Re-expression of tumorigenicity after attenuation of human synaptotagmin 13 in a suppressed microcell hybrid cell line

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Abstract. Human chromosome 11p11.2 contains a putative liver tumor suppressor locus that was identified using a microcell hybrid cell line-based model of tumor suppression. Transcription mapping of repressed microcell hybrid cell lines suggests that human SYT13 represents a strong candidate for the 11p11.2 tumor suppressor gene. Other evidence suggests that the putative 11p11.2 tumor suppressor gene (SYT13 or some other) may modulate the tumorigenic potential of neoplastic liver cell lines through direct or indirect regulation of the rat Wt1 tumor suppressor gene. To characterize a functional role for SYT13 in liver tumor suppression, we employed RNAi to attenuate SYT13 expression in a suppressed microcell hybrid cell line (GN6TF-11neoCX4). SYT13-attenuated cells display aggressive phenotypic properties that are similar to or indistinguishable from the parental tumor cells (GN6TF), including altered cellular morphologies, disrupted contact inhibition, elevated saturation densities, and decreased tumorigenicity in vivo. Moreover, SYT13 attenuation and re-expression of tumorigenicity in GN6TF-11neoCX4-derived cell lines was accompanied by a significant decrease of Wt1 expression. In contrast, the phenotypic properties of scrambled-control cells were similar to the suppressed microcell hybrid cells and Wt1 expression was unaffected. These observations combine to establish that: i) human SYT13 functions as a liver tumor suppressor gene that complements a molecular defect in GN6TF rat liver tumor cells resulting in a normalized cellular phenotype in vitro and suppression of tumorigenicity in vivo; ii) RNAi-mediated attenuation of SYT13 expression restores the neoplastic phenotype of GN6TF-11neoCX4 microcell hybrid cells, consistent with the function of a liver tumor suppressor gene; and iii) loss of Wt1 expression is important for the re-establishment of tumorigenic potential by GN6TF-11neoCX4 microcell hybrid cells after attenuation of SYT13.

Introduction

Hepatocellular carcinogenesis is a multi-step process that results from altered expression of multiple genes as a consequence of genetic mutations, epigenetic mechanisms, and/or chromosomal aberrations affecting a number of chromosome arms (1,2), including chromosome 11p (3-6). Comparative mapping studies suggest strongly that hepatocarcinogenesis in humans and rodents may be governed by orthologous tumor suppressor genes (7). We exploited this supposition to identify human liver tumor suppressor genes using a functional model for tumor suppression based on microcell-mediated transfer of human chromosome 11 into the aggressive rat liver tumor cell line GN6TF (8). The resulting microcell hybrid cell lines exhibit normalized cellular morphology, re-expression of contact inhibition, complete loss of anchorage-independent growth potential, and decreased tumorigenicity in vivo, suggesting that human chromosome 11 contains one or more liver tumor suppressor genes (8). Genomic mapping localized the liver tumor suppressor locus to a small (<1 Mbp) region of human chromosome 11p11.2 containing a number of candidate genes (9,10). Subsequently, transcription mapping identified several candidate 11p11.2 tumor suppressor genes (including TP53I11, PRDM11, LOC219638 and SYT13) that were expressed in a panel of suppressed microcell hybrid cell lines (11,12). SYT13 mRNA was detected in 100% of suppressed microcell hybrid cell lines and was absent in all non-suppressed clones, including a non-suppressed microcell hybrid cell line that contains chromosome 11 with an...
interstitial deletion at the suppressive 11p11.2 locus (11,13). Finally, SYT13 expression was lost in all microcell hybrid-derived tumor cell lines examined, coordinate with re-expression of the tumorigenic phenotype (11). Based on its differential expression among microcell hybrid cell lines, SYT13 emerged as an excellent candidate for the human 11p11.2 liver tumor suppressor gene. The mechanism of SYT13-mediated tumor suppression in this model system is not yet known. However, induction of rat Wt1 gene expression accompanies suppression of tumorigenicity by human 11p11.2 (14), suggesting that the 11p11.2 liver tumor suppressor gene (SYT13 or some other gene) may directly or indirectly regulate Wt1 gene expression. Based upon the results of these studies, we propose that: i) SYT13 represents an excellent candidate for the human 11p11.2 liver tumor suppressor gene; and ii) the tumor suppressor function of SYT13 may be mediated through (or require) expression of the rat Wt1 gene.

To characterize the mechanistic role of SYT13 in this model of liver tumor suppression, we analyzed phenotypic alterations in the suppressed microcell hybrid cell line GN6TF-11^exo^CX4 after stable silencing of human SYT13 utilizing RNA interference (RNAi). Cells were transfected with an expression vector encoding a SYT13-specific short hairpin dsRNA (shRNA), resulting in a severe attenuation of SYT13 protein expression. SYT13-targeted cell lines display aggressive phenotypic properties similar to the parental GN6TF tumor cells, consistent with the suggestion that SYT13 functions as a tumor suppressor in this model system. Cell morphologies were altered, saturation densities were elevated, anchorage-independent growth was restored, and tumorigenicity in vivo was significantly increased. In addition, rat Wt1 gene expression levels were depressed in SYT13-attenuated cell lines, suggesting that loss of Wt1 may contribute to the expression of the aggressive phenotypic characteristics. In contrast, cell lines expressing a scrambled control SYT13 shRNA retain a robust level of Wt1 expression and display a phenotype both in vitro and in vivo similar to that of the suppressed GN6TF-11^exo^CX4 cells. These observations combine to establish that: i) human SYT13 functions as a liver tumor suppressor gene that complements a molecular defect in GN6TF rat liver tumor cells resulting in a normalized cellular phenotype in vitro and suppression of tumorigenicity in vivo; ii) RNAi-mediated attenuation of SYT13 expression restores the neoplastic phenotype of GN6TF-11^exo^CX4 microcell hybrid cells, consistent with the function of a liver tumor suppressor gene; and iii) loss of Wt1 expression is important for the re-establishment of tumorigenic potential by GN6TF-11^exo^CX4 microcell hybrid cells after attenuation of SYT13, consistent with the possibility that Wt1 is the ultimate tumor suppressor gene in this model system.

Materials and methods

Cell lines and culture. The rat liver epithelial tumor cell line GN6TF (15) was derived from a normal rat liver epithelial cell line (termed WB-F344) that has been described previously (16). Microcell hybrid cell lines were established from GN6TF rat liver tumor cells (8,13) by microcell-mediated chromosome transfer of human chromosome 11, using established methods (17,18). Clonal GN6TF-11^exo^CX4 microcell hybrid cell lines used for chromosome deletion and transcription mapping have been described (11). In the current study, GN6TF-11^exo^CX4 cells were transfected with shRNA expression constructs (as described below). All phenotypic and gene expression-related comparisons were made relative to GN6TF tumor cells and GN6TF-11^exo^CX4 microcell hybrid cells. All cell lines were cultured in Richter’s minimal essential medium supplemented as described (19). GN6TF-11^exo^CX4 cells (and derived cell lines) were maintained in medium containing 800 μg/ml G418 (8). After transfection with shRNA vectors, cell lines derived from GN6TF-11^exo^CX4 cells were maintained in medium containing 180 μg/ml Zeocin (Invivogen, San Diego, CA).

Short hairpin-RNA expression vectors. Expression vectors for SYT13 shRNA (and scrambled control shRNAs) were constructed using psiRNA-hH1zeo (InvivoGen). This plasmid contains a Bbs1 cloning site downstream of the H1 promoter which drives the expression of a short dsRNA hairpin structure, as well as an Sh Ble expression cassette (under the control of the EM7 promoter) which confers resistance to Zeocin. Targeting oligodeoxynucleotides (designated SYT13-i), designed according to guidelines provided by InvivoGen, were directed against a 21 base pair target in exon 2 of hSYT13 (Fig. 1) which has 71% (15/21) sequence identity to the rat Syt13 gene. Control oligodeoxynucleotides (designated SYT13-s) were generated by scrabblesnucleotides of the SYT13-i targeting sequence (SYT13-i, 5'-AGA CTA TTC ACT GAG GTC TAC-3'; SYT13-s, 5'-A GC ATA TTC CAT GAG GTC TAC-3') (Fig. 1). Oligodeoxynucleotides corresponding to sense and anti-sense strands for both the SYT13-i and SYT13-s sequences were synthesized by the UNC Oligodeoxynucleotide Core Facility (Chapel Hill, NC). These oligodeoxynucleotides were denatured, annealed, cloned into the psiRNA-hH1zeo plasmid and propagated in E. coli GT116 (InvivoGen). Plasmids were purified using the Wizard Midiprep kit from Qiagen (Madison, WI) and sequenced by the UNC DNA Core Sequencing Facility to verify the construction of the shRNA expression cassettes.

Production of stably transfected SYT13-targeted and control cell clones. Transfection and stable integration of DNA vectors into the GN6TF-11^exo^CX4 cell line was achieved using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the protocol provided by the manufacturer. Purified plasmid DNA (5 μg) was linearized by SwaI digestion and then transfected into 8.6x10^3 cells. Successfully transfected cells were selected in 180 μg/ml Zeocin (InvivoGen), and individual cell lines (designated with the prefix SYT13-i and SYT13-s, respectively) were cloned isolated and maintained in culture as previously described (11).

Gene expression analysis. Transcriptional analyses of SYT13-i and SYT13-s cell lines included semi-quantitative RT-PCR and/or quantitative real-time RT-PCR for various gene targets. Total cellular RNA was isolated from cultured
cells using RNeasy (Qiagen, Valencia, CA) according to the manufacturer’s guidelines. For quantitative reverse transcription-PCR, oligodeoxynucleotides to rat Wt1 were designed around intron-exon boundaries using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). Each multiplex real-time RT-PCR was carried out in triplicate in a 30 μl volume [2X ABsolute QPCR ROX Mix (ABgene, Rochester, NY), 0.1 μg/μl of each primer, 20 μM TaqMan probe, 5 U Reverse-iT Rtase Blend (ABgene), 100 ng total RNA] for 30 min at 48˚C for reverse transcription, 10 min at 95˚C for initial denaturing, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min in the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Values for Wt1 expression were subtracted from the same-tube expression of Gapdh and relative quantitation of the individual reactions were normalized to GN6TF-11 neoCX4 mRNA using the Comparative (2ΔΔCt) method described in User Bulletin #2: ABI Prism 7700 Sequence Detection System. The following primers and probes were used: rat Gapdh forward, 5'-ATG GGT GTG AAC CAC GAG AA-3', reverse, 5'-GGC ATG GAC TGT GGT CAT GA-3', probe, 5'-fam CAC CAC CAA CTG AGG TAG TCG CTC TAT ACC-3' tamra, and rat Wt1 forward, 5'-TCT TCC GAG CAA TTC AGG AT-3', reverse, 5'-TTC AGT TGA GCC AGG AAC-3', probe, 5'-fam CTC CAG GTA CAG GCA GCC GCA-3' tamra. Semi-quantitative RT-PCR was performed as described previously (11). RNA (1 μg) was reversed-transcribed with RETRO script (Ambion, Austin, TX). PCR reactions consisted of 2 μl RT reaction in 50 μl of a buffer consisting of 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2 mM MgCl₂, 200 μM of each dNTP (EasyStart PCR Mix-in-a-Tube, Molecular Bio-Products, San Diego, CA), 0.4 μM of each primer, and 2.5 U AmpliTaq enzyme (Perkin-Elmer Applied Biosystems). Amplifications were carried out in a Perkin-Elmer Thermo-cycler using a step-cycle program consisting of 30 cycles of 94˚C for denaturing (1 min), 55-65˚C for annealing (1 min) and 72˚C for extension (2 min). PCR products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1 mM EDTA (pH 8.0) and visualized by ethidium bromide staining. The following primers were used: h SYT13 forward, 5'-TTA ACA ATG TGG ACA TCT GTT TAGA-3', reverse, 5'-TTA GTC TAT GAC ATC TGG CTA CATG-3' (11); PRDM11 forward, 5'-CTT GGG GAT GAC CTC GTT TA-3', reverse, 5'-AAA GCT TCC AGC AAG TGG AC-3'; TP53I1 forward, 5'-TGT GGA ACG CTC TCT ACA CG-3', reverse 5'-TTC GGC CGA CTT GGT AAT AG-3'; and β-actin forward, 5'-AGA GAT GGC CAC GGC TGC TT-3', reverse, 5'-ATT TGC GGT GGA CGA TGG AG-3' (11). Unless otherwise noted, oligodeoxynucleotide primers were designed using the Primer3 PCR primer program (http://frodo.wi.mit.edu/) (20).

Affymetrix microarray analysis. Large-scale gene expression analysis was performed by Expression Analysis (Durham, NC) (www.expressionanalysis.com) using the Affymetrix GeneChip Rat Genome 230 2.0 Array (Affymetrix, Santa Clara, CA). Target RNA was prepared and hybridized
according to the Affymetrix Technical Manual as previously described (21) and statistical analysis was performed by Expression Analysis proprietary methods. Expression levels of select genes (including interferon-associated genes and potential off-target genes) were mined from the microarray data.

**Western blot analysis.** A rabbit polyclonal antibody directed against hSYT13 was generated by Anaspec (San Jose, CA). The antibody was raised against a 14 amino acid sequence (RDQDPPPLEAKPSSL) located in the linker region between the transmembrane and C2A domains of hSYT13 (corresponding to amino acids 41-57). This peptide target was chosen based on its antigenicity and divergence from the rSyt13 homolog (57%, 8/14 amino acids). Rabbits (n=2) were initially immunized with 4 mg KLH-conjugated peptide in complete Freund’s Adjuvant, and subsequently immunized at 3, 6 and 10 weeks with the KLH-conjugated peptide in incomplete Freund’s Adjuvant. Serum was collected 7 and 11 weeks after the initial immunization.

Cells intended for Western blot analysis were lysed in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] and subjected to Western analysis using standard methods. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL). Lysates (50 μg protein) were fractioned by SDS-PAGE on 10% polyacrylamide resolving gels and transferred to nitrocellulose membrane (BA85, 0.45 μm pore size, Schleicher & Schuell, Keene, NH). The hSYT13 antibody was used at a dilution of 1:1000 in 5% milk/TBST [10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.05% Tween-20]. Bound primary antibody was detected using a goat anti-rabbit IgG peroxidase conjugate (Sigma Chemical Company, St. Louis, MO, A-9169; 1:10,000) and visualized by chemiluminescence using the Amersham ECL kit (GE Healthcare, Buckinghamshire, UK).

**Phenotypic characterization of shRNA-transfected cell lines.** The morphologies of cells in culture were evaluated by phase contrast microscopy. For determination of saturation densities in monolayer cultures, cells were plated at a density of 1.25x10^5 cells/60-mm dish and maintained in culture with medium changes every 3 days. At the end of 14 days, cells were harvested and counted using a Model Z1 particle counter (Beckman Coulter, Fullerton, CA). Anchorage-independent growth was assessed as described previously (15). To determine tumorigenicity of the cell lines, suspensions containing 1x10^6 cells were injected into dorsal subcutaneous tissue of 1-day-old Fischer 344 rats (Charles River Laboratories, Inc., Wilmington, MA). Animals were euthanized when tumors reached an approximate diameter of 1 cm or after 10 months. Studies involving the use of animals were carried out in accordance with federal and institutional guidelines put forth by the National Institutes of Health and the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Statistical analysis.** Data in the text, table and figures represent mean values ± the standard error of the mean. Statistical analysis of real-time PCR data was accomplished using one-way ANOVA (KaleidaGraph, Reading, PA). The statistical significance of linear correlation coefficients was calculated using VasserStats Significance of Correlation Coefficient Calculator (http://faculty.vassar.edu/lowry/rsig.html). The statistical significance of data from saturation density and anchorag-independent growth assays was assessed using the Student’s t-test for unpaired data with unequal variance (KaleidaGraph). Statistical significance associated with Kaplan-Meier survival analysis was determined using the log-rank test (GraphPad Prism, San Diego, CA).

**Results**

**SYT13 expression in SYT13-i and SYT13-s cell lines.** Introduction of human chromosome 11 into the tumorigenic GN6TF cell line resulted in suppressed microcell hybrid cell lines, including GN6TF-11neoCX4 (8). Selected GN6TF-11neoCX4-derived transfected cell lines that carry the psiRNA vectors for SYT13-targeting (designated SYT13-i3, SYT13-i5, SYT13-i6 and SYT13-i12) or scrambled-control (designated SYT13-s1 and SYT13-s8) were expanded and SYT13 knockdown was assessed by Western blot. The SYT13 protein is approximately 66 kDa in size (22,23), but synaptotagmin protein family members are known to form SDS-resistant dimers (24). Both monomeric and dimeric forms of immunoreactive hSYT13 protein were detected in GN6TF-11neoCX4 (Fig. 2a). GN6TF cells express significantly reduced levels of rSyt13 protein compared to GN6TF-11neoCX4 cells, and there is a complete absence of the dimeric form of the protein. The amount of SYT13 detected in GN6TF-11neoCX4 cells reflects the contribution of the human allele in the microcell hybrid cell line. All SYT13-i1 cell lines examined (SYT13-i3, SYT13-i5, SYT13-i6 and SYT13-i12) express less SYT13 protein than the GN6TF-11neoCX4 cells (Fig. 2a). The diminished SYT13 protein level among these cell lines was most pronounced when the dimeric form of the protein is considered (Fig. 2a). In contrast, the SYT13-s1 cell lines examined (SYT13-s1 and SYT13-s8) express levels of SYT13 protein that are similar or indistinguishable from that observed in GN6TF-11neoCX4 cells (Fig. 2a).

**Short hairpin RNA-mediated interferon response and potential off-target effects.** Transfection of cells with shRNA can result in an interferon response (25-27) or off-target effects (28). In studies that have shown an interferon response to shRNA transfection, numerous interferon-associated genes were found to be induced 2- to 5-fold (27). The gene expression profile of both SYT13-i1 and SYT13-s1 cell lines (including SYT13-i3, SYT13-i5, SYT13-i12 and SYT13-s1) were evaluated by microarray analysis to determine the levels of expression of 35 interferon-associated genes. There was minimal change in gene expression for these genes among SYT13-i1 and SYT13-s1 cell lines when compared to GN6TF-11neoCX4 cells; 34/35 (97%) interferon-associated genes were expressed at levels equal to or less than that
observed in GN6TF-11neoCX4 cells (data not shown), and the other (Isg20, interferon stimulated exonuclease 20) was modestly induced (2-fold). These results suggest that minimal interferon-associated gene expression changes accompany the transfection of SYT13-i or SYT13-s shRNA expression vectors in GN6TF-11neoCX4 cells. siRNAs have been reported to affect translation of unintended transcripts containing partial complementarity to targets (29,30). A BLAST search (http://www.ncbi.nlm.nih.gov/blast/) of the 21 nucleotide sequences corresponding to targets (29,30) revealed that human genes (PRDM11 and TP53I11) carried on human 11p11.2 were located within a ~350 kbp region of human 11p11.2. Semi-quantitative RT-PCR analysis of PRDM11 and TP53I11 failed to detect any change in expression of these genes between GN6TF-11neoCX4 cells and SYT13-i and SYT13-s cell lines analyzed (including SYT13-i5, SYT13-i6, SYT13-i12, SYT13-s1 and SYT13-s8), suggesting that knockdown of SYT13 does not result in secondary alterations in the mRNA expression of these genes (data not shown).

Our previous studies identified a correlation between human 11p11.2-mediated rat liver tumor suppression and induction of rat Wt1 (14). Based on this observation we hypothesized that the molecular mechanism governing rat liver tumor suppression by human 11p11.2 may directly or indirectly involve this previously identified tumor suppressor gene (14). Thus, if the microcell-mediated introduction of SYT13 into the cells is responsible for the induction of Wt1, we expect the expression of Wt1 to be diminished in SYT13-attenuated cell lines. The parental GN6TF tumor cells express Wt1 at a significantly lower level than the suppressed GN6TF-11neoCX4 cells (Fig. 2b). Likewise, the level of Wt1 mRNA was significantly decreased in cell lines transfected with SYT13-i constructs, but was unchanged (or higher) in SYT13-s cell lines (Fig. 2b). The loss of Wt1 expression concurrent with attenuation of SYT13 was most pronounced in SYT13-i6 and SYT13-i12 (Fig. 2b). Furthermore, the expression of Wt1 significantly correlates with the level of SYT13 protein found in the SYT13-i and SYT13-s cell lines evaluated (R=0.79, p=0.035). These data: i) support our previous hypothesis that expression of Wt1 is directly or indirectly regulated in response to hSYT13 in this model system; and ii) suggest strongly that Wt1 is an important down-stream effector of SYT13-mediated tumor suppression.

Phenotypic and growth characteristic analyses of SYT13-i and SYT13-s cell lines in vitro. Analysis of morphology, contact inhibition, and anchorage-dependent growth in GN6TF-11neoCX4 cells revealed that human 11p11.2 normalizes the aggressive phenotype of the GN6TF cells in vitro (13,14). After targeting human SYT13 in GN6TF-11neoCX4 cells with shRNAs, we re-examined these characteristics to assess the contribution of SYT13 to the suppression of the neoplastic cell phenotype of GN6TF cells in vitro. GN6TF cells are spindle-shaped and grow in multiple layers, whereas GN6TF-11neoCX4 cells exhibit a flattened and polygonal cell morphology and produce a

shRNAs match 33 off-target genes with >15/21 (71%) sequence identity. However, microarray analysis failed to show significant alterations in mRNA levels of these potential off-target genes, suggesting minimal off-target effects from these shRNAs.

Molecular analysis of SYT13-i and SYT13-s cell lines. RNA samples corresponding to Zeocin-resistant SYT13-i and SYT13-s clones were analyzed by semi-quantitative and real-time RT-PCR to determine RNAi-mediated alterations in the expression of several genes, including human genes (PRDM11 and TP53I11) carried on human 11p11.2 (proximal to SYT13) and rat Wt1, which has been implicated in 11p11.2-mediated tumor suppression in our model system (14). SYT13, PRDM11 and TP53I11 are located within a ~350 kbp region of human 11p11.2. Semi-quantitative RT-PCR analysis of PRDM11 and TP53I11 failed to detect any change in expression of these genes between GN6TF-11neoCX4 cells and SYT13-i and SYT13-s cell lines analyzed (including SYT13-i3, SYT13-i5, SYT13-i6, SYT13-i12, SYT13-s1 and SYT13-s8), suggesting that knockdown of SYT13 does not result in secondary alterations in the mRNA expression of these genes (data not shown).
contact-inhibited growth pattern (Fig. 3a). SYT13-i cell lines retain some polygonal features similar to GN6TF-11™CX4 cells, but exhibit a multilayer growth pattern after reaching confluence in culture (Fig. 3a). The morphologies of control-transfected cell lines (SYT13-s1 and SYT13-s8) were similar to that of the parental suppressed cell line GN6TF-11™CX4 (Fig. 3a). Fig. 3b shows the saturation densities of the SYT13-i and SYT13-s cell lines analyzed. SYT13-i5 and SYT13-i12 grow to an elevated saturation density that is indistinguishable from GN6TF, reflecting multilayer growth. In contrast, the saturation densities of SYT13-s1 and SYT13-s8 were indistinguishable from that of the GN6TF-11™CX4 cells. Fig. 3c shows the anchorage-independent growth potential of the SYT13-i and SYT13-s cell lines analyzed. All of the SYT13-i cell lines efficiently produced colonies in soft agarose, indicative of the restoration of anchorage-independent growth.
Table I. Tumorigenic potential of GN6TF-11neoCX4 and derived SYT13-i and SYT13-s cell lines following transplantation into neonatal Fischer 344 rats.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Tumorigenicity* (%)</th>
<th>Average latency (days)</th>
<th>Latency range (days)</th>
<th>Latency significance (P)</th>
<th>Survival significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN6TF-11neoCX4</td>
<td>4/6 (67)</td>
<td>274±11</td>
<td>256-293</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SYT13-i3</td>
<td>1/5 (20)</td>
<td>201</td>
<td>NA(^d)</td>
<td>NA</td>
<td>0.10 (NS(^e))</td>
</tr>
<tr>
<td>SYT13-i5</td>
<td>6/7 (86)</td>
<td>144±14</td>
<td>110-201</td>
<td>&lt;0.0001</td>
<td>0.04</td>
</tr>
<tr>
<td>SYT13-i6</td>
<td>1/8 (12)</td>
<td>201</td>
<td>NA</td>
<td>NA</td>
<td>0.07 (NS)</td>
</tr>
<tr>
<td>SYT13-i12</td>
<td>8/10 (80)</td>
<td>164±25</td>
<td>87-293</td>
<td>0.002</td>
<td>0.15 (NS)</td>
</tr>
<tr>
<td>SYT13-s1</td>
<td>1/4 (25)</td>
<td>256</td>
<td>NA</td>
<td>NA</td>
<td>0.28 (NS)</td>
</tr>
<tr>
<td>SYT13-s8</td>
<td>3/4 (75)</td>
<td>216±41</td>
<td>153-293</td>
<td>0.28 (NS)</td>
<td>0.48 (NS)</td>
</tr>
</tbody>
</table>

*Tumorigenic potential is expressed as the percentage of animals forming tumors per total animals receiving cell transplants. *Compared to GN6TF-11neoCX4 cells using the Student’s t-test. *Compared to GN6TF-11neoCX4 cells using the log-rank test. *Not applicable; *not significant.

Discussion

Synaptotagmin XIII is an atypical member of the synaptotagmin family of membrane-associated synaptic vesicle transport proteins which largely serve as Ca\(^{2+}\) sensors in vesicular trafficking and exocytosis (22,31). The function of SYT13 is unknown, but appears to lack some conserved functions of other family members (22,23), including calcium-dependent phospholipid binding in vesicle transport and calcium-independent binding to target SNARE heterodimers during calcium-triggered membrane fusion (32). We have identified a potential role for SYT13 in liver tumor suppression. The human chromosome 11 liver tumor suppressor locus was identified through deletion mapping of microcell hybrid cell lines derived from GN6TF rat liver tumor cells (9). The minimal liver tumor suppressor region of human 11p11.2 contains a number of potential candidate tumor suppressor genes (10-12). However, transcription mapping of suppressed and non-suppressed microcell hybrid cell lines reduced the number of viable candidate genes and produced evidence suggesting that SYT13 is the best candidate for the human 11p11.2 liver tumor suppressor (11). hSYT13 is expressed in suppressed microcell hybrid cell lines, but not in non-suppressed microcell hybrid cell lines (11). Additionally, hSYT13 was not expressed in one microcell hybrid cell line that was determined to have an interstitial deletion in the human 11p11.2 liver tumor suppressor region (11,13). Thus, our studies indicate that expression of hSYT13 accompanies 11p11.2-mediated liver tumor suppression, strongly suggesting that SYT13 contributes directly or indirectly to tumor suppression in this model system. GN6TF cells are highly tumorigenic in vivo (100% of host animals develop tumors with short latency) and exhibit aggressive phenotypic characteristics in vitro, including loss of contact inhibition, growth to elevated saturation density and anchorage-independent growth (15,33). Suppression of the neoplastic phenotype of GN6TF cells by introduction of human chromosome 11 results in reduced tumorigenic potential (fewer tumors formed with longer latency), and normalization of the cell phenotype in vitro (restoration of contact inhibition, reduction of...
mediated silencing of expression (11). In the current study, we show that RNAi-mediated tumor suppression in GN6TF rat liver tumor cells. The major phenotypic properties of GN6TF tumor cells, GN6TF-11p11.2 chromosome (8), and the related loss of human SYT13 expression and suppression of tumorigenicity in suppressed microcell hybrid cell lines are summarized. In addition, the relative gene expression levels of SYT13 and Wt1 are indicated for each cell type.

Figure 4. Evidence for SYT13-mediated tumor suppression in GN6TF rat liver tumor cells. The major phenotypic properties of GN6TF tumor cells, GN6TF-11p11.2 chromosome (8), and the related loss of human SYT13 expression and suppression of tumorigenicity in suppressed microcell hybrid cell lines are summarized. In addition, the relative gene expression levels of SYT13 and Wt1 are indicated for each cell type.

satisfaction density, and loss of anchorage-independent growth potential) (8). As summarized in Fig. 4, when human 11p11.2 is introduced into GN6TF cells (resulting in suppressed microcell hybrid cell lines), hSYT13 is expressed coordinate with suppression of tumorigenicity and normalization of the cellular phenotype. In previous studies, re-expression of tumorigenicity in suppressed microcell hybrid cell lines followed loss of the suppressive human 11p11.2 chromosome (8), and the related loss of hSYT13 expression (11). In the current study, we show that RNAi-mediated silencing of SYT13 results in re-expression of the neoplastic phenotype of GN6TF tumor cells (Fig. 4). This result suggests strongly that SYT13 directly or indirectly regulates the growth properties of these cells in vivo. GN6TF tumor cells express the monomorphic species of rSyt13 at easily detectable levels (Fig. 2a), but this protein fails to form dimers. Expression of exogenous hSYT13 (from human 11p11.2) results in the production of both monomeric protein and dimeric complexes (Fig. 2a). In the presence of a dimeric form of SYT13, a molecular defect in GN6TF tumor cells is complemented (and corrected) by a gene from human 11p11.2, resulting in suppression of tumorigenicity. These observations suggest that: i) the endogenous rSyt13 may be defective or non-functional, possibly as a consequence of a missense mutation in the rSyt13 gene; ii) the dimeric form of the rSyt13 protein is required for expression of its tumor suppressive functions; and iii) the endogenous rSyt13 in GN6TF cells is unable to dimerize, resulting in loss of tumor suppressor function.

The molecular mechanism for human 11p11.2-mediated liver tumor suppression has not yet been fully elucidated. However, our previous studies identified a precise correlation between Wt1 expression and suppression of tumorigenicity among microcell hybrid cell lines, suggesting that the molecular mechanism governing human 11p11.2-mediated liver tumor suppression may directly or indirectly involve the induction of rat Wt1 (14). The current study supports this hypothesis. In response to RNAi-mediated attenuation of SYT13, Wt1 expression levels decline coordinate with re-expression of the neoplastic phenotype. This observation suggests a mechanistic linkage between SYT13 and Wt1. The function of SYT13 is unknown. However, Fukuda and Mikoshiba have suggested that this membrane-associated protein is involved in a constitutive pathway that docks transport vesicles to plasma membranes (22). Thus, SYT13 may function in cellular signaling, relaying extracellular signals to the nucleus and eventually contributing to the regulation of transcription. The current study suggests that the rSyt13 expressed in GN6TF cells may be defective (non-functional) as a consequence of some as yet unidentified mutation that results in a failure (or inability) of the protein to dimerize. It is intriguing to speculate that the loss of rSyt13 function interrupts normal cell signaling, resulting in loss of expression of other genes, such as Wt1. It follows that expression of a functional hSYT13 in these cells would restore normal signaling, resulting in re-expression of Wt1.

However, additional studies will be required to establish a direct linkage between SYT13 (either the monomeric or dimeric form) and regulation of Wt1 transcription.

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