Trop-1 Are Conserved Growth Stimulatory Molecules That Mark Early Stages of Tumor Progression

Paola Zanna, PhD[1]
Marco Trerotola, BSc[1,2]
Giovanna Vacca, PhD[1,2]
Veronica Bonasera, PhD[2,3]
Barbara Palombo, RA[1]
Emanuela Guerra, PhD[1,2]
Cosmo Rossi, RA[4]
Rossano Lattanzio, MD[2]
Mauro Piantelli, MD[2]
Saverio Alberti, MD, PhD[1,2]

[1] Laboratory of Experimental Oncology, Consorzio Mario Negri Sud, S.M. Imbaro, Chieti, Italy.

[2] Unit of Cancer Pathology, Department of Oncology and Neurosciences and CESI, Fondazione "G. D' Annunzio," University of Chieti, Italy.

[3] Unit of Cancer Pathology, Department of Oncology and Neurosciences and CESI, Fondazione "G. D' Annunzio," University of Chieti, Italy.

[4] Animal Care Facility, Consorzio Mario Negri Sud, S.M. Imbaro, Chieti, Italy.

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Address for reprints: Saverio Alberti, Unit of Cancer Pathology, Center of Excellence for Research on Aging, University "G. D' Annunzio," via Colle dell' Ara, 66013 Chieti, Italy; Fax: (011) 0871-541-551; E-mail: s.alberti@unich.it

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BACKGROUND. Trop-1 is a cell-cell adhesion regulatory molecule that is overexpressed by a large fraction of tumors in man.

METHODS. To identify fundamental, conserved functional features of Trop-1 in transformed cells, a search was performed for evolutionarily conserved structure, expression patterns, and function by gene cloning, DNA array and serial analysis of gene expression (SAGE), Northern and Western blotting, flow cytometry, and immunohistochemistry of sequential stages of tumor progression in experimental systems and in man.

RESULTS. *TROP1* genes demonstrate conserved structure and promoter regions with parallel expression patterns (high expression in the small intestine and colon; lower expression in prostate, thyroid, salivary glands, breast, kidney, lung, liver, and spleen; very low levels in skin and stomach; no expression in heart, muscle, and brain). Progenitor cells of different tissues were shown to express Trop-1. Hence, the expression and functional role of Trop-1 were analyzed at successive stages of tumor progression in vitro and in vivo. The findings show that Trop-1 is expressed at early stages of tumor development, eg, in dysplastic lesions and immortalized cells, is sufficient to stimulate cell growth of expressing transformed cells, and is required for tumor growth in vivo.

CONCLUSIONS. The findings identify Trop-1 as a novel determinant of cell growth at early stages of tumor development and as a marker of early stages of development in normal tissues and in cancer, making this molecule a candidate for novel diagnostic and therapeutic procedures. ***Cancer*** **2007;110:452–64.** © *2007 American Cancer Society.*

KEYWORDS: cancer, cell growth, cell transformation, tumor progression.

Trop-1 is a 38 kDa transmembrane type I glycoprotein,[1–3] that is encoded by a single-copy gene (*TROP1/TACSTD1*) in man (h), mouse (m), and rat.[3–5] The extracellular domain of Trop-1 contains an EGF-like domain and a thyroglobulin repeat[2,3] that plays a role in regulating cell-cell adhesion via a 2-step homophilic oligomerization.[6] Homotetramerization occurs first intracellularly; tetramers subsequently bind cognate oligomers at the surface of adjacent cells.[6,7] Notably, the Trop-1 protein is expressed by a large fraction of human cancers,[1,8] among them breast tumors[9,10] (ms. in prep.), squamous lung cancers,[11] and most colon carcinomas,[12] suggesting a role in tumor development.

To identify fundamental, conserved functional features of Trop-1 in transformed cells, we searched for evolutionarily conserved structure, expression patterns and function by gene cloning, DNA array and serial analysis of gene expression (SAGE), Northern and Western blotting, flow cytometry, and immunohistochemistry of sequential stages of tumor progression in experimental systems and in man. Our

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findings demonstrate that Trop-1 expression plays a direct growth-stimulatory role at early stages of tumor development and identify Trop-1 as a marker of stem cells in normal tissues and in cancer.

MATERIALS AND METHODS

Nomenclature

The Trop-1 protein[13] is known under different names, frequently derived from the diverse monoclonal antibody (mAb) used to identify it. The most common ones are GA733–2 (antigen), as recognized by the GA733 or 17–1A mAb[14]; KS-1A[2]; Ep-CAM[6]; ESA (antigen), as recognized by the 323/A3 mAb[15]; MH99, M104, HT-29/26, EGP2, or EGP40.[1,6] The murine Trop-1 is also called G8.8[16] or EGP-314.[5]

Cells

Cell lines representative of different stages of skin carcinogenesis were analyzed.[17,18] C5N is a nontumorigenic, immortalized cell line isolated by single-cell cloning of MCA3D cells. MSC P6 (P6) and MSC P1 (P1) are papilloma lines isolated from dimethylbenzanthracene (DMBA) / TPA-treated *spretus* × CBA F1 mice.[19] B9 is a squamous cell carcinoma from a multiple DMBA-treated *spretus* × CBA F1 hybrid mouse, and A5 is a spindle cell carcinoma variant isolated from the same tumor. CarB is a highly aggressive spindle cell carcinoma isolated from an NIH mouse after DMBA/TPA treatment. MTE 4–14 is a murine immortalized, nontumorigenic epithelial cell line of thymic origin.[20] The human MCF-7 breast cancer and the murine L fibrosarcoma and 3LL Lewis lung carcinoma[21] cell lines are all tumorigenic in vivo. All cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum.

Embryonic carcinoma (ES) cells were obtained from 129S2/SvPas mice.[22] ES cells were cultured over a layer of mytomycin-C-treated mouse embryonic fibroblasts (MEF) in DMEM supplemented with 15% fetal bovine serum FBS and 1000 U/mL recombinant human leukemia inhibitory factor (Chemicon, Milan, Italy). MEF were obtained from C57BL/6 × CD1 mice.

Fresh murine keratinocytes were obtained from shaved skin of Balb/c mice. Briefly, skin samples were treated with collagenase type IV (2 times for 30 minutes). Cell suspensions were filtered and were immediately analyzed by flow cytometry to prevent induction of cell differentiation by in vitro culture.

Mice

Apc^{Min} mice[23] were obtained from Jackson Laboratories (Bar Harbor, Me).[24] C57BL/6 J Min/+ males and C57BL/6 J +/+ females (Charles River, Calco, Italy) were mated and the Min/+ offspring were identified by an allele-specific polymerase chain reaction (PCR)-assay, as previously described.[25] Animals were sacrificed and the small intestine was removed at different stages of disease. Formalin-fixed, paraffin-embedded samples were analyzed as described below.

Transformed cell lines and *TROP1*-transfectants were injected subcutaneously into nude CD1 female mice (Charles River, Portage, Mich). Larger and smaller equatorial tumor diameters were measured weekly. Tumor volume was calculated as described previously.[26]

Procedures involving animals and their care were conducted in compliance with institutional guidelines and with national and international laws and policies.[26,27]

Plasmids

The EGP-314 (*mTrop1*) cDNA (AC: M76124; MGI: 106653) in pGEM4z[5] was a kind gift of Dr. M. Kuehl. The pBJI-neo vector was provided by Dr. M. Davis and was used to express the *mTrop1* and *hTROP1* genes. The pBluescript vector was obtained from Stratagene (La Jolla, Calif). The pEYFP expression vector was obtained from ClonTech (Palo Alto, Calif). The coding sequence of EYFP was removed from the latter and substituted with the *mTrop1* and *hTROP1* sequences.

siRNA design was based on Tuschl criteria,[28] a proprietary Invitrogen algorithm (rnaidesigner.invitrogen.com/rnaiexpress/), predicted binding energies (jura.wi.mit.edu/bioc/siRNAext/), or data mining in validated siRNA data-banks (sonnhammer.cgb.ki.se/siSearch/).[29] Oligos that were identified by more than 1 method or were considered optimal by any individual procedure were subcloned in the pSUPER vector.[30]

Gene Cloning and Mapping by Fluorescence In Situ Hybridization (FISH)

A mouse genomic 129SV FixII l phage library (kindly donated by Dr. M. Davis) was plated and hybridized at high stringency (43°C, 5× SSPE, 50% formamide)[31] with the EGP-314 *mTrop1* cDNA.[5] The 602bp Eco RI/Ban II EGP-314 fragment (3′ end of the *mTrop1* cDNA) was used as a probe for a second round of screening of the same genomic library. Accession numbers of the cloned *mTrop1* gene exons are: AJ489452 (VI), AJ489453 (VII), AJ489454 (VIII), and AJ489455 (IX).

The isolated clones were used to map *mTrop1* in the murine karyotype. A probe specific for the centromeric region of chromosome 17 was used to identify this chromosome. Probes were labeled with digoxigenin-dUTP by nick translation, combined with sheared mouse DNA, and hybridized to normal metaphase chromosomes from mouse embryo fibroblast cells in 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were revealed with fluoresceinated antidigoxigenin antibodies. Counterstaining was performed with DAPI.

DNA Transfection

The *mTrop1* or *hTROP1* cDNAs[2,5] in the pBJI-neo or pEYFP expression vectors and siRNAs were transfected in the MTE 4–14 and L cells by lipofection (Lipofectamine 2000, Invitrogen, San Diego, Calif), following the manufacturer's instructions. Transfected cells were selected in medium containing 500 μg/mL geneticin.

DNA Microarray and Serial Analysis of Gene Expression (SAGE) Analysis

DNA microarray data from fresh murine tissues (U74A, Affymetrix, Santa Clara, Calif) were analyzed as previously described[32] (Gene Expression Atlas, Scripps Clinics, expression.gnf.org/). SAGE analysis[33] was conducted as described previously (cgap.nci.nih.gov/SAGE/) (Fig. 3).

Northern Blot Analysis

The cell pellets or tissue fragments were lysed in GITC/sarkosyl. The cell lysate was loaded on a 4-mL cushion of 5.7 M cesium chloride and centrifuged at 30,000 rpm for 17 hours at 20°C in a SW-41 rotor.[34] RNA samples were electrophoresed in 1% agarose, 2.2 M formaldehyde gels, and transferred to nylon membranes (Bio-Rad Laboratories, Hercules, Calif) for hybridization.[31]

Real-Time PCR

One μg of total RNA was reverse-transcribed with the ImProm-II Reverse Transcriptase (Promega, Madison, Wis) according to standard protocols. Quantitative real-time PCR was performed using an ABI-PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, Calif) and Assay-on-Demand TaqMan probes (Applied Biosystems; Hs00158980: TROP-1; Hs99999905: GAPDH).[35] Each sample was assayed in triplicate and the $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression.[36]

Antibodies

The G8.8 antimurine Trop-1[16] and the HT-29/26 and H-99 antihuman Trop-1 mAb[1] were purified by affinity chromatography of mouse ascites over protein-G sepharose.

Rabbit polyclonal anti-mTrop-1 antisera were generated by subcutaneous immunization with recombinant mTrop-1 produced in bacteria.[37] Anti-mTrop-1 reactive antibodies were purified by binding to a NHS-Sepharose columns (Pharmacia, Piscataway, NJ) conjugated to recombinant mTrop-1, and sequentially eluted with glycine buffer at pH 2.5 and 1.5.

Immunofluorescence Analysis, Cell Sorting, and Bulk Cell Separation

Cell staining was performed in suspension with 0.3 μg of specific primary antibody (or isotype-matched control Ig) per 2×10^5 for 30 minutes at 4°C. Binding was revealed with goat $F(ab)_2$ FITC-conjugated antimouse or antirat immunoglobulin (Coulter-Immunotech, Milan, Italy) for 30 minutes at 4°C.[21] Fluorescence analyses and sorts[38,39] were made with a fluorescence-activated cell sorter (Vantage, Becton Dickinson, Sunnyvale, Calif). Immunofluorescence profiles are from the analysis of 5000 cells.

In Vitro Cell Growth Assays

MTE 4–14 cells were seeded at 3×10^3 cells/well in 96-well plates (5 replica wells) and quantified at daily intervals with the MTT colorimetric assay (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. Cell numbers were quantified in parallel by staining with crystal violet.[40] Briefly, adherent cells were fixed in 10% formalin in phosphate-buffered saline (PBS). A stock solution of 20% crystal violet (Sigma, St. Louis, Mo) in ethanol was diluted to 0.1% in water. Cells were stained for 1 hour, washed extensively with water, and dried. The cell-bound crystal violet was solubilized with 0.2 mL of acetic acid and optical density was read at 550 nm.

Immunohistochemical Analysis

Paraffin sections from human and murine samples were dewaxed in xylene, rehydrated, and incubated in a steam cooker for 20 minutes with 1× basic antigen retrieval buffer (AR-10, BioGenex, San Ramon, Calif). The slides were rinsed with PBS and the endogenous peroxidase was inactivated with 3% hydrogen peroxide. After blocking with 1% bovine serum albumin (BSA), 2% rabbit serum, sections from human samples were incubated overnight with a 1:200 dilution of the H-99 anti-Trop-1 antibody asci-

tes at 4°C. Sections from murine samples were incubated overnight with a 1:250 dilution of a rabbit anti-mTrop-1 at 4°C. After washes in 1× PBST (1× PBS + 0,05% Tween-20), the sections were probed for 30 minutes with a 1:100 diluted biotinylated rabbit antimouse or goat antirabbit IgG, respectively (Vector Laboratories, Burlingame, Calif) and reacted with peroxidase-labeled streptavidin/biotin complexes (DAKO, Glostrup, Denmark). Samples were incubated for 20 seconds with diaminobenzidine and counterstained with hematoxylin.

Computer Sequence Analysis

DNA sequences were analyzed using Genetics Computer Group programs.[41] Ambiguities in exon border assignments were solved by manual curation. The 5′ region of the *mTrop1* gene was cloned in silico by a BLAST analysis of the mouse genome (www.ensembl.org/Mus_musculus/), using the 5′ region of the *mTrop1* cDNA as a query. Due to the poor overall sequence quality of this region several incomplete matches were obtained, with unordered contigs mapping to different chromosomes. Those mapping to chromosome 17E5 were retrieved and manually aligned with the experimental sequences. Murine and human chromosome maps were compared using the Mouse Genome Server (Sanger Center, EBI) (www.ensembl.org/Mus_musculus/), the Human-Mouse Homology Maps (www.ncbi.nlm.nih.gov/Homology/), and the Mouse Genome Informatics Database (www.informatics.jax.org). The consensus sequences for the transcription factor binding sites were identified with the program MatInspector V2.2 (transfac.gbf.de/TRANSFAC/).

RESULTS

Cloning and Chromosome Mapping of the *mTrop1* Gene

Clones of the *mTrop1* gene were isolated by high-stringency hybridization of a 129SV genomic library with an EGP-314 probe.[5] The human *TROP1* and the murine gene were shown to possess a high degree of coding sequence identity (73%) and exact conservation of exon-intron boundaries (Fig. 1). Transcription factor binding sites were identified in the 400 bases that precede the transcription start site of the genes (Fig. 1). Four of the 5 transcription factor binding sites (Sp1, AP-1, deltaEF1, and MZF1) are conserved between the human and murine promoters, but present with a different order in the 2 species, suggesting convergent evolution for a parallel regulatory activity.

Hybridization of murine metaphases with *mTrop1* genomic probes resulted in the specific labeling of chromosome 17, band 17E5 (Fig. 2). This region exactly corresponds to the 2p21 band in the human karyotype where the *TROP1* gene maps.[4] Eighteen genes mapping to the syntenic regions in both species were identified. The closest ones to the *TROP1* genes are Calm1 (Calmodulin 1), MSH2 (*E. coli* MutS homolog), and MSH6 (MutS-alpha 160 kDa subunit) (Fig. 2).

Expression of the Murine *Trop1* Gene in Normal Tissues

Little information is available on the distribution of Trop-1 in murine tissues[5,16] and the issue was raised whether and to what extent the expression and function of *TROP1* in rodents correspond to those in man.[16,42] Thus, the expression of the *mTrop1* gene in normal mouse organs was analyzed.

Oligonucleotide microarrays from murine normal tissues were interrogated for expression of the *mTrop1* mRNA. A 3× median value was utilized as threshold to detect significant overexpression. High levels of expression (>10× median value) were demonstrated in small intestine > colon > prostate > thyroid (Fig. 3). Values between 3× and 10× median were shown in the breast > salivary gland > uterus > epidermis > ovary > stomach. Kidney and bladder > trachea and lung showed *mTrop1* mRNA expression levels just below the 3× median cutoff (Fig. 3). SAGE analysis[33] (cgap.nci.nih.gov/SAGE/) was conducted using *mTrop1*-specific tags (Fig. 3 and data not shown). Tag abundance was normalized per library size. Highest expression was revealed in small intestine, prostate, colon, pancreas, and kidney; expression was detected also in breast, lung, pituitary, and skin.

Significant expression was detected in embryo libraries at very early developmental stages, in embryo stem cells and endodermal tissues precursors. Expression was seen at the earliest stages of embryo development (0.5 days postcoitum, Theiler stages 1–5). A subsequent peak of expression was detected at Day 8.5 of pregnancy in the foregut and hindgut endoderm. This was followed by a second peak at Day 16.5 in the epithelium of the urogenital sinus, large intestine, and small intestine (Fig. 3). Lower levels were detected in the kidney, bladder, ovary, pancreas, lung, thymus, and spleen. After birth, increasing levels were detected in prostatic tissue and in breast during pregnancy.

To extend the suggested relevance of mTrop-1 expression at the earliest stages of embryo development, we analyzed its expression in ES cells, which are totipotent/multipotent stem cells that retain the capacity to integrate in mouse embryos as early as at the 8-cell stage.[43] Remarkably, all ES cells were

FIGURE 1. The *TROP1* promoter. (a,b) Sequence and structure of the murine (a) and human (b) *TROP1* promoters. The first exon, the region upstream of the transcription start site, and the 5′ region of the first intron (in italics) are shown. In bold is the donor splice site of the first intron. Bent arrows indicate the transcription start sites.[5,14] The consensus sequences for the binding sites of known transcription factors are indicated. The core regions of the consensus sites are underlined. Kozak initiation of translation sequences are underlined, the ATG Met translation start codons are doubly underlined. The stricken-through sequence in (b) was identified as an 'insertion sequence' by Linnenbach et al,[3] is not present in other *hTROP1* mRNA sequences[2] and has no correspondence in the mouse. (c) Gap comparison of the murine and human *TROP1* transcribed regions. Exon-exon borders are indicated by red arrows. The 5′ poly-A / cleavage signal sequences are underlined.

stained with the monoclonal G8.8 (unpubl.) or specific rabbit polyclonal antibodies with a bimodal (medium to bright) pattern of expression of the murine Trop-1 (Fig. 3).

Northern blot analysis was utilized to further validate the DNA microarray and SAGE data. Northern blotting confirmed that the *mTrop1* mRNA is highly expressed in the small intestine and colon > duodenum. Clear expression was demonstrated in the kidney > lung. *mTrop1* transcripts were barely detectable in the liver and spleen and were completely absent from brain, heart, and muscle (Fig. 4). Parallel findings were obtained in man[44] (unpublished observation).

Flow cytometry and Western blot analysis detected high Trop-1 expression in normal colon > endometrium > gall bladder > exocrine pancreas > prostate > liver (bile ducts) > kidney. Lower levels of expression were detected in small intestine, lung, breast, and thyroid. Expression was detectable in stomach and skin (Figs. 3, 4, and data not shown). mTrop-1 protein expression levels largely paralleled those of the *mTrop1* transcripts. A notable exception was that of the small intestine, where the relative

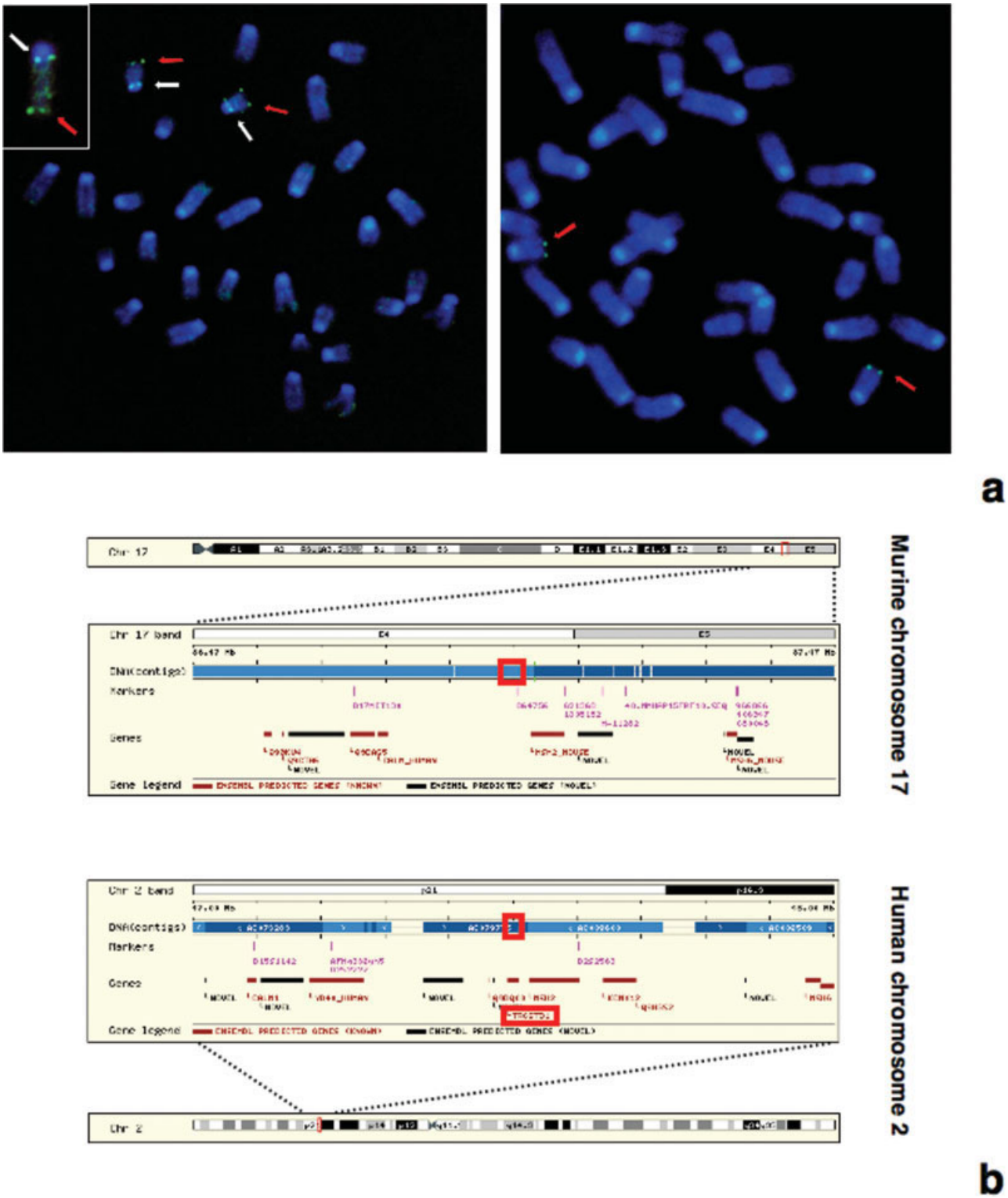

FIGURE 2. *TROP1* gene location and chromosome region maps. (a) Chromosome mapping of the *mTrop1* gene by fluorescence in situ hybridization (FISH); mouse embryo fibroblasts metaphase spreads. Left: Cohybridization with a probe specific for the centromeric region of chromosome 17 (white arrows), and λ phage probes containing the *mTrop1* gene (E5 region; red arrows). A magnified chromosome 17 is shown in the inset. Right: Hybridization of mouse metaphase chromosomes with the *mTrop1* probes only (red arrows). (b) Map of the syntenic region of mouse chromosome 17 and of human chromosome 2. The regions flanking the *TROP1* genes are magnified. Red rectangles indicate the map location of the human (*TACSTD1*, chromosome 2) and murine (*mTrop1, Tacstd1*, chromosome 17) *TROP1* genes.

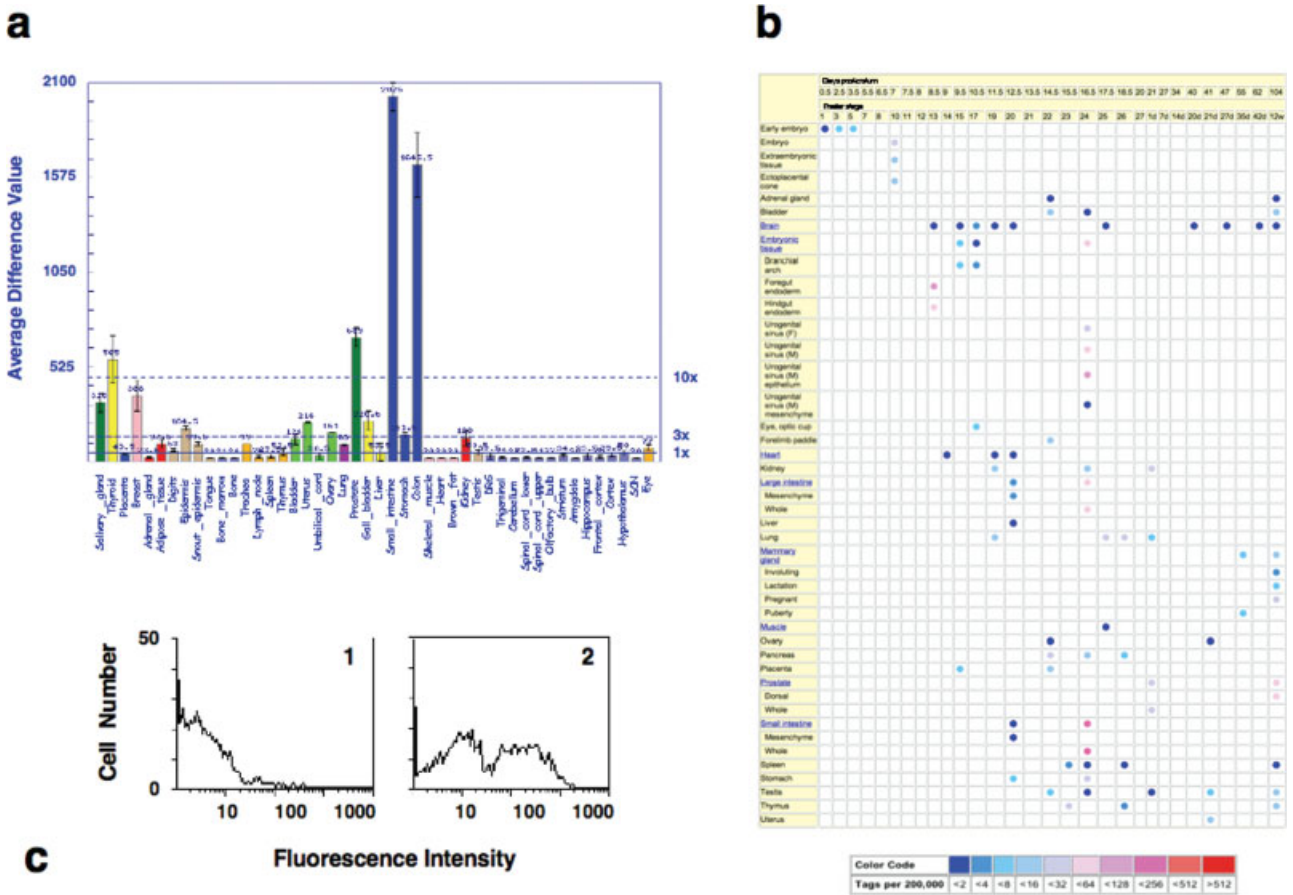

FIGURE 3. Murine *Trop1* mRNA expression during development. (a) DNA microarray analysis. The median level of expression for the whole organism (1×) is indicated by a solid line. A threshold of 3× median identifies the highest expressors of the *mTrop1* gene. Standard deviations of measurements are shown. (b) SAGE expression matrix of *mTrop1* expression throughout development. Each library is represented as a circle defined by tissue and developmental stage; the shading of each circle indicates the levels of gene expression. Days of pregnancy, Theiler stage vs postnatal period are shown. (c) Flow cytometry analysis of ES cells. (1) Goat-antirabbit-FITC antiserum alone; (2) antimurine Trop-1 rabbit antiserum / goat-antirabbit-FITC.

expression levels of the mTrop-1 protein were considerably lower than those of the corresponding mRNA (Figs. 3, 4, 7).

Expression of the Murine *Trop1* Gene During Tumor Progression

Fresh keratinocytes, immortalized keratinocytes, and cell lines at different stages of skin carcinogenesis were analyzed for the expression of *mTrop1*.[17] Fresh keratinocytes display barely detectable levels of the mTrop-1 protein and mRNA (Fig. 4), whereas the highest *mTrop1* levels were shown by the C5n immortalized keratinocytes, with a striking induction of expression upon immortalization. Clear expression of *mTrop1* was detected in the 2P6 papilloma cells and in the SN161 and B9 squamous carcinomas (Fig. 4). No expression was detectable in the Carc-C, Carc-B, and A5 undifferentiated spindle cells and in the 3LL Lewis lung carcinoma line, indicating less stringent requirements for mTrop-1 expression at later stages of tumor progression.

Stimulation of Cell Growth by *mTrop1* and *hTROP1*

The expression of *mTrop1* at early stages of tumor progression was consistent with a potential growth advantage, as was the expression at early stages of embryo development, that are characterized by a rapid growth rate. Hence, we investigated if *mTrop1* could stimulate the growth of expressing cells.

Immortalized MTE 4–14 epithelial cells were transfected with the *mTrop1* and *hTROP1* genes and selected by flow cytometry (Fig. 4). The growth of the *mTrop1* and *hTROP1* transfectants was markedly stimulated compared with vector-alone transfected cells (Fig. 5). No difference was found in the rate of apoptosis of Trop-1-expressing MTE 4–14 cells

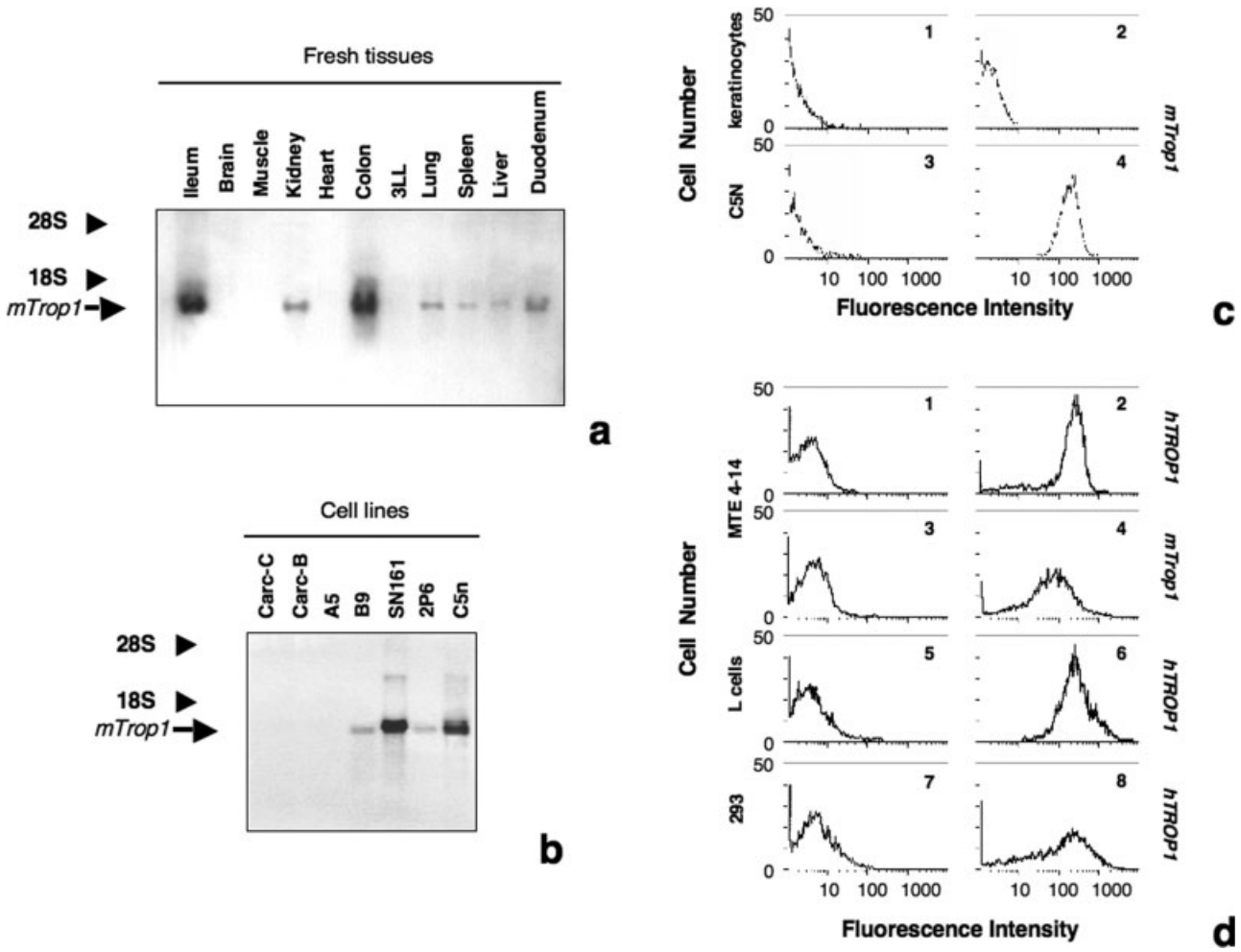

FIGURE 4. Expression of the murine *Trop1* mRNA during tumor progression. (a,b) Northern blot analysis. (a) Fresh murine tissues. 3LL: lung carcinoma cell line; (b) Cell lines. Carc-C, Carc-B, A5: spindle cell carcinomas; B9, SN161: squamous carcinomas; 2P6: papilloma; C5n: immortalized keratinocytes. Filters were hybridized with an EGP-314 cDNA probe[5] (arrow). 28S and 18S rRNA are indicated (arrowheads). (c,d) Flow cytometry analysis. (c) Keratinocyte expression. (1, 3) fresh keratinocytes; (2, 4) immortalized cells. (1, 2) unstained cells; (3, 4) G8.8 anti-mTrop-1 mAb-stained cells. (d) Transfectants. (1, 3, 5, 7) vector-transfected cells and (2, 4, 6, 8) Trop-1 transfectants stained with anti-Trop-1 mAbs.

(unpubl.). The increase in growth rate was of similar magnitude for the human and murine *TROP1* transfectants. Levels of mTrop-1 lower than those of immortalized cells (Fig. 4) were sufficient to stimulate growth, supporting a physiological significance of the growth-stimulatory activity of mTrop-1. Revertant transfectants, ie, cells that had randomly lost the expression of the *mTrop1* gene over time in culture, showed growth rates identical to those of vector-alone transfected cells, confirming that the expression of the *mTrop1* gene is a true inducer of faster cell growth. As MTT assays measure metabolic activity, and may be affected by Trop-1-induced metabolic changes, cell growth was measured with crystal violet assays. Essentially identical results were obtained, confirming that Trop-1 molecules induce a real increase in cell growth rate in vitro.

Inhibition of Cell Growth by *hTROP1* siRNA

siRNA were used to silence *hTROP1* in expressing MCF-7 cells. *hTROP1* mRNA levels in wildtype cells and transfectants were determined by quantitative real-time PCR. Linear response and accuracy of RT-PCR measurements were preliminarily verified. Transfection was performed at time 0 and the growth of the MCF-7 transfectants was measured (Fig. 6). Marked inhibition of cell growth was induced by specific siRNAs, which paralleled the extent of reduction of *TROP1* mRNA levels (Fig. 6). Death of siRNA-transfected cells was also observed, indicating that

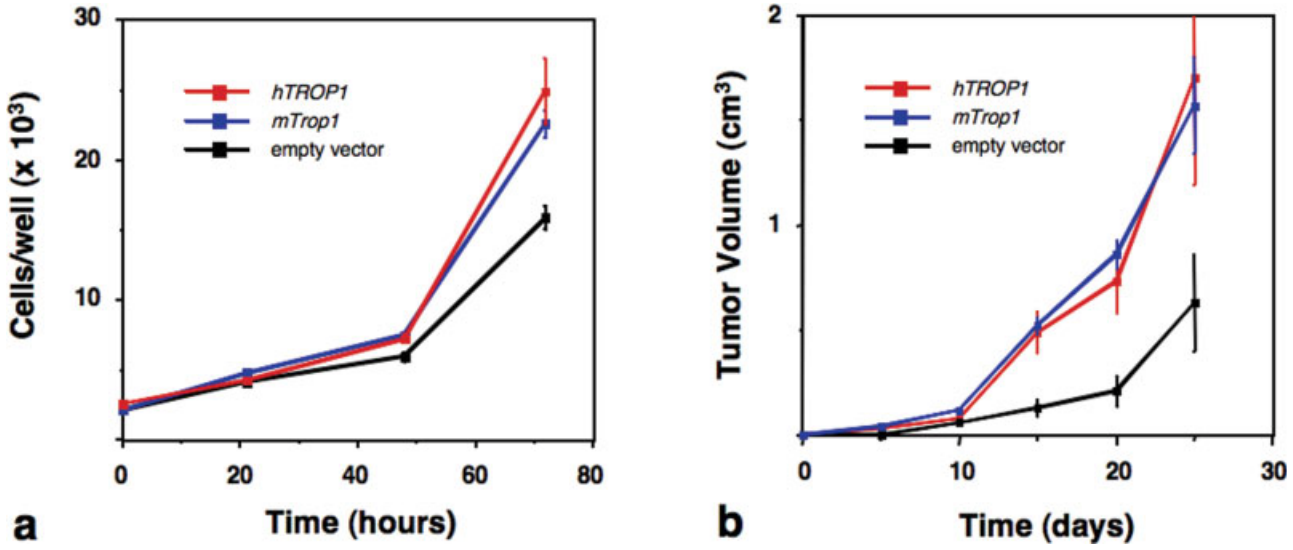

FIGURE 5. Cell growth stimulation by the murine and human Trop-1. (a) In vitro growth rates of MTE 4–14 cell transfectants. (b) In vivo growth rates of L cell transfectants. Cells transfected with vector alone are in black; murine *Trop1* transfectants are in blue; human *TROP1* transfectants are in red.

Trop-1 expression is required for both cell growth and survival.

Expression of Trop-1 at Early Stages of Cell Transformation in Mouse and Man

Investigation of primary in vivo lesions, ie, tumors and preneoplastic lesions, was undertaken in mouse and man to verify a potential relevance of Trop-1 expression at early stages of tumor development in vivo.

Dysplastic aberrant crypt foci demonstrate close relation with tumor development in Apc^{Min} mice.[45] The dysplastic crypts of Apc^{Min} mice are characterized by increased crypt diameter, loss of differentiation of crypt cells, pseudostratification, loss of goblet cells, and increase of nuclear diameter (Fig. 7). Marked induction of Trop-1 protein was observed in Apc^{Min} intestinal glands with early focal hyperplasia / low-grade dysplasia. Increased staining was observed both at the cell membrane and in the cytoplasm.

The expression of hTrop-1 was similarly quantified by immunohistochemistry in preneoplastic lesions of the human stomach (Fig. 8). Immunoreactivity for hTrop-1 is essentially absent in normal gastric mucosa. Conversely, a switch-on of the expression of hTrop-1 was observed in areas of intestinal metaplasia. Metaplastic areas of the gastric mucosa are characterized by well-differentiated intestinal-like epithelium, with goblet cells and enterocytes, both strongly expressing the hTrop-1 protein. Patterns of hTrop-1 immunoreactivity in metaplastic glands include 1) prevalent membrane staining, 2) cytoplasmic staining, or 3) granular patterns. The correspondence between hTrop-1 induction and metaplastic appearance is striking, as stained areas essentially coincide with metaplastic lesions. Highest levels of Trop-1 expression were observed in areas of higher grading/dysplasia and with higher mitotic index.

Stimulation of Tumor Growth by *mTrop1* and *hTROP1*

Transformed cells overexpressing *mTrop1* and *hTROP1* were assayed for growth in immunosuppressed animals (Fig. 5). Strikingly, a dramatic increase in tumor growth was demonstrated in both cases, with an essentially identical stimulation pattern for the murine or the human *TROP1*. A measure of the intensity of the stimulation is provided by comparison with wildtype L cells, which generate aggressively growing tumors that grow to a detectable size with a short latency of 1 week.

DISCUSSION

To identify fundamental, conserved functional roles of Trop-1 in cell transformation, we searched for evolutionarily conserved structure, expression patterns, and function by gene cloning, DNA array and SAGE analysis, Northern and Western blotting, flow cytometry, and immunohistochemistry analysis of sequential stages of tumor progression in experimental systems and in man.

Sequence comparison of the murine *Trop1* with its human homolog demonstrated high conservation of the coding sequence and an exact match of exon-

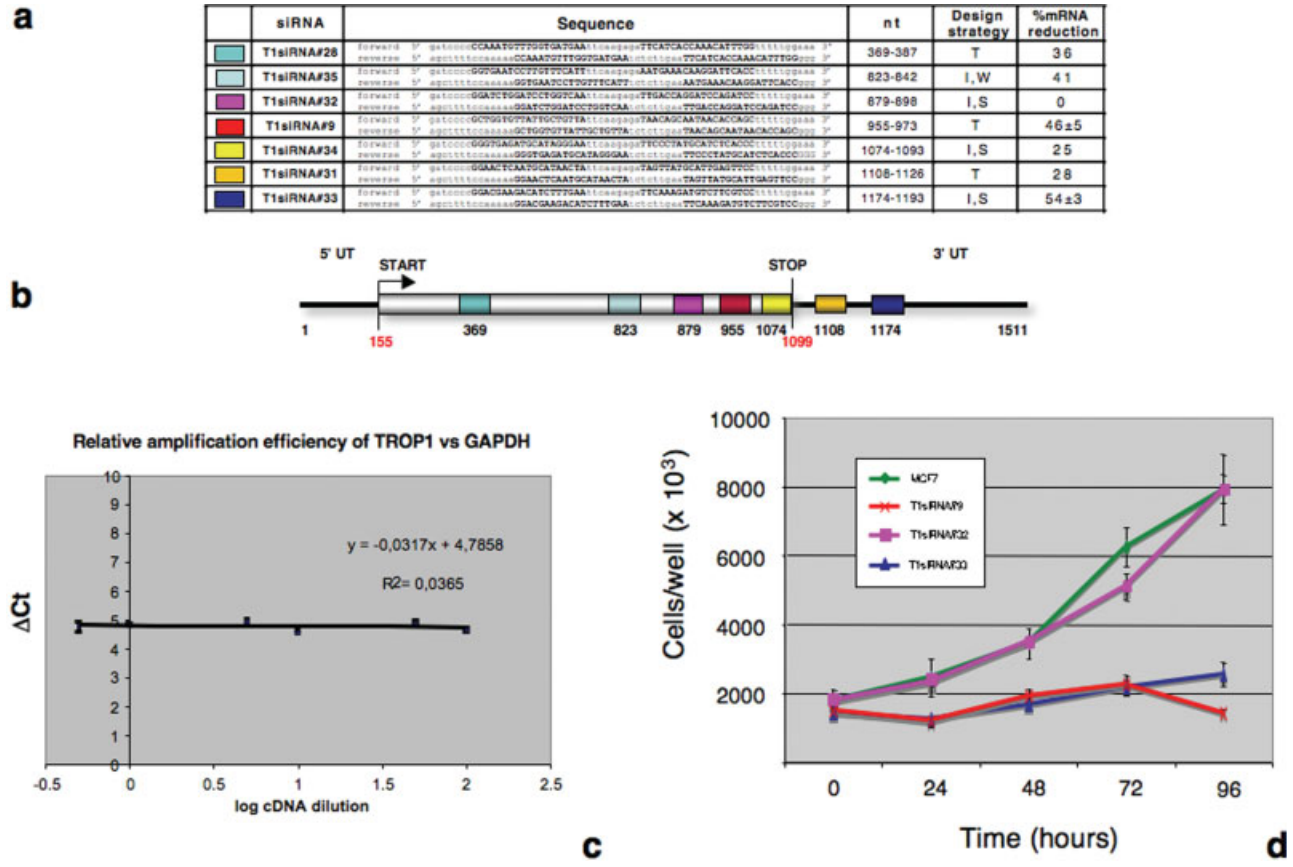

FIGURE 6. Cell growth inhibition by *hTROP1* siRNA. (a)*hTROP1* siRNA oligonucleotide sequences and mRNA location (nt) are indicated. S: Sonnhammer (sonnhammer.cgb.ki.se/siSearch/); W: Whitehead Institute (jura.wi.mit.edu/bioc/siRNAext/); I: Invitrogen (rnaidesigner.invitrogen.com/rnaiexpress/); T: Tuschl criteria.[28] Real-time polymerase chain reaction (PCR) analysis of *hTROP1* mRNA levels in siRNA-transfected cells; mean ± standard error of the mean of the reverse-transcriptase PCR (RT-PCR) measurements are indicated. (b) Box diagram of the *hTROP1* mRNA. Color codes correspond to the siRNA in the top diagram. (c) Linear response of real-time PCR quantification. ΔC_T ($C_{T,\ TROP1} - C_{T,\ GAPDH}$) was calculated for serial cDNA dilutions.[36] (d) Cell growth inhibition by the *TROP1* siRNA. Wildtype MCF-7 cells are in green. Cells transfected with a nonfunctional *TROP1* (*T1*) siRNA (#32) are in magenta. Cells transfected with efficient *TROP1* siRNA are in red (#9) and blue (#33). Standard deviation bars of cell number measurements are shown.

intron boundaries. The 17E5 *mTrop1* map region was shown to correspond in localization and structure to the human 2p21 band where the *hTROP1* gene resides,[4] consistent with a parallel selective pressure for the conservation of the structure/function of the gene in the 2 species.

The promoter regions of the *TROP* genes share key transcription factor binding sites (Sp1, AP-1, deltaEF1, and MZF1), suggesting a common regulatory scheme in mouse and man, for a parallel expression pattern in the 2 species. This was indeed shown to be the case by DNA microarray, SAGE, and Northern blot analysis.[8] Parallel expression levels of the *mTrop1* transcript and protein were observed in most cases. An exception was the small intestine, where the expression of the Trop-1 protein was considerably lower than that of the corresponding mRNA. This suggested a regulated utilization of the *TROP1* mRNA for protein synthesis, eg, for a rapid induction of expression of the protein under specific functional requests.

Of interest, Trop-1 was found expressed by totipotent ES cells and by tissues at very early stages of differentiation (eg, embryonic stem cells, endodermal precursors, urogenital sinus). This expression pattern suggested a requirement for Trop-1 expression in progenitor/early-stage differentiated cells. The expression of Trop-1 by germinal cells[46] and by progenitor cells of diverse epithelial, eg, epidermis, breast, pancreas, liver,[1,47–49] and hematopoietic[50] tissues is consistent with this model.

The findings above and the presence of Trop-1 molecules in highly proliferating tissues (eg, early embryo, fetal and adult colon, breast during preg-

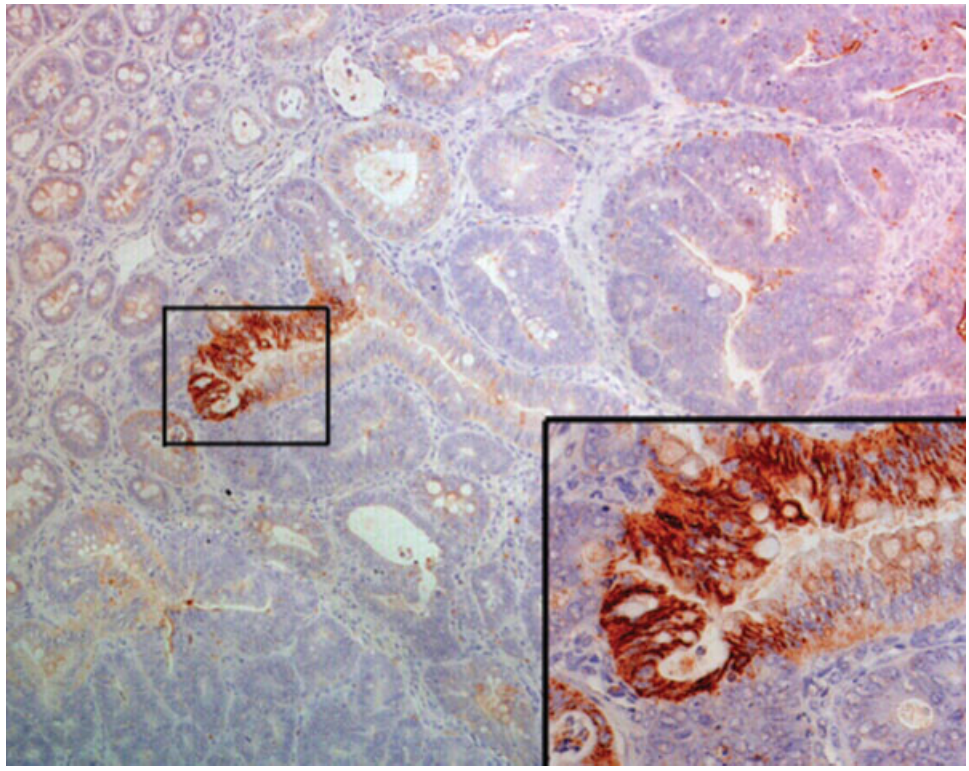

FIGURE 7. Small intestine of *Apc*^{Min} mice: immunohistochemical staining for mTrop-1. Membranous and cytoplasmic staining for Trop-1 protein in murine intestinal glands with early focal hyperplasia / low-grade dysplasia is shown (×20 magnification). A neighboring adenomatous lesion, largely devoid of mTrop-1 expression, is present (right side of the field). Strong immunoreactivity for Trop-1 is restricted to a focal area in the context of dysplastic glands. The positivity, granular and diffuse, is essentially cytoplasmic (insert, ×40). Nondysplastic, hyperplastic glands (upper left corner) only show weak, diffuse cytoplasmic staining. A few neoplastic scattered cells contain some specifically Trop-1 immunostained granules (upper right corner). Trop-1-positive material can also be found within the gland lumina.

nancy) suggested that the requirement for Trop-1 expression resided in its capacity to stimulate cell growth. This was shown to be true in epithelial cell transfectants. The induced increase in growth rate was similar for the murine and human gene transfectants, consistently with a conserved structure of the 2 molecules,[2,3,5,51] and of the corresponding signaling mechanisms in man and mouse. siRNA silencing assays demonstrated the requirement of *TROP1* expression for sustained tumor cell growth and survival. A direct role in tumor development in vivo was demonstrated by the aggressive growth *TROP1*-expressing cells in immunosuppressed animals. More in detail, analysis of cells at various stages of transformation showed very little expression of Trop-1 in fresh keratinocytes, at variance with the high levels of expression in their immortalized counterparts. Of interest, Trop-1 expression is induced by transformation in vitro by the SV-40 T antigen or activated ras,[1] indicating that it is associated with early events of cell immortalization and transformation. Consistently, membrane and cytoplasmic staining for the Trop-1 protein were detected in focal hyperplasia/low-grade dysplasia in intestinal glands, which are precursors of neoplastic lesions in *Apc*^{Min} mice.[45] Similarly, preneoplastic lesions of the stomach in man showed a dramatic induction of hTrop-1, at stark variance with the absence of expression in the normal gastric mucosa. A link with early/causal stages of tumor progression is supported by the expression of Trop-1 in stem cells, eg, totipotent ES cells, which can give rise to tumors with wide differentiative capacity like teratomas.[52] The analysis of breast tumor cells according to patterns of expression of CD24, CD44, and Trop-1/ESA was consistent with this model, as cancer stem cells were found to reside only in the population expressing CD44 and Trop-1/ESA.[53]

Taken together, our findings identify Trop-1 as a marker of proliferating cells in normal tissues and in cancer, and as a novel determinant of cell growth at early stages of tumor development. They also candi-

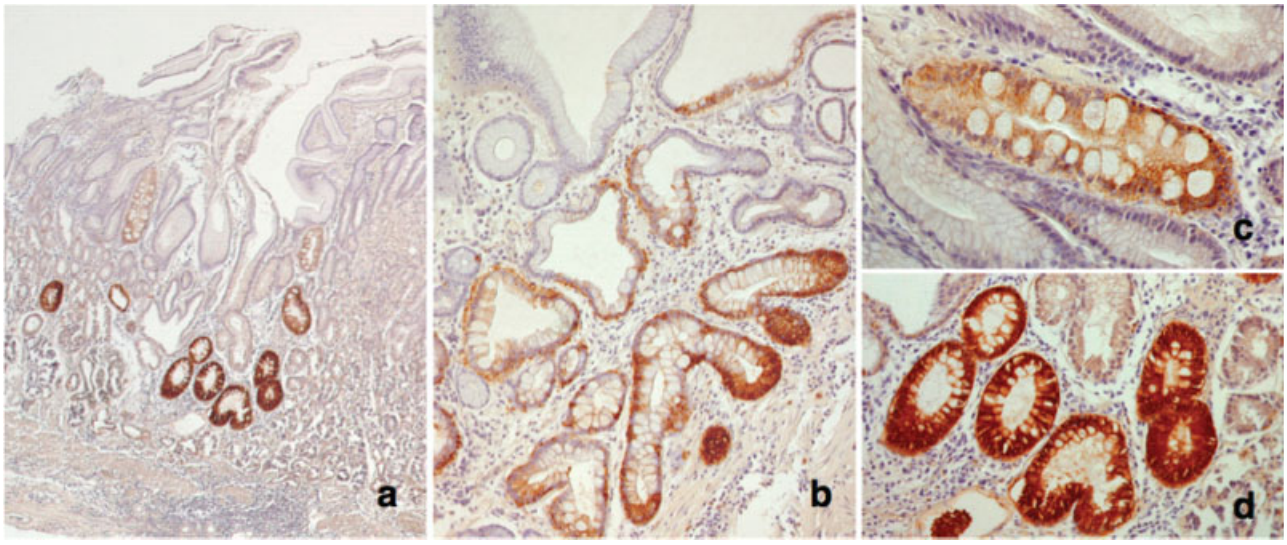

FIGURE 8. Immunohistochemical staining for hTrop-1 in human fundic gastric mucosa with intestinal metaplasia. (a) Immunoreactivity for hTrop-1 is absent in normal gastric mucosa. Positivity for Trop-1 is confined to glands showing intestinal type metaplasia (left panel, ×10). (b) Gastric mucosa changes to well-differentiated intestinal epithelium, as characterized by fully developed goblet cells and enterocytes with strong expression for hTrop-1 protein (central panel, ×20). (c,d) Expression of hTrop-1 is highest in the most hyperplastic areas of intestinal metaplasias; in these a rim of immunoreactivity at cell membrane becomes readily apparent (right panel, ×40). Cell membrane staining for hTrop-1 can be prevalent (c,d) or can be accompanied by an even cytoplasmic staining (a) or by granular patterns (b).

date hTrop-1 as a novel target of diagnostic/screening procedures for early cancer detection.

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