Characterization of the murine 5T4 oncofoetal antigen: a target for immunotherapy in cancer

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Human 5T4 oncofoetal antigen defined by the murine 5T4 monoclonal antibody is a highly glycosylated protein expressed by trophoblast and a few specialized adult epithelia. Up-regulation of 5T4 expression in some cancers is associated with poor clinical outcome; overexpression of human 5T4 cDNA in epithelial cells can alter their morphology and motility, supporting a role for such functions in cancer and development. A murine model to study 5T4 biology and tumour immunology would be useful. The production of m5T4-specific antibodies, their use in establishing transfected cells and documenting their biological properties *in vitro* are described. A rat monoclonal antibody specific for mouse 5T4 molecules by ELISA, flow cytometry, immunohistochemistry and immunoprecipitation was isolated and epitope mapped. Similar to its human counterpart, murine 5T4 antigen is a 72 kDa glycoprotein (immunoprecipitation and Western blot analysis) and exhibits punctate cell surface expression, dependent upon the integrity of the actin cytoskeleton. Likewise, overexpression of autologous murine 5T4 by B16 F10 melanoma cells and A9 L fibroblasts accentuates the 5T4 phenotype, which is characterized by a spindle-like morphology, increased motility, and reduced adhesion and proliferation rate. Immunohistochemical analysis of adult mouse tissues shows a restricted pattern of expression similar to that of human 5T4 antigen. The murine 5T4 antigen-expressing cell lines and antibody reagents are now being used to explore novel immunotherapies in pre-clinical models and the biology of 5T4 in development.

Key words: cell biology, epitope, immunohistochemistry, leucine-rich repeat.

INTRODUCTION

Normal placental trophoblast and malignant tumour cells share a number of common physiological processes, including invasion of the local tissue and the avoidance of immune surveillance. Such shared characteristics stimulated the drive to identify molecules whose expression was common both to normal trophoblast and to cancer. Monoclonal antibody 5T4 (MAb5T4), raised against purified human trophoblast glycoproteins, identifies a 72 kDa transmembrane glycoprotein, which is also expressed on number of tumours of different origin [1,2]. The 5T4 oncofoetal antigen is strongly expressed in trophoblast of the placenta throughout pregnancy, but shows relatively limited expression in certain specialized epithelia in the adult [2–5]. However, its overexpression in a number of different tumour types, notably ovarian, gastric and colorectal, is associated with poorer clinical outcome [4,6–8]. This association suggests a role for 5T4 in the progression of malignancy.

Sequence analysis of the human 5T4 (h5T4) cDNA identified the antigen as a member of the leucine-rich repeat (LRR) family of proteins [9]. The extracellular domain comprises two LRR regions and associated cysteine-containing flanking regions, separated by a hydrophilic domain. All of the seven consensus NXS/T N-glycosylation sites in the extracellular domain are glycosylated with a combination of complex glycans, including two high mannose chains and five sialylated, bi- to tetra-antennary complex chains with minor quantities of core fucosylation [10]. LRR proteins are a diverse family of approx. 60 members, which have in common a repeating structure of aXXaXaXXN/C/T, where a is an aliphatic residue such as leucine [11]. The tertiary structure of porcine ribonuclease inhibitor, which is comprised entirely of LRRs, has been solved by X-ray crystallography [11]. Ribonuclease inhibitor folds into a horseshoe-like structure of repeating units of α-helix and β-pleated sheets; this resolved structure has formed the basis of structural models for other family members [12,13]. However, the precise structure may vary due to differences in the lengths of the LRRs and the presence of other functional domains. Despite no common function having been ascribed, many are involved in protein–protein interactions and overall it is likely that the LRR domains provide a scaffold for a variety of functions [11,14].

5T4 antigen is expressed on microvillous projections of cells and when the h5T4 cDNA is constitutively overexpressed in fibroblasts, there are alterations in motility and morphology which are consistent with a role in both tumour and trophoblast invasion [15,16]. Sequence comparisons between the h5T4 and mouse 5T4 (m5T4) cDNAs [17] indicate the highly conserved structure of 5T4 molecules between species. These molecules share 81 % amino acid identity with the cytoplasmic and transmembrane domains being completely conserved. Of the seven N-linked glycosylation sites in the human antigen, six are conserved in the mouse. The most N-terminal site (N^{81}) is absent, but an additional site (N^{334}) in the C-terminal flanking region is present, predicting a similar level of glycosylation to the human molecules. The murine protein contains an additional six amino acids adjacent to the glycosylation site in the hydrophilic domain,

Abbreviations used: CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; hm, human–mouse; HRP, horseradish peroxidase; h5T4, human 5T4; LRR, leucine-rich repeat; MAb5T4, monoclonal antibody 5T4; α-MEM, α-minimal essential medium; mh, mouse–human; m5T4, mouse 5T4; m5T4ex, extracellular domain of m5T4; m5T4vac WR, recombinant m5T4 expressing Western Reserve vaccinia virus; Rabαm5T4, rabbit anti-m5T4-Fc polyclonal antiserum; 5T4-Fc, extracellular domain of 5T4 fused to the Fc domain of human IgG_1.

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which is a direct repeat of the preceding six amino acids. Despite the high degree of identity between the two species, the monoclonal antibody against the human molecule does not recognize the mouse antigen [10]. The monoclonal antibody that defines the human antigen, MAb5T4, recognizes a conformational epitope that is dependent on carbohydrate and the correct formation of intramolecular disulphide bonds [10,18]. The epitope lies solely within the extracellular domain of the molecule and is localized within the more membrane-proximal LRR2.

The h5T4 oncofoetal antigen is under development as a target for tumour immunotherapy, for both antibody-based targeted therapy [5,19] and as a cancer vaccine. The prognostic association between 5T4 expression and clinical outcome, taken together with its effects on cell physiology when overexpressed, are consistent with a role for 5T4 molecules in tumour progression. To facilitate the further development of immunotherapies and the investigation of 5T4 biology, both in tumours and during normal foetal development, we have generated m5T4 antigen-specific serological reagents. These have been used to characterize m5T4 molecules: the adult tissue and tumour cell line expression pattern and the biological properties of m5T4 cDNA-transfected cell lines.

MATERIALS AND METHODS

5T4-Fc fusion proteins

A 1004 bp cDNA fragment encoding the extracellular domain of m5T4 antigen was generated by PCR and cloned by restriction digestion into the signal-pIg plus expression vector (Ingenious; R&D Systems). Stable expression in Cos-7 cells [19] was achieved by selection in G-418 at 1 mg/ml. Mouse and human 5T4-Fc fusion proteins (containing the extracellular domain of mouse or human 5T4 fused to the Fc domain of human IgG_1) were fractionated from tissue culture supernatant by ammonium sulphate precipitation and purified by wheatgerm agglutinin and Protein G affinity chromatography. The concentration was determined by anti-human Fc-capture ELISA [19] and modified Bradford assay [20]. Purity was assessed by silver-stained SDS/PAGE. The Fc domain of m5T4-Fc was removed by overnight digestion with factor Xa protease (Roche). m5T4 extracellular domains (m5T4ex) were then enriched by negative selection on a Protein G column and concentrated by centrifugal spin filter [10].

ELISA

Plates were coated with 50 μl of antigen at 1 μg/ml in 0.1 M sodium carbonate buffer, pH 9.3, overnight at 4 °C. Plates were washed with PBS containing 0.05 % Tween 20 (PBST) three times between each layer. Non-specific binding sites were blocked with 5 % skimmed milk powder in PBST for 1 h at 37 °C. Plates were incubated successively for 1 h at 37 °C with 50 μl/well of each of the following; test sample, biotinylated mouse anti-rat κ/λ (1:3000; Sigma) and streptavidin-conjugated horseradish peroxidase (HRP; 1:6000; Dako). Reactions were developed with 100 μl of tetramethyl benzidine at 0.1 mg/ml in 50 mM citrate phosphate buffer, pH 5.5, stopped by the addition of 50 μl of 1 M sulphuric acid and read at 450–650 nm.

Polyclonal antisera

Rabbits were immunized subcutaneously with 100 μg of purified m5T4-Fc in Freunds complete adjuvant and boosted on a fortnightly regime using Freunds incomplete adjuvant. Anti-m5T4 activity was assessed by ELISA-based assay against m5T4ex on alternate weeks. Upon acquisition of significant anti-m5T4ex activity, rabbits were terminally bled by cardiac puncture, serum harvested, aliquoted and stored at −20 °C.

Cell culture

Non-adherent cells were grown in RPMI 1640 and adherent cells in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 2 mM L-glutamine and 10 % fetal calf serum (FCS). Transfected cell lines were maintained under selection with 1 mg/ml G-418. Cells were maintained in a humidified atmosphere of 5 % CO_2/95 % air at 37 °C and passaged on reaching 90 % confluence. Four-day conditioned medium was prepared from confluent cultures of Y3Ag1.2.3. Fusion media comprised RPMI supplemented to 20 % FCS, 50 % conditioned medium, 2 mM L-glutamine, 2 mM sodium pyruvate and 1 × DMEM non-essential amino acids (Sigma). Hybridoma cloning was performed in fusion media supplemented with 10 ng/ml human epidermal growth factor.

Flow cytometry

Adherent cells were removed from flasks with trypsin and washed three times at 4 °C with FACS buffer (PBS plus 0.1 % BSA and 0.1 % sodium azide). Aliquots of 10^5 cells were transferred to a 96-well v-bottom plate, pelleted by centrifugation and the supernatant aspirated. All subsequent steps were incubated on ice for 30 min and cells washed three times with FACS buffer between layers. Tissue-culture supernatants were tested neat and purified antibodies were tested at 10 μg/ml. Rat and mouse immunoglobulins were detected with rabbit anti-rat or -mouse FITC direct conjugate respectively (1:30; Dako). Prior to analysis cells were fixed for 10 min at 4 °C by the addition of an equal volume of 3.7 % paraformaldehyde in PBS.

Cell lines

A9 fibroblastic cells expressing h5T4 [15] or chimaeric human–mouse (hm) and mouse–human (mh) 5T4 were generated as described previously [10]. Lipofectamine was used to transfect A9 cells with m5T4 cDNA in pCMVα. Bulk cultures were grown for 2 weeks with G-418 at 1 mg/ml and then assessed for m5T4 antigen expression with rabbit anti-m5T4-Fc polyclonal antiserum (Rabαm5T4) by flow cytometry. Positive cultures were cloned by limiting dilution, assessed for m5T4 antigen expression as before and positive wells re-cloned. The murine melanoma B16 F10 was transfected by electroporation with human or m5T4 cDNA in pCMVα. Stable expression was achieved by the addition of G-418 at 1 mg/ml and clones were established following two rounds of limiting dilution.

m5T4 Western Reserve vaccinia

The full-length 5T4 cDNA [17] was cloned into a transfer vector and m5T4 Western Reserve vaccinia virus (m5T4vac WR) prepared as described previously for LacZ expression [21].

Immunization

LOU Rats (Harlan) were immunized twice intramuscularly with 10^8 p.f.u. m5T4vac WR at 4-week intervals and test bled 2 weeks later. Then, 4 weeks after test bleeds were taken 10^8 syngeneic splenocytes were infected overnight with m5T4vac WR at a multiplicity of infection of 2 and used to boost the highest responder. Day 4 post-boost this animal was terminally bled and splenectomized.

Fusion

Cell fusion was performed by the poly(ethylene glycol) method as described previously [22]. Fused plasmablasts were plated at a density of 10^6/ml in 96-well plates (100 μl/well). After 24 h in culture 100 μl of fusion medium containing 2 × HAT (hypoxanthine/aminopterin thymidine; Sigma) was added. The cells were fed at days 4, 7 and 12 by 50 % change of 1 × HAT medium and on day 14 weaned into HT (hypoxanthine/thymidine) medium. At day 21, tissue culture supernatant was removed from wells positive for growth and assayed for anti-m5T4 activity by flow cytometry versus B16 F10-m5T4 or B16 F10-Neo control plasmid-transfected cells and by ELISA versus m5T4-Fc fusion protein. Positive wells were cloned four times by limiting dilution and re-screened as before. Isolated anti-m5T4 antibody isotypes were determined with a rat monoclonal antibody isotyping kit according to the instructions of the manufacturer (The Binding Site).

Antibody production

Clarified tissue culture supernatant was brought to 45 % ammonium sulphate and stirred overnight at 4 °C. The precipitate was pelleted, resuspended in PBS to 10 % of the original volume and dialysed at 4 °C against five changes of 100 vol. of PBS. The immunoglobulin was purified by Protein G affinity chromatography and the purified antibody extensively dialysed against PBS.

Immunoprecipitation, SDS/PAGE and Western blotting

Cells were lysed at 10^7/ml in PBS and 0.5 % Nonidet P40 containing 1 × Complete™ protease inhibitors (Roche). Lysates were pre-cleared at 4 °C for 4 h with 5 μg of control rat IgG_1. Proteins coupled to rat IgG_1 were complexed with 50 μl of a 50 % suspension of Protein G–Sepharose (Amersham Biosciences) and removed by centrifugation (1000 ***g***, 1 min). Immunoprecipitations were performed with 5 μg of test antibody, and 50 μl of a 50 % suspension of Protein G–Sepharose. Immunoprecipitates were washed five times with lysis buffer, resuspended in 50 μl of 1 × SDS sample buffer and boiled for 3 min. Carbohydrates were removed according to the instructions of the manufacturers in the absence of β-mercaptoethanol. Briefly, 1 μg of m5T4-Fc was digested overnight with either Endo-F (500 units; New England Biolabs) to remove N-linked glycans or neuraminidase (50 units; New England Biolabs) followed by O-glycosidase (0.5 m-units; Roche) to remove O-linked glycans. Samples were separated by SDS/PAGE using an Atto minigel system according to methods of Laemmli [23]. Proteins were transferred electrophoretically to nitrocellulose with a Bio-Rad Transblot semi-dry transfer system and blocked overnight at 4 °C in PBST containing 5 % skimmed milk powder. All antibodies were applied for 1 h at room temperature with agitation and blots washed five times for 5 min between layers [rat IgG_1 and 9A7 (10 μg/ml), goat anti-human-IgG Fc HRP (1:10000; Sigma), rabbit anti-rat HRP (1:2000; Dako) and streptavidin-HRP (1:6000; Dako)]. Antibody binding was detected by chemiluminescence (Amersham Biosciences) according to the instructions of the manufacturer.

Immunofluorescence microscopy

Cells (10^4) were seeded on to acid-washed 16 mm glass coverslips in α-minimal essential medium (α-MEM) containing 1 % FCS and grown for 48 h. Cells were washed three times with FACS buffer and either fixed with 3.7 % paraformaldehyde in PBS for 15 min prior to labelling or, alternatively, labelled at 4 °C in FACS buffer, washed and then fixed. Antibodies were applied as follows; 9A7 (10 μg/ml), MAb5T4 (5 μg/ml), rat IgG_1 (10 μg/ml) or mIgG (5 μg/ml) and the second layer rabbit anti-rat or mouse-FITC conjugate (1:30; Dako; as appropriate) for 30 min. Non-fixed samples were then washed and fixed as described previously. Samples were mounted in PBS/80 % glycerol/2 % 1,4-diazabicyclo[2.2.2]octane, and sealed with clear nail lacquer. To investigate the effect of cytoskeletal disruption upon 5T4 distribution, samples were incubated with 10 μg/ml of either demecolcine or cytochalasin D for 2 h prior to labelling [15].

Cell attachment

Aliquots of 3×10^5 cells were seeded in α-MEM containing 0.25 %, 1 % or 5 % FCS in each well of a 6-well plate and incubated for 24 h. Wells were washed three times with PBS to remove non-adherent cells and adherent cells trypsinized and counted by haemocytometer. The effect of extracellular matrix proteins upon cell attachment was assessed in 96-well plates. Each well was coated with 10 μg of laminin, fibronectin, collagen IV or matrigel in PBS overnight at 4 °C. Plates were washed three times with PBS and seeded at 10^3 cells/well in 100 μl of serum-free α-MEM containing 25 μg/ml transferrin (Sigma). Plates were incubated for 24 h, washed three times with PBS and stained with 0.01 % Crystal Violet in PBS for 15 min. Excess dye was removed by extensive washing, plates air dried and residual dye dissolved by agitation for 30 min with 100 μl/well of 10 % acetic acid at room temperature. The attenuance was then read at 570 nm.

Proliferation

Proliferation assays were performed as described [15,16]. Briefly, 10^4 cells were seeded in duplicate in 6-well plates in DMEM containing 10 % FCS. Twenty-four hours later the cells were washed three times and the medium replaced with α-MEM containing 0.5 %, 1 % or 5 % FCS. Cells were trypsinized and absolute numbers determined at 24 h intervals with a Coulter counter.

Motility and invasion assay

Motility and invasion assays were performed as described previously [15,16]. Falcon cell culture inserts with a non-coated 8 μm porous polyethylene teraphthalate membrane were used for motility assays, and coated with 10 μg of Biocoat Matrigel for invasion assays (Becton Dickinson). α-MEM containing 0.25 % FCS, used for all assays, was conditioned by incubation with NIH 3T3 fibroblasts for 2 h. Conditioned medium (0.5 ml) was placed in the lower compartment and 10^4 cells seeded in 250 μl of non-conditioned medium in the upper compartment in multiples of four. Then, 24 h later wells were washed and fixed with 3.7 % paraformaldehyde in PBS for 20 min. Migration to the lower chamber was assessed by removal of cells from the upper chamber of membranes (with a cotton bud) and comparison with the total number of cells remaining on both surfaces. Cells were stained with 0.01 % Crystal Violet and then processed as for cell attachment.

Immunohistochemistry

Murine tissues examined were obtained in triplicate from both male and female mice. These included 17.5 day placenta and adult heart, lung, liver, spleen, kidney, large intestine, small intestine, brain, testes and ovary. Immunohistochemistry was performed on 5 μm cryostat sections of snap-frozen tissues.

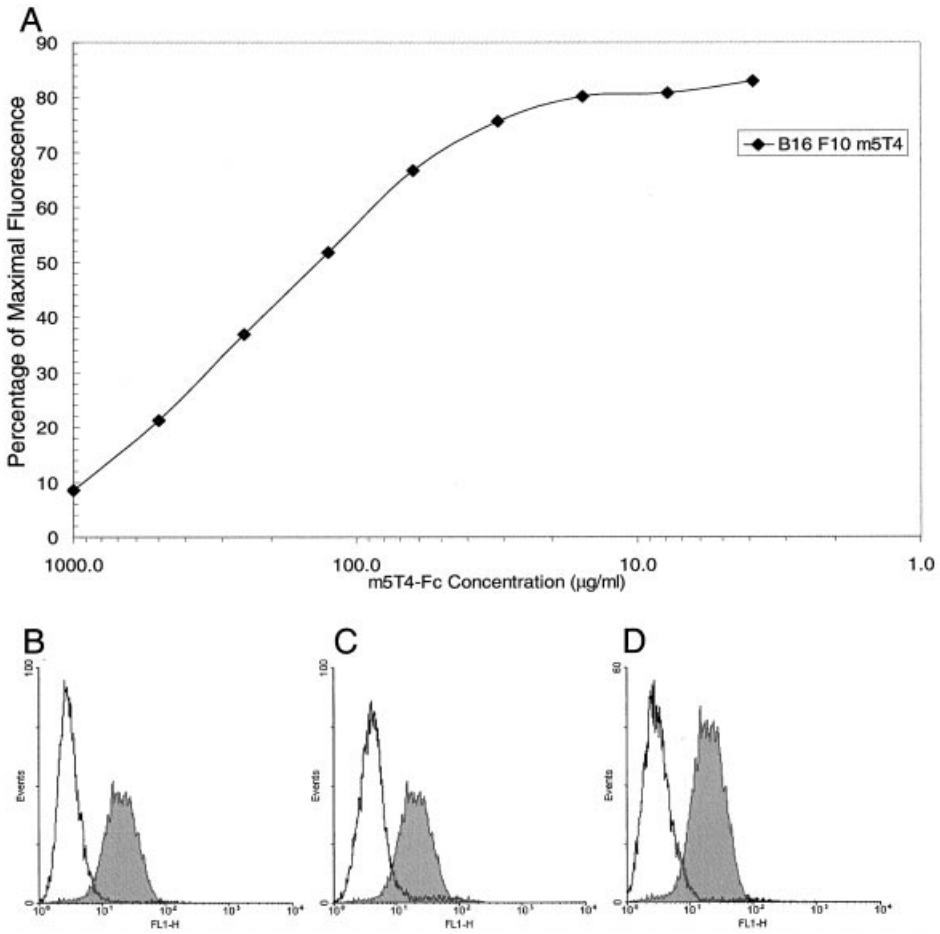

Figure 1 The rabbit anti-m5T4 polyclonal antiserum is specific for m5T4 by FACS

(**A**) Shows the effect of an increasing concentration of m5T4-Fc upon the binding of a constant concentration of Rabαm5T4 to B16 F10-m5T4 cells. Cells were analysed by FACS and results expressed as a percentage of the maximal geometric mean. (**B**–**D**) Black profiles show A9-m5T4 transfectants stained with Rabαm5T4 (1:300; **B**–**D**); white profiles show A9-m5T4 (**B**), A9H12 neomycin control (**C**) and A9-h5T4 (**D**) stained with either rabbit pre-immune serum (**B**; 1:300) or Rabαm5T4 (**C** and **D**; 1:300).

Slides were fixed at room temperature for 5 min in acetone and air-dried prior to re-hydration in Tris-buffered saline (TBS; 50 mM Tris, pH 7.6/140 mM NaCl). Endogenous peroxidase activity was blocked by incubation in TBS containing 0.1% sodium azide and 0.1% hydrogen peroxide at room temperature for 10 min. The sections were blocked with 10% normal rabbit serum for 30 min, and all subsequent steps were in TBS containing 1% normal rabbit serum for 30 min at 30 °C. Sections were stained with either 9A7 or a rat IgG_1 at 10 μg/ml followed by the secondary antibody, rabbit anti-rat HRP direct conjugate (1:100; Dako). Anti-mouse immunoglobulin activity in the secondary antibody was neutralized by the addition of 10% of mouse serum. Immediately prior to use, reagents were spun at 4 °C for 30 min at 13800 ***g*** in a bench-top microfuge. Antibody labelling was visualized with diaminobenzidine and slides counter-stained, cleared, fixed and mounted as described by Southall et al. [2].

RESULTS

Polyclonal rabbit anti-m5T4-Fc

To facilitate cloning and preliminary characterization of m5T4-transfected cell lines, a rabbit antiserum was raised against m5T4-Fc. The fourth test bleed from this rabbit showed significant anti-m5T4 activity by ELISA and, after boosting, the rabbit was terminally bled and the serum harvested. The resulting antiserum (Rabαm5T4) had a titre of 1:5000 by ELISA for m5T4ex (results not shown). The rabbit pre-immune serum showed no activity versus control or m5T4-transfected cells by flow cytometry (Figure 1). However, the Rabαm5T4 antiserum labelled pCMVα m5T4 cDNA-transfected B16 F10-m5T4 melanoma cells and A9-m5T4 fibroblasts, but did not label control plasmid-transfected A9H12 cells or h5T4 cDNA-transfected A9 fibroblasts (Figure 1).

The binding of Rabαm5T4 to m5T4-Fc or B16 F10-m5T4 cells, as measured by ELISA and flow cytometry respectively, was inhibited by pre-incubation with the m5T4-Fc fusion protein (Figure 1). This effect was titratable and could not be replicated with either hIgG or h5T4-Fc (results not shown). These results establish the specificity of Rabαm5T4 antiserum for m5T4 by ELISA and flow cytometry (1:300 dilution) and of the expression of m5T4 molecules on the transfected B16 melanoma and A9 fibroblast cell lines.

Although specific at the cell surface, immunohistochemical analysis with Rabαm5T4 showed widespread and non-specific staining of mouse placental and liver sections (results not shown).

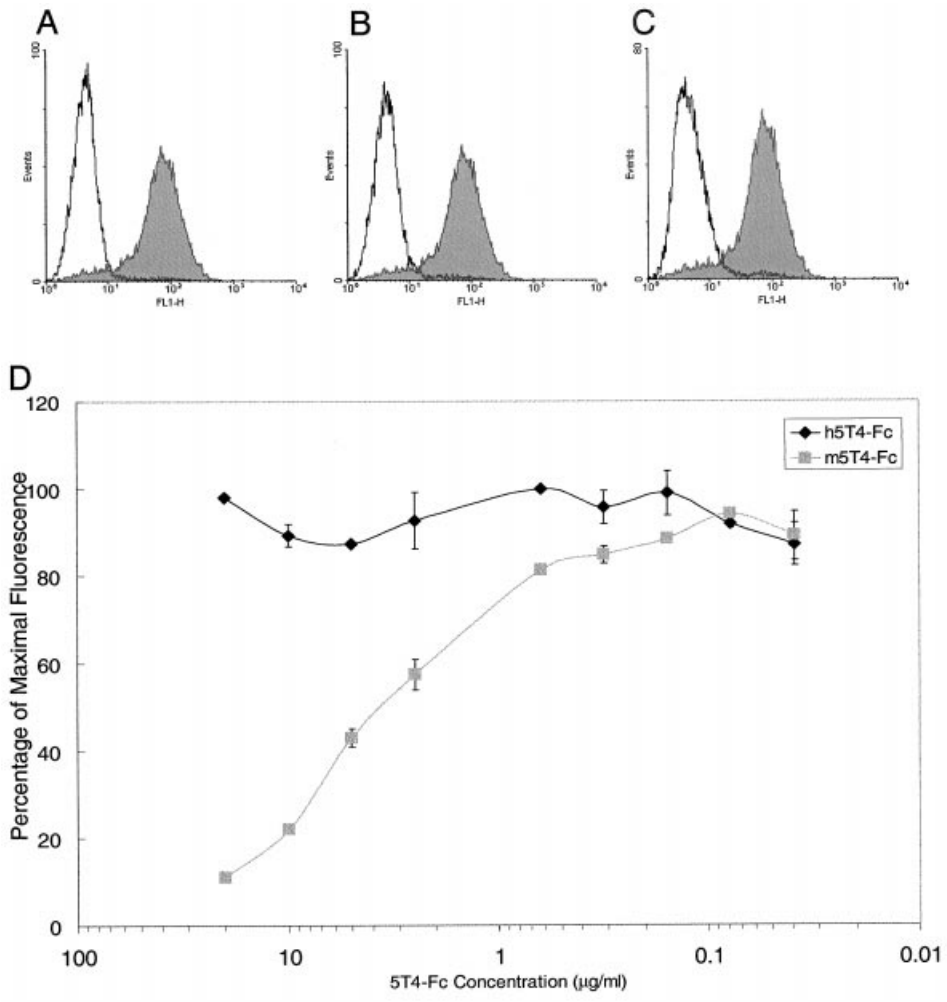

Figure 2 Specificity of the 9A7 antibody for m5T4 cDNA-transfected cells by FACS

Black profiles show A9-m5T4 (9A7; **A**–**C**). White profiles show A9-m5T4 (rat IgG; **A**), A9H12 neomycin (9A7; **B**) and A9-h5T4 (9A7; **C**) transfectants. (**D**) Shows the effect of a decreasing concentration of human or murine 5T4-Fc upon the ability of a constant concentration of 9A7 to stain A9m5T4 cells. Cells were analysed by FACS and results expressed as a percentage of the maximal geometric mean.

These reactivities could not be removed by exhaustive absorption with normal liver tissue and m5T4-specific antibodies proved impossible to purify by affinity chromatography. For these reasons monoclonal rat anti-m5T4 antibodies were generated.

Generation of m5T4-positive cell lines

The establishment of mouse cell lines that showed stable m5T4 expression was not straightforward. In the A9 cells, flow-cytometric analysis showed stable expression of the m5T4 antigen over 20–25 passages. However, after passage 25 the cells began to show evidence of reduced levels of m5T4 in the population, decreased attachment and proliferation after passage and failure to propagate. These problems were not encountered during the generation of other A9-transfected cell lines expressing human or chimaeric 5T4 molecules. Similarly, B16 F10-h5T4-positive cells were relatively easy to produce and maintain whereas B16 F10-m5T4 cell lines required exhaustive selection to produce cells with stable expression and behaviour *in vitro*. However, as the B16 F10-m5T4 cell line showed uniform growth properties and stable expression of m5T4 in culture, it was used to screen hybridoma fusions for rat anti-m5T4 antibodies by flow cytometry.

Monoclonal antibody isolation and characterization

Rats were immunized with a recombinant strain of Western Reserve vaccinia, which encoded m5T4 (m5T4vac WR) and provided antigen expression in the context of a strong adjuvant effect. Two weeks post-boost, tail bleeds showed titres of 1:3000 against m5T4-Fc by ELISA with no cross-reactivity towards h5T4-Fc or hIgG (results not shown). Test sera specifically stained m5T4-transfected cells by flow cytometry and could only be blocked from doing so by pre-incubation with m5T4-Fc (results not shown).

The best responder was boosted and the resultant plasmablasts harvested and fused with the Y3 Ag1.2.3 partner cell line. Of the 960 plated wells, 151 were positive for growth and 104 of these contained rat antibodies, three of which reacted specifically with the m5T4-Fc fusion protein by ELISA. These wells were designated 8C7, 9A7 and 10F4 by location. However, flow-cytometric

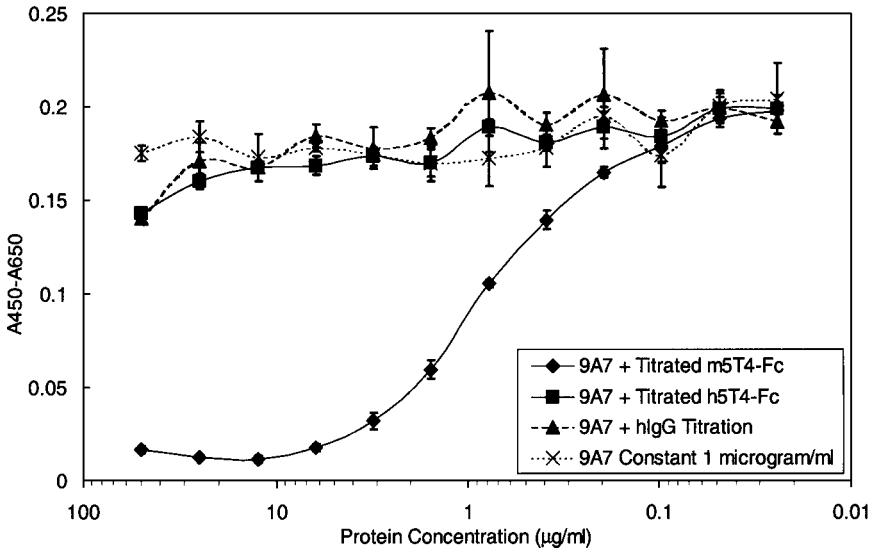

Figure 3 9A7 is specific for m5T4 by ELISA

The capacity of different antigens to inhibit the binding of 9A7 to m5T4-Fc was investigated. Antigen was titrated in a constant concentration of 9A7 (1 μg/ml) and immediately applied to m5T4-Fc-coated plates (1 μg/ml).

analysis with the B16 F10-m5T4 cell line, showed that only well 9A7 reacted and therefore further analysis was limited to this antibody. 9A7 activity was specific for A9 cell lines transfected with the m5T4 cDNA and did not react with A9 cell lines transfected with either neomycin control plasmid (A9H12) or h5T4 cDNA (Figure 2). Antibody labelling could be titrated and was inhibited by pre-incubation with a 5-fold molar excess of m5T4-Fc (Figure 2). Similar results were seen for B16 transfected cells (results not shown).

By ELISA, 9A7 only recognized m5T4 as antigen and this recognition could be specifically inhibited by simultaneous incubation with a 5-fold molar excess of m5T4-Fc (Figure 3). The inhibition of 9A7 binding to m5T4-Fc was titratable and was not affected by either hIgG or h5T4-Fc. Together, these results confirm the specificity of 9A7 for m5T4-Fc antigen.

Epitope mapping

Chimaeric A9-5T4 cell lines (Figure 4) were used to map the 9A7 epitope to a specific region of the m5T4 molecule. Flow-cytometric analysis showed that the 9A7 and MAb5T4 antibodies labelled the A9-hm5T4 and A9-mh5T4 chimaeras respectively, in a non-reciprocal fashion (Figure 4). Therefore, both these cell lines expressed antigenically competent chimaeric 5T4 molecules. These results localized the MAb5T4 and 9A7 epitopes to the membrane proximal regions of the h5T4 and m5T4 molecules respectively.

Western blotting and immunoprecipitation

Reduced and non-reduced Western blots of the mouse and human 5T4-Fc fusion proteins were probed with either 9A7 or a polyclonal rat anti-m5T4 (Ratαm5T4; Figure 5). Ratαm5T4 reacted specifically with both reduced and non-reduced m5T4-Fc (Figure 5A). However, the 9A7 antibody was only specific for

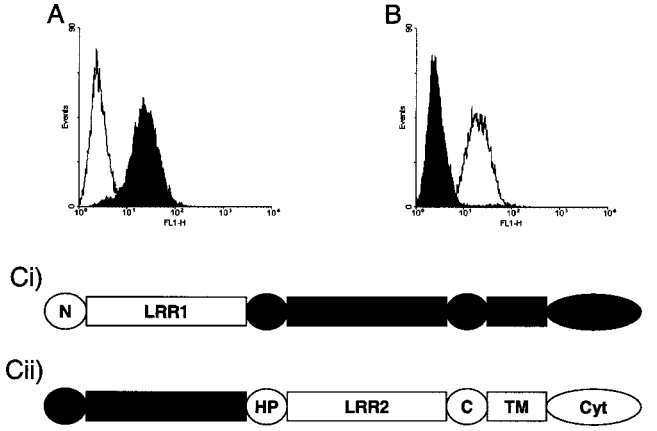

Figure 4 The 9A7 epitope maps to the membrane proximal region of m5T4

A9 cell lines expressing hm5T4 (**A** and **Ci**) or mh5T4 chimaeric cDNA constructs (**B** and **Cii**) in a stable manner, were labelled with 9A7 (black profiles) or MAb5T4 (white profiles). (**C**) Shows a diagrammatic representation of the 5T4 chimaeric molecules. Mouse sequences are shown in black and human sequences in white. From the N-terminus, the domains are labelled N (N-terminal flanking region), LRR1, HP (hydrophilic region), LRR2, C (C-terminal flanking region), TM (transmembrane region) and Cyt (cytoplasmic domain).

m5T4-Fc under non-reducing conditions, giving a small but significant signal with reduced h5T4-Fc (Figure 5Aii).

The contribution of carbohydrate moieties to the integrity of the 9A7 epitope was assessed by Western blot analysis of de-glycosylated m5T4-Fc molecules (Figure 5B). Treatment of m5T4-Fc fusion protein with neuraminidase followed by O-glycosidase produced incremental reductions in molecular mass, indicating the presence of both sialylation and O-linked glycans. Neither treatment, however, reduced the antigenicity of the 9A7 epitope. However, removal of N-linked carbohydrate

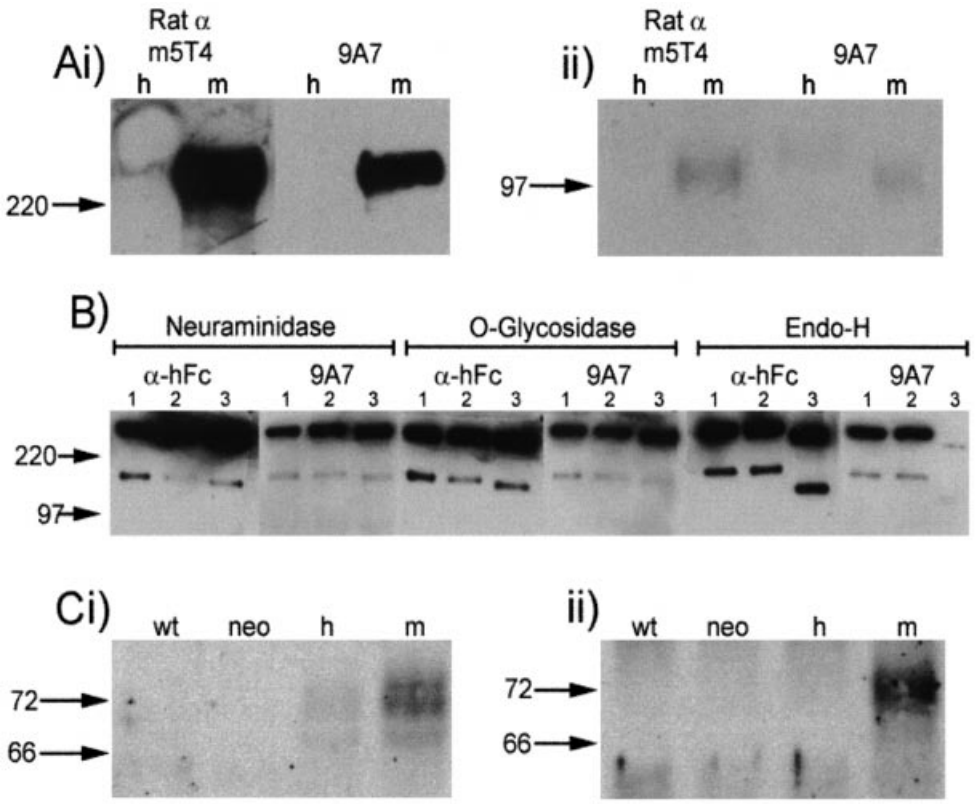

Figure 5 Biochemical analysis of the 9A7 epitope by Western blot

(**A**) 9A7 specificity. Lanes were loaded with 50 ng of human (h) or mouse (m) 5T4-Fc fusion protein under reducing (**Ai**) or non-reducing (**Aii**) conditions and probed with a rat anti-m5T4 polyclonal antiserum (1:200) or 9A7 (5 μg/ml). (**B**) Carbohydrate and the 9A7 epitope. Lanes were loaded with 50 ng of m5T4-Fc pre-treated with either nothing (1), sham-treatment (2) or enzyme (3), run under non-reducing conditions and probed with anti-human IgG-Fc HRP (1:2000) to confirm protein loading or 9A7 (5 μg/ml) to confirm epitope integrity. (**C**) Full-length m5T4. Non-reduced Western blot of cell lysates (**Ci**) and a 9A7 immunoprecipitation (**Cii**) from A9 cells; wild type (wt), neomycin control (neo), human (h) or mouse (m) 5T4. Cell lysates were loaded at 4×10^5 cell equivalents/lane (i), and 10^6 cell equivalents were immunoprecipitated with 5 μg of 9A7 with the entire reaction loaded (ii). Both panels (**Ci** and **Cii**) were probed with Rabαm5T4 (1:3000).

moieties with endoglycosidase H led to a significant reduction in molecular mass of the m5T4-Fc molecules with the concomitant ablation of the 9A7 epitope (Figure 5B). The precise glycosylation patterns of the fusion protein may not reflect the pattern of glycosylation of the native molecule, but the antigenicity clearly depends on N-linked sugars.

By comparison to m5T4-Fc, detection of full-length m5T4 antigen by Western blotting of m5T4 cDNA-transfected cell lysates with 9A7 is relatively insensitive. However, partial purification of membrane glycoproteins by wheatgerm agglutinin enrichment from transfected A9 cell-lysates reveals a broad 72 kDa band specific to the m5T4 cDNA-transfected cells (results not shown). To corroborate this data, non-reduced Western blots of 9A7 immunoprecipitates from A9 cell lysates were probed with the Rabαm5T4 antiserum. As this antiserum cross-reacts with full-length h5T4 (Figure 5Ci), it can be used to determine the specificity of 9A7 immunoprecipitation reactions for human or murine 5T4 molecules. The resultant 72 kDa band was only present in A9-m5T4 cell lysates, indicating that 9A7 was specific for m5T4 and did not immunoprecipitate h5T4 antigen (Figure 5Cii).

Cellular distribution of m5T4

The A9-m5T4 and B16-m5T4 cell lines show a punctate pattern of labelling when stained with 9A7 (Figure 6), which was independent of pre- or post-fixation and therefore not due to antibody-induced antigen redistribution. Similar patterns of staining were seen by confocal microscopy for the murine mammary carcinoma-derived cell lines C127I and EMT6, confirming that punctate labelling was independent of cytomegalovirus (CMV) immediate-early promoter-driven expression.

Disruption of the actin cytoskeleton with cytochalasin D led to a redistribution of punctate staining away from the periphery of the cell. This effect was not seen upon disruption of the microtubule network suggesting that the integrity of the actin cytoskeleton is an important factor in maintaining the distribution of m5T4 molecules (Figure 7). Cell lines derived from murine tumours were assessed by flow cytometry for staining with 9A7 (Table 1). Positive lines included three derived from mammary tissue, a colon carcinoma, a squamous lung carcinoma and an embryonic carcinoma. Those that did not stain with 9A7 included a fibroblastoid cell line, two melanomas, a lymphoma, two lung carcinomas, a rectal cell line, a breast carcinoma, an ovarian carcinoma and also an embryonic stem cell line.

Patterns of cell growth

Under low serum conditions A9H12 fibroblasts grow as a 'pavement'-type monolayer with many cell–cell contacts with little space between cells (Figure 8). Transfection of h5T4 into mouse fibroblasts results in a more dendritic morphology, fewer

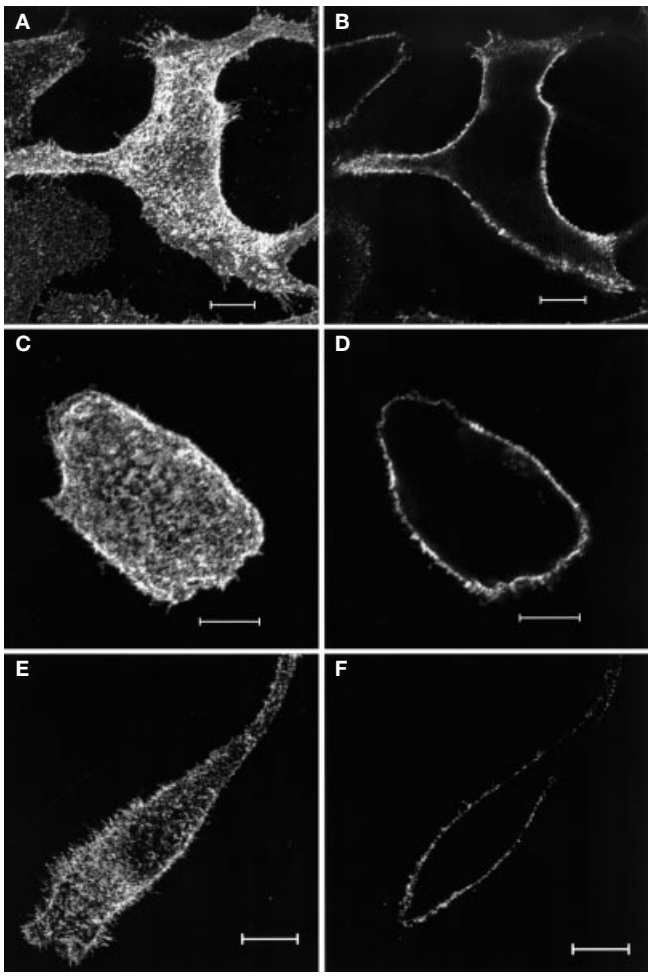

Figure 6 Distribution of m5T4 at the cell surface

A9h5T4 (**A** and **B**), A9m5T4 (**C** and **D**) and B16 F10-m5T4 (**E** and **F**) cells were pre-fixed and stained with 9A7. Cells were analysed by confocal microscopy. Panels show the entire Z-stack projection (**A**, **C**, **E**) or a single Z slice at the midpoint of the Z stack (**B**, **D**, **F**). Scale bars, 10 μm.

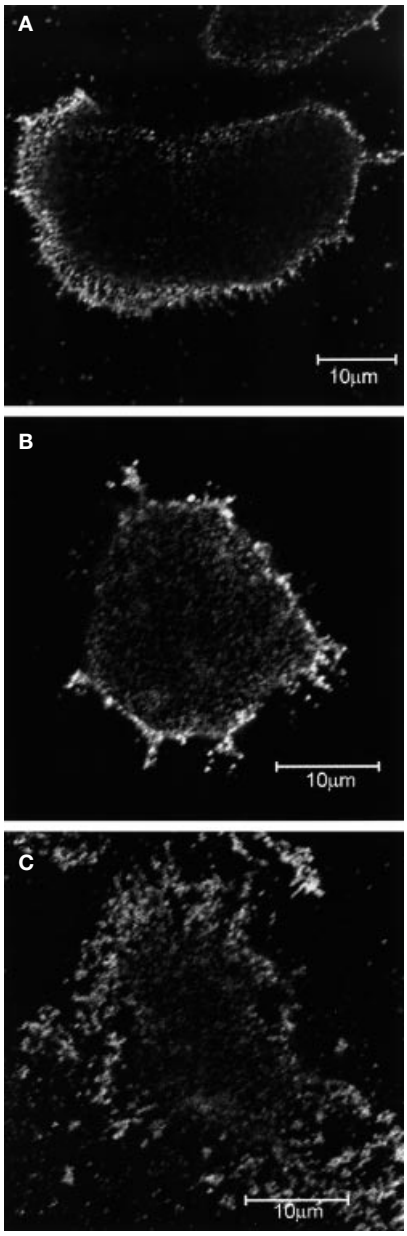

Figure 7 The distribution of m5T4 after disruption of the cytoskeleton

Cells were left untreated (**A**) or treated with the cytoskeletal poisons demecolcine (**B**) or cytochalasin D (**C**) to disrupt the microtubule network or the actin filaments respectively. Cells were labelled 2 h later with 9A7 and analysed by confocal microscopy. Scale bars, 10 μm.

cell–cell contacts and an increased tendency to disperse (Figure 8). The expression of m5T4 by A9 fibroblasts resulted in long spindle-shaped cells compared with plasmid control-transfected cells (Figure 8). m5T4-transfected cells form colonies that stack vertically and align in a parallel fashion along the axis of the spindle. This results in the formation of 'fibres' that grow by extension to connect with others, after which they spread outwards to cover the remaining free surface. This was seen in many experiments, throughout the passage window and with several independently derived clones.

A9-m5T4 antigen-positive cells showed reduced proliferation when compared with the A9H12 neomycin control cell line. Of the A9-h5T4, A9-m5T4 and A9H12 cell lines, only the A9H12 neomycin cell line could be maintained in serum-free media with a doubling time of 75 h. Addition to the media of FCS (0.5 %) allowed all cell lines to be maintained. Proliferation rates were in the order A9H12 > A9-h5T4 > A9-m5T4 with doubling times of 62, 120 and 146 h respectively. Increasing the concentration of foetal calf serum to 5 % did not alter this rank order, but did decrease the differences in doubling times between the lines; A9H12, A9-h5T4 and A9-m5T4 at 53, 62 and 67 h respectively.

Transfection of the B16 and A9 murine cell lines with m5T4 resulted in a 7 % reduction of forward scatter as assessed by flow cytometry (Table 2). This implies an average reduction in cell volume upon transfection of cells with autologous 5T4. This effect was not observed in A9 fibroblasts transfected with the h5T4 cDNA, the neomycin control cassettes or the hm5T4 or mh5T4 chimaeric constructs. All cultures showed good viability with homogeneous 5T4 antigen expression by flow cytometry.

Adhesion

A9 cell lines exhibit serum concentration-dependent attachment to plastic (Figure 9). The degree of this effect lessened as the

Table 1 FACS analysis of 9A7 activity against a panel of murine cell lines

10^5 cells of each line were stained with 9A7 and analysed by FACS. Results are representative of three individual cultures and staining experiments.

Cell line	Origin	Flow cytometry
A9 neo	Lung fibroblast L cells	−
A9-m5T4	Lung fibroblast L cells	++++
B16 F10 Neo	Melanoma	−
B16 F10-m5T4	Melanoma	++
EMT6	Mammary adrenocarcinoma	+++
C127I	Mammary carcinoma	+++
Clone M3	Melanoma	−
EL4	Lymphoma	−
KLN-205	Squamous cell lung carcinoma	+/−
JC	Breast adenocarcinoma	−
LL/2	C57BL Lewis lung carcinoma	−
Mosec	Ovarian carcinoma	−
Nulli 2A	Embryonic carcinoma	+
129 ES	Embryonic stem cell	−
CL-S1	BALB/c mammary pre-neoplastic alveolar nodules	+/−
CMT-93	Rectal carcinoma	−

serum concentration was increased but the relative differences between cell lines remained. The capacity of A9-m5T4 cells to adhere to plastic shows the most pronounced sensitivity to serum concentration followed by A9-h5T4 and then A9H12. The extracellular matrix components collagen IV, laminin and fibronectin showed little differential effect upon adhesion of cells and followed the same trend as for adhesion to plastic (Figure 9). However, matrigel-coated wells resulted in increased adhesion of all cell lines tested but did not alter their relative propensities to adhere.

Motility and invasion

The effect of the stable expression of human and murine 5T4 molecules on the ability of A9 cells to actively move and invade was compared with that of the A9H12 neomycin control cell line. The stable expression of human or mouse 5T4 by A9 cells did not significantly alter their propensity to invade but did increase their motility 3-fold and 7-fold respectively (Figure 10). These experiments were repeated three times using cells of low passage number with uniform growth and 5T4 expression. The data presented are representative of these results. Interestingly, cultures of A9-m5T4-positive cells, heterogeneous in their m5T4 expression and older than 25 passages, show reduced motility in comparison with homogeneous cultures of lower passage number.

Immunohistochemistry

As the h5T4-oncofoetal antigen was identified in placental tissue, the immunohistochemical reactivity of anti-m5T4 monoclonal antibodies was assessed against frozen sections of 17.5 day mouse placenta (Figure 11). This showed that the 9A7 antibody specifically labelled placental tissue of foetal origin. Cells of the syncytio- and cytotrophoblast showed discrete staining and, in addition, the amnion was also positive.

Adult tissues examined were isolated from three individual male and female adult mice. These included heart, lung, liver, spleen, kidney, large intestine, small intestine, brain, testes and ovary. Limited staining of specialized subsets of cells was seen in a variety of adult tissues. In order of intensity these were the choroid plexus in the lateral ventricles of the brain (Figure 11), the outer epithelial lining of the ovary, the glandular mucosal cells of the large and small intestine, the glomeruli of the kidney, the sinusoids of the liver and the lining of the bronchi. Adult tissues completely negative for 9A7 staining included the spleen, testis and heart. 9A7 failed to specifically label paraformaldehyde-fixed wax-embedded mouse placenta.

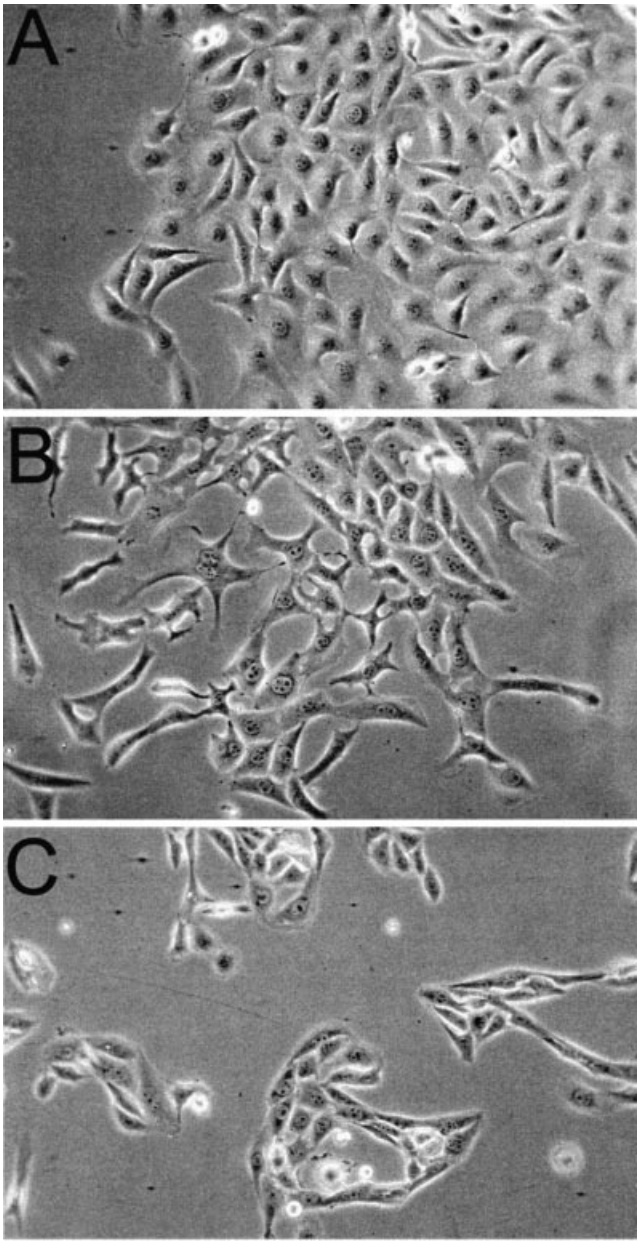

Figure 8 5T4 antigen expression affects the proliferation and growth patterns of A9 cells

Panels **A**–**C** show typical fields of view of A9H12 neomycin control cells (**A**), A9-h5T4 cells (**B**) and A9-m5T4 cells (**C**) at × 200 magnification. All cultures were seeded in 10% FCS. The medium was changed 24 h later to 1% MEM-α and cells cultured for a further 2 days before image capture.

DISCUSSION

The production of m5T4-positive cell lines and the description of m5T4 expression in the adult mouse required the development

Table 2 Expression of m5T4 antigen mediates a reduced mean cell volume

FACS was used to assess the forward scatter (FSC) profile of mid-exponential-phase cultures of the cell lines listed. The geometric mean of the forward scatter was taken as a measure of average cell volume. These results are representative of three separate experiments.

Cell line	Mean FSC	S.D.	Percentage of A9H12 FSC
B16 F10-Neo	547.1	2.1	100
B16 F10-m5T4	508.9	2.1	93.0
B16 F10-h5T4	550.7	0.6	100.6
A9H12	577.9	1.0	100
A9-m5T4	538.4	6.6	93.1
A9-h5T4	573.2	5.2	99.2
A9-mh5T4	573.4	13.6	99.2
A9-hm5T4	572.5	8.9	99.1

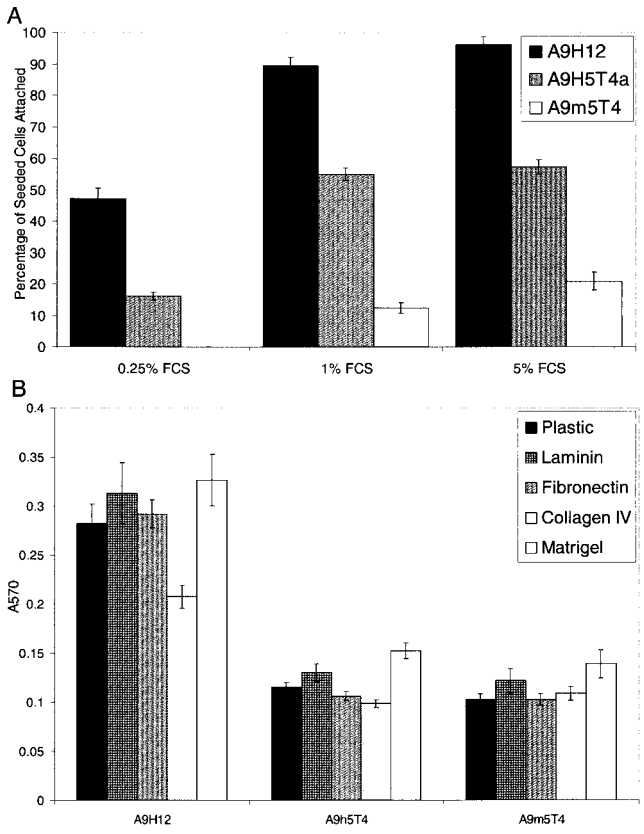

Figure 9 5T4 expression and cell adhesion

(**A**) 10^6 cells were seeded into 6-well plates in medium supplemented with 0.25, 1 and 5% FCS. 24 h later the percentage of seeded cells attached was calculated. (**B**) Extracellular matrix proteins and adhesion. 10^3 cells were loaded into protein-coated wells in serum-free α-MEM containing 25 μg/ml transferrin. Wells were washed 24 h later and adhesion measured by staining wells with Crystal Violet and reading the A_{570}.

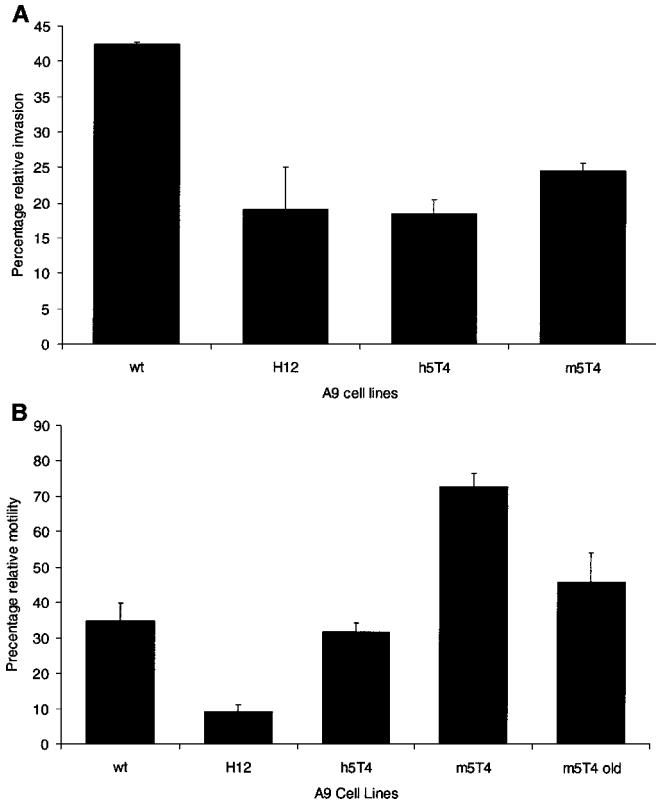

Figure 10 The expression of 5T4 cDNA by A9 fibroblasts enhances their motility but does not affect their capacity to invade

The relative capacity of various A9 cell lines to pass across matrigel-coated (**A**; invasion) or non-coated (**B**; motility) tissue culture inserts was assessed. Cells numbers were scored by measurement of incorporated Crystal Violet. Results are expressed as the percentage of all cells, which were present on the lower membrane.

of a specific rabbit anti-m5T4-Fc polyclonal serum (Rabαm5T4). Previous observations had demonstrated the antigenic integrity of the h5T4-Fc fusion protein with both mono- and polyclonal reagents [19]. Therefore, rabbits were immunized with a m5T4-Fc fusion protein and the resultant Rabαm5T4 antiserum was shown to be specific for the m5T4 antigen at the cell surface in B16 F10 and A9 transfected cell lines. However, Rabαm5T4 could not be used for immunohistochemistry due to high levels of background labelling. Therefore, rats were immunized with a vaccinia virus encoding m5T4 antigen and a hybridoma fusion performed, which was then screened by ELISA and flow cytometry against the m5T4-Fc fusion protein and the B16 F10-m5T4 cell line respectively.

Screening of this fusion resulted in isolation of the rat anti-m5T4 antibody 9A7. Here we have demonstrated its specificity for the m5T4 antigen by flow cytometry, ELISA and immunoprecipitation. The labelling of tumour and transfected cell lines with 9A7 confirmed expression of 5T4 antigen by m5T4 mRNA-positive cells [17]. The epitope recognized by 9A7 was shown to possess a conformational component that was dependent upon N-linked glycosylation and mapped to the membrane proximal region of the m5T4 molecule.

We have performed a preliminary screen on a limited panel of murine tumour-derived cell lines. However, there is a requirement to examine 5T4-antigen expression in a wider variety of tumour cell lines including *in vivo* models of carcinogenesis such as the Min mouse. Expression of either mouse or human 5T4 cDNA by transfected mouse tumour cell lines increased their motility but reduced their rate of proliferation and capacity to adhere. The magnitude of these effects was shown to be serum-concentration-dependent and was greater when cells were transfected with autologous 5T4-cDNA. Finally, the 9A7 antibody was used to describe the distribution of m5T4 in adult mouse tissues by immunohistochemistry.

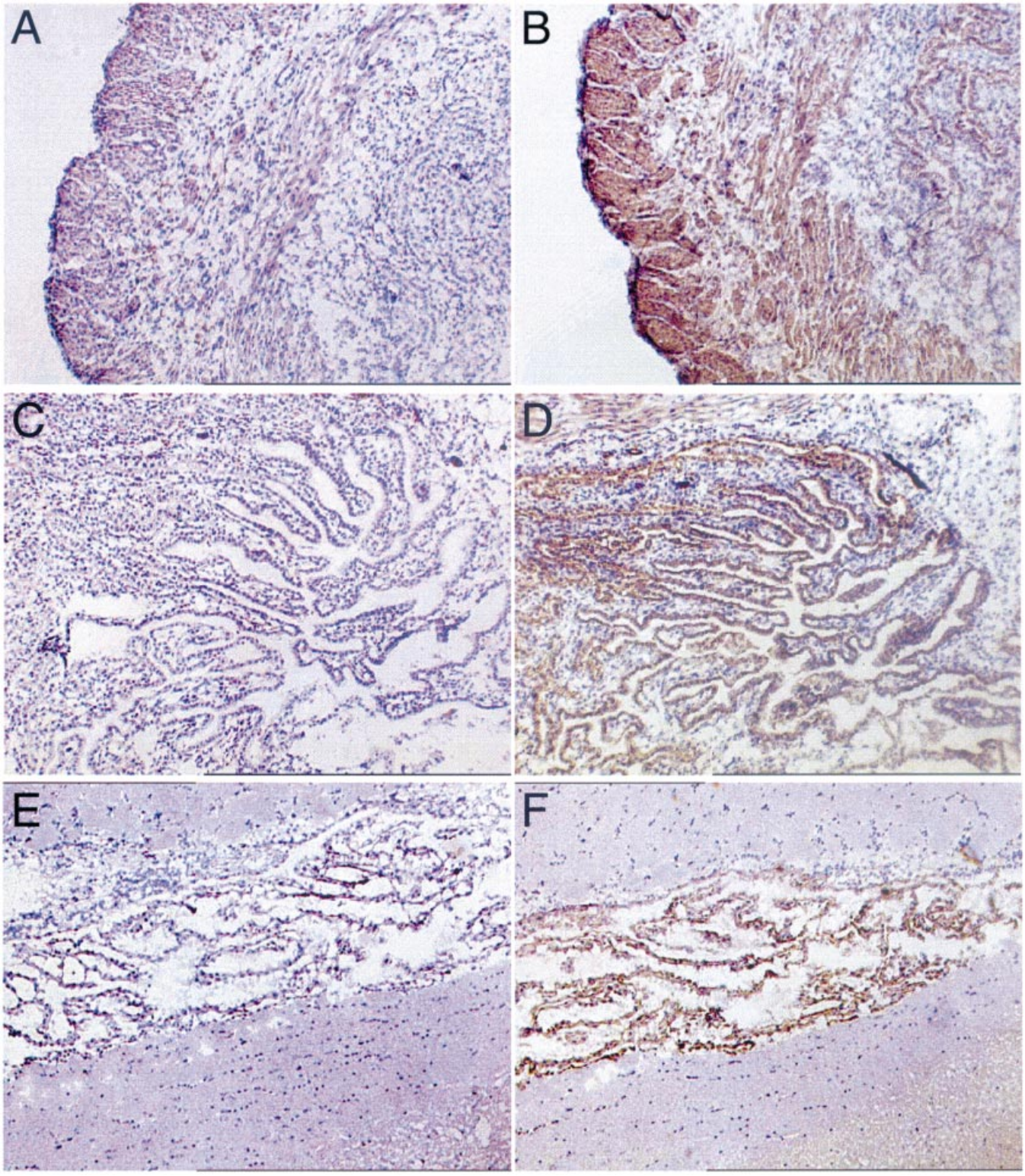

Figure 11 Immunohistochemical analysis of murine tissues with 9A7

Transverse sections of 17.5 day mouse placenta (**A**–**D**) and longitudinal sections of adult mouse brain (**E** and **F**) were labelled with rat IgG_1 (**A**, **C**, **E**) or 9A7 (**B**, **D**, **F**). Images were captured at $\times 200$ magnification.

Selection for stable growth and expression of the m5T4 antigen by murine cell lines was relatively difficult. However, the stable expression of human or chimaeric 5T4 molecules by these cells was, in comparison, relatively straightforward; stable and long-term expression beyond 25 passages. It is possible that over-expression of autologous 5T4 molecules may deliver negative effects (e.g. through proliferation rate and adhesion changes), which are more pronounced because of species-specific influences of 5T4 antigen expression.

The rat anti-m5T4 antibody, 9A7, was used to describe the expression of m5T4-antigen in adult mice by immunohistochemistry. The specificity of 9A7 for m5T4 was confirmed by direct binding and inhibition-based assays *in vitro* (by ELISA) and at the cell surface where binding of 9A7 to m5T4 mRNA-positive cells [17] could only be inhibited by the m5T4-Fc fusion protein. Western blots of m5T4-Fc fusion protein show that the reduction of disulphide bridges or removal of N-linked carbohydrate both significantly lower its recognition by 9A7, implying that the 9A7 epitope, like that of MAb5T4, may be conformational in nature. However, reduced Western blots of h5T4-Fc revealed a cryptic epitope within the human molecule, which can be recognized by 9A7. As the amino acid sequences of h5T4 and m5T4 show over 81 % identity [9] it is likely that the 9A7 epitope, or one very similar, is present in an altered conformation within h5T4. Reduction, electrophoresis and blotting may allow this cryptic epitope to re-fold into a conformation that facilitates recognition by 9A7.

Western blot analysis of full-length m5T4 antigen from cell lysates was not very sensitive with 9A7 and required enrichment of membrane glycoproteins by either immunoprecipitation or wheatgerm agglutinin affinity chromatography. Western blots of such enriched cell lysates showed a broad 72 kDa band when probed with the Rabαm5T4 antiserum. These results were similar to those previously demonstrated for h5T4 [18] and were limited to m5T4 mRNA-positive cell lysates [17]. As the Rabαm5T4 antiserum used to probe 9A7 immunoprecipitation reactions also detects the h5T4 antigen by Western blotting, the lack of a 72 kDa band from h5T4-transfected cell lysates indicates that 9A7 specifically immunoprecipitated the m5T4 antigen.

The 9A7 epitope was mapped to a region of m5T4 spanning the hydrophilic domain to the plasma membrane. The MAb5T4 epitope was also shown to map to this region of h5T4 and also shows sensitivity to reduction [10,18]. Both m5T4 cDNA-transfected and murine tumour-derived cell lines exhibited a punctate pattern of labelling with 9A7, which localized to the cell membrane. This pattern was independent of overexpression driven by the CMV immediate-early promoter and not induced by antibody-mediated re-organization. However, the disruption of the actin cytoskeleton resulted in the redistribution of 9A7 staining, which is consistent with results reported for h5T4 antigen [15].

Transfection of cells with heterologous 5T4 had a pleiotropic effect [15,16], which was more pronounced upon transfection with autologous 5T4. The morphological, adhesive and proliferative differences between cell lines were clear under low serum conditions but became less apparent at higher FCS concentrations. However, under all FCS concentrations examined the morphology, adhesive capacity and proliferation of the A9 cell lines was always greatest for A9H12 cells followed by A9-h5T4 and then A9-m5T4. Typically, A9H12 cells show the most adhesive morphology with a pavement-like appearance and many cell–cell contacts [16], while A9-m5T4 cells show the least adhesive morphology with a spindle-like shape and little contact with the growth support. Both the A9-m5T4 and A9-h5T4 cell lines required > 0.1 % FCS for growth, whereas A9H12 could be grown in the short term with no FCS when supplemented with transferrin. It is likely that the difference in the ability of these cells to proliferate is linked to their morphology and adhesion to the substratum.

The stable expression of human or murine 5T4 by A9 cells did not alter their invasive capacity but doubled their motility when compared with that of A9-h5T4 cDNA-transfected cells. Both the A9 and B16 F10-m5T4 cDNA-transfected cell lines show a reduced mean volume after transfection in comparison to neomycin control-transfected cells. The human ovarian tumour cell line Hoc-8 also shows a similar reduction in volume when overexpressing h5T4. As the cytoplasmic and transmembrane domains of the human and m5T4 molecules are completely conserved at the amino acid level it is possible that specific interactions resulting from the extracellular domain of autologous 5T4 molecules may be involved. Mechanisms reported to affect cell volume include accelerated cell-cycle progression [24], modulation of the actin cytoskeleton [25] and ion-channel-mediated regulation of cell hydration [26,27].

The immunohistochemical distribution of m5T4 antigen in the majority of adult murine tissues and murine 17.5 day placenta was consistent with that reported for h5T4 antigen [2,5]. 9A7 recognized both syncytio- and cytotrophoblast in term murine placental tissue, as well as amnion. The 9A7 antibody was also shown to label discrete subsets of cells within adult murine tissues. The observation of reactivity in the choroid plexus of the lateral ventricles of the brain is novel, as is the above background signal around the sinusoids of the liver, both of which were not seen in the human immunohistochemistry. However, whereas murine brain has been shown to be positive for m5T4 mRNA, murine liver was shown to be negative for m5T4 mRNA by RNase-protection assay [17]. The lack of m5T4 mRNA detection in murine liver may result from a minor population of cells transcribing the gene and the relatively long half-life of the protein. Therefore, *in situ* hybridization of m5T4 mRNA may provide further information. Another explanation for the reaction of 9A7 with murine liver is the transport of m5T4 antigen from a distant site. Some human colorectal tumour biopsies show staining for h5T4 antigen in the surrounding stroma, the intensity of which decreases with distance from the tumour cells. This has been attributed to the release of h5T4 antigen from the tumour cells [4,7,28]. However, it is also possible that the reactivity demonstrated in liver is due to an unknown cross-reaction of the 9A7 antibody, perhaps with another member of the LRR family of proteins.

Here we have characterized m5T4 molecules, their tissue expression and tools (antibodies, tumour cell lines) for preclinical mouse models relevant to studies of anti-5T4-directed immunotherapy. The 9A7 antibody isolated for this work will also be instrumental in the study of the biology of 5T4 in embryonic development.

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