of haemoglobin-expressing cells was significantly increased, 1.3-fold in cells expressing shRNA-*SCA2* compared to the control cells transfected with the empty vector, grown 2 days in differentiation medium (Fig. 3d). The effect of shRNA-*SCA2* was not observed after 3 days of culture (Fig. 3c,d) in accordance with the recovery of *SCA2* expression at this time (Fig. 3a). A similar effect was observed with a second-independent shRNA-*SCA2* (data not shown). These data were confirmed with morphological analysis of T2ECs (Fig. 3e). We observed an increase of the percentage of more mature erythrocytic progenitors, in particular early reticulocytes, in T2ECs, grown 2 days in differentiation medium, expressing shRNA-*SCA2*, compared to the empty vector.

In order to check the specificity of this effect, we constructed a two-nucleotide mutation in the shRNA-*SCA2* (shRNA-*SCA2* mutated). The mutant did not affect the level of *SCA2* mRNA (Fig. 3a), the proliferation rate (Fig. 3c), or weakly affected the differentiation of T2ECs (Fig. 3e).

Figure 2. Expression of *SCA2* in T2ECs following a TGF- β deprivation-stimulation. (a) Effect of a TGF- β stimulation on *SCA2* expression. *SCA2* expression was measured after 48 h of TGF- β deprivation in comparison with 48 h of TGF- β deprivation followed by addition of 1 ng/mL TGF- β during 1, 8 or 24 h. The folds of variation are represented as TGF- β stimulation/TGF- β deprivation ratios. (b) Effect of the cycloheximide (CHX) on the expression level of *SCA2*. *SCA2* expression was measured after 48 h of TGF- β deprivation in comparison with 48 h of TGF- β deprivation followed by addition of 10 μ g/mL CHX supplemented or not with 1 ng/mL TGF- β during 24 h. The folds of variation are represented as CHX $\pm$ TGF- β stimulation/TGF- β deprivation ratios. (a and b) The bars correspond to the mean with standard deviations of three independent experiments. In each case, the ratio represents a mean calculated using two reference genes *T-Complex 1* and *hnRNP*. Ratios having *P* values = 0.024 (**) and 0.061 (*) are considered statistically significant.

To conclude, these results demonstrate that down-regulation of *SCA2* decreases proliferation and promotes differentiation of T2ECs.

Overexpression of *SCA2* inhibits differentiation of T2ECs

We next explored the effect of *SCA2* overexpression on proliferation and differentiation of T2ECs. To this end, the full-length *SCA2* cDNA was cloned into the defective retroviral expression vector pCRNCM, which was then transfected into T2ECs. As no antibody against the chicken *SCA2* is available, we constructed a second vector in which a FLAG-tag was introduced into the avian *SCA2* coding sequence at the N-terminal, immediately after the signal peptide sequence which extends to residue 15. Based on different reports concerning other Ly-6 proteins, we supposed that *SCA2*-FLAG is normally processed in a way similar to wild-type *SCA2* in transfected cells (Rai *et al.* 2001; Gauczynski *et al.* 2002; Koch-Nolte *et al.* 2005). Expression of *SCA2*-FLAG was confirmed by Western blot analysis using an anti-FLAG antibody (Fig. 4a). Then, we analysed the effect of the up-regulation of *SCA2* expression on proliferation of T2ECs by using the WST-1 assay. We observed that neither *SCA2* nor *SCA2*-FLAG overexpression in T2ECs modified their proliferation rate (Fig. 4b). On the contrary, overexpression of *SCA2* and

Figure 3. Effect of a knockdown of *SCA2* expression by RNAi on the proliferation and differentiation of T2ECs. T2ECs were transfected with the RNAi expression vectors expressing either a shRNA complementary to the *SCA2* mRNA (shRNA-SCA2) or a two-nucleotide mutated shRNA-SCA2 (shRNA-SCA2 mutated), or with the empty expression plasmid (mock). (a) Expression of *SCA2* in T2ECs after a specific inhibition of its expression by RNAi. RNA was extracted from T2ECs grown in LMI, 1, 2 and 3 days after the selection of transfected cells. The ratios of *SCA2* mRNA accumulation in T2ECs expressing the RNAi vectors in comparison with T2ECs expressing the mock vector are represented. The bars correspond

to the mean with standard deviations of two (shRNA-SCA2 mutated) to three (shRNA-SCA2) independent experiments. In each case, the ratio represents a mean calculated using two reference genes *elf5* and *hnRNP*. (b) Expression of *SCA2* in T2ECs after a specific inhibition of its expression by RNAi. RNA was extracted from T2ECs grown in LMI, 2 days after the selection of transfected cells. *SCA2* mRNA expression is assayed by semiquantitative RT-PCR analysis. Amplification of *hnRNP* in samples served as an internal standard. (c) Effect of the repression of *SCA2* expression on proliferation rate of T2ECs. The number of viable cells was determined 1, 2 or 3 days after the selection of transfected cells, grown in LMI, by WST-1 assay. The results show the proliferation of T2ECs with the RNAi conditions in comparison with the mock condition and correspond to the mean values with standard deviations of two (shRNA-SCA2 mutated) to four (shRNA-SCA2) independent experiments. Statistical significance was inferred for *P* values less than 0.05 (*). (d) Effect of the repression of *SCA2* expression on the differentiation of T2ECs. T2ECs were maintained in differentiation medium and stained with acidic benzidine after 2 or 3 days of culture. The percentages of haemoglobin-expressing cells in RNAi conditions in comparison with the mock condition were reported and represented the mean with standard deviations of two (shRNA-SCA2 mutated) to four (shRNA-SCA2) independent experiments. Statistical significance was inferred for *P* values less than 0.05 (*). (e) Effect of the repression of *SCA2* expression on morphological characteristics of T2ECs in differentiation. After the selection of transfected cells, T2ECs were maintained 2 days in differentiation medium and stained with Wright–Giemsa. The graph represents the percentages of erythroblasts (Ebl), early reticulocytes (ER) and late reticulocytes (LR) determined by counting cells. One representative experiment is shown.

Figure 4. Effect of *SCA2* overexpression on the proliferation and differentiation of T2ECs. T2ECs were either transfected with the empty expression vector pCRNCM (mock) or with the pCRNCM-*SCA2* (SCA2) or with the pCRNCM-*SCA2*-FLAG (SCA2-FLAG). (a) Expression level of SCA2-FLAG in T2ECs. T2ECs grown in LMI were lysed 2 days after the selection of the transfected cells. Protein extracts (20 µg) were analysed by SDS-PAGE electrophoresis and two separate Western blots were probed with an anti-FLAG antibody or anti-GAPDH antibody. (b) Effect of the overexpression of *SCA2* on proliferation rate of T2ECs. The number of viable cells was determined 1, 2 or 3 days after the selection of transfected cells, grown in LMI, by

WST-1 assay. The results show the proliferation of T2ECs between overexpression of *SCA2* and mock conditions. Data are average of three (SCA2-Flag) to four (SCA2) independent experiments and error bars indicate maximum and minimum values of different sets of data points. (c) Effect of the overexpression of *SCA2* on the differentiation of T2ECs. T2ECs were maintained in differentiation medium and aliquots of cells were stained with acidic benzidine after 2 or 3 days of culture. The percentages of haemoglobin-expressing cells in *SCA2* overexpression conditions in comparison with the mock condition were reported and represented the mean with standard deviations of two (3 days), three (SCA2-Flag, 2 days) or four (SCA2, 2 days) independent experiments. Percentages having P values = 0.074 (*) and 0.014 (**) are considered statistically significant. (d) Effect of the overexpression of *SCA2* on morphological characteristics of T2ECs in differentiation. After the selection of transfected cells, T2ECs were maintained 2 days in differentiation medium and stained with Wright-Giemsa. The graph represents the percentages of erythroblasts (Ebl), early reticulocytes (ER) and late reticulocytes (LR) determined by counting cells. One representative experiment is shown.

SCA2-FLAG both resulted in a significant inhibition of T2ECs' differentiation (2–4-fold) (Fig. 4c,d).

These data demonstrate that *SCA2* overexpression inhibits the differentiation but does not affect the proliferation rate of T2ECs. Altogether, these results suggest that *SCA2* expression is required at a certain threshold to maintain self-renewal, with its function being more directly implicated in inhibition of differentiation.

Expression of *SCA2* gene is increased in malignant cells

We analysed the expression level of *SCA2* in different human cell types. We used the SQUAT (SAGE Querying and Analysis Tools) application, developed in our laboratory (see the Material and Methods section), to find libraries where the *SCA2* tag (CACTTCAAGG) is present. We discovered that *SCA2* is expressed in 326 human SAGE libraries, including 196 cancer and 112 normal libraries (Fig. 5a,b). *SCA2* is overexpressed in a large proportion of cancer libraries (215.27 mean tags per million), mostly in carcinomas (chondrosarcomas and mesotheliomas), as compared to normal libraries (113.98 mean tags per million) (Fig. 5a,b). We have confirmed by quantitative PCR, that *SCA2* is indeed overexpressed in human cancer of the colon and in malignant kidney tissues as compared to normal tissues (Fig. 5c). Then, we searched with SQUAT for all genes that are simultaneously overexpressed together with the human *SCA2* gene in cancer libraries. We observed that different ribosomal proteins, like ribosomal proteins L8 and L31, are the proteins coded for by some of the most frequent genes. This finding strengthens the importance of remodeling of translation apparatus during the transformation process. Otherwise, transcriptional regulation of genes encoding ribosomal proteins is an emerging pattern in numerous large-scale transcriptomic analyses (Becquet *et al.* 2002). Finally, we used the L2L application (Newman & Weiner 2005) to find relevant biological patterns in gene expression data by comparing our own list of genes, simultaneously overexpressed, with *SCA2* in human cancer SAGE libraries, with lists of genes that have been determined by microarrays to be co-expressed in response to different conditions. We observed that our list significantly overlaps the list of genes found to be enriched in mouse neural stem cells, compared to differentiated brain and bone marrow cells (P value = $8.99\text{e-}19$). These results suggest that *SCA2* might be involved in some property of the stem state.

These results show that *SCA2* is overexpressed in different human malignant cells compared to normal cells, and genes simultaneously overexpressed with *SCA2* in human cancer SAGE libraries seem to be involved in the stemness. These findings confirm that *SCA2* is involved in the self-renewal process, which is a specific stem cell trait.

DISCUSSION

In this study, we have demonstrated that *SCA2* is a target gene of TGF- β , a growth factor essential for the self-renewal of T2ECs. Moreover, we demonstrate for the first time the biological role of *SCA2* in erythroid progenitor cells, which was initially identified by SAGE analysis (Damiola *et al.* 2004). The emerging concept is that *SCA2* functions in a dose-dependent manner to influence avian erythroid progenitors' fate. Our results indicate that a modest reduction in *SCA2* expression is sufficient to decrease the proliferation rate and to trigger differentiation of T2ECs. However, its overexpression inhibited differentiation only of T2ECs, in a manner similar to activation of TGF- β signalling (Gonin-Giraud *et al.* 2008). Moreover, this study illustrates, by using a new bioinformatic tool and by quantitative PCR, that *SCA2* is also overexpressed in

Figure 5. Expression of *SCA2* in different human tissues. Expression level of *SCA2* in 354 different human SAGE libraries is assessed *in silico* by SQUAT. 122 normal libraries (a) and 214 cancer libraries (b) are represented. For each library, the *SCA2* expression level is displayed (tags per million). The average of *SCA2* expression in normal and cancer libraries is calculated ($\bar{X}^Y$). *SCA2* expression is also measured by real-time PCR in normal and cancer human tissues like kidney and colon (c). The folds of variation are represented as cancer/normal ratios calculated using one reference gene the *18 s ribosomal RNA*.

different human malignant cells compared to normal cells. These observations suggest that *SCA2* maintains self-renewal and that perturbation in its expression could participate in development of some cancers.

Previous studies suggest the involvement of *SCA2* in cells with malignant characteristics. Indeed, the *SCA2* gene was first identified as one of several that were up-regulated in bone marrow cells transformed with v-Rel, a NF- κ B transcription factor (Petrenko *et al.* 1997). It was also reported that expression of *SCA2* is enhanced in chicken embryo fibroblasts susceptible to

infection by the Marek's disease virus, responsible for a lymphoproliferative disorder (Liu *et al.* 2003). Furthermore, expression of *SCA2* is also elevated in hepatocellular carcinoma cells (He & Chang 2004).

Finally, we believe that *SCA2* has an intracellular threshold level below which differentiation can occur. Taken together, our results are consistent with reports that indicate a role for other genes, *Oct4* and *Nanog*, in maintaining the self-renewal state by preventing differentiation (Hough *et al.* 2006). However, the regulatory mechanism by which *SCA2* promotes self-renewal remains to be elucidated. It is possible that *SCA2* may function as a signal transduction molecule as reported for several GPI-linked molecules in haematopoietic cells (Robinson 1991). However, it is not known how *SCA2* transmits signals into the cytoplasm of the cell, but it has been suggested that GPI-anchored proteins transmit signals to the cell interior by interacting with non-receptor type tyrosine kinases, p56^{lck} and p59^{fyn} (Shenoy-Scaria *et al.* 1992).

In conclusion, we have reported that *SCA2*, identified in previous studies by SAGE as up-regulated in self-renewing cells (Damiola *et al.* 2004), is functionally involved in the decision process between self-renewal and differentiation states. These findings enhance our understanding of how self-renewal may be controlled in erythroid progenitors.

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