

# Development of New Monoclonal Antibodies for Immunocytochemical Characterization of Neural Stem and Differentiated Cells

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## 1. Introduction

Neural stem cells are present in both the developing and adult nervous system of all mammals, including humans. Due to their therapeutic promise, considerable attention has been focused on identifying the sources of stem cells, the signals that regulate their proliferation and the specification of neural stem cells towards more differentiated cell lineages (Bauer et al., 2006). Presently, neural stem cells are often identified based upon the presence of molecular markers that are correlated with the stem and/or progenitor state along with the absence of a more differentiated phenotype as assessed through marker analysis. Nevertheless, from the very beginning of NSC research the frame was set by the search for such markers and some have been identified, which, at least, allow for the identification of NSC clonal cells in cell culture. Still, the task proves to be difficult because of the changing identity that NSC can undergo and the demands that are imposed on markers. A reliable marker should identify NSC not only in the embryonic brain but also in the adult brain. Generally, markers may either be selected for cell function or for some phenotypic differences. There exist several commonly used immunomarkers for the identification of cells of neural lineages. CD 15 (SSEA1), CD133 (Prominin-1), CD184 (CXCR4), CD271 (p75-NTR), CDw338 (ABCG2), Ki67, Musashi-1, Musashi-2, nestin, Notch-1, PAX-6, SOX-1, SOX-2 are known as neural stem cell markers; PSA-NCAM and CD271(p75-NTR) as neuronal restricted progenitor markers; CD56 (N-CAM), MAP2, DCX,  $\beta$ -III-A2B5 and NG2. GFAP, FGFR3, Ran-2, S100B and CD44 (H-CAM) are known as Type 1 astrocyte, and GFAP, A2B5, CD44 and S100B as Type 2 astrocyte markers. The tubulin and neurofilament NF-H are used as neuron markers. Glial restricted progenitor markers are differentiation of glial restricted progenitors to oligodendrocyte progenitors has been marked by A2B5, NG2, Olig2 and CD140a (PDGFR $\alpha$ ). GalC, MBP, CD140a (PDGFR $\alpha$ ), O1, O4, Olig1, Olig2 and Olig3 have been used as mature oligodendrocyte markers. In addition CD57, CD271 (p75-NTR), MASH1, Neurogenin 3 and Notch-1 have been used as neural

crest stem cell markers (Kennea & Mehmet, 2002; Schwartz et al., 2008; Yuan et al., 2011). The expression overlap of markers requires a use of different immunomarkers for the identification of specific cells in neural lineages. The most problematic point, however, is the potential pitfall in identifying the phenotype of any newborn cell by a single marker. There exists a great need for more and specific monoclonal antibodies as immunomarkers for the characterization of both normal and malignant neural cells.

## 2. Aim of the study

The main aim of the study is the development of new monoclonal antibodies (MAbs) against neuronal tissue cells to investigate the differentiation and malignization of human nerve cells. The antibody producing hybridomas were obtained by immunizing Balb/c mice with the native fragments of human glioblastoma and foetal neural stem/progenitor cells (see Fig. 1).

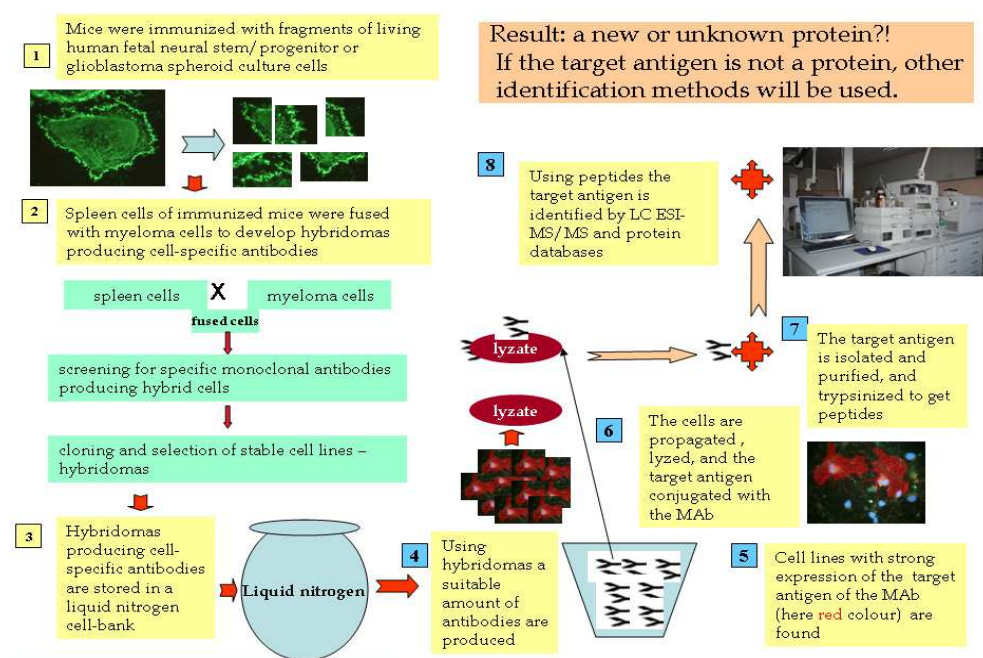


Fig. 1. Flow chart of developing hybridomas and monoclonal antibodies.

In this way it is possible to obtain MAbs against all kinds of antigenic determinants (proteins, glycans and lipids and conformational complex epitopes) that are co-expressed in the living cell, also including those determinants that are expressed on the cell surface. Used immunization scheme allows to obtain MAb panels which characterise the given cell type, including tumour cells. It is possible to obtain MAbs, differentiating between normal and tumour cells. Such specific antibodies against cell surface antigens may be the primary candidates for therapeutic antibodies and/or diagnostic purposes.

### 3. Methods

#### 3.1 The isolation of human fetal neural stem/progenitor and glioblastoma spheroid cells

Human fetal neural stem/progenitor and glioblastoma spheroid cell lines were developed as described earlier (Kalev et al., 2006). In a greater detail, stem cells were isolated from the brains of 18-21-weeks old fetuses aborted due to medical indications (pregnancy problems). The study was approved by the Ethics Review Committee on Human Research of the University of Tartu. The tissue was mechanically dispersed into a cell suspension in the DMEM/F12 medium, containing gentamicin as antibiotic (Gibco BRL, Gaithersburg, USA). The cell suspension was centrifuged and washed once with the same medium and seeded into 6-well tissue culture plates with a density of 5000-10000 living cells per ml in the medium composed of DMEM/F12, B27 supplement (Gibco BRL) and growth factors bFGF (20 ng/ml; Peprotech), EGF (20 ng/ml; Peprotech), LIF (20 ng/ml, Chemicon) and gentamicin (Gibco BRL). The stem/progenitor cells were grown as neurospheres, the medium was changed every three days, the spheres were dissociated by mechanical trituration after every 12-15 days. Glioblastoma biopsy materials (obtained from Dr E.Jõeeste, Department of Pathology, North-Estonian Regional Hospital, Tallinn) were from patients who had signed a consent form. Materials were manipulated by the same method as in the isolation of CNS stem/progenitor cells. The isolation of both CNS stem/progenitor cells and neurosphere-like growing cells from glioblastoma biopsies were performed earlier in LabAs Ltd. In this study five neural stem/progenitor cell lines (hBrSc003, hBrSc004, hBrSc005, hBrSc006 and hBrSc009) and two glioblastoma spheric cell lines (glioblastoma TiVi M-cells and glioblastoma OtAi M-cells) were used for the development and characterization of new monoclonal antibodies (Fig. 2). During differentiation all these cell lines produced three main neural cell types: neurons, astrocytes and oligodendrocytes as it was shown by staining cells with  $\beta$ -III-tubulin, GFAP and GalC (Fig. 3) although in glioblastoma spheroid cultures the cellular and nuclear heterogeneity was significantly higher than in fetal neural stem cell cultures (unpublished). Differentiation of fetal neurospheres and neurosphere-like growing glioblastoma cells (spheroids) was initiated by plating cells onto laminin-coated cover-glasses in the growth media containing all-trans retinoic acid (RA; 10<sup>-6</sup> M) and dibutyryl cyclic AMP (dBcAMP; 1mM) and the cells were fixed on different days after the initiation of differentiation.

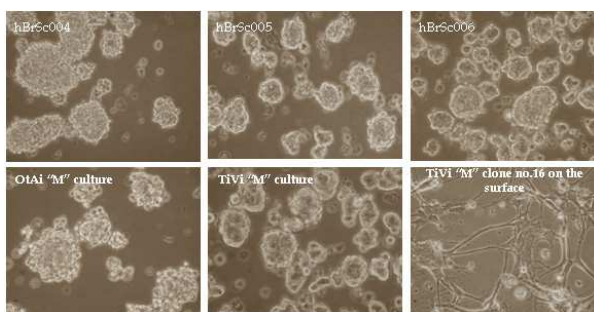


Fig. 2. Neurospheres in fetal neural stem/progenitor cell lines (hBrSc004, hBrSc005 and hBrSc006) and spheroids in glioblastoma OtAi "M" and TiVi "M" cell lines. The last picture shows a clone of TiVi M culture, adapted to grow on surface (Inverted microscope, obj. LWD A20PL 0.40 160/1.2).

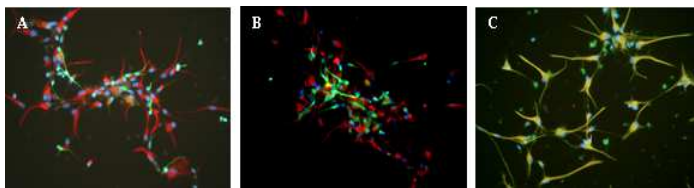


Fig. 3. Expression of different neural immunomarkers in fetal neural stem/progenitor cell line hBrSc006. Neurospheres were differentiated for 7 days. Cells were fixed with 4% PFA and permeabilized with Tritone X-100. (A) Double staining for  $\beta$ -III-tubulin (green - Alexa 488) and GFAP (red - Alexa 594); (B) Double staining for  $\beta$ -III-tubulin (green - Alexa 488) and GalC (red - Alexa 594); (C) Double staining for GFAP (green - Alexa 488) and GalC (red - Alexa 594), note co-location of GFAP and GalC - yellow staining. Obj. 40x. DAPI is used for nuclei staining (blue).

### 3.2 Development of new mouse monoclonal antibodies

#### 3.2.1 Preparation of immunogen

The three neural stem/progenitor cell lines (hBrSc003, hBrSc004 and hBrSc005) and one glioblastoma spheric cell line (glioblastoma TiVi M-cells) were used as immunogens in this study. The stem/progenitor cells were grown as neurospheres, the DMEM/F12 medium with B27 supplement (Gibco BRL) and growth factors bFGF (20 ng/ml; Pepro Tech, Princeton, USA), EGF (20 ng/ml; Pepro Tech, LIF (20 ng/ml, Chemicon, Temecula, USA) and gentamicin (Gibco BRL) was changed every 3 days. Two-three-week old neurospheres from one 25cm<sup>2</sup> cell culture flask were mechanically dispersed into cell suspension and thereafter repeatedly frozen and thawed to get cell fragments.

#### 3.2.2 Development of mouse hybridoma cells

About 100  $\mu$ l of the disrupted cell suspension were injected intraperitoneally into 6-week-old normal female Balb/c mice. The injections were repeated 4 times at 4-week intervals.

The cells from the spleens of immunized Balb/c mice were fused with the Sp2/0 myeloma cells by standard procedure (Mikelsaar et al., 2009). Hybridomas were grown in the RPMI 1640/HAT medium containing 10% FCS (Gibco BRL) and gentamicin. Primary screening of supernatants from hybridomas was performed starting from the 10<sup>th</sup> day of growth. Both unfixed and/or non-permeabilized paraformaldehyde (PFA) fixed human fetal stem/progenitor cells were tested immunocytochemically for the reaction with MAbs. The unfixed cells were incubated with hybridoma supernatants for 1 h at 37°C or overnight at 4°C, washed three times with PBS and a specific reaction of the MAb was revealed by incubating cells with fluorochrome ALEXA 594<sup>®</sup> - conjugated secondary goat anti-mouse IgG antibody (Molecular Probes, Eugene, USA) for 1 h. The cell fixation without permeabilization was performed with 8% PFA in PBS for 15 min at room temperature (RT), washed three times with PBS, the excess of aldehyde being quenched with 50mM NH<sub>4</sub>Cl in PBS (10 min) and blocked (0.3% casein, 0.01% Tween 20 in PBS) for 1 h at RT or overnight at 4°C. Ice-cold 100% methanol producing permeabilization was used for a further characterization of MAbs. The cells were incubated for 15 min at RT, washed three times with PBS and blocked. From plenty of MAb-producing clones, only those that revealed heterogeneity of reaction with stem/progenitor

cells, and glioblastoma spheroid cultures, were further investigated. The expression of antigens of selected monoclonal antibodies was further characterized besides neural stem/progenitor cells also on other living and/or fixed cells of different origin. It is important to find the cells where the target antigen of MAbs has the strongest expression in order to use these cells for molecular characterization of the antigen.

### **3.2.3 MAb cloning and isotyping**

Selected hybridomas were cloned by limiting dilution and isotypes of the MAbs were determined by using goat anti-mouse Ig isotype specific antibodies developed in LabAs Ltd. Cloned hybridomas are stored in a liquid nitrogen cell bank.

## **3.3 Immunocytochemical characterization of monoclonal antibodies**

### **3.3.1 Cells and cell lines used to characterize monoclonal antibodies**

Besides the cells used as immunogens (fetal neural stem/progenitor and glioblastoma spheroid cells) other types of cells and cell lines were used to characterize monoclonal antibodies. This was necessary to study the specificity of antibodies and is also useful to find out the positive cell lines with shorter duplication time. These cell lines were often used for getting more cellular material in shorter time for the identification of target antigens of MAbs (see also 3.4.2). The following additional cells and cell lines were used for the characterization of monoclonal antibodies: normal human cells - human blood thrombocytes, human sperms, normal fetal and adult skin fibroblast cell lines NL011 (LabAs Ltd) and SA-54, respectively, human normal amniotic epithelial cell line KM (LabAs Ltd.); malignant cell lines - human glioblastoma TiViMNBFCs10 cell line, glioblastoma TiVi M clone 16, glioblastoma OjArMNBFCs and OjFeMNBFCs cell lines (all developed in LabAs Ltd), Bowes melanoma cell line; cells of other species - COS-1 cell line (simian origin), rat granulosa cell culture (kindly provided by Prof. A. Žarkovský).

### **3.3.2 Fixation methods**

Paraformaldehyde or methanol fixation methods were used. In PFA fixation the coverslips were transferred without any previous washing into dishes containing prewarmed 4% PFA in PBS and left for 5 min at RT. Then the coverslips were washed for 3x 5 min with PBS and the excess of aldehyde was quenched with 50mM of NH<sub>4</sub>Cl in PBS (10 min). After washing twice with PBS, the cells were permeabilized for 10 min with 0.1% Tritone X-100 in PBS, washed with PBS and blocked. (In methanol fixation the coverslips were treated for 5 min with ice cold methanol and washed with PBS). The coverslips were then transferred into a blocking solution (0.3% casein, 0.01% Tween 20 in PBS) for 1 h at RT or overnight at 4°C.

### **3.3.3 Staining methods**

The blocking solution was removed by aspiration and the cells were stained as follows: they were incubated for 1 hour at RT with the MAb supernatant. The coverslips were washed at least for 3x5 min with PBS and immunolabeling was visualized by incubating the cells with the goat anti-mouse IgG antibody conjugated with fluorochrome Alexa 594 (Molecular Probes)

for 1 h at RT. In all cases, the coverslips were washed at least for 3x5 min with PBS, 10  $\mu$ l of DAPI solution 1 $\mu$ g/ml was added into the last PBS and then the coverslips were incubated for 5 minutes at RT. After quick rinsing in distilled water, the coverslips were mounted in the anti-fading mounting medium Prolong Gold Antifade (Molecular Probes). The cells were checked by a visual microscoping system (Olympus BX, using objectives UplanFI 20x/0.50, 40x/0.75, or 100x/1.30 Oil Iris and the Olympus DP50-CU Photographing System).

### **3.4 Characterization and identification of target antigens of new monoclonal antibodies**

#### **3.4.1 Strategy for target antigen identification**

Our strategy for the identification of target antigens of monoclonal antibodies was the following: (1) lysing, electrophoresis and the immunoblotting of suitable cellular material were performed to identify where the bands reacting with specific monoclonal antibodies are located; (2) for immunoprecipitation antibodies were caught to protein-G-conjugated Sepharose beads from the culture medium or used DVS-activated beads for purified antibodies; (3) the immunoprecipitated antigen was separated from the antibody and nonspecifically associated material by using electrophoresis in the SDS-PAGE gel; (4) the bands containing antigen were identified by using immunoblotting from the same gel; (5) the bands containing the antigen were cut out from the gel and trypsinized; (6) tryptic peptides and target antigens were identified with aid of mass-spectroscopy (LC ESI-MS-MS) and protein databases.

#### **3.4.2 Cells and cell lines used for identification of target antigens**

For molecular identification of the target antigens of MAbs different cells and cell lines were used (see also 3.3.1). However, in this study mainly the Bowes melanoma cell line (neural crest origin) and glioblastoma cell line TiViMNBFC10 (glioblastoma TiVi M spheroid culture, but adapted to growing on the surface) were used. Both the cell lines were propagated in medium DMEM/F12 with 10% FCS and gentamicin (Gibco BRL).

#### **3.4.3 Purification of antigens**

##### **3.4.3.1 Purification of antigens from cells growing on surface**

The cells of the human Bowes melanoma or TiViMNBFC10 cell lines were lysed in 8M urea, 3% SDS, 50mM Tris-HCL, pH 6.8 and diluted with 30 volumes of TBS. About 50  $\mu$ l of Sepharose-bound MAbs were added to 30 ml of the diluted sample solution and incubated overnight at 4°C. The beads were washed, incubated in an electrophoresis sample buffer for 10 min at 95°C and loaded onto the top of the 10% SDS-PAGE gel. After electrophoresis immunopositive bands were located by immunoblotting an one part of the gel, whereas the rest of the gel was stained with colloidal Coomassie G-250 for the confirmation of protein location. This electrophoresis step was absolutely necessary to avoid interferences with non-specifically bound proteins. The immunopositive band was cut out, minced, washed and dried (about 15 min at RT) by CentriVac. The dried pieces of the gel were rehydrated and the proteins trypsinized overnight at 37°C with sequencing grade trypsin (Promega, Madison WI, USA).

### 3.4.3.2 Purification of human blood thrombocytes

Purification of platelets was performed essentially according to the method of P.J. Canvar and co-workers (Canvar et al., 2007). Shortly, human platelet-rich plasma from the blood center of Tartu University Clinicum was further purified by centrifuging at 1400g for 15 min to pellet any remaining white or red blood cells and the platelet-rich plasma was decanted. Protease inhibitors (Roche Complete, Roche Diagnostics GmbH, Mannheim, Germany) were added as recommended by manufacturer and incubated 10 min at RT. Platelets were pelleted by centrifuging in at 4°C and washed once in Tyrode's buffer by centrifuging at 2400g for 15 min at 4°C. The pellets were stored in Tyrode's buffer at -80°C until used.

### 3.4.3.3 Cell lysis

Radioimmunoprecipitation assay buffer (RIPA) was used containing 50 mM of Tris/HCl pH 7.4 (ultrapure, AppliChem, Darmstadt, Germany); 0.1% SDS (ultrapure, AppliChem, Darmstadt, Germany); 1% NP40 (Octylphenoxy polyethoxy ethanol, reagent grade, AMRESCO, Solon Ind. Ohio, USA); 1% Tritone X-100 (Schuchardt, München, Germany); 0.5% DOC (Natriumdeoxycholat, AppliChem, Darmstadt, Germany); 500 mM NaCl (AppliChem, Darmstadt, Germany); protease inhibitor tablets (Roche Complete, Roche Diagnostics GmbH, Mannheim, Germany). For more gently lysis of the cells simply 1% NP40 solution in PBS (AppliChem, Darmstadt, Germany) was used.

### 3.4.3.4 SDS-polyacrylamid gel electrophoresis (SDS-PAGE)

Cells for electrophoresis were lysed in the RIPA buffer or with 1% NP-40 in PBS and centrifuged at maximum speed in Eppendorf centrifuge 5415C for 10 min at 4°C. The supernatant was diluted 1:1 in the SDS-electrophoresis sample buffer with DTT (AppliChem, Darmstadt, Germany) and heated for 10 min at 95°C or at 60°C for sensitive antigens. Before electrophoresis 8M of urea was added to the sample solution until 2M of the end concentration. Electrophoresis was run in a mighty small Hoefer electrophoresis system (Hoefer Scientific Instruments, CA, USA) with the glasses of 10 x 12 cm and the spacers of 0.75 mm. The end concentration of the gel buffer was 0.43 M and pH 8.4. Usually the samples were separated in the gradient (8-24%) SDS-PAGE gel for achieving sharper bands, yet the concentrating gel was not used. The parameters for prerunning electrophoresis were 60 V and 30 min. Electrophoresis was run for 4 hours at max 210V and 16mA.

### 3.4.3.5 Colloid Coomassie G250 staining

Colloid Coomassie G250 staining was performed essentially according to the D.Kang and co-workers (Kang et al., 2002). Shortly, the SDS-PAGE gel was fixed in a mixture of 30% ethanol and 2% phosphoric acid for 15 min and washed in distilled water 3x for 15 minutes. Staining was performed in a solution containing 5% w/v aluminium sulphate (Applichem, Darmstadt, Germany), 0.02% Coomassie G-250 (Serva Electrophoresis GmbH, Heidelberg, Germany), 2% phosphoric acid and 10% ethanol for 1 hour. Destaining was performed in distilled water until the bands became clearly visible.

### 3.4.3.6 Western blot

The method described in the Millipore manual for immunoblotting to the PVDF membrane was used. Buffer components and the stain were from AppliChem, Darmstadt, Germany. The procedure was as follows: the membrane was wetted in methanol for 1 to 3 seconds.

Methanol was eluted incubating the membrane for 5 min in distilled water on a shaker. Thereafter the membrane was equilibrated in the transfer buffer for 2 to 3 min. SDS-PAGE gel was equilibrated in the cathode buffer for 15 min on the shaker. GEHealthcare electrophoresis unit Multiphor II and a semidry immunoblotting kit for electroblotting were used. The anode buffers were Tris 0.3 M (no.1) and Tris 15 mM (no.2); the cathode buffer contained 40 mM of glycine and 25 mM of Tris. Two sheets of chromatography papers (Whatman CHR17, Mainstone, England) were soaked in anode buffer no.1 and one sheet in the anode buffer no.2. The wetted PVDF membrane was placed on the top of the soaked anode sheets and then equilibrated SDS-PAGE gel was placed on the top of them. After that three chromatography papers with the cathode buffer were located on the top of the gel and immunoblotting was performed at 0.8 mA /cm<sup>2</sup> for 2 hours. After electroblotting nonspecific binding of antibodies was blocked by incubating the membrane in TBS with 0.1% Tween 20 (Sigma, MO, USA). For vimentin-specific antibody GB26 10G3 a mixture of 1% horse serum and 0.1% Tween 20 in TBS was used as a blocking solution. The membrane was incubated with monoclonal antibody in the blocking buffer for overnight at 4°C. After washing 3 x for 10 min in PBS 0.1% Tween 20 the membrane was incubated with a secondary antibody (goat anti-mouse IgG polyclonal antibody conjugated with horseradish peroxidase) in PBS 0.1% Tween 20 for one hour at RT. After that the membrane was washed with 0.1% Tween 20 in PBS 3 times for 10 min and then 1 x for 10 min in PBS. The membrane was incubated for 10 minutes in the staining solution containing 50 mg of DAB (diaminobenzidin trihydrochloride, AppliChem, Darmstadt, Germany), 6 ml chloronaphtole (Sigma, MO,USA) solution (3 mg/ml) in ethanol and 20 µl of 30% H<sub>2</sub>O<sub>2</sub> in PBS and washed 3 times for 5 minutes in distilled water. After drying the membrane can be saved in the archive.

#### **3.4.3.7 Conjugation of antibody with horseradish peroxidase**

Antibodies were conjugated to HRP as described previously (Tjissen, 1985). Shortly, 1 mg of horseradish peroxidase (Boehringer Mannheim, Germany) was solubilized in the Eppendorf tube in 0.1 ml of the freshly prepared 0.1M NaHCO<sub>3</sub> solution and 0.1 ml of 8-16 mM NaIO<sub>4</sub> (Merck, Darmstadt, Germany) was added and incubated for 2 hours in the dark room at RT. 3 mg of antibody was solubilized in 1 ml of the sodium carbonate buffer pH 9.2 and dry Sephadex G-25 (GE Healthcare, Sweden) was added of about 1/6 from the total amount of the solution and the mix was incubated for 3 hours in the dark room at RT. The conjugate was eluted from Sephadex and mixed (1/20 from the total volume) with the freshly prepared NaBH<sub>4</sub> (Sigma, MO, USA) solution in 0.1M of NaOH (5 mg/ml) on a shaker. After 30 min an additional amount of the NaBH<sub>4</sub> solution (1/10 from the total volume) was added and the mix was incubated for 1 hour at 4°C. Then 50% of glycerol (AppliChem, Darmstadt, Germany) was added and the conjugate was stored at -20°C. For immunoblotting HRP-conjugated antibodies were diluted from 1:500 to 1:1500.

#### **3.4.3.8 Activating of Sepharose granules with divinylsulphone (DVS) and binding of antibodies to activated granules**

Sepharose CL-4B granules (Pharmacia, Uppsala, Sweden) were washed with distilled water of 4 to 5 gel volumes. The granules were equilibrated with 4 to 5 gel volumes of 0.5 M carbonate buffer (pH 11), 10 ml of the 0.5 M carbonate buffer (pH 11) containing 1 ml of divinylsulphone (Sigma, MO, USA) were added to 10 ml of the gel and incubated on a shaker for 1.5 hr. The activated granules were washed once with the carbonate buffer, then 2



x with distilled water and stored in PBS with 0.01%  $\text{NaN}_3$  at 4°C. Activated granules are useable during about 1 year. For the binding of antibodies the granules were washed with 4 to 5 volumes of distilled water and then equilibrated in 0.1 M carbonate buffer pH 9.0. The buffer for antibodies was changed to the carbonate buffer pH 9.0 using the PD 10 column (GE Healthcare, Sweden). DVS-activated Sepharose CL-4B granules (200  $\mu\text{l}$ ) were added to antibody solution (1-2 mg in 3.5 ml) in the carbonate buffer and incubated for 10 min at RT. Then 5% PEG 20,000 was added and the granules were incubated overnight at RT on a shaker. Afterwards the granules with conjugated antibodies were washed and blocked in TBS buffer and stored in TBS at 4°C with 0.01%  $\text{NaN}_3$  until used.

#### 3.4.3.9 Immunoprecipitation

For immunoprecipitation the lysates in RIPA buffer, PBS with 1% NP40 or PBS/TBS diluted 1:30 electrophoresis sample buffer were used. Before immunoprecipitation MABs from 1.5 ml of the hybridoma medium (sometimes more) were conjugated to 50  $\mu\text{l}$  of Protein-G Sepharose 4 Fast Flow granules (GE Healthcare, Sweden). The granules were washed 3 times in the immunoprecipitation buffer and incubated with cell lysate at 4°C overnight. Then the granules were washed in one of the immunoprecipitation buffers 4 times and once with distilled water. The washed granules (50  $\mu\text{l}$ ) were diluted in 150  $\mu\text{l}$  of the SDS-PAGE sample buffer and incubated for 5 min at 95°C or in case of sensitive antigens for 10 min at 60°C. Then 50  $\mu\text{l}$  of 8M urea were added and the granules were incubated for 15 min at RT on a shaker and thereafter put on the top of the electrophoresis gel. When purified monoclonal antibodies not hybridoma supernatants were used for immunoprecipitation, MABs were directly conjugated to the divinyl-sulphone-activated granules.

#### 3.4.3.10 Trypsinization of isolated antigens

After electrophoresis, immunopositive bands were located by immunoblotting a part of the gel, whereas the rest of the gel was stained with colloidal Coomassie G-250 for the confirmation of protein location. This electrophoresis step is necessary to avoid interferences with non-specifically bound proteins. The immunopositive band was cut out from the gel and minced to pieces of about 1 x 1 mm using a scalpel. Pieces of the gel were washed once with methanol on a shaker for 1-2 minutes, then with 10 mM  $(\text{NH}_4)_2\text{CO}_3$  2 times for 5 min and thereafter with 50% acetonitrile in 10 mM  $(\text{NH}_4)_2\text{CO}_3$ . The gel pieces were dried with the CentriVac for 15 min and treated for 10 min with the solution containing 2  $\mu\text{g}$  of trypsin in 1 ml of 50 mM  $(\text{NH}_4)_2\text{CO}_3$  with 0.05% ProteasMax surfactant (Promega, USA). 10 mM of  $(\text{NH}_4)_2\text{CO}_3$  was added to fully coated gel pieces and incubated at 37°C overnight. The solution from the top of the gel and the washing solutions were collected into one tube. The gel pieces were washed two times with 50% acetonitrile in 10 mM  $(\text{NH}_4)_2\text{CO}_3$  and once in pure acetonitrile. The solution containing peptides was concentrated until the amount of 100  $\mu\text{l}$  and analyzed using mass-spectroscopy. Sometimes for confirming the exact band location in the gel direct staining of proteins on the membrane is needed. To that end colloid Coomassie stain diluted 1:1 in distilled water was used. After that we destained the membrane washing with distilled water about ten times 10 min or until protein bands became visible. On the dry membrane we saw strongly stained black bands containing the antigen or antibody components and other blue-stained bands containing different co-immunoprecipitated nonantigenic proteins.

### 3.4.3.11 Peptide analysis by LC ESI-MS/MS and protein identification with Mascot and the Global Proteome Machine

The Agilent 1100 Series chromatograph with LC/MSD Trap XCT (Agilent, Santa Cruz, USA) was used for LC/MS experiments applying the 2.1 x 150 mm Agilent 300Extend C18 column of 3.5  $\mu\text{m}$  particle size. 50  $\mu\text{l}$  of the peptide mixture was injected into the column and eluted with a gradient from 0.1% HCOOH/5% acetonitrile to 75% acetonitrile during 120 minutes at 0.3 ml/min. The column was thermostated at 35°C. Positive ions were detected in a "smart mode" with the target mass set to 1000 m/z, whereas doubly charged ions were preferred. The data were analyzed with both Mascot [www.matrixscience.com](http://www.matrixscience.com) and GPM [www.thegpm.org](http://www.thegpm.org) search engines (1000 most abundant ions). For the positive identification of the antigen the consent of both search engines was necessary.

## 4. Results

During our study we have developed several hundreds of new monoclonal antibodies. The characterization and identification of the target antigens of them are in progress. Some results of this work have been recently published (Mikelsaar et al., 2009). Here we present data on the five new monoclonal antibodies (see Table 1), which we have developed according to the strategy described in this chapter.

Name of monoclonal antibodies	Ig sub-class	Immunogen	Identified target antigen	Neural cell types identified by antibody
E14G2	IgG1	Mix of fetal neural cell lines hBrSc003/ hBrSc004/ hBrSc005	Annexin A1	Annexin A1- and + cells among differentiated fetal neural stem cells, glioblastoma spheroid cells and in glioblastomas; microglia; annexin A1+ glial cells and Purkinje (?) cells in rat granule cell culture
E15F10.B9	IgG1	Mix of fetal neural cell lines hBrSc003/ hBrSc004/ hBrSc005	Calnexin	Calnexin- and + cells among differentiated fetal neural stem cells and glioblastoma spheroid cells; shows cellular heterogeneity in glioblastomas
W4A8.F4	Ig2a	hBrSc003	14-3-3 $\zeta/\delta$	Both human and rat glial and neuronal cells
GB26 10G3	IgG1	Mix of different glioblastoma cells	Vimentin	The precursors cells of both neuronal and glial lineages; shows cellular heterogeneity in glioblastomas
A3G2.B4	IgG1	hBrSc003	Lupus La protein	La protein- and + cells among differentiated fetal neural stem cells and glioblastoma spheroid cells; shows cellular heterogeneity in glioblastomas

Table 1. Summary of new monoclonal antibodies described in this chapter. All these antibodies may be useful for detailed analysis of the expression of target antigens in all types of neural cells .

## 4.1 New monoclonal antibodies

### 4.1.1 Monoclonal antibody E14G2 (MAb E14G2)

Here we describe the results of the immunocytochemical analysis of MAb E14G2 (Table 1) with different cell types and the biochemical/molecular analysis for identifying the target antigen of the antibody. In Fig. 4 we can see a distinct heterogenous expression of MAb E14G2 antigen in differentiated fetal neural stem/progenitor (A,B,C,D), in glioblastoma spheroid cells (E) and also in the rat granule primary culture (H). However, the expression of the target antigen of MAb E14G2 was strong and homogenous in glioblastoma OjAr secondary culture and in Bowes melanoma cell lines (F and G, respectively).

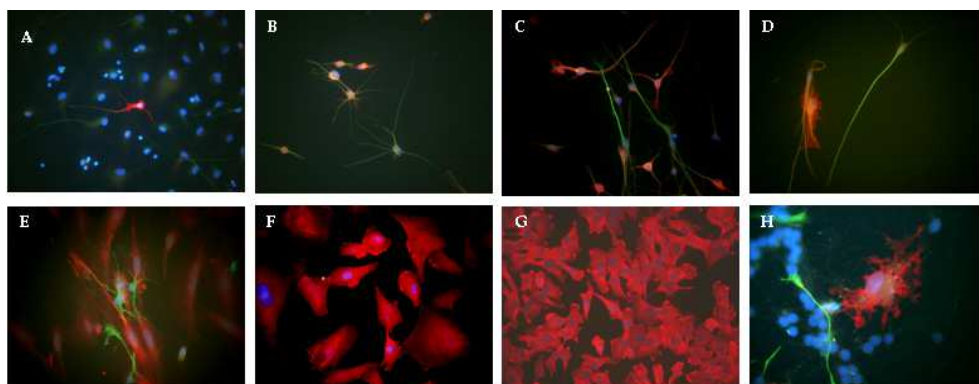


Fig. 4. Presence of MAb E14G2 target antigen (Annexin A1) in different cells. (A) Human fetal hBrSc003 neurosphere cells differentiated for 11 days. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Annexin A1 expression was revealed by red staining – Alexa 594,  $\beta$ -III-tubulin by green staining – Alexa 488, obj. 40x. (B-D). Human fetal hBrSc005 neurosphere cells differentiated for 3 days (B,C) and 7 days (D). Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Annexin A1 expression was revealed by red staining – Alexa 594,  $\beta$ -III-tubulin by green staining – Alexa 488, obj. 40x. (E) Human glioblastoma TiVi spheroid cell line differentiated for 7 days. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Annexin A1 expression was revealed by red staining – Alexa 594,  $\beta$ -III-tubulin by green staining – Alexa 488, obj. 40x. (F) Human glioblastoma OjAr secondary cell culture on the 6th day of cultivation. Cells were fixed with 4% PFA and permeabilized. Annexin A1 expression was revealed by red staining – Alexa 594, obj. 40x. (G) Bowes melanoma cell line on the 2nd day of cultivation, fixed with 4%PFA, no permeabilization. Specific Annexin A1 staining was revealed by red staining – Alexa 594, obj. 40x. (H) Rat granulare cell culture. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Annexin A1 expression was revealed by red staining – Alexa 594, GFAP by green staining – Alexa 488, obj. 100x. DAPI is used for nuclei staining (blue).

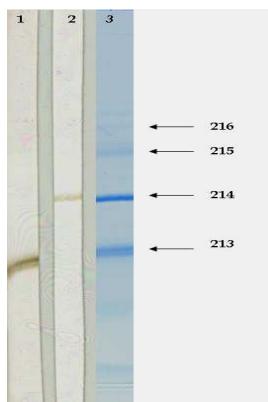


Fig. 5. Electrophoresis and Western blot of MAb E14G2 target antigen. (1) negative control, MAb Y1C7 directly conjugated with peroxidase; (2) MAb E14G2 directly conjugated with peroxidase; (3) gel stained with colloidal Coomassie G-250. Arrows with numbers indicate bands analyzed by LC ESI-MS/MS.

In Fig. 5 we present the data on electrophoresis and immunoblot of the target antigen of MAb 14G2. The antigen was immunoprecipitated from the Bowes melanoma cell lysate and the precipitated cells (100  $\mu$ l) were lysed in 10 ml of RIPA buffer. MAb E14G2 was conjugated directly to the divinylsulphone-activated Sepharose granules. Then 50  $\mu$ l of the activated granules were incubated in 10 ml of the cleared Bowes melanoma cell lysate overnight at 4°C, washed 4x with the buffer and 1x with distilled water. Then 150  $\mu$ l of SDS-PAGE sample buffer was added and the sample heated 5 min at 95°C before electrophoresis.

The bands, which were positive according to the reaction with specific monoclonal antibody MAb E14G2 (Fig. 4, arrows 213-216) were analyzed by LC ESI-MS/MS and the proteins were identified using Mascot and the GPM software databases (see Table 2).

Band	Identified proteins	Mass	Mascot score	Sequence coverage %
213	ANXA1 - Annexin A1	38690	41	8
	KV401 - Ig kappa chain V-IV region	13372	37	7
214	ANXA1 - Annexin A1	38690	2274	71
215	ANXA1 - Annexin A1	38690	198	34
	TBB5 - Tubulin beta chain	49639	113	24
	K2C1- Keratin, type II cytoskeletal 1	65999	84	6
216	DHSA- Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mit	72645	103	26
	K2C1- Keratin, type II cytoskeletal 1	65999	82	7
	ANXA1 - Annexin A1	38690	65	18

Table 2. LC ESI-MS/MS and Mascot software analysis of immunoprecipitated with MAb 14G2 proteins in bands 213, 214, 215 and 216 (Fig. 4, arrows 213-216).

1 MAMVSEFLKQ **AWFIENESEQE YVQTVKSSKG GPGSAVSPYP TFPNSSDVAA**  
 51 **LHKAIMVKGV DEATIIDILT KRNNARQQI KAAYLQETGK PLDETLKKAL**  
 101 **TGHLLEEVLA LLKTPAQFDA DELRAAMKGL GTDEDTLIEI LASRTNKEIR**  
 151 **DINRVYREEL KRDLAKDITS DTSGDFRNAL LSLAKGDRSE DFGVNEDLAD**  
 201 **SDARALYEAG ERKRGTDVNV FNTILTTRSY PQLRRVFQKY TKYSKHD MNK**  
 251 **VLDLELKGDI EKCLTAIVKC ATSKPAFFAE KLHQAMKGVG TRHKALIRIM**  
 301 **VSRSEIDMND IKAFYQKMYG ISLCQAILDE TKGDYEKILV ALCGGN**

Fig. 6. Amino acid sequences of Annexin A1, ANXA1. In red are marked amino acids sequences identified by mass-spectroscopy (band 214 in Fig. 5).

### Conclusion

On the basis of data presented above we conclude that the target antigen of MAb E14G2 is protein Annexin A1. According to UniProtKB/Swiss-Prot database Annexin A1 is a calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. This protein regulates phospholipase A2 activity. ([http://www.uniprot.org/uniprot/P04083#section\\_comments](http://www.uniprot.org/uniprot/P04083#section_comments)).

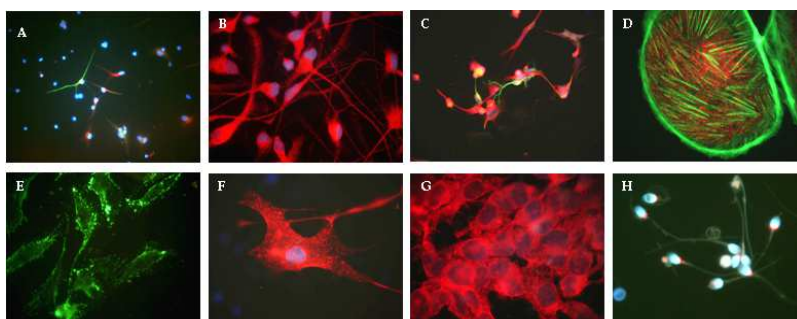


Fig. 7. Presence of MAb E15F10.B9 target antigen (calnexin) in different cells. (A) Human fetal hBrSc003 neurosphere cells differentiated for 11 days. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Calnexin expression was revealed by red staining - Alexa 594,  $\beta$ -III-tubulin by green staining - Alexa 488, obj. 40x. (B) Human fetal hBrSc003 neurosphere cells differentiated for 7 days, cells were fixed with 4% PFA and permeabilized. Calnexin expression was revealed by red staining - Alexa 594, obj. 100x. (C) Human glioblastoma TiVi spheroid cell line differentiated for 7 days. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Calnexin expression was revealed by red staining - Alexa 594,  $\beta$ -III-tubulin by green staining - Alexa 488, obj. 40x. (D) Human normal amniotic epithelial cell line KM on the fifth days of cultivation. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. Calnexin expression was revealed by red staining - Alexa 594, actin by green staining with Alexa 488 conjugated Phalloidin, obj. 100x. (E) Unfixed cells of adult skin fibroblast cell line SA-54 on the 3rd day of cultivation. Specific calnexin staining revealed by green staining - Alexa 488, obj. 20x. (F) Human glioblastoma TiViMNBFC510 cells on the 3rd day of cultivation, fixed with 4%PFA, no permeabilization. Specific calnexin staining revealed by red staining - Alexa 594, obj. 100x. (G) Bowes melanoma cell line on the 2nd day of cultivation, fixed with 4%PFA, no permeabilization. Specific calnexin staining revealed by red staining - Alexa 594, obj.100x. (H) Unfixed sperms of a normal human male. Specific calnexin staining revealed by red staining - Alexa 488, obj. 100x (note a red staining between the sperm head and tail). DAPI is used for nuclei staining (blue).

#### 4.1.2 Monoclonal antibody E15F10.B9 (MAb E15F10.B9)

In Fig. 7 we can see different expression of MAb E15F10.B9 (Table 1) detected antigen in differentiated fetal neural stem/progenitor (A) and glioblastoma spheroid cells (C). In some regions of hBrSc003 cell line differentiated for 7 days there was a very strong expression of MAb E15F10.B9 target antigen (B). Strong expression of the antigen one can see also in unfixed adult skin fibroblasts and in fixed, but not permeabilized glioblastoma and Bowes melanoma cells. Interestingly, in unfixed human sperms the staining of MAb E15F10.B9 was seen as two separate points between the head and neck of the sperm (H). Double staining with actin in normal amniotic epithelial cells (D) shows an independent staining of actin and target antigen of MAb E15F10.B9, whereas the last one shows nuclear membrane and punctate cytoplasm staining.

The LC ESI-MS/MS and Mascot database analysis of peptides from the immunoprecipitated band 208 (Fig. 8) identified two possible candidates for the target antigen of MAb E15F10.B9 – calnexin and Keratin, type II cytoskeletal 1. However, as immunocytochemical analysis on different cell types including normal epithelial amniotic cells (Fig. 7) showed no typical cytoskeletal staining for cytokeratin we consider the keratin to be in immunoprecipitate as a contaminant substance. In Fig. 8 and 9 and Table 3 the results of Western blot and molecular identification of the target antigen of MAb E15F10.B9 are shown.

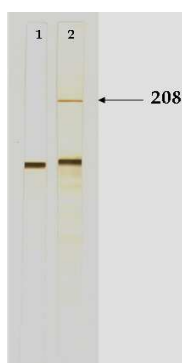


Fig. 8. Western blot of MAb E15F10.B9 target antigen (calnexin). MAb E15F10.B9 from hybridoma supernatant was conjugated to Protein G granules and thereafter target antigen immunoprecipitated from Bowes melanoma cell line lysed with RIPA buffer. Proteins were separated in SDS-PAGE 4-12% gradient gel. (1) negative control with HRP-conjugated goat anti-mouse IgG secondary antibody; (2) incubation with MAb E15F10.B9 and secondary antibody. Arrow indicates the band analyzed by ESI-MS/MS.

Band	Identified proteins	Mass	Mascot score	Sequence coverage %
208	CALX - Calnexin	67526	384	22
	K2C1- Keratin, type II cytoskeletal 1	65999	313	20

Table 3. LC ESI-MS/MS and Mascot software analysis of the proteins in the band which was immunoprecipitated with MAb E15F10.B9 proteins (Fig. 8).

```

1 MEGKWLCLML LVLGTAIVEA HDGHDDDDVID IEDDLLDDVIE EVEDSKPDFTT
51 APPSSPKVTY KAPVPTGEVY FADSFDRGTL SGWILSKAKK DDTTDEIAKY
101 DGKWEVEEMK ESKLPGDKGL VLMSRAKHHA ISAKLNKPFLL FDTKPLIVQY
151 EVNFQNGIEC GGAYVKLLSK TPELNLDQFH DKTPYTIMFG PDKCGEDYKL
201 HFIFRHKNPV TGIYEEKHAK RPDADLKYF TDKKTHLYTL ILNPDNSFEI
251 LVDQSVVNSG NLLNDMTPPV NPSREIEDPE DRKPEDWDER PKIPDPEAVK
301 PDDWDEDAPA KIPDDEATKP EGWLDDEPEY VPDPAEKPE DWDEDMDGEW
351 EAPQIANPRC ESAPGCGVWQ RPVIDNPNYK GKWKPPMIDN PSYQGIWKPR
401 KIPNPDDFFED LEPFRMTPFS AIGLELWSMT SDIFFDNFII CADRRIVDDW
451 ANDGWGLKKA ADGAAEPGVV GOMIEAAEER PVLWVVYILT VALPVFLVIL
501 FCCSGKKQTS GMEYKTDAP QPDVKEEEEE KEEKDKGDE EEEGEEKLEE
551 KQKSDAEEEDG GTVSQEEEDR KPKAEDEEIL NRSRNRKPR RE

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Fig. 9. Amino acid sequences of Calnexin. With red colour are marked amino acids sequences identified by mass-spectroscopy (band 208 in Fig. 8).

### Conclusion

On the basis of data presented above we conclude that the target antigen of MAb E15F10.B9 is protein Calnexin. According to UniProtKB/Swiss-Prot database Calnexin is a calcium-binding protein that interacts with newly synthesized glycoproteins in the endoplasmic reticulum. It may act in assisting protein assembly and/or in the retention within the ER of unassembled protein subunits. It seems to play a major role in the quality control apparatus of the ER by the retention of incorrectly folded proteins (<http://www.uniprot.org/uniprot/P27824>).

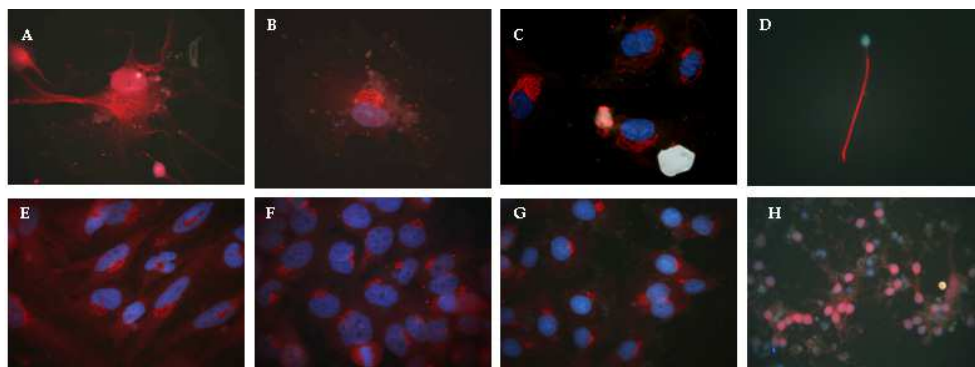


Fig. 10. Presence of MAb W4A8.F4 target antigen (protein 14-3-3) in different cells. Specific staining of MAb W4A8.F4 was revealed by red staining –Alexa 594. (A,B) Human fetal hBrSc006 (A) and hBrSc009 (B) neurosphere cells differentiated for 3 days. Cells were fixed with 4% PFA and permeabilized, obj. 100x. (C) Human fetal skin fibroblast culture on the 3rd day of cultivation. Cells were fixed with 4% PFA and permeabilized, obj. 100x. (D) Unfixed sperms of a normal human male, obj. 100x. (E-F) Human glioblastoma cell line TiViMNBFC510 (E), Bowes melanoma cell line (F), COS-1 cell line and rat granulosa cell culture (H) on the 3rd day of cultivation. Cells were fixed with 4% PFA and permeabilized, obj. 100x. DAPI is used for nuclei staining (blue).

### 4.1.3 Monoclonal antibody W4A8.F4 (MAb W4A8.F4)

In Fig. 10 we can see the expression of MAb W4A8.F4 (Table 1) in the fetal neural stem/progenitor cell lines hBrSc006 (A) and hBrSc009 (B). The strong staining was seen in the cytoplasm in Golgi region but also in cell projections and nuclei. In the fetal skin fibroblasts, glioblastoma, Bowes melanoma and COS-1 cell lines the cytoplasm, especially Golgi region was strongly stained (C,E,F and G respectively). In the rat granular cell culture there was a heterogeneous staining of the cytoplasm and nuclei (H). Interestingly, in the unfixed human sperms the entire tail, except the neck, was strongly and homogeneously stained (D).

In Fig. 11 and 12 and Table 4 the results of Western blot and molecular identification of the target antigen of MAb W4A8.F4 are shown.

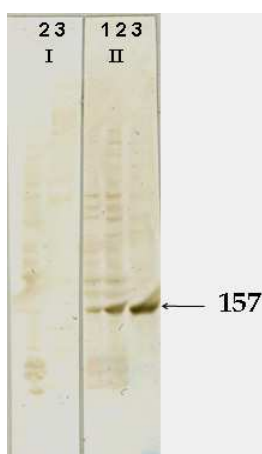


Fig. 11. Western blot with MAb W4A8.F4. Proteins in RIPA lysate were separated electrophoretically in SDS-PAGE gradient (8-25%) gel and transferred to PVDF membrane: (1) Bowes cell line, (2) glioblastoma TiViMNBFC510 cell line and (3) human thromocytes. I - negative control with HRP-conjugated goat anti-mouse IgG secondary antibody; II - reaction with MAb W4A8.F4 and secondary antibody. Arrow indicates the band 157, analyzed by ESI-MS/MS.

Band	Identified proteins	Mass	Mascot score	Sequence coverage %
157	14-3-3 protein zeta/delta	27728	139	40
	14-3-3 protein gamma	28295	64	18
	14-3-3 protein eta	28201	64	19
	14-3-3 protein sigma	27757	64	12
	Chloride intracellular channel protein 1	26906	62	19

Table 4. LC ESI-MS/MS and Mascot software analysis of immunoprecipitated with MAb W4A8.F4 proteins in band 157 (Fig. 11).



```
1 MDKNELVQKA KLAEQAERYD DMAACMKSVT EQGAELSNEE RNLLSVAYKN
51 VVGARRSSWR VVSSIEQKTE GAEKKQOMAR EYREKIETEL RDICNDVLSL
101 LEKFLIPNAS QAESKVFYLK MKGDYYRYLA EVAAGDDKKG IVDQSQQAYQ
151 EAFEISKKEM QPTHPIRLGL ALNFSVFYIE ILNSPEKACS LAKTAFDEAI
201 AELDTLSEES YKDSTLIMQL LRDNLTLWTS DTQGDEAEAG EGGEN
```

Fig. 12. Amino acid sequences of protein 14-3-3 protein zeta/delta. With red colour are marked amino acids sequences identified by mass-spectroscopy (band 157 in Fig. 11).

### Conclusion

On the basis of data presented above we conclude that most probably the target antigen of MAb W4A8.F4 is protein 14-3-3 zeta/delta. According to UniProtKB/ Swiss-Prot database protein 14-3-3 zeta/delta is an adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways. Binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner (<http://www.uniprot.org/uniprot/P63104>).

#### 4.1.4 Monoclonal antibody GB26 10G3 (MAb GB26 10G3)

In Fig. 13 we can see the filamentous staining of MAb GB26 10G3 (Table 1) antigen in human adult skin fibroblasts (A) and in glioblastoma cell line TiViMNBFC10 (B). In glioblastoma cell lines the staining pattern was very heterogeneous, some cells did not show any signs of staining. Double staining for  $\beta$ -III-tubulin and the MAb GB26 10G3 (C) showed an independent staining pattern, some cells were double stained, some cells showed only  $\beta$ -III-tubulin staining and the majority of cells was only MAb GB26 10G3 positive. In D the double staining of target antigen of MAb GB26 10G3 and glial fibrillar acid protein (GFAP) is shown. Note that the both antigens are located in the same cells, but show an independent staining pattern.

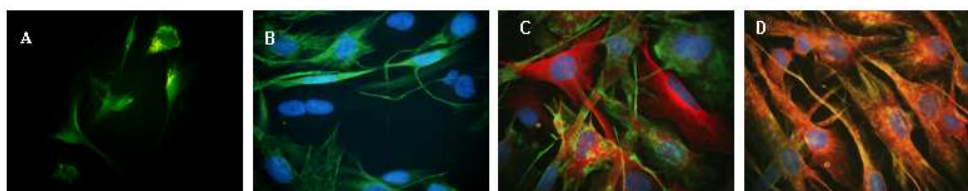


Fig. 13. Presence of MAb GB26 10G3 target antigen in different cells. (A,B) Human adult skin fibroblast cell line SA-54 (A) and human glioblastoma cell line TiViMNBFC10 (B) on the 3rd day of cultivation. Cells were fixed with 4% PFA and permeabilized. The target antigen of MAb GB26 10G3 expression was revealed by green staining - Alexa 488, obj. 100x. (C) Human glioblastoma cell line TiViMNBFC10 on the 3rd day of cultivation. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. The target antigen of MAb GB26 10G3 expression was revealed by green staining - Alexa 488,  $\beta$ -III-tubulin by red staining - Alexa 488, obj. 100x. (D) Human glioblastoma cell line TiViMNBFC10 on the 3rd day of cultivation. Double immunofluorescent staining, cells were fixed with 4% PFA and permeabilized. The target antigen of MAb GB26 10G3 expression was revealed by green staining - Alexa 488, GFAP by red staining - Alexa 488, obj. 100x. DAPI is used for nuclei staining (blue).

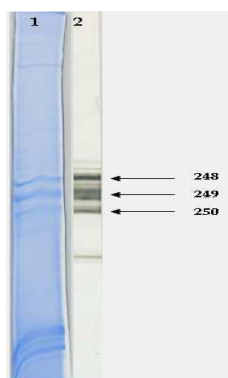


Fig. 14. SDS-PAGE gradient gel (8-24%) (1) and immunoblot (2) with MAb GB26 10G3. The sample was in RIPA buffer insoluble fraction of glioblastoma TiViNBFC510 cells that was heated with SDS-PAGE sample buffer with DTT at 60°C for 10 min and 8M urea was added to 2M final concentration. Arrows with numbers indicate bands analyzed by ESI-MS/MS.

As molecular masses of antibody light chain and vimentin are similar and vimentin binds nonspecifically to the secondary antibody, we could not purify vimentin by immunoprecipitation as we did for other MABs. Only gradient electrophoresis of lysate and immunoblotting were used to separate the target antigen of MAB GB26 10G3.

In Fig. 14 and 15 and Table 5 the results of electrophoresis, Western blot and molecular identification of the target antigen of MAB GB26 10G3 are shown.

Band	Identified proteins	Mass	Mascot score	Sequence coverage %
248	Vimentin	53619	198	39
249	Glial fibrillar acid protein - GFAP Vimentin	49850 53619	135 123	32 28
250	Actin, cytoplasmic 1	41710	43	14

Table 5. LC ESI-MS/MS and Mascot software analysis of proteins in bands 248- 250 (Fig. 14).

```

1  MSTRSVSSSS YRRMFGGPGT ASRPSSRSY VTTSTRTYSI GSALRPSTSR
51 SLYASSPGGV YATRSSAVRL RSSVPGVRL QDSVDFSLAD AINTEFKNTR
101 TNEKVELQEL NDRFANYIDK VRFLEQQNKI LLAELEQLKG QGKSRLGDLY
151 EEEMRELRQ VDQLTNDKAR VEVERDNLAE DIMRLREKLQ EEMLREEAE
201 NTLQSFQDV DNASLARLDL ERKVESLQEE IAFLKKLHEE EIQELQAQIQ
251 EQHVQIDVDV SKPDLTAALR DVRQQYESVA AKNLQEAEEW YKSKFADLSE
301 AANRNNDALR QAKQESTEYR RQVQSLTCEV DALKGTNESL ERQMREMEEN
351 FAVEAANYQD TIGRLQDEIQ NMKEEMARHL REYQDLLNVK MALDIEIATY
401 RKLLEGEESR ISLPLPNFSS LNLRETNLDS LPLVDTHSKR TLLIKTVETR
451 DGQVINETSQ HHDDLE

```

Fig. 15. Amino acid sequences of Vimentin. With red colour are marked amino acids sequences identified by mass-spectroscopy (band 248 in Fig. 14).

## Conclusion

On the basis of data presented above we conclude that the target antigen of MAb GB26 10G3 is protein Vimentin. According to UniProtKB/Swiss-Prot database vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells (<http://www.uniprot.org/uniprot/P08670>).

### 4.1.5 Monoclonal antibody A3G2.B4 (MAb A3G2.B4)

In Fig. 16 we can see the expression of MAb A3G2.B4 antigen (Table 1) in differentiated fetal neural stem/progenitor cell lines hBrSc003 (A,B) and hBrSc005 (B). Note a clear heterogeneous staining of cell nuclei. In D, E,F and G the staining of glioblastoma spheroid cell line differentiated for 7 days, normal epithelial amniotic cells, adult skin fibroblasts and glioblastoma OjFe secondary cell culture are shown, respectively. Note the heterogeneous nuclear and nucleolar staining and a weak cytoplasmic staining. However, in Bowes melanoma cell line both the nuclear and strong granular cytoplasmic staining was observed (H).

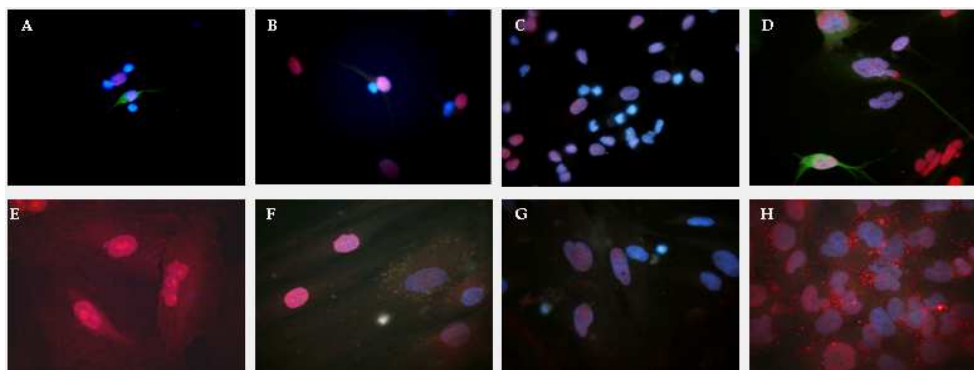


Fig. 16. Presence of MAb A3G2.B4 antigen in different cells. In all cases the cells were fixed with 4% PFA, permeabilized with Tritone X-100 and specific staining of MAb A3G2.B4 revealed by red staining –Alexa 594. (A,B) Human fetal hBrSc003 neurosphere cells differentiated for 11 days. Double immunofluorescent staining.  $\beta$ -III-tubulin is revealed by green staining – Alexa 488, obj. 40x. (C) Human fetal hBrSc005 neurosphere cells differentiated for 7 days, obj. 40x. (D) Human glioblastoma TiVi spheroid cell line differentiated for 7 days. Double immunofluorescent staining.  $\beta$ -III-tubulin is revealed by green staining – Alexa 488obj. 100x. (E-H) Human normal amniotic epithelial cell line KM on the 2nd days of cultivation, human adult skin fibroblast cell line SA-54 on the 3rd day of cultivation, human glioblastoma OjFe secondary cell culture on the 3rd day of cultivation, and Bowes melanoma cell line on the 2nd day of cultivation, respectively, obj. 100x. DAPI is used for nuclei staining (blue).

In Fig. 17 and 18 the results of Western blot and molecular identification of the target antigen of MAb A3G2.B4 are shown.

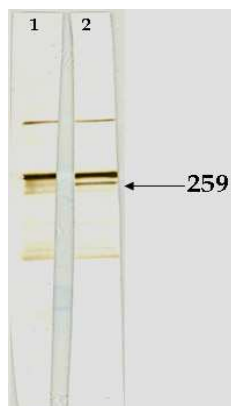


Fig. 17. Western blot with MAb A3G2.B4. Target antigen Lupus La antigen was first immunoprecipitated with MAb A3G4.B4 in 1% NP-40 PBS solution from the concentrated lysate of Bowes melanoma cells. Precipitate was heated at 60°C 15 min, added urea up to end concentration of 2M and proteins separated in SDS-PAGE gradient (8-24%) gel. 1 - negative control with HRP-conjugated goat anti-mouse IgG secondary antibody; 2 - reaction with MAb A3G2.B4 and secondary antibody.

```

1  MAENGDNEKM  AALEAKICHQ  IEYYFGDFNL  PRDKFLKEQI  KLDEGWVPLE
51  IMIKFNRLNR  LTTFENVIVE ALSKSKAELM  EISEDKTKIR  RSPSKPLPEV
101 TDEYKNDVKN  RSVYIKGFPT  DATLDDIKEW  LEDKGQVLNI  QMRRTLHKAF
151 KGSIFVVFDS IESAKKFVET  PGQKYKETDL  LILFKDDYFA  KKNEERKQNK
201 VEAKLRAKQE  QEAKQKLEED  AEMKSLEEKI  GCLLFSGDL  DDQTCREDLH
251 ILFSNHGEIK  WIDFVRGAKE  GIILFKEKAK  EALGKAKDAN  NGNLQLRNKE
301 VTWEVLEGEV  EKEALKKII E  DQQESLNKWK  SKGRRFPKGK  KGNKAAQPGS
351 GKGVQFQGK  KTKFASDDEH  DEHDENGATG  PVKRAREETD  KEEPASKQQK
401 TENGAGDQ

```

Fig. 18. Amino acid sequences of Lupus La protein. With red colour are marked amino acids sequences identified by mass-spectroscopy (band 259 in Fig. 17).

### Conclusion

On the basis of data presented above we conclude that the target antigen of MAb A3G2.B4 is protein Lupus La protein. According to UniProtKB/Swiss-Prot database Lupus La protein binds to the 3' poly(U) terminus of nascent RNA polymerase III transcripts, protecting them from exonuclease digestion and facilitating their folding and maturation (<http://www.uniprot.org/uniprot/P05455>).

### 5. Discussion and conclusions

The main aim of present study is the development of new monoclonal antibodies against neuronal tissue cells to investigate the differentiation and malignization of human nerve cells. The antibody producing hybridomas were obtained by immunizing mice with the native fragments of human glioblastoma and foetal neural stem/progenitor cells to obtain MAbs against all kinds of antigenic determinants, that are expressed in the living cells, including those determinants, that are expressed on the cell surface. From plenty of MAb-

producing clones only those that revealed heterogeneity of reaction with stem/progenitor cells and glioblastoma spheroid cultures were further characterized and the target antigens identified. In this way there is possible to obtain panels of MAbs, that characterizes the given cell types, including tumour cells. The spectrum of the monoclonal antibodies obtained using our method is quite large, including antibodies against proteins as well as against their different modifications. Previously, using described approach we have developed MAb F10H2.B3 specific to Ku80 (ATP-dependent DNA helicase 2 subunit 2). We suggest this antibody could be used in certain conditions as a proliferation marker for cells of different origin (Mikelsaar et al., 2009). In this chapter we present the data about development of the five new monoclonal antibodies against neural antigens

Annexin A1 has been reported to take part in different functions as both inhibition of phospholipase A2, acute inflammation, pituitary hormone regulation, fever, neutrophil migration, cell proliferation, and stimulation of cell proliferation, differentiation, apoptosis, membrane repair, macrophage phagocytosis and neuroprotection (Solito et al., 2008). Less information is about annexin A1 expression in the developing brain. It seems to have limited neuronal distribution, but is strongly expressed in glia and ependymocytes (Fava et al., 1989). The studies have shown that annexin A1 (LC-1) positive cells carry other microglial markers and are quite distinct from astrocytes identified by S100B immunoreactivity (McKanna, 1993). It has been also proposed that LC1 can be a comprehensive and reliable marker for microglia (McKanna & Zhang, 1997). Annexins are generally cytosolic proteins, soluble or reversibly associated with components of the cytoskeleton or proteins that mediate interactions between the cell and the extracellular matrix (matricellular proteins) (Moss & Morgan, 2004). In certain cases, annexins may be expressed at the cell surface, despite the absence of any secretory signal peptide (Solito et al., 1994). In differentiated fetal neural stem/progenitor (Fig. 4, A,B,C,D) and glioblastoma spheroid cells (Fig.4, E) MAb E14G2 antigen (annexin A1) is expressed mainly in glial cells but not in  $\beta$ -III-tubulin positive neuronal cells. This observation is in accordance with data on the developing brain obtained by R.A.Fava and coworkers (Fava et al., 1989). Similar picture of expressing of annexin A1 only in limited cell types we have seen also in rat granule primary culture (Fig. 4, H). We propose that the cell expressing annexin A1 might be a Purkinje cell, which have been shown to be annexin A1 positive in adult rat cerebellum (Solito et al., 2008). In rat granule primary culture we have also seen a small amount of GFAP<sup>+</sup>/annexin A1<sup>+</sup> double positive glial cells (data not shown here). The majority of glial cells were only GFAP positive. The expression of annexin A1 was strong and homogenous in glioblastoma OjAr secondary culture and in Bowes melanoma cell lines (Fig. 4, F and G respectively). We propose that MAb E14G2 may be a perspective marker for some distinct neural cell types..

Calnexin is a calcium-binding protein that interacts with newly synthesized glycoproteins in the endoplasmic reticulum (Ellgaard & Helenius, 2003; Ou et al, 1995). Like calreticulin, calnexin is predominantly located in the ER but it has also been identified at the cell surface of a number of cells. Okazaki Y. and co-workers (Okazaki et al., 2000) reported that a small fraction of calnexin is normally expressed on the surface of various cells. The results of these authors suggest that there is continuous exocytosis and endocytosis of calnexin, and the amount of calnexin on the plasma membrane results from the balance of the rates of these two events. The findings suggest that the surface expression of calnexin depends on the association with glycoproteins and that calnexin may play a certain role as a chaperone on the plasma membrane as well (Okazaki et al., 2000). Our observations are in good

accordance with previous data. We observed the expression of MAb E15F10.B9 detected calnexin on the surface of unfixed human adult skin fibroblasts and on fixed, but not permeabilized cells of glioblastoma TiViMNBFCs10 and Bowes melanoma cell line, respectively (Fig. 7, E-G). In Fig. 7 we can see a different expression of MAb E15F10.B9 detected calnexin in differentiated fetal neural stem/progenitor (A) and glioblastoma spheroid cells (C) in which  $\beta$ -III-tubulin-positive cells are negative or very weakly calnexin-positive. In the human fetal hBrSc003 neurosphere culture differentiated for 11 days we see at least three populations of cells, namely the calnexin<sup>+</sup>/ $\beta$ -III-tubulin-cells, calnexin<sup>-</sup>/ $\beta$ -III-tubulin<sup>+</sup> cells and also cells which are negative for both antigens. It shows that MAb E15F10.B9 may be used for detection of some distinct population of neural cells (see also a strongly calnexin<sup>+</sup> cell among other totally calnexin<sup>-</sup> cells in TiViMNBFCs10 glioblastoma cell culture, Fig.7, F). Interestingly, in the unfixed human sperms we see two separate points between the head and neck of the sperm (H). This phenomenon needs further investigation.

14-3-3 proteins. There are seven genes that encode 14-3-3s in most mammals (Takashi, 2003). 14-3-3 proteins are abundantly expressed in the brain and have been detected in the cerebrospinal fluid of patients with different neurological disorders. By their interaction with more than 100 binding partners, 14-3-3 proteins modulate the action of proteins that are involved in cell cycle and transcriptional control, signal transduction, intracellular trafficking and regulation of ion channels. The study of some of these interactions is shedding light on the role of 14-3-3 proteins in processes such as apoptosis and neurodegeneration (Berg et al., 2003). The immunohistological and subcellular location of the 14-3-3 proteins was studied using different isoform-specific antisera (Martin et al., 1994). The immunohistochemical examination using the specific antibody showed significant staining of the cytoplasm, including neuronal axons and dendrites. This result was confirmed by the ultracentrifugal cellular fractionation method, indicating that 14-3-3 is mainly localized in the neuronal cytoplasm and a portion of 14-3-3 may be bound to the plasma membrane, endoplasmic reticulum, and Golgi membrane. This is in good accordance with our *in vitro* study on different cell lines. In Fig. 10 we see the expression of the protein 14-3-3 in the fetal neural stem/progenitor cell lines hBrSc006 (A) and hBrSc009 (B). The strong staining was seen in the cytoplasm in Golgi region, but also in cell projections and nuclei in many cells. In the rat granular cell culture there are two different 14-3-3 stained cell populations, detected by MAb W4A8.F4: negatively stained cells and cells with positively stained cytoplasm and nuclei (Fig.10, H). It shows that the MAb W4A8.F4 may work in certain conditions as a marker for some types of neural cells. In the fetal skin fibroblasts, glioblastoma, Bowes melanoma and COS-1 cell lines the cytoplasm, especially Golgi region was strongly stained (Fig.10, C,E,F and G respectively).

Interestingly, in the unfixed human sperms the entire tail, except the neck, was strongly and homogeneously stained (Fig.10, D). This is a very interesting fact and needs further investigation.

Vimentins are class-III intermediate filaments found in various non-epithelial cells, especially mesenchymal cells. During the development of the nervous system, vimentin is transiently expressed in virtually all the precursors cells of both neuronal and glial lineages. In the astroglial cell lineage, vimentin is the only IF protein expressed in radial glia and immature astrocytes in the embryonic nervous system (Alonso, 2001; Colluci-Guyon et al., 1999; Schnitzer et al., 1981). The expression of glial fibrillary acidic protein (GFAP), vimentin

and fibronectin (Fn) was studied in cells cultured from human glioma and fetal brain by indirect immunofluorescence (IIF) microscopy and multiple labelling experiments (Paetau 1988). The results of the study demonstrate a general coexpression of GFAP and vimentin in cultured astroglial cells, in addition to cells expressing only vimentin. This is in good accordance with our data. In Fig. 13 we see the filamentous staining of the vimentin with MAb GB26 10G3 in human adult skin fibroblasts (A) and in glioblastoma cell line TiViMNBFCs10 (B). In glioblastoma cell lines the staining pattern was very heterogeneous, some cells did not show any signs of staining. Double staining for  $\beta$ -III-tubulin and vimentin (Fig. 13,C) showed an independent staining pattern, some cells were double stained (vimentin<sup>+</sup>/ $\beta$ -III-tubulin<sup>+</sup>), some cells showed only  $\beta$ -III-tubulin staining (vimentin<sup>-</sup>/ $\beta$ -III-tubulin<sup>+</sup>) and the majority of cells were only vimentin positive. The double staining of vimentin and glial fibrillar acid protein (GFAP) is shown in Fig. 13, D. Note that all the cells are vimentin<sup>+</sup>/GFAP<sup>+</sup>, but show an independent staining pattern. We propose that our MAb GB26 10G3 may be a good additional tool for detecting and characterization of expression of vimentin in different types of neural cells, including glioblastomas.

Lupus La protein (known also as Sjogren syndrome antigen B and autoantigen La) is ubiquitous in eukaryotic cells and associates with the 3' termini of many newly synthesized small RNAs. The La protein protects the 3' ends of these RNAs from exonucleases (Wolin & Cedervall, 2002). The immunohistochemical location of La antigen was shown to be the nucleus but an intense staining of the nucleolus was seen in human cerebral cortical neurons as well as a subset of neurons of rat brain (Graus et al., 1985). Further it was shown that La ribonucleoproteins (RNP) exist in distinct states that differ in subcellular localization (Intine et al., 2003). This is in good accordance with our results of the immunocytochemical study. In Fig. 16 we see the staining of La protein with MAb A3G2.B4 in nuclei of all cell lines studied. However, the staining of nuclei was very heterogeneous in differentiated fetal neural stem cells (A-C), clearly showing the existence of two cell population for MabA3G2.B4 detected La protein - La protein<sup>+</sup> and La protein<sup>-</sup> cells. This may be a sign of real existence of two different cell populations and needs further investigation. A clear nucleolar staining was also seen in many cells, especially in normal amniocytes and glioblastoma cells (E-G). In the Bowes melanoma cell line both the nuclear and strong granular cytoplasmic staining was observed (H).

Further perspectives. The characterization of the target antigenes and epitopes of all other monoclonal antibodies obtained during our main project is in progress.

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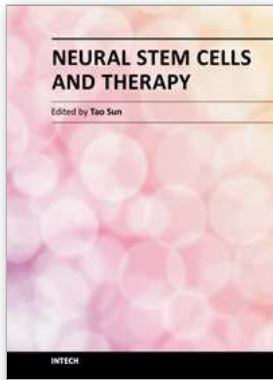
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This book is a collective work of international experts in the neural stem cell field. The book incorporates the characterization of embryonic and adult neural stem cells in both invertebrates and vertebrates. It highlights the history and the most advanced discoveries in neural stem cells, and summarizes the mechanisms of neural stem cell development. In particular, this book provides strategies and discusses the challenges of utilizing neural stem cells for therapy of neurological disorders and brain and spinal cord injuries. It is suitable for general readers, students, doctors and researchers who are interested in understanding the principles of and new discoveries in neural stem cells and therapy.

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