MRPS18–2 protein immortalizes primary rat embryonic fibroblasts and endows them with stem cell-like properties

Elena Kashuba[a,b,c], Surya Pavan Yenamandra[a,b], Suhas Deoram Darekar[a], Mariya Yurchenko[c], Vladimir Kashuba[a,d], George Klein[a,1,2], and Laszlo Szekely[a,b,1]

[a]Department of Microbiology, Tumor, and Cell Biology, Karolinska Institutet, BOX 280, S-17177 Stockholm, Sweden; [b]Center for Integrative Recognition in the Immune System, Karolinska Institutet, S-17177 Stockholm, Sweden; [c]R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, 45 Vasylkivska str, Kyiv-22, 03022 Ukraine; and [d]Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, 150 Acad Zabolotny str, Kyiv-680, 03680 Ukraine

Contributed by George Klein, October 7, 2009 (sent for review July 2, 2009)

We report that the overexpression of mitochondrial ribosomal protein MRPS18–2 (S18–2) can immortalize primary rat embryonic fibroblasts (REFs). The immortalized cells (18IM) lose contact inhibition, form foci, and are capable of anchorage-independent growth. Concurrently, mesodermal markers, such as vimentin, smooth muscle actin, and Fut4, disappear completely. 18IM cells express embryonic stem cell markers, such as SSEA-1, Sox2, and Oct3/4. In confluent cultures, a portion of cells also express ectoderm- and endoderm-specific pan-keratin, ectoderm-specific beta-III-tubulin, mesoderm-specific MHC class II, and become stainable for fat with Oil red O. None of these changes was detected in c-myc+Ha-ras (MR)-transformed cells. In immunodeficient mice, 18IM cells formed small transiently growing tumors that have down-regulated SSEA-1 and showed pan-keratin staining. We conclude that S18–2 can immortalize REFs and induces them to express stem cell traits.

immortalization | mitochondrial ribosomal protein

Recently, we reported that the mitochondrial ribosomal protein MRPS18–2 (S18–2, NP_054765) could bind to EBNA-6 (EBNA-3C), an Epstein-Barr virus (EBV)-encoded transforming protein (1). EBV-encoded latent proteins immortalize B-cells after infection by interfering with normal cellular pathways (2). Therefore, we examined the role of S18–2 in cell transformation, using rat embryonic fibroblasts (REF) as targets. We found that overexpression of human S18–2 can immortalize REFs and induces them to express stem cell markers.

Results

Overexpression of S18–2 Immortalizes REFs. A plasmid, encoding GFP-fused S18–2 (GFP-S18–2) carrying a neomycin resistance gene, was transfected into REFs either alone or in combination with a *Ha-Ras* and/or *GFP-EBNA6* (in triplicate) vectors. The transfected cells were selected on 0.5 mg/mL G418 for 2 weeks. Surprisingly, GFP-S18–2 induced massive transformation of the REFs when introduced on its own. Large colonies were formed in the Petri dishes (Fig. 1*A*). This unexpected finding was confirmed with two other constructs, *c-myc-tagged S18–2* (MT-S18–2) and *pBabe-S18–2* (in triplicate). The latter clones were selected with G418 (0.5 mg/mL) and puromycin (1 μg/mL), respectively. All cells on the control plates died during the first 3–5 days, while 2–4 $\times$ 10^5 S18–2 transfected cells produced 150–200 large colonies per plate by 14 days. Primary REFs did not form any foci on non-selective media during an observation period of 2 weeks. *GFP-S18–2*-transfected cells could be propagated in selective medium during the entire observation period of 8 months. GFP-S18–2 protein showed mainly nuclear localization (Fig. 1*B*).

Deletion from the cDNA of *S18–2* of either one or two of the initial bases in the reading frame generated a frameshift mutated protein in pBabe vector that failed to induce colony formation (Table 1). To further assess the transforming effect of S18–2, cells immortalized by S18–2 (18IM) were transfected with a mixture of four different small interfering RNA (siRNA) oligos that were designed to specifically antagonize S18–2 mRNA. The decrease of the S18–2 protein level (Fig. 1*C*) was followed by massive cell death after 48 h. An introduction of non-specific siRNA had no such effect.

S18–2-Immortalized Cells Show Embryonic Stem Cell-Like Properties. S18–2 immortalized (18IM) cells grew as large multilayered foci in ordinary IMDM medium, supplied with 10% FBS and 0.5 mg/mL G418. The foci consisted of small cells with a high nucleo-cytoplasmic ratio and prominent nucleoli. The periphery of these cells was morphologically diversified, in contrast to the morphologically more uniform REFs (Fig. 1*D*). The 18IM cultures had a higher frequency of mitotic figures than the control REFs (Movie S1).

In bacterial Petri dishes, 18IM cells formed large compact aggregates, reminiscent of embryoid bodies (Fig. 1*E*, *Left*). When transferred back to ordinary tissue culture flasks, they grew again as foci like those shown in Fig. 1*D*, with a compact inner core and larger scattered cells at the periphery. The S18–2 immortalized cells were designated as 18IM.

For comparison with a well established transformation system, REFs transformed by c-myc and mutated Ha-ras (MR cells) (described in ref. 3) were tested under similar conditions. MR cells formed clumps in bacterial Petri dishes. There were no embryoid body-like aggregates (Fig. 1*F*).

18IM cells were analyzed for the ability to grow in soft agar, upon the seeding of 100–1,000 cells per 3.5-cm plate, in comparison with control REFs and MR (c-myc- and Ha-ras-transformed) cells. Cloning efficiency of 18IM cells was 94% (Fig. 1*G*). Normal REFs did not grow in soft agar. MR cells proliferated well, with the cloning efficiency >98%.

18IM Cells Express Embryonic Stem Cell Markers. 18IM cells were tested for stem-cell marker expression by immunostaining and RT-PCR. For immunostaining, cells were grown on coverslips in parallel with MR and normal REF controls. All cells were fixed in a mixture of cold (−20 °C) methanol and acetone (1:1) and stained for stage-specific embryonic antigens 1 (SSEA-1) and 4

Author contributions: E.K. and L.S. designed research; E.K., S.P.Y., S.D.D., M.Y., and V.K. performed research; E.K., S.P.Y., M.Y., V.K., G.K., and L.S. analyzed data; and E.K. and G.K. wrote the paper.

The authors declare no conflict of interest.

[1]G.K. and L.S. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: georg.klein@ki.se.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911545106/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.0911545106

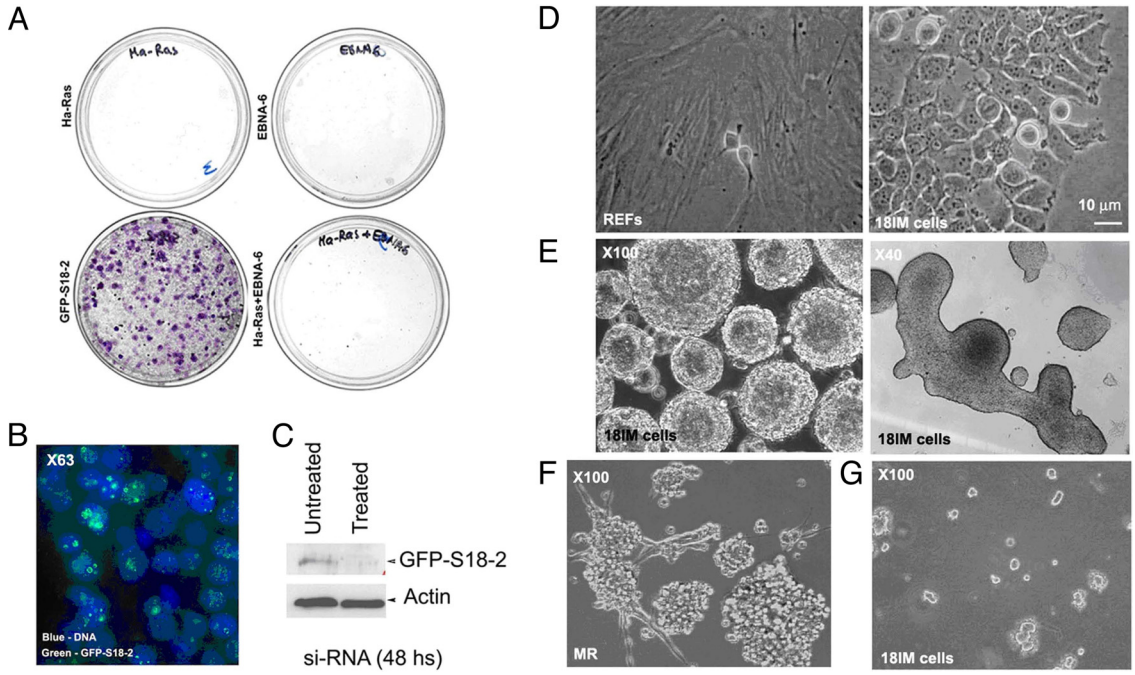

Fig. 1. Growth characteristic of S18–2 immortalized cells. (*A*) Colony formation assay. Primary rat embryonic fibroblasts were transfected with the different plasmids. After 2 weeks of selection on 0.5 mg/mL G418, the cells were fixed with formaldehyde and stained with crystal violet. (*B*) Fusion GFP-S18–2 protein expression (in green) in the 18IM cells. They were stained with mouse monoclonal anti-GFP antibody. DNA was stained with Hoechst (blue signal). Notice the mainly nuclear localization of GFP-S18–2 protein. (*C*) si-RNA treatment of the 18IM cells. Cells were treated with si-RNA for 48 h. Western blots were probed with rabbit polyclonal anti-S18–2 antibody for GFP-S18–2 fusion protein. (*D*) Growth of GFP-S18–2 transformed cells. Freshly passaged REFs did not form foci (first from the *Left*). The 18IM cells formed foci. Cells of different shapes were observed in the periphery (second from the *Left*). Notice increased number of mitoses in the 18IM cells. (*E*) Growth of GFP-S18–2 transformed cells. The 18IM cells formed embryoid body like structures in bacterial Petri dishes (*Left*). After 2–3 days these bodies fused with each other, forming large agglomerates after 3–5 days (*Right*). (*F*) Growth of c-myc and mutated Ha-ras transformed cells. REFs transformed by c-myc and mutated Ha-ras (MR cells) formed clumps in bacterial Petri dishes. (*G*) Growth of GFP-S18–2 transformed cells. The 18IM cells grew in soft agar (0.6% on the *Bottom*, 0,5% *Top* agarose) in contrast to primary REFs.

(SSEA-4). Between 30–40% of the 18IM cells showed strong membrane staining for SSEA-1 (Fig. 2*A*, *Top*). In contrast, REFs failed to express this antigen, while <0.3% of the MR cells expressed it. The SSEA-4 signal was quite strong in control REFs and increased in the 18IM and MR cells. Among mesenchymal cell markers, vimentin (NP_003371) was highly expressed in the control REFs and MR cells but was undetectable in the 18IM cells (Fig. 2*A*, second row). Smooth muscle actin (SMA, NP_001135417) was highly expressed by REFs but only by a small portion (≈5%) of the MR cells and not at all by the 18IM cells (Fig. 2*A*, *Bottom* row). All 18IM cells expressed an increased level of alkaline phosphatase compared to REFs.

Transfection of 18IM cells with an *S18–2* specific siRNA led to a dramatic reduction of the level of S18–2 protein (Fig. 1*C*) and a concomitant disappearance of SSEA-1-positive cells as gauged by immunostaining (Fig. 2*B*). Non-specific siRNA had no effect on SSEA-1 expression level.

RT-PCR data have confirmed the high level of vimentin mRNA (NM_031140.1) expressing in the control REFs and its virtually complete extinction in the 18IM cells. *FUT4*, another marker of mesenchymally differentiated cells, (NM_022219.2), was strongly expressed in the control REFs but was absent from 18IM cells. The mRNA encoding the embryonic stem-cell marker *Sox2* (NM_001109181.1) gave only a very low signal in RT-PCR analysis

Table 1. Colony formation assay

Plasmid	Selection	Observation
Ha-Ras	G418 (0.5 mg/ml)	No colonies*
Ha-Ras + GFP-S18–2	G418	100–150 colonies per plate**
Ha-Ras + GFP-EBNA-6	G418	Tiny non proliferating colonies
GFP-EBNA-6	G418	No colonies
GFP-S18–2 + EBNA-6	G418	150–200 large colonies per plate
GFP-S18–2	G418	150–200 large colonies per plate
C-myc-tag-S18–2	G418	50–100 colonies per plate
pBabe-S18–2	Puromycin, (1 μg/ml)	20–80 colonies per plate
pBabe-S18–2 (frameshift mutant, 1 b.p. deletion after start codon)	Puromycin	No colonies
pBabe-S18–2 (frameshift mutant, 2 b.p. deletion after start codon)	Puromycin	No colonies

REFs were grown for two weeks on selective medium after transfection. *, 2–4 × 10^5 cells were plated prior to transfections; **, 7.5-cm plate.

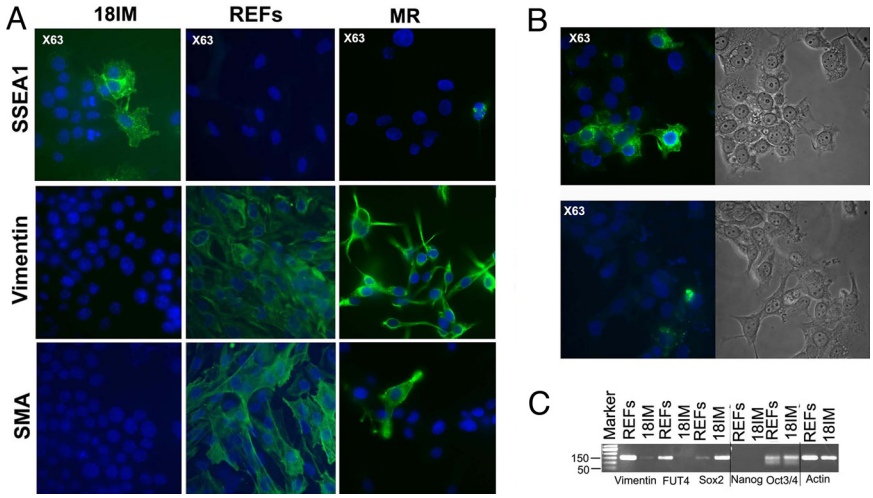

Fig. 2. Comparative expression of differentiation markers by 18IM, MR cells, and REFs. (*A*) Immunostainings on primary rat embryonic fibroblasts (REFs) and GFP-S18–2 transformed cells (18IM). Green signal, marker expression. Blue signal, DNA (stained with Hoechst). (*B*) si-RNA treatment of GFP-S18–2 cells grown on cover slips. *Top*, untreated cells stained with anti-SSEA-1. Green, SSEA-1 signal; blue, DNA staining. *Lower*, cells treated with si-RNA for 24 h. Green, SSEA-1 signal; blue, DNA staining. SSEA-1 staining decreased significantly. (*C*) RT-PCR on primary rat fibroblasts (REFs) and GFP-S18–2 transformed cells (18IM). Marker (100-bp) was loaded in the first lane.

of RNA prepared from the control cells, but was highly expressed by the 18IM cells. *Oct3/4* (NM_001009178.1) was expressed at similar levels in both cell types. *Nanog* (NM_001100781.1) was not expressed in either REFs or the 18IM cells (Fig. 2*C*).

18IM Cells Can Differentiate In Vitro. 18IM cells were grown on coverslips or in ordinary flasks at high density. Some of the highly proliferating cells detached from these substrates and were collected after 10 days and examined on cytospin slides. Many of them had large cytoplasmic vacuoles that were positive for fat by Oil Red O staining (Fig. 3*A*). No fat cells were detected in the control REF and MR cells grown under similar conditions. Rat-specific MHC class II (hematopoetic marker; Fig. 3*B*) and beta-III-tubulin-expressing cells (ectoderm marker; Fig. 3*C*) were also found in the 18IM but not in REF and MR cells.

Embryoid-like bodies formed by 18IM cells were replated from Petri dishes in ordinary flasks. They expressed pan-keratin when grown on coverslips (Fig. 3*D*). Single cells at the periphery of the colonies were most intensively stained (see arrows in Fig. 3*D*). Control REF and MR cells did not show pan-keratin staining. Frozen sections of the 18IM cell aggregates, arising following propagation in bacterial Petri dishes (Fig. 1*E*, *Right*), were stained for cytokeratins and SSEA-1 (Fig. 3*E*). The frequency of SSEA-1-positive cells was far lower in the aggregates than in the 18IM cells propagated under standard condtions of tissue culture (≈2% compared to >30%) (Fig. 2 *A* and *B*, *Top* compared to Fig. 3*E*, *Top*). In contrast, keratin-positive cells appeared mainly on the surface of the multicell aggregates (Fig. 3*E*, *Bottom*). We concluded that in our experimental system mesoderm-derived fibroblasts were induced to differentiate into cells that expressed ectoderm- or endoderm-specific markers.

18IM Cells Can Differentiate in SCID Mice. 18IM cells were tested for tumorigenicity by inoculating 5×10^6 cells into 5 SCID mice s.c. Small tumors appeared at the site of inoculation in all mice and reached a maximum volume of ≈20 mm^3 after 9–13 days. However, by 3–4 weeks later they had decreased in volume to 2–3 mm^3 in three mice of the inoculated mice and had disappeared in the other two. All mice were killed on day 44; the tumors were removed and fixed in 10% neutral buffered formalin. Histological sections (5-μm) were prepared from paraffin embedded samples and stained with hematoxylin and eosin. They contained morphologically heterogeneous anaplastic cells aggregated in 50- to 100-μm clusters of eosinophile pearls embedded in a fibrous stroma. Solitary, large neoplastic cells with prominent nucleoli and cytoplasmic vacuoles were also seen. (Fig. 4, *Left*). Vimentin, SMA, and pan-keratin were stained by indirect immunofluorescence, counterstained with crystal violet (4). Rat and mouse cells could be distinguished by the presence of Hoechst positive pericentromeric heterochromatin foci in all mouse nuclei that were absent from the rat cells (for review, see ref. 5). No vimentin or SMA was detected in the rat cells. Pan-keratin staining detected cytoplasmic filaments in a fraction of the rat cells (Fig. 4). The original REFs and the in vitro cultured 18IM cells before embryoid body formation did not express keratin. The small tumor size, the lack of progressive growth and the pan-keratin staining suggested that 18IM cells have transdifferentiated in vivo.

Discussion

Primary rat and mouse fibroblasts can be transformed by the overexpression of c-myc (NP_002458.2) and mutated ras (NP_001123914.1) proteins (6). The phenotypic changes include immortalization, loss of contact inhibition, anchorage-independent growth, and tumorigenicity.

Mouse embryonic and adult fibroblasts can be reprogrammed by overexpression of defined sets of genes, such as *SOX2*, *OCT3/4*, *KLF4* (NM_004235), and *C-MYC* (NM_002467.3) [or *N-MYC* (NM_005378.4) (7)] and turned into iPSC, induced pluripotent stem cells (8–10). This reprogramming is associated with changes in DNA methylation, chromatin structure and gene expression (11–13), mimicking embryonic cells. The resulting iPSC can develop into teratomas and other types of tumors in SCID and NUDE mice (for review, see ref. 14)

Recently, authentic embryonic stem cells were also produced from rat blastocysts (15). Induced pluripotent stem rat cells were also recently generated (16), by lentiviruses expressing four genes, *OCT4*, *SOX-2*, *C-MYC*, and *KLF4*. Attempts to produce rat iPSC with another set of genes, namely *OCT4*, *SOX-2*, *NANOG*, and *LIN28* (NM_024674.4) was unsuccessful (16).

Here, we have found that introduction of the human mitochondrial ribosomal gene *MRPS18B* (GenBank gene accession number NM_014046) into rat primary fibroblasts immortalized them. S18–2 protein is encoded by a cellular gene, located on human chromosome 6p21.3, adjacent to the MHC class II gene

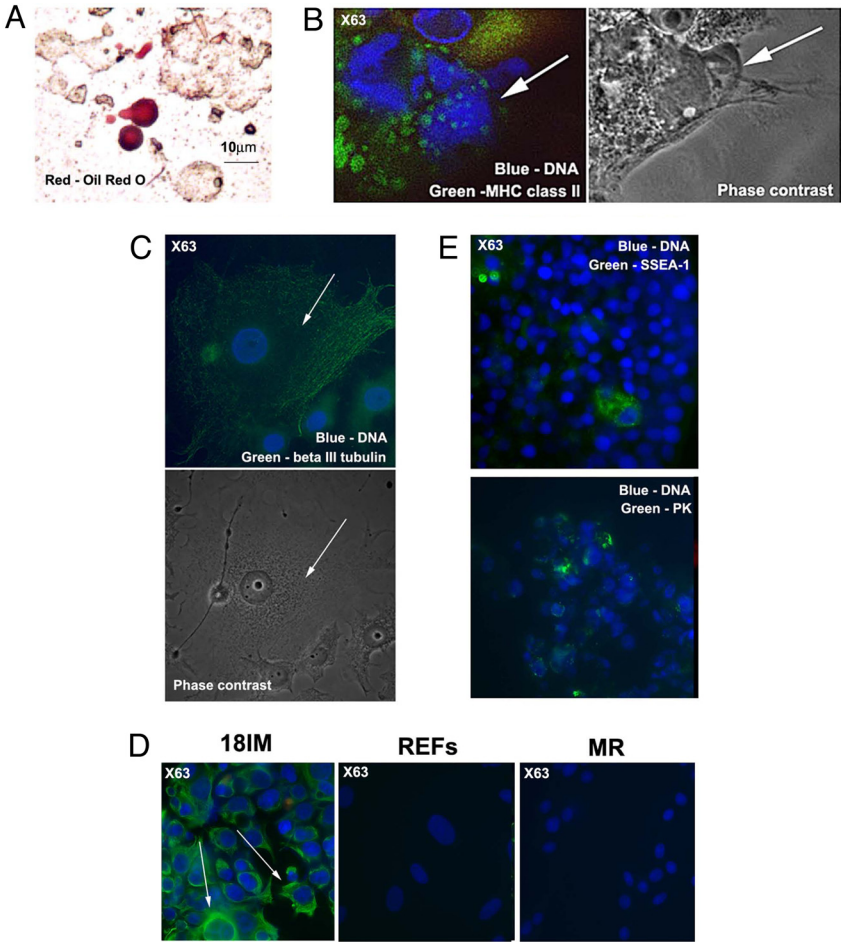

Fig. 3. Expression of differentiation markers by 18IM cells. (*A*) Staining of the 18IM cells in confluent cultures. Suspended cells were collected and spun onto glass slides. Oil Red O staining. Red, fat cells. (*B*) Immunostaining of the suspended cells. Suspended 18IM cells were collected and spun onto glass slides. MHC class II staining. Green, MHC class II; blue, DNA. (*C*) Immunostaining of the 18IM cells in confluent cultures. The 18IM cells grown on coverslips were stained with antibody against beta III-tubulin. Green, beta III tubulin; blue, DNA. (*D*) Immunostaining of the 18IM cells. Pan-keratin is expressed in the differentiated from embryoid bodies 18IM cells, in contrast to REF and MR cells. (*E*) Immunostaining of cell agglomerates on frozen sections. *Top*, SSEA-1 expression. Notice decrease in the frequency of SSEA-1 positive cells (green) compared to the correspondingly cultured cells (Fig. 2 *A* and *B*, *Top*). DNA was stained with Hoechst (blue signal). *Bottom*, pan-keratin staining. Notice positive cells at the edges of the agglomerate (green signal). DNA is stained in blue.

cluster. S18–2 cDNA was cloned during an analysis of 300 previously undefined genes with ORFs expressed in CD34+ hematopoietic stem/progenitor cells by Zhang and coauthors (17). It encodes one of the three MRPS18 family proteins localized on the surface of the small subunit (28S) of the mammalian mitochondrial ribosome (18, 19). The human genome encodes three *S18* genes, in contrast to one in bacteria. S18–2 homologs are found in mammals, *D.* melanogaste,r and *C. elegans*. We have found earlier, that S18–2 binds specifically to retinoblastoma protein (pRb), but not to other pocket proteins, such as p107 and p130 (20). In EBV-infected cells we have observed a transfer of S18–2 to the nucleus by the EBV-encoded transforming protein EBNA-6. This was accompanied by increasing levels of free E2F1 and inhibition of S-phase entry block

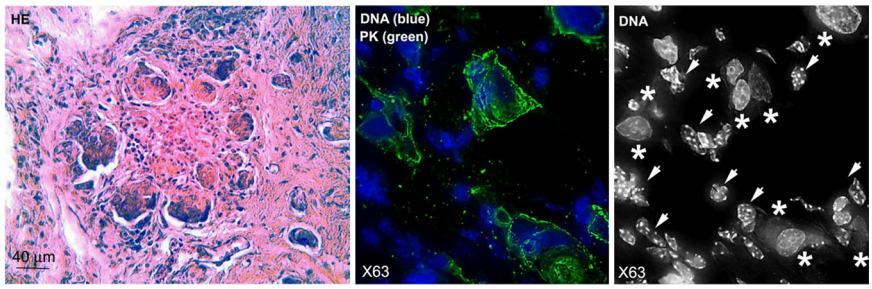

Fig. 4. Immunostaining of the tumors produced by GFP-S18–2 transformed cells in SCID mice. Formalin fixed paraffin embedded tissue was sectioned (5-μm). Paraffin was removed by xylene, followed by ethanol. Epitopes were recovered by boiling in microwave oven in citric buffer. *Left*: hematoxylin and eosin staining. *Center*: green, pan-keratin; blue, DNA. Heterochromatin in mouse cells forms aggregates in the nucleus, but is distributed evenly in rat cells. Only rat cells express pan-keratin. *Right*, DNA staining. Arrowheads, mouse cell nuclei; stars, rat nuclei.

by pRb (1). As seen from the Fig. 1*B*, S18–2 protein shows mainly nuclear localization in the 18IM cells.

We found that S18–2-transfected cells are immortalized, lose contact inhibition, and become capable of anchorage-independent growth. Their tumorigenicity was minimal. This was in line with the marker changes detected both in vitro and in vivo. 18IM cells expressed embryonic stem cell markers, such as SSEA-1 and SOX2 in vitro that were not present on either the control or the transformed MR cells. In contrast, the mesodermal markers vimentin, smooth muscle actin, and FUT4, characteristic of fibroblastic differentiation, were downregulated. This is consistent with the recent finding that S18–2 is highly expressed in embryonic stem cells but not in differentiated tissues (21).

The (c-myc+Ha-ras) transformed cells were different from S18–2 immortalized cells, since they did not lose vimentin expression (Fig. 2) nor did they produce embryoid bodies (Fig. 1). Moreover, 18IM cells expressed markers in confluent culture and in mice, such as ectoderm- and endoderm-specific pan-keratin, ectoderm specific beta-III-tubulin, and mesoderm specific MHC class II. They could also differentiate into Oil red O stainable fat cells.

Mitochondrial ribosomes, encoded by the mammalian genome, have attracted increasing interest (22). More than 70 mitochodrial ribosomal proteins are encoded by the human genome (23). MRPS29, one protein of the small mitochondrial ribosomal subunit can bind GTP and may be involved in the control of apoptosis (24). As mentioned above, S18–2 binds to pRb and EBNA-6, and can change its cellular localization (1, 20). The present findings will serve as the basis for further studies on other binding partners and on the role of S18–2 in other cell types.

Methods

Plasmids and Transfections. Construction of the fusion GFP-S18–2 and c-myc-S18–2 was described in ref. 1. S18–2 cDNA was subcloned from GFP-S18–2 into pBabe vector (that contained moderately strong Moloney leukemia virus long terminal repeat (LTR) promoter and puromycin resistance gene), using the following sets of primers: for wild-type: Forward - TTAAGGATCCATGGCG-GCGTCTGTATTAAACACC GT, Reverse (the same for all three constructs) - TAATTGAATTCTTACAGGAG TGAGCCACCACG; for 1-bp deletion mutant: Forward - TTAAGGATCC ATGCGGCGTCTGTATTAAACACCGT; for 2-bp deletion mutant: Forward - TTAAGGATCCATGGGCGTCTGTATTAAACACCGT. All constructs were verified by direct sequencing using forward and reverse primers and an Applied Biosystems sequencer (PerkinElmer). The GFP-EBNA-6 construct was kind gift of Tom Sculley, Queensland Institute for Medical Research, Brisbane, Australia (25). Ha-Ras-pEVJB plasmid was kind gift of Marie Arsenian Henriksson, Department of Microbiology, Tumor, and Cell Biology, Karolinska Institutet, Stockholm (described in ref. 3).

Rat embryonic fibroblasts were isolated from 13- to 14-day-old fetuses, as described in ref. 3. They were plated at the density 0.2–0.4 $\times$ 10^6 cells per Petri dish of 7.5 cm in diameter. Transfections were performed using Lipofectamine 2000 TR (Life Technologies), according the manufacturer's protocol. Selection of the transfected cells was carried out on 0.5 mg/mL G418 and 1 μg/mL puromycin, purchased from Sigma–Aldrich for 2 weeks. Expression of S18–2 in selected cells was shown by Western blot analysis, using anti-S18–2 rabbit polyclonal serum and anti-GFP mouse monoclonal antibodies.

REFs transformed by c-myc and mutated Ha-ras (MR cells) were kind gift of Marie Arsenian Henriksson, MTC, KI, Stockholm (described in ref. 3).

Antibodies. The following primary antibodies were used: mouse monoclonal anti-beta-III isoform of tubulin (Millipore); anti-keratin, Pan Ab-1 (Thermo Scientific); anti-rat MHC class II (kind gift of Helena Erlandsson-Harris, CMM, Karolinska Hospital, Stockholm); anti-smooth muscle actin (DAKO); anti- stage specific embryonic antigen SSEA-1, detecting lactoseries oligosaccharide antigen that is expressed on the surface of mouse embryonal carcinoma and embryonic stem cells, and anti-SSEA-4, (R&D Systems); anti-vimentin (DAKO), anti-GFP (Cell Signaling Technology); anti-S18–2 [clone 75–5 (1)] antibodies; Oil Red-O (Sigma–Aldrich). The rabbit anti-mouse FITC-conjugated (DAKO) was used as a secondary antibody in immunostaining.

Cell Culture, Immunostaining, and Imaging. All cells (primary and transfected) were cultured at 37 °C, in Iscove's medium containing 10% FBS and appropriate antibiotics. Before immunostaining experiments, the cells were grown on coverslips. Immunostaining and digital image capturing was performed as described earlier (26). Briefly, cells on coverslips or on slides after cytospin were fixed in a 1:1 mixture of cold methanol and acetone (−20 °C). After rehydration in phosphate buffer saline, cells were stained with antibodies. Hoechst 33258 (Sigma–Aldrich) was added at a concentration of 0.4 μg/mL to the secondary antibody for DNA staining when necessary. The images were captured using DAS microscope Leitz DM RB with a dual mode cooled charged coupled device (CCD) camera C4880 (Hamamatsu) or Zeiss LSM 510 laser scanning confocal microscope with ORCA-ER CCD camera (Hamamatsu).

Western Blot Analysis. Proteins were separated on the SDS-polyacrylamide gel. ECL kit, anti-mouse and anti-rabbit horseradish peroxidase conjugated secondary antibodies, produced in sheep and donkey correspondingly (GE Healthcare Bio-Sciences AB), were used to visualize the protein bands.

Reverse Transcription PCR. RNA was isolated from about 5 $\times$ 10^6 primary and transformed cells, using GenElute mammalian total RNA miniprep kit (Sigma–Aldrich). First-strand cDNA synthesis was carried using Enhanced avian RT first-strand synthesis kit (Sigma–Aldrich) according manufacturer's protocol. RT-PCR performed using GeneAmp PCR system 2700 (Applied Biosystems).

PCR was performed, using following 35 cycles: 15 s at 95°, 30 s at 60°, and 1 min at 72°. The following rat-DNA specific primers were used: for *Fut4* (NM_022219.2): Forward - CTTCGAATCACCCTCCCACA, Reverse - CAGACCTGAAGGCTGTTCGG; for *Nanog* (NM_001100781.1): Forward - AGGCCCAGTTGTGTGCACTC, Reverse - TGCCACCTCTTGCACTTCATT; for *Oct3/4* (NM_001009178.1): Forward - CTCT-GGGCCCTGTACTGCC, Reverse - GGTGTACCCCAAGGTGATCCT; for *Sox2* similar (NM_001109181.1): Forward - AACCAGAAGAACAGCCCGG, Reverse -ACAAAAGTTTCCACTCGGCG; for Vimentin (NM_031140.1): Forward - CAATGCT-TCTCTGGCACGTC, Reverse - GGAAACGTCCACATCGATCTG. Expression of beta-actin gene (NM_031144.2) was used as a control with the primers Forward - AGTACCCCATTGAACACGGC and Reverse - TTTTCACGGTTGGCCTTAGG.

si-RNA Targeting. The transformed cells were grown on coverslips and transfected with the mixture of four predesigned siRNAs (ON-Targetplus SMART pool L-013043–01: J-013043–09, J-013043–10, J-013043–11, and J-013043–12) using DharmaFECT 2 and 3 siRNA transfection reagents according to manufacturer's protocol (Thermo Scientific). All four oligos were targeting the coding region of the S18–2 gene.

ACKNOWLEDGMENTS. We thank Robert Weinberg for the critical discussion, Marie Henriksson for MR cells, and Kenth Andersson and Katalin Benedek for technical assistance. The Swedish Cancer Society, a matching grant from the Concern Foundation (Los Angeles), and the Cancer Research Institute (New York), Swedish Institute, and Swedish Foundation for Strategic Research supported this work.

1. Kashuba E, et al. (2008) EBV-encoded EBNA-6–6 binds and targets MRS18–2 to the nucleus, resulting in the disruption of pRb-E2F1 complexes. *Proc Nat Acad Sci USA* 105:5489–5494.
2. Klein G (2002) Perspectives in studies of human tumor viruses. *Front Biosci* 7:d268–274.
3. Cerni C, et al. (2002) Repression of in vivo growth of myc/ras transformed tumor cells by MAD1. *Oncogene* 21:447–459.
4. Buchynska L, Kashuba E, Szekely L (2008) Immunofluorescence staining of paraffin sections: Creating DAB staining like virtual digital images using CMYK color conversion. *Exp Oncol* 30:1–3.
5. Maison C, Almouzni G (2004) HP1 and the dynamics of heterochromatin maintenance. *Nat Rev Mol Cell Biol* 5:296–304.
6. Land, H, Parada, LF, Weinberg, RA (1993) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304:596–602.
7. Blelloch R, Venere M, Yen J, Ramalho-Santos M (2007) Generation of induced pluripotent stem cells in the absence of drug selection. *Cell Stem Cell* 1:245–247.
8. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
9. Nakagawa M (2008) Generation of induced pluripotent stem cells without myc from mouse and human fibroblasts. *Nat Biotechnol* 26:101–106.
10. Wernig M, Meissner A, Cassady JP, Jaenisch R (2008) C-myc is dispensable for direct reprogramming of mouse fibroblasts. *Cell Stem Cell* 2:10–12.
11. Wernig M, et al. (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 448:318–324.
12. Maherali N, et al. (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* 1:55–70.

13. Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. *Nat Biotechnol* 25:1177–1181.
14. Liu SV (2008) IpS cells: A more critical review. *Stem Cell Devel* 17:391–397.
15. Buehr M, et al. (2008) Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135:1287–1298.
16. Liao J, et al. (2009) Generation of induced pluripotent stem cell lines from adult rat cells. *Cell Stem Cell* 4:11–15.
17. Zhang QH, et al. (2000) Cloning and functional analysis of cDNAs with open reading frames for 300 previously undefined genes expressed in CD34+ hematopoietic stem/progenitor cells. *Genome Res* 10:1546–1560.
18. Suzuki T, et al. (2001) Proteomic analysis of the mammalian mitochondrial ribosome. Identification of protein components in the 28S small subunit. *J Biol Chem* 276:33181–33195.
19. Cavdar Koc E, Burkhart W, Blackburn K, Moseley A, Spremulli LL (2001) The small subunit of the mammalian mitochondrial ribosome. Identification of the full complement of ribosomal proteins present. *J Biol Chem* 276:19363–19374.
20. Snopok B, Yurchenko M, Szekely L, Klein G, Kashuba E (2006) SPR-based immunocapture approach to creating an interfacial sensing architecture: Mapping of the mrs18–2 binding site on retinoblastoma protein. *Anal Bioanal Chem* 386:2063–2073.
21. Hailesellasse SK, et al. (2007) Gene function in early mouse embryonic stem cell differentiation. *BMC Genomics* 8:85.
22. O'Brien TW (2003) Properties of human mitochondrial ribosomes. *IUBMB Life* 55:505–513.
23. Schieber GL, O'Brien TW (1985) Site of syntheis of the proteins of mammalian mitochondrial ribosomes: Evidence from cultured bovine cells. *J Biol Chem* 260:6367–6372.
24. Matthews DE, Hessler RA, Denslow ND, Edwards J, O'Brien TW (1982) Protein composition of bovine mitochondrial ribosomes. *J Biol Chem* 257:8788–8794.
25. Krauer KG, Kienzle N, Young DB, Sculley TB (1996) Epstein–Barr nuclear antigen-3 and -4 interact with RBP-2N, a major isoform of RBP-J kappa in B lymphocytes. *Virology* 226:346–353.
26. Mattsson K, et al. (2001) Proteins associated with the promyelocytic leukemia gene product (PML)-containing nuclear body move to the nucleolus upon inhibition of proteasome-dependent protein degradation. *Proc Nat Acad Sci USA* 98:1012–1017.