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SHORT COMMUNICATION

Suppression of KCMF1 by constitutive high CD99 expression is involved in the migratory ability of Ewing's sarcoma cells

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High *CD99* expression levels and rearrangements of the *EWS* gene with *ETS* transcription factor genes characterize the Ewing's sarcoma family of tumors (ESFT). CD99 is a cell surface glycoprotein whose engagement has been implicated in cell proliferation as well as upregulation and transport of several transmembrane proteins in hematopoietic cells. In ESFT, antibody ligation of CD99 induces fast homotypic cell aggregation and cell death although its functional role in these processes remains largely unknown. Here, using an RNAi approach, we studied for the first time the consequences of modulated *CD99* expression in six different ESFT cell lines, representing the most frequent variant forms of *EWS* gene rearrangement. *CD99* suppression resulted in growth inhibition and reduced migration of ESFT cells. Among genes whose expression changes in response to *CD99* modulation, the potassium-channel modulatory factor *KCMF1* was consistently upregulated. In a series of 22 primary ESFT, *KCMF1* expression levels inversely correlated with CD99 abundancy. Cells forced to express ectopic KCMF1 showed a similar reduction in migratory ability as *CD99* silenced ESFT cells. Our results suggest that in ESFT, high *CD99* expression levels contribute to the malignant properties of ESFT by promoting growth and migration of tumor cells and identify *KCMF1* as a potential metastasis suppressor gene downregulated by high constitutive *CD99* expression in ESFT.
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The Ewing's sarcoma family of tumors (ESFT) is a group of primitive small-round-cell tumors of bone and soft tissue that show an extremely aggressive clinical course but with an as yet unknown histogenic origin. Common to this class of tumors is the presence of a *EWS* gene-rearrangement with *FLI1, ERG* or, in rare cases, other *ETS* genes resulting in the expression of potent chimeric transcription-factors with both activating and repressing properties (Arvand and Denny, 2001). There is some variability in the architecture of these fusion-proteins that may be of biological importance. The most frequent rearrangements are *EWS-FLI1* type 1 and 2 occuring in 51 and 27%, respectively, and *EWS-ERG* in 10% of cases. A further hallmark of ESFT is a consistent high-level expression of the cell-surface glycoprotein CD99 (Kovar *et al*., 1990; Ambros *et al*., 1991). Introduction of EWS-FLI1 into neuroblastoma or rhabdomyosarcoma cells turns on CD99 expression (Rorie *et al*., 2004; Hu-Lieskovan *et al*., 2005) while silencing of *EWS-FLI1* in ESFT cells does not affect high-level *CD99* expression (our unpublished results). Thus, the functional relation between EWS-FLI1 and CD99 in ESFT remains unclear.

CD99 is a ubiquitous, 32 kDa transmembrane sialoglycoprotein that is encoded by the pseudoautosomal *MIC2* gene (Goodfellow *et al*., 1988). Among normal tissues, CD99 has been shown to be highly expressed on pancreatic islet cells, Leydig and Sertoli cells (Ambros *et al*., 1991). In the hematopoietic system, the level of CD99 expression decreases with the degree of differentiation, with early T- and B-precursors showing the highest CD99 positivity (Gelin *et al*., 1989; Dworzak *et al*., 1994, 1999). Similarly, an inverse association of CD99 with differentiation has been demonstrated recently for osteoblasts (Bertaux *et al*., 2005). Conversely, downregulation of CD99 is a major requirement for generating Hodgkin's and Reed-Sternberg cells in Hodgkin's disease (Kim *et al*., 1998, 2000). With the exception of lymphoblastic lymphoma, ESFT remain the only class of tumors that most consistently expresses CD99, a feature widely employed in the differential diagnosis of ESFT among small-round-cell tumors of childhood (Fellinger *et al*., 1991). CD99 shares no structural homology with any known protein family and putative rodent orthologs significantly diverge from human CD99 (Park *et al*., 2005). Available functional data on CD99 derive from triggering CD99 by agonistic monoclonal antibodies. In hematopoietic cells, CD99 ligation has been implicated in induction of homotypic aggregation, cell adhesion, migration (Bernard *et al*., 1995; Hahn *et al*., 1997; Schenkel *et al*., 2002), upregulation of TCR expression (Choi *et al*., 1998),

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vesicular transport of MHC molecules (Sohn *et al*., 2001), and costimulation to induce a Th1-type cytokine production (Waclavicek *et al*., 1998). In immature thymocytes and ESFT cells, CD99 antibodies induce massive apoptosis (Bernard *et al*., 1997; Sohn *et al*., 1998) as well as caspase-independent cell death (Cerisano *et al*., 2004) and increase sensitivity to chemotherapeutic agents (Scotlandi *et al*., 2000). Recent studies have provided fragmentary information about the involvement of protein kinases in CD99 triggering of intracellular signal-transduction (Hahn *et al*., 2000; Kasinrerk *et al*., 2000). In most tissues, CD99 is expressed in two isoforms generated by alternative splicing. In the B lymphoblastoid cell line IM-9, the short-isoform which lacks the cytoplasmic domain, has been demonstrated to act antagonistically to the major long-isoform in the induction of cellular adhesion (Hahn *et al*., 1997). In addition, expression of the major form in a CD99-deficient Jurkat T cell line was sufficient to promote cell adhesion, whereas coexpression of the two isoforms was required to trigger T-cell death (Alberti *et al*., 2002). The diversity of biological responses to antibody-ligation of CD99 in the absence of mechanistic insights into CD99 function and the lack of rodent homologues that might be knocked-out for genetic studies prompted us to investigate the role of CD99 in ESFT by an RNA-interference approach.

Four different siRNAs targeting different regions of both CD99 mRNA isoforms were designed and tested for silencing CD99 expression after two consecutive transfections in the ESFT cell lines SK-N-MC, TC252 and A673 expressing type 1 *EWS-FLI1*, STA-ET-7.2 and RDES carrying a type 2 *EWS-FLI1* gene-fusion, and RM82 with a *EWS-ERG* gene rearrangement. As monitored by flow-cytometry on day 5 after the first transfection (Figure 1a), all four siRNAs modulated *CD99* expression in SK-N-MC to variable extents. The most effective siRNA targeting nucleotides 249–267 of

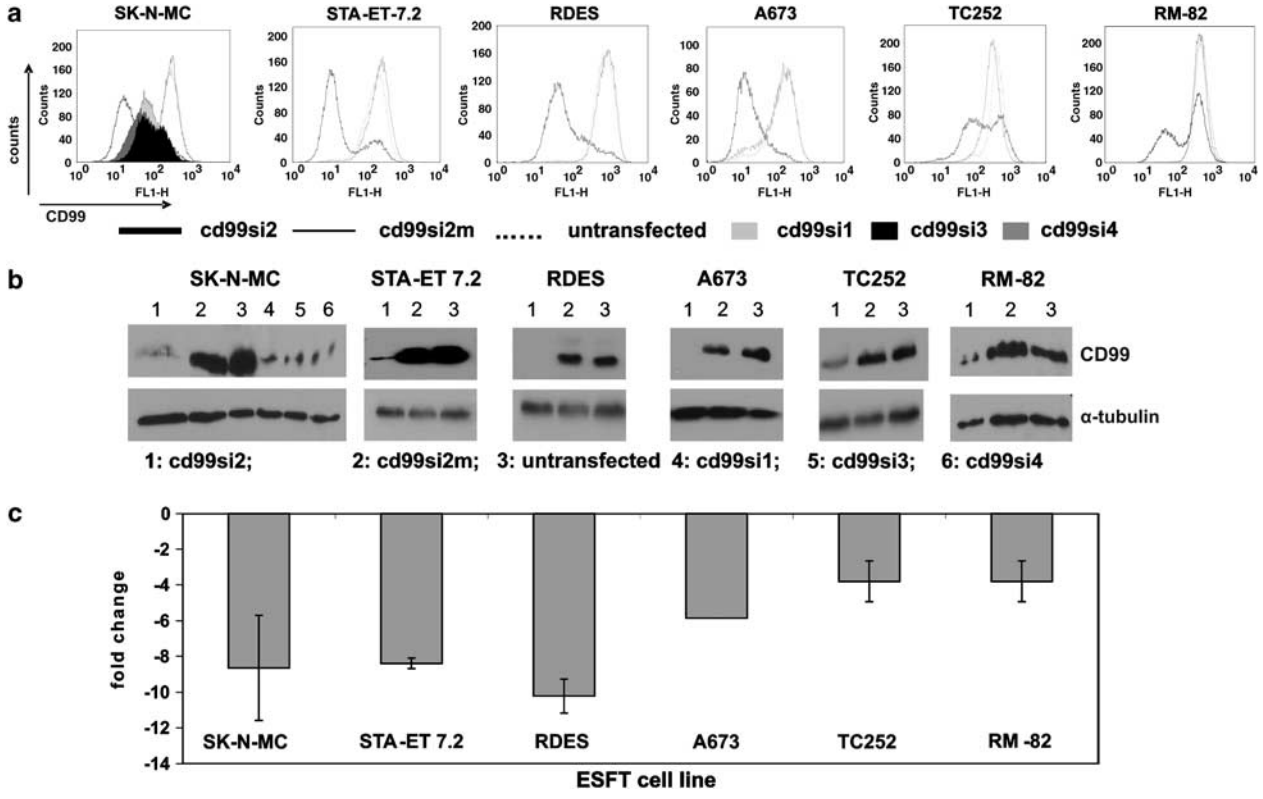

Figure 1 RNAi-mediated silencing of *CD99* in ESFT cell lines. SK-N-MC, STA-ET-7.2, RDES, A673, TC252 and RM82 were transfected twice with *CD99* specifc siRNAs as indicated on days 1 and 3 with a final siRNA concentration of 200 nM using Oligofectamine™ (Invitrogen, Carlsbad, CA). SiRNA sequences (all with dTdT 3′overhangs) were: cd99si1 5′-ACCCAGUGCUGGG GAUGAC-3′; cd99si2 5′-CCCUAGUUCCUCCGGUAGC-3′; cd99si3 5′-AUGCCAACGCAGAGCCAGC-3′; cd99si4 5′-CCCACC CAAACCGAUGCCA-3′; cd99si2m (mismatched control) 5′-CCCUAGUUCAGACGGUAGC-3′; The cells were collected on day 5 after the first transfection and analysed for CD99 expression by either (**a**) flow-cytometry on a FACS-Calibur (BD Biosciences, San Jose, CA), or (**b**) immunoblotting or (**c**) RQ–PCR. Protein expression of CD99 was monitored by monoclonal antibody 12E7 with tubulin as a loading-control. Specific primers and probes for RQ-PCR were: for CD99: sense-primer 5′-TAGGAGATGCTGTTGTT GATGGA-3′, antisense-primer 5′-GGATTTGGCATCGGTTTGG-3′, probe 5′-AAAATGACGACCCACGACCACCGAA-3′; for beta-2-microglobulin: sense-primer 5′-TGAGTATGCCTGCCGTGTGA-3′, antisense-primer 5′-TGATGCTGCTTACATGTCTC GAT-3′, probe 5′-CCATGTGACTTTGTCACAGCCCAAGATAGTT-3′. Cycling parameters on the ABI 7700 or 7900 sequence detection system: 2 min at 50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 60 s at 60°C. The beta-2-microglobulin values were used for normalization. *CD99*-silencing is expressed as the fold-decrease deduced from the difference between C_t-values of cd99si2 and cd99si2m transfected cells.

the CD99 mRNA (cd99si2) downregulated CD99 surface-expression by 85% in up to 75% of transfected cells as compared to untreated cells and cells transfected with a mismatched control siRNA (cd99si2m). This result was confirmed by immunoblot analysis of total cell extracts from all transfected cell lines (Figure 1b) and, on the RNA level, by real-time quantitative PCR (RQ-PCR) (Figure 1c). A four- (TC252) to 10-fold (RDES) reduction in CD99 expression was achieved at day 5 after the first transfection. Efficient silencing persisted until day 10 when CD99 levels were still suppressed by more than 50% (not shown).

To investigate the effect of *CD99*-silencing on surfacedependent and independent cell proliferation, equal numbers of cd99si2 or cd99si2m transfected or untreated SK-N-MC cells were either seeded on plastics or in soft-agar. In monolayer cultures, only a small but highly reproducible growth difference was observed three days after seeding (Figure 2a). Identical results were obtained with the cell line STA-ET-7.2 (not shown). No other obvious changes in cell morphology and growth-patterns were observed under this condition (Figure 2b). This result was confirmed by histological examination of paraffin-embedded thin sections

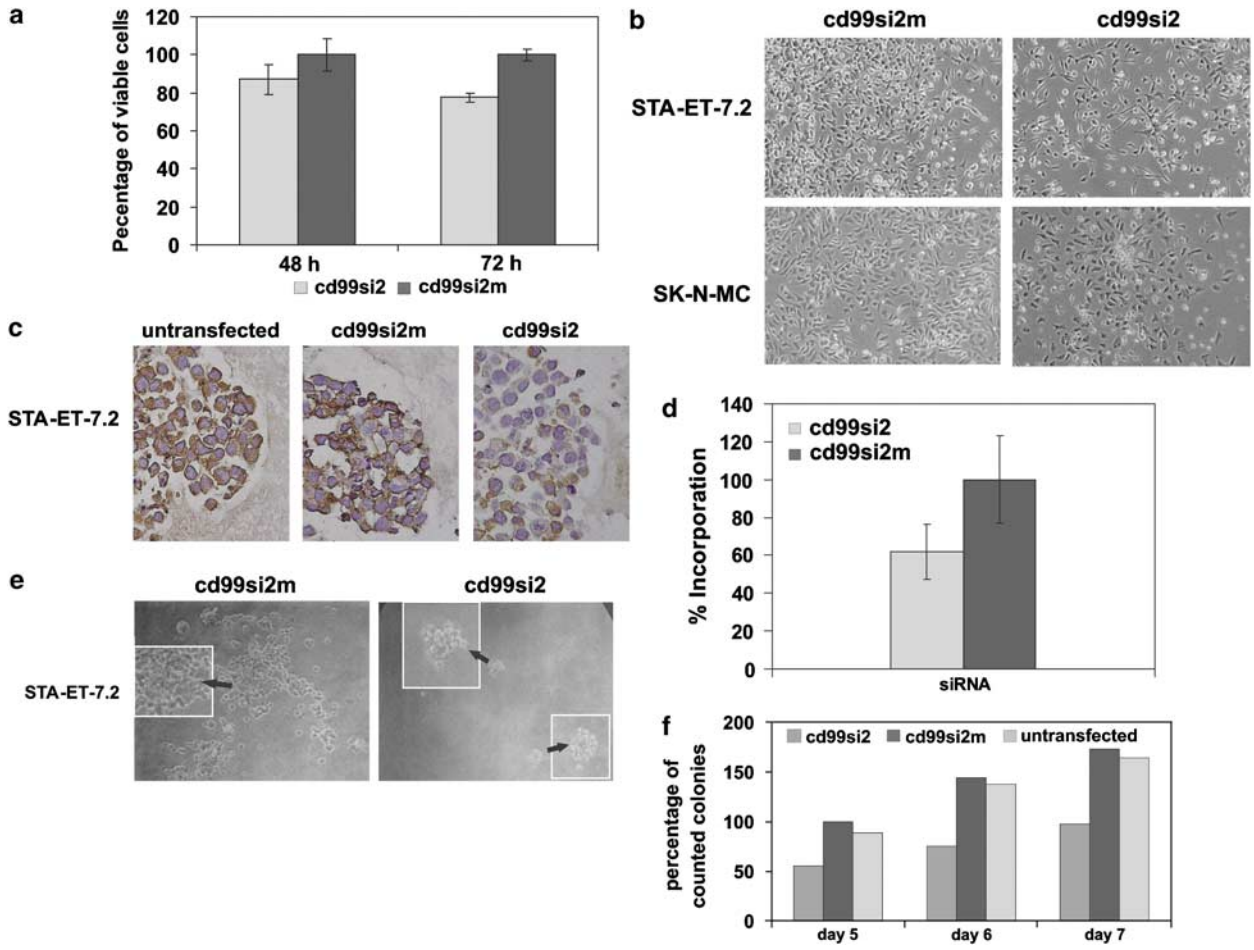

Figure 2 *CD99*-silencing reduces ESFT cell growth. (**a**) SK-N-MC cells transfected with cd99si2 and cd99si2m (negative control) were collected directly after the second transfection and equal numbers seeded on cell culture flasks to assess surface-dependent growth. Vital (Trypan-blue negative) cells were manually counted in a Buerker-Tuerk chamber at 48 and 72 h post-seeding. Mean-values and s.d. from three independent experiments are shown. (**b**) Surface-dependent growth pattern of SK-N-MC and STA-ET-7.2 cells treated as in (**a**) ($\times 10$ magnification). (**c**) Immunohistology of STA-ET-7.2 cells left untreated or treated with cd99si2 or cd99si2m and grown as spheroid cultures for 3 days (day 7 after the first transfection). Spheroids were fixed with 4% paraformaldehyde, sedimented, and embedded in 1.5% agarose to maintain morphology. Samples were subsequently embedded in paraffin and thin sections stained with CD99 antibody 12E7 with hemalum counterstain. (**d**) [^{3}H]thymidine-incorporation assay of cd99si2 and cd99si2m-treated SK-N-MC. Cells were transferred to 96-well flat-bottom tissue-culture plates and incubated with 1 μCi (3.7×10^4 Bq) [^{3}H]thymidine (ICN Biomedicals, Costa Mesa, CA) per well for 18 h. [^{3}H]thymidine-incorporation was assessed by β scintillation counting. Thymidine-incorporation in cd99si2-transfected cells was calculated from three independent determinations and is displayed relative to control transfection with cd99si2m. (**e**) Anchorage-independent growth was determind by seeding of siRNA-transfected STA-ET-7.2 cells into soft-agar on top of a 0.5% agarose-containing basal layer. Colonies were manually counted 5, 6 and 7 days postseeding. Results from a representative experiment are shown. (**f**) Morphological analysis of EFST cell colonies growing in soft-agar. Colonies of cd99si2 and cd99si2m-transfected STA-ET-7.2 cells were checked for morphological features 6 days postseeding ($\times 10$ magnification; details displayed as inserts at $\times 32$ magnification).

obtained at day 7 from cd99si2 and cd99si2m transfected STA-ET-7.2 cells grown as spheroid cultures for 4 days (Figure 2c). Except for the expected difference in CD99 membrane staining decorating the cells contours, immuno staining for mesenchymal (vimentin), epithelial (cytokeratin mix), and neural (monoclonal S100, neurofilament, chromogranin A, synaptophysin, monoclonal neuron-specific enolase) markers was negative except for vimentin in both control and cd99si2 treated cells (data not shown). Together with the apparent lack of neurite-like cellular processes these results are consistent with the absence of differentiation in response to *CD99* silencing. No evidence for increased cell death was obtained by propidium iodide and Annexin V staining (data not shown), but thymidine-incorporation assays suggested that, upon *CD99* silencing, the observed growth-reduction was due to decreased proliferation (Figure 2d). When seeded into 0.35% soft-agar, *CD99* suppressed STA-ET-7.2 cells showed a marked decrease in colony numbers as compared to controls (Figure 2e). Moreover, the morphology of colonies was different. Until day 10 (day 6 after seeding), while untransfected (not shown) and mismatch control transfected cells gave rise to loose colonies with cells penetrating the soft-agar occasionally attaching to the basal layer underneath, cd99si2 transfected cells grew as clumpy compact colonies which were always smaller than the controls (Figure 2e). Afterwards, *CD99*-suppressed colonies started to grow normally and to spread-out consistent with the loss of RNAi-suppression. These results may be considered in context with previous reports on homotypic aggregation and impaired cellular migration of hematopoietic and ESFT cells when CD99 was ligated to antibody (Bernard *et al*., 1995; Scotlandi *et al*., 2000; Schenkel *et al*., 2002).

To directly test the effect of *CD99*-silencing on the mobility of ESFT cells, SK-N-MC and STA-ET-7.2 cells were transfected with cd99si2 and cd99si2m, or were left untreated, and tested for their migration-potential in transwell-chamber assays. Upon modulation of CD99, a 50% reduction of migrating cells was consistently obtained in both cell lines 1 day after seeding (Figure 3). It should be noted that cd99si2 has the potential of modulating both CD99 isoforms. Thus, the observed changes in proliferation and migration of ESFT cells cannot be assigned to a shift in relative expression levels of full-length and truncated CD99 isoforms but is due to the general loss of *CD99* expression. The combined data indicate that CD99 plays a role in both ESFT growth and migration.

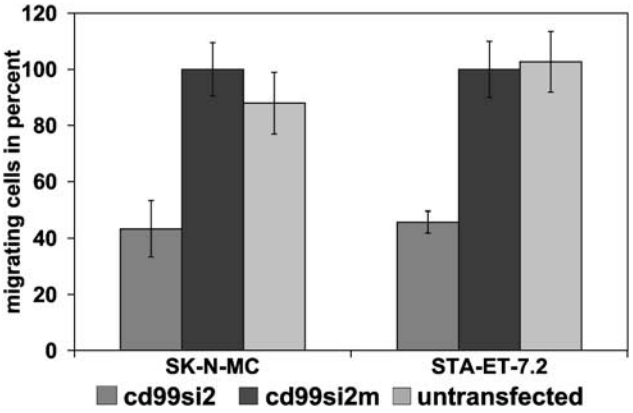

Figure 3 *CD99*-silencing effect on the *in vitro* migratory ability of ESFT cells. 5×10^5 siRNA-transfected and untransfected SK-N-MC and STA-ET-7.2 cells were seeded into the upper compartment of transwell-chambers (Corning, MA) and were incubated for 24 h at 37°C. Cells that migrated through the filter to reach the lower chamber were fixed with 2.5% glutaraldehyde, stained with hemalaun and counted using an inverted-microscope. The number of cd99si2 and untransfected cells that have passed through the filter is presented in percent relative to cd99si2m control transfected cells.

Table 1 Expression-profiling of *CD99*-suppressed cells

Gene name	*Cellline*	*Average fold change*	*UniGene*	*GenBank*
Genes upregulated upon CD99 silencing				
Potassium channel modulatory factor 1	1; 2; 3	2.5	Hs.345694	AI743396
Placental growth factor, vascular endothelial growth factor-related protein	1; 2; 3	2.0	Hs.252820	BC001422
F-box protein 9	2; 3	1.9	Hs.216653	AL137520
DnaJ (Hsp40) homolog, subfamily A, member 2	1; 3	1.8	Hs.368078	AI697792
Myeloid cell leukemia sequence 1 (BCL2-related)	1; 2	1.7	Hs.532826	BF981280
Proenkephalin	1; 2	1.7	Hs.339831	BC010527
SK-N-MC… 1; STA-ET-7.2… 2; TC252… 3				
Genes downregulated upon CD99 silencing				
START domain containing 4, sterol regulated	1; 2	−1.9	Hs.93842	AA628398
BCL2-like 2	2; 3	−1.8	Hs.410026	BC021198
Insulin induced gene 1	1; 2	−1.8	Hs.520819	BG292233
RAB GTPase activating protein 1-like	1; 2	−1.7	Hs.495391	AB019490
Kinesin family member 21B	1; 2	−1.7	Hs.169182	NM_017596
Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, *Drosophila*)	1; 2	−1.7	Hs.258855	Z69744
Early B-cell factor	1; 2	−1.7	Hs.308048	W73890
Homeodomain interacting protein kinase 3	1; 2	−1.7	Hs.201918	AF305239
SK-N-MC… 1; STA-ET-7.2… 2; TC252… 3				

RNA extracted at day 5 post-transfection of cell lines TC252, SK-N-MC, and STA-ET-7.2 with cd99si2 or cd99si2m and tested for efficient CD99-silencing was subjected to comparative gene-expression profiling-analysis on Affymetrix U133A (one experiment) and U133 Plus 2.0 microarrays (three experiments). Only genes displaying a more than 1.6-fold mean expression difference in the comparative analysis of cd99si2 and cd99si2m-transfected cells in at least two of the three cell lines are shown and were considered as putative CD99 downstream genes.

In order to gain insight into the mechanisms by which CD99 regulates ESFT cell growth and migration, we aimed at defining changes in gene-expression patterns associated with modulated *CD99* expression thus determining the end point of *CD99*-signalling. SK-N-MC, STA-ET-7.2 and TC252 cells were transiently transfected twice consecutively with cd99si2 and, for comparison, cd99si2m, in four independent experiments and subjected to expression-profiling by microarray analysis. Using a cutoff value of 1.6-fold, we found 37 probe sets representing 14 genes which were modulated by CD99-silencing in at least two of the three tested cell lines (Table 1). Only two genes, the potassium-channel modulatory factor 1 (*KCMF1*) and placental growth factor (*PGF*), showed consistent upregulation upon RNAi-mediated *CD99*-silencing in all three cell lines. To validate *KCMF1* as a consistent downstream gene of CD99 in ESFT, we performed RQ-PCR analyses on 6 ESFT cell lines with different *EWS* gene-rearrangements 5 days after the first (2 days after the second) transfection. Figure 4a clearly indicates that *KCMF1* was consistently induced up to six-fold by transient CD99 silencing independent of the type of diagnostic *EWS* gene-rearrangement. Reproducibility of this result with all four siRNAs to CD99, as demonstrated for SK-N-MC cells, confirmed the specificity of the effect. These observations suggest that CD99, by an unknown mechanism, generally represses *KCMF1* expression in ESFT.

In addition, we performed Affymetrix GeneChip-based gene expression patterning on a series of 22 primary ESFT (16 localized and six metastatic). Results obtained for *KCMF1* and *CD99* indicated a statistically significant inverse correlation between the expression levels of these two genes (Pearson correlation coefficient $r=-0.558$, $P=0.007$) consistent with the experimentally observed CD99 dependent modulation of *KCMF1* (Figure 4b).

To test, whether *KCMF1* contributes to the observed changes in the migratory ability of ESFT cells, KCMF1 cDNA was cloned from *CD99*-silenced SK-N-MC cells with a C-terminal His-tag into a CMV promoter-based expression vector and was stably introduced into the cell lines SK-N-MC and STA-ET-7.2. When control (empty vector transfected) and KCMF1 expressing polyclonal cell populations were tested in transwell-chamber assays, a marked difference in the migratory ability of ESFT cells was observed (Figure 4c). *KCMF1*-transfected cells showed a consistent reduction in migration similar to ESFT cells with silenced *CD99*. Similar results were obtained with the cell line RDES (not shown). Taken together, these results imply that constitutive CD99 expression supports ESFT cell migration by suppression of *KCMF1* expression in the absence of additional stimulatory CD99 interactions. Since migratory ability is an essential component of tumor-dissemination and ESFT are intrinsically highly metastatic, our results identify *KCMF1* as a CD99-regulated putative metastasis suppressor gene. Future studies will have to test this hypothesis in a xenograft mouse model.

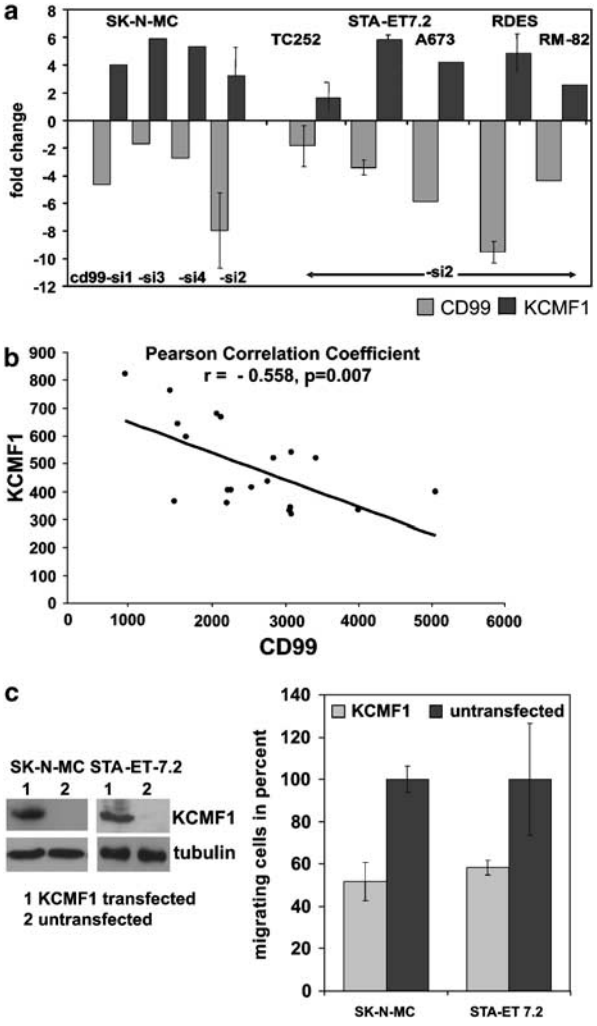

Figure 4 Silencing of constitutive *CD99* expression results in upregulation of *KCMF1* and reduced ESFT cell-migration. (**a**) RNAi-mediated silencing of CD99 upregulates *KCMF1* RNA levels. Cell lines SK-N-MC, STA-ET-7.2, RDES, A673, TC252 and RM-82 were transfected with the indicated siRNAs and, for control with cd99si2m and analysed on day 5 by RQ–PCR for *CD99*, *KCMF1* and control beta-2-microglobulin expression as described for Figure 1c. Primers and probe for *KCMF1* analysis were: sense-primer 5′-CTTGAACACAGAGCCCCTAGAGA-3′, antisense-primer 5′-CGGCCAGGGTGAAACATTC-3′, probe 5′-GATGAATCGAGTGGTGTTCGACATGTACG-3′. Mean values and s.d. of relative *CD99* and *KCMF1* expression-changes in suppressed versus cd99si2m control-transfected cells from three independent determinations performed in triplicate (except for cd99si1, 3, and 4, and A673 and RM82 – single experiments each) are shown. (**b**) Affymetrix GeneChip results for KCMF1 (probe set 217938_s_at) and CD99 (probe set 201029_s_at) expression for 22 primary ESFT at diagnosis (16 localized, 6 metastatic). (**c**) Effect of *KCMF1* overexpression on the *in vitro* migratory ability of ESFT cells. KCMF1 cDNA was amplified from cd99si2-transfected SK-N-MC cells using primers 5′-ATGTCCCGACATGAAGTT GTCA-3′and 5′-AAGAGGAGGTGGTGGAGGCTC-3′, cloned into pcDNA3.1/V5-His©TOPO®TA (Invitrogen, Carlsbad, CA) and transfected into SK-N-MC and STA-ET-7.2 cells. 5×10^5 empty vector or KCMF1 expression vector-transfected cells were seeded into transwell-chambers and analysed for their migratory ability as described in the legend to Figure 3. Expression of ectopic *KCMF1* was monitored in parallel on the immunoblot using anti-His_6-Peroxidase antibody (Roche, Mannheim).

KCMF1, also known as *FIGC* (bFGF-induced in gastric cancer) (Jang, 2004) and, in mice, *DEBT91* (differentially expressed in branching tubulogenesis) (Li *et al*., 2003), encodes a 381 amino-acid molecule with high Ser and Thr composition. As a result of the presence of a putative ion-channel binding-domain, a role in the regulation of potassium-channels has been inferred. On the other hand, sequence analysis suggests that it is a coiled-coil nuclear phosphoprotein with zinc-finger motifs at the N-terminal conserved region forming a novel ring finger motif that displays ubiquitin-ligase activity *in vitro* (Jang, 2004). Modulators of K^+-channels have been demonstrated to have the ability to impair cancer cell proliferation *in vitro* and to counteract cancer progression *in vivo* (Conti, 2004). It has also been reported that K^+-channel modulators are effective inhibitors of brain tumor cell growth and that their growth regulation may be due to the interference with intracellular Ca^{2+}-signaling mechanisms (Lee *et al*., 1993). Considering the reported activities of ubiquitin ligases in signaling and protein stability and of Zn-coordinated ring fingers, the one responsible for the observed inhibitory effect of KCMF1 on ESFT cell migration remains to be established. So far, our results suggest that high CD99 expression levels may contribute to tumor cell-growth and dissemination by interfering with the migration-inhibitory activity of KCMF1. Thus, *KCMF1* represents a good candidate for a metastasis-suppressor gene and strategies to reactivate its expression in ESFT might have the potential to interfere with tumor dissemination, the most dangerous threat to ESFT patients.

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