

Production of Monoclonal Antibody against Human Nestin

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Abstract

We have employed a peptide-based antibody generation protocol for producing antibody against human nestin. Using a 12-mer synthetic peptide from repetitive region of human nestin protein devoid of any N- or O-glycosylation sequences, we generated a mouse monoclonal antibody capable of recognizing human, mouse, bovine, and rat nestin. A wide variety of nestin proteins ranging from 140-250 *kDa* was detected by this antibody. This antibody is highly specific and functional in applications such as ELISA, flow cytometry, immunocytochemistry, and Western blot assays.

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Introduction

Nestin is a protein of about 240 *kDa* which belongs to type VI Intermediate Filament (IF) proteins. Nestin is considered as a hallmark for proliferating neural progenitor cells during early embryonic development. Although it is reported not to be expressed in adult tissues, (1) recently expression of nestin in the hair follicle stem cells has been reported (2). Nestin is evolutionary conserved within the variety of species indicating its important role in early development.

Recent advances in cancer research have revealed the expression of nestin in some

cancer cells. Expression of nestin has been reported in pancreatic carcinoma (3, 4), breast cancer (5), glioblastoma (6), high-grade astrocytoma (7), dermatofibrosarcoma protuberans (8), and also thyroid tumors (9) with different molecular weights ranging from 180-240 *kDa*. This variation in electrophoretic mobility is mainly because of differential post-translational modifications like phosphorylation and glycosylation (10).

It appears that a wide variety of tumors are expressing nestin transcript without its expression at the protein level. One reason for

such discrepancy might be due to the lack of a proper anti-nestin antibody recognizing all different types of nestin. To overcome this problem and also producing better research tools we decided to generate a monoclonal antibody capable of distinguishing all different variants of nestin. To do this a peptide-based antibody generation approach was selected and regions devoid of any N- or O-linked glycosylation were selected to target. These regions should also be structurally exposed to the surface of native protein for antibody binding. Regions of a protein molecule (rich in hydrophilic amino acids) tend to dissolve in water and consequently more exposed to outer part of molecule was selected. In this regard internet-based online databases such as Ensembl (<http://www.ensembl.org/index.html>), and CBS prediction server (<http://www.cbs.dtu.dk/services/>) was used.

Materials and Methods

Peptide conjugation

The amino acid sequence of human nestin was carefully analyzed and compared to all other species. A 12-mer peptide of PEVGDEEALRPL from human nestin corresponding to amino acids 681-692 (NM_006617) was selected as immunogen. A cysteine residue was added to the C-terminus end of the peptide to facilitate the conjugation to carrier protein. Immunograde peptide was purchased from Thermo Electron Corporation, GmbH, Ulm, Germany. Keyhole Limpet Hemocyanin (KLH)-conjugated peptide was used for generating mouse monoclonal antibody according to standard protocol with minor modifications^(11, 12).

Briefly, 5 mg of Keyhole Limpet Hemocyanin (KLH) (Sigma, cat. no: H7017) was dissolved in 1 ml of deionized water. One mg of Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) dissolved in 200 µl of Dimethyl Formamide (DMF) was then added to the carrier protein solution. The mixture was incubated at room temperature for 2 hrs with gentle stirring followed by dialysis against large volume of Phosphate Buffer Saline

(PBS) overnight. In a separate tube 5 mg of peptide was dissolved in 1 ml of Phosphate Buffered Saline (PBS). MBS-activated protein was then added to the peptide solution and the mixture was incubated for 4 hrs at room temperature. After overnight dialysis against PBS, the conjugate was stored at -20 °C for later use. All chemicals for bioconjugation were purchased from Sigma, St. Louis, USA. The same procedure was performed for conjugation of peptide to BSA.

Evaluation of conjugated peptide by SDS-PAGE

To check the efficiency of conjugation, 10 µg of peptide-BSA was mixed with 10 µl of sample buffer, boiled for 2 - 5 min and cooled on ice. Electrophoresis was performed in a 10% SDS-PAGE gel with a mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, Hercules, CA, USA) 100 mA for 1 hr. The gel was stained with Coomassie Blue R-250 (Sigma). The change in mobility shift of conjugated BSA represented the efficiency of conjugation.

Immunization protocol

Four BALB/c mice were used for peptide immunization. Each mouse was immunized 5 times with an interval of two weeks. The first immunization was performed using Freund's complete adjuvant. Incomplete Freund's adjuvant was used for the 2nd, 3rd, 4th, and 5th immunization.

For the first immunization, 100 µg of conjugated KLH-peptide was mixed with an equal volume of Freund's complete adjuvant and injected Intra Peritoneally (IP) not exceeding 100 µl total volume. For the subsequent immunizations 50 µg of peptide-KLH were injected with Freund's incomplete adjuvant. Three days before the cell fusion, 20 µg of KLH-peptide (without any adjuvant) was injected intravenously.

Bleeding and titration of antibody

One week before the last immunization, mice were bled by a vertical incision of the tail vein. Serum ELISA assay was performed as follows:

Wells of ELISA plate (Nunc, Germany) were coated with 100 μ l of the immunizing peptide (20 μ g/ml in PBS) at 37 °C for one hr followed by overnight incubation at 4 °C. Next day the plate was washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) for 5 min. The plate was blocked with 2.5% BSA at 37 °C for 1.5 hr. Wells were then washed 3 times as above and mice sera were added to the wells in two fold serial dilutions starting from 1:100. The plate was incubated at 37 °C for 1.5 hr and washed again with PBS-T.

At the next step, 100 μ l of 1:1000 dilution of HRP-conjugated rabbit anti-mouse Ig (Avicenna Research Institute, Iran) was added to the wells and incubation was continued for 1.5 hr at 37 °C. After washing, 100 μ l of Tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated at room temperature in a dark place. After 15 min, the reaction was stopped by adding 30 μ l of stopping solution (0.16 M H₂SO₄) to each well.

The Optical Density (OD) of the reactions was measured at 450 nm by an ELISA reader (BioTek, USA). Negative controls included omission of coating layer, serum (as primary antibody) or combination of both. Mice with higher titer of specific antibody were selected for fusion.

Hybridoma production

Mouse myeloma SP2/0 cell line was used as fusion partner. Cells were cultured in RPMI (GIBCO) and 10% FBS until reaching to >70% confluency. To collect mouse peritoneal macrophages, 5 ml of RPMI was injected into the peritoneal cavity of unimmunized BALB/c mouse with subsequent aspiration and collecting the peritoneal cells at sterile conditions (~3x10⁶ cells). The collected peritoneal fluid was washed twice with RPMI. The cells were incubated in RPMI with 20% FBS for 24-48 hrs at 37 °C with 5% CO₂. Spleen of the immunized mouse was removed at sterile conditions. To separate spleen cells, 10 ml of RPMI was injected to the spleen from different angles. The col-

lected cells were washed twice with RPMI for 10 min and centrifuged at 1000 rpm.

Cell fusion with polyethylene glycol (PEG)

A 50 ml sterile Falcon tube was selected and SP2/0 cells were mixed with the spleen cells at a ratio of 1:10 (1 SP2/0 and 10 spleen cells). Mixture was washed twice with RPMI. 800 μ l of pre-warmed (37 °C) 50% PEG 1500 (Sigma) was added to the cell pellet slowly by mixing at the same time. Immediately after adding PEG, 20 ml of pre-warmed RPMI was added to the tube to dilute out PEG. Cells were centrifuged at 22 °C for 5 min and at 500 rpm. The cells were washed twice. HAT medium was added based on the number of spleen cells for fusion. Selective HAT medium was added to the pellet (2x10⁶ cells/ml) and cells were seeded into a 96-well plate. The cells were incubated at 37 °C with 5% CO₂ for 2-3 days. Cell growth and colony formations were examined daily. Colonies appeared after 5-10 days. Once the colony diameter reached to 1 mm the presence of antibody against the immunized peptide was determined by ELISA. After two weeks of incubation 100 μ l of supernatant from each well were separated and ELISA assay was performed using peptide alone, KLH-peptide, BSA-peptide, and KLH only as coating antigen.

Antibody purification

The monoclonal antibodies were purified from culture supernatants by affinity chromatography based on its isotype. Briefly, culture supernatants were filtered through 45 μ m filter and pH was adjusted to 7.5. Antibodies were affinity purified using a column of CNBr-activated sepharose 4B (GE Healthcare, Sweden) conjugated to immunogenic peptide. The elution was performed using 0.1 mol/l glycine pH= 2.7. The pH of eluted antibody was adjusted to 7.0 with 1 mol/l Tris buffer pH= 9.0. The eluted antibodies were dialyzed overnight against PBS pH= 7.5 and the reactivity of the antibodies were measured by ELISA as described above.

Western blot

Western blotting was performed to see pattern of reactivity of anti-nestin monoclonal antibodies with different isoforms of this protein according to the protocol we reported elsewhere with minor modification⁽¹²⁾.

Briefly, a panel of cell lines with different origin known to express nestin (Table 1) (all from ATCC, USA), were cultured in their recommended medium, harvested and lysed in 1 ml of lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH= 7.4, 150 mM NaCl, 5 mM EDTA, and 1% protease inhibitor cocktail (Sigma) for 1 hr on ice with a 15 min interval of vortexing for 30 sec. The protein concentration of lysate was measured by Thermo Scientific BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Scientific, IL, USA). Twenty μ g of cell lysates were run under both reducing and non-reducing conditions on a 6% SDS-PAGE gel at 100 V for 2 hr. After electrophoresis, resolved proteins were transferred onto Immobilon-PVDF membranes (Millipore Corporation, USA). The membranes were blocked for overnight at +4 °C with 5% non-fat

milk in PBS plus 0.05% Tween 20 (PBS-T). All immunostainings were performed in PBS-T supplemented with 3% non-fat milk. Filters were incubated with 3 μ g of affinity-purified anti-nestin monoclonal antibody 1.5 hr at room temperature. After extensive washing with PBS-T the filters were incubated with peroxidase-conjugated sheep anti-mouse immunoglobulins (ACECR, Tehran, IRAN) for 1 hr at room temperature followed by washing and developing with ECL chemiluminescence detection system (GE Healthcare, Uppsala, Sweden).

RNA preparation and RT-PCR amplification of nestin

Total RNA was extracted from cell lines and tissue samples using RNA-Bee reagent (BioSite, Täby, Sweden) according to the manufacturer's instruction. The quality of the RNA samples was determined by agarose gel electrophoresis after staining with ethidium bromide, and visualization under UV light. Total RNA was unfolded at 65 °C for 10 min. cDNA was then synthesized using 5 μ g of total RNA in 20 μ L reaction mixture consisting of 4 μ L of 5x reaction buffer, 1 μ L of

Table 1. Summary of expression profile of nestin in different cell lines

Cell line/Tissue	Description	Species	This report			Literature
			RT-PCR	ICC	W.B	Reference
PaCa2	Pancreatic carcinoma	Human	+	+	+	[3]
A172	Glioblastoma	Human	+	+	+	[17]
PC3	Prostate cancer	Human	-	-	-	[14]
ESTDAB-075	Melanoma	Human	+	+	+	NF
T47D	Breast cancer	Human	-	-	-	[16]
U373	Glioblastoma	Human	+	+	+	[18]
GL261	Glioma	Mouse	+	+	ND	[19]
COS-7	Kidney	Monkey	ND	+	+	NF
CHO	Ovary	Hamster	-	-	-	[15]
4T1	Breast cancer	Mouse	+	+	+	[20]
CT26	Colon carcinoma	Mouse	+	+	+	NF
Bovine sertoli cells	Primary testis tissue	Cow	+	+	+	NF
Bovine brain	24 day embryo	Cow	+	+	+	NF

ICC=Immunocytochemistry, W.B=Western blot, ND=Not determined, NF=Not found in the literature

10 mM dNTPs, 1 μ L of 10 pmol/mL random hexamers (N6), and 200 U M-MuLV reverse transcriptase (Fermentas GmbH, St. Leon-Rot, Germany). The reaction mixture was incubated at 42 °C for 45 min. The amplification was performed with 1 min denaturing at 95 °C followed by 35 cycles of 94, 52 and 72 °C, 1 min each with a 2.5 mM MgCl₂, using AmpliTaq Gold DNA polymerase (Applied Biosystems, USA) using species-specific nestin primers (Table 2).

Immunocytochemistry (ICC)

The cells were harvested using 0.5% trypsin and 0.1% EDTA (Gibco) loaded 1-2 $\times 10^4$ cells on 8 well laminated glass slide (Marienfeld, Germany) that homogenized in RPMI 1640 contain 20% FBS with subsequent incubation in moisturized conditions for overnight.

After overnight incubation the medium was removed and the cells were washed with TBS for three times (3 \times 3 min). Slides were dried at room temperature for 15 min, acetone-fixed (at -20 °C), permeabilized for 2 min and kept at 4 °C for 30 min until slides were dried. Slides were washed with Tris-Buffered Saline, pH= 7.4 containing 5% bovine serum albumin (TBS-Glycerol) three times (3 \times 3min).

Slides were blocked with 5% sheep serum for 10 min at room temperature. The primary antibody Mab anti-nestin (4G10G8) diluted with TBS-BSA to a final concentration of 5 μ g/mL, respectively, were incubated at room temperature for 60 min and then washed with TBS-BSA three times (3 \times 3 min).

Fluorescein Isothiocyanate (FITC)-conjugated Sheep anti-mouse (ACECR, Tehran, Iran) was diluted with TBS-BSA in a ratio of 1:50 and incubated at room temperature for 45 min. Negative antibody control slides were incubated with mouse IgG1 at a final concentration of 10 μ g/mL in TBS-BSA. After washing with TBS-BSA, the nuclei were counterstained by 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Calbiochem, USA) at 1 μ g/ml for 5 min, then the slides were washed, mounted in TBS-glycerol 80% and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry analysis

Bovine sertoli cells were harvested by 0.5% trypsin and 0.1% EDTA (Gibco) and permeabilized by permeabilizing solution (Becton Dickinson, USA). According to the related protocol, sample analysis and data acquisition were performed by Flomax flow cytometry analysis software (Partec, Germany).

Table 2. Nestin species-specific primers

Species	Sequence	g.b. accession
Human-F	TACTGAAAAGTTCAGCTG	NM_006617
Human-R	TCTGATCTCTGCATCTACA	
Mouse-F	TACATACAGGACTCTGCTG	NM_016701
Mouse-R	CTCACTGGATTCTCTATCTT	
Bovine-F	ACGTCAAGACCGCTAGA	XM_600663
Bovine-R	TTCCACAGACCCAGTG	
Chicken-F	AGAAAATCAGCTCGGTC	NM_205033.1
Chicken-R	GCTTGCTCTACTATCT	
Rat-F	TCTGGAGGTGGCTACATA	NM_012987
Rat-R	TCAGATATAGGTGGGATG	
Hamster-F	GATCTAGCATATCCAG	AF110498
Hamster-R	CTCTACCTCTACATCCT	

F=Forward, R=Reverse

Results

Several anti-nestin monoclonal antibody producing hybridomas were obtained. Among them one clone designated as "NES-4G10G8" had a very high reactivity with immunogenic peptide in ELISA assay. Further characterization of this antibody showed that it is of an IgG1 isotype with a kappa light chain. Due to stronger reactivity of this clone in ELISA assay, all subsequent tests were carried out with this clone.

Cell lines of human, mouse, and bovine were tested by western blot (Figures 1A, B and C), immunofluorescent staining (Figures 2A, B and C) RT-PCR (Figure 3) and flow cytometry (Figure 4) for expression of nestin at both gene and protein levels. Results of RT-PCR showed that except CHO, T47D and

Anti-nestin Monoclonal Antibody

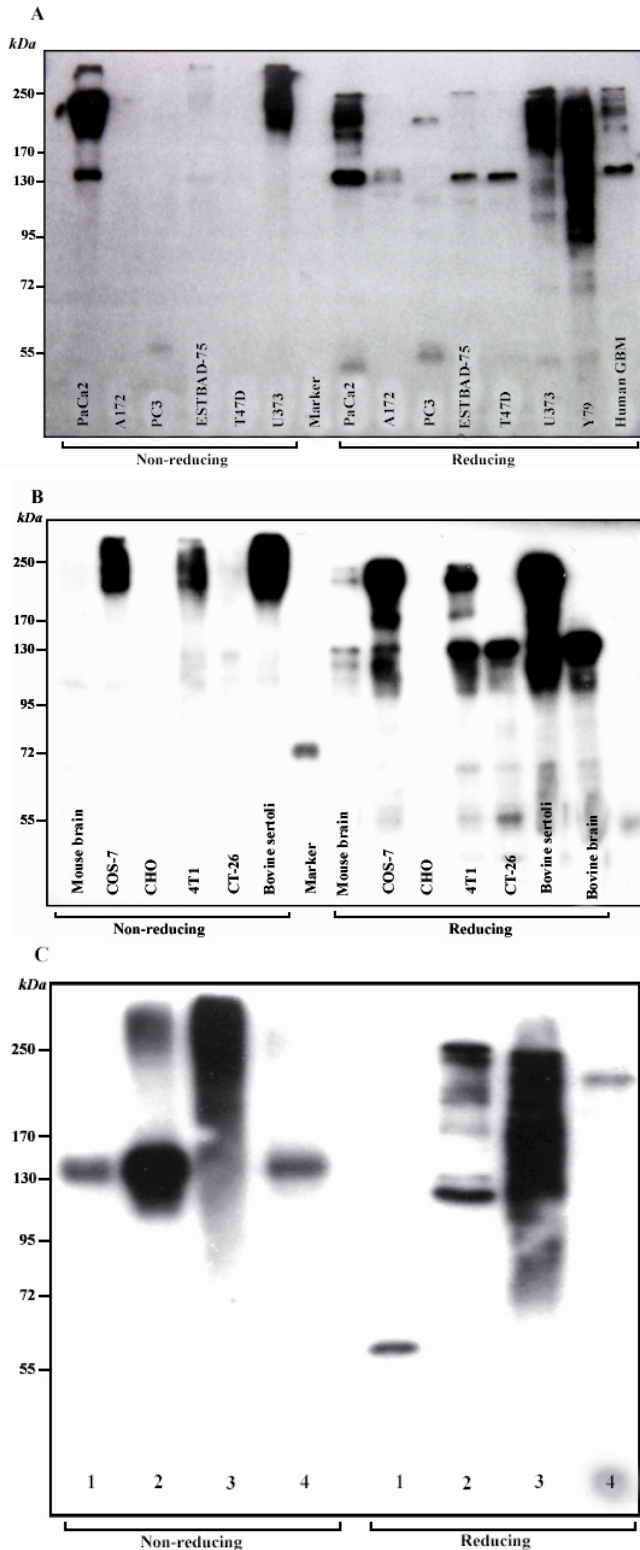


Figure 1. Western blot assay using 3 μ g affinity-purified anti-nestin (4G10G8). A) Cell lysates from a human glioblastoma multiforme (GBM) and several human malignant cell lines were run in a 6% SDS-PAGE under reducing and non-reducing conditions. B) Cell lysates and several cell lines from other species were also analyzed. Several bands ranging from 150-300 kDa was observed. C) Cell lysates from 1- Rat hippocampus, 2- Mouse brain (12 days embryo), 3- Bovine brain (24 days embryo), 4- Human cerebral tumor

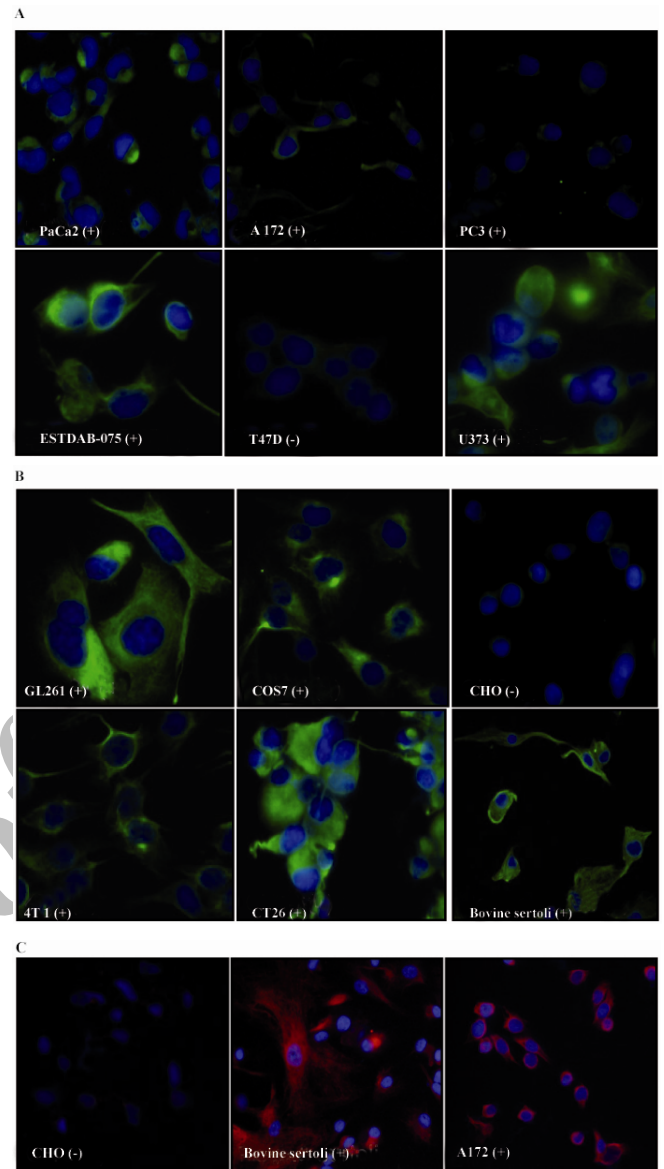


Figure 2. Immunocytochemistry (ICC) assay using 5 μ g of affinity-purified anti-nestin (4G10G8). FITC-conjugated sheep anti-mouse antibody was used as secondary antibody. DAPI was used for staining the nucleus (blue). The green fluorescence represents nestin expressed in cytoplasm and the blue color represents the nucleus. A) Human cancer cell lines. B) Cell lines derived from other species and also a primary sertoli cells. C) To confirm the ICC results, a negative (CHO), a strong positive (bovine sertoli cells), and a weak positive (bovine sertoli cells) were stained with anti-nestin 4G10G8 conjugated with Alexa Fluor 568 (Invitrogen). Red immunofluorescence represents nestin

PC3, all cell lines express nestin-specific message. Based on the sequences amplified, band sizes differed accordingly.

In western blot analysis, nestin-specific bands ranging from 140-250 kDa were observed (Figure 1). More interestingly, the

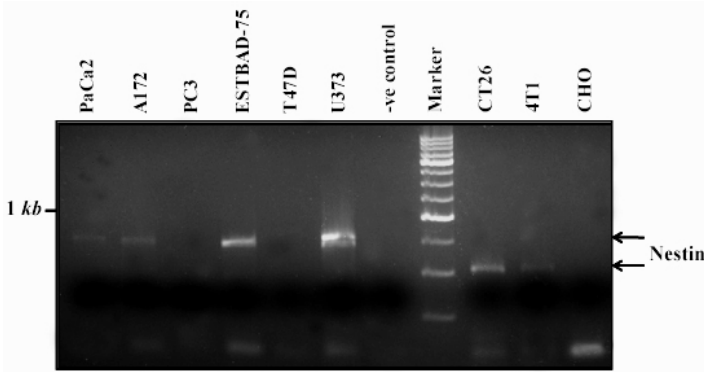


Figure 3. Expression of nestin in different cell lines by RT-PCR. The standard marker is DNA molecular weight marker XVI from Roche. The negative (-ve) control was PCR reaction without any template

pattern of bands in reducing and non-reducing conditions differed almost in all cell lines examined. This was especially the case for 140 kDa band which appeared or became stronger under reducing condition.

To examine the expression pattern of nestin, cell lines were subjected to immunofluorescent staining. The results clearly showed that our antibody strongly reacts with nestin intracellularly (Figures 2A, B, C and 4).

In line with RT-PCR and western blot analysis, PC3, T47D and CHO cell lines failed to express nestin, while other cell lines showed excellent reactivity with nestin-specific monoclonal antibody.

In some settings, anti-nestin antibody was conjugated with Alexa fluor 568 and used for direct staining of cell lines. As shown in figure 2B, bovine sertoli cells and A172 cell

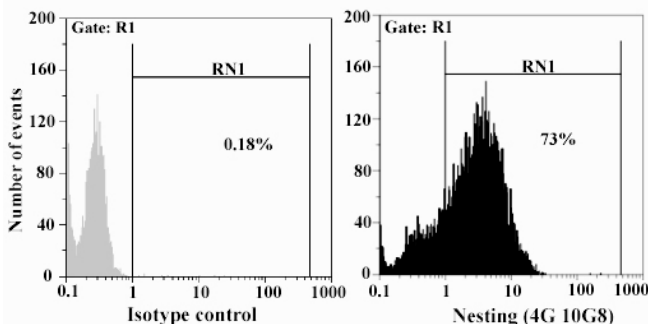


Figure 4. Indirect intracellular staining of bovine sertoli cells and flow cytometry analysis using anti-nestin (4G10G8) antibody. FITC-conjugated rabbit anti-mouse was used as secondary antibody. An irrelevant mouse IgG1 (ACECR, Tehran, Iran) was used as isotype control

Species	Sequence
	* . * + * * . * + *
Human	PEVGDEEALRPL
Bovine	LEEENQELLRPL
Mouse	PEEEDQQAFRL
Hamster	PEEEDQETDRPL
Pig	QDIENQEALRPL
Horse	LEKENQEPLRPL
Dog	LEDEKQETLRPL
Rat	PEEEDQEAGRSL
Consensus	<u>XEEXQEXRPL</u>

Figure 5. The sequence of human nestin immunogenic peptide was aligned with corresponding regions of nestin protein from other species. The conserved amino acids are highlighted in gray and potential antigenic epitope is underlined

line (as positive controls) strongly expressed nestin, while CHO cell line showed no reactivity. Summary of nestin expression in different cell lines employing three readout systems are depicted in table 2.

To further characterize our antibody, bovine sertoli cells were subjected to intracellular staining and tested by flow cytometry. The results showed that 73% of the cells were positive for expression of nestin (Figure 4).

Discussion

Peptide-based antibody generation gave rise to identification of several hybridomas producing anti-nestin antibody. One antibody of IgG1 isotype was generated which showed to be very specific to nestin. The antibody could react with nestin protein in both non-reducing and reducing conditions with some variations. Examples of such variations are prostate cancer cell line PC3 and melanoma cell line ESTBAD-75 which did not react in non-reducing conditions but could react in reducing conditions as well as in ICC experiments (Figures 1A and 2A).

This variation might be due to recognition of antigenic epitope in different conformation by the antibody. Apparently this antibody recognizes a linear epitope which is structurally folded and appears and exposes to the antibody upon dissociation by 2-mercapto-

ethanol (reducing conditions). Furthermore, reactivity of this antibody to nestin from pancreatic carcinoma cell line PaCa2 at both reducing and non-reducing conditions (Figure 1A), indicates that the structure of nestin expressed by PaCa2 cells might be different with the structure of nestin expressed by A172 or ESTBAD-75 cell lines.

This discrepancy might be due to altered Post-Translational Modification (PTM) mainly glycosylation of nestin in cancer cells,⁽¹³⁾ which affects the electrophoretic mobility of protein. Our results showed expression of nestin with different molecular weight ranging from 140-250 *kDa* confirming the previously described nestin variants⁽¹⁰⁾.

Our results are in accordance to the results on expression of nestin in the literature. The reference data are shown in table 1. The only case which differs from the literature is expression of nestin in prostate cancer cell line PC3. Unlike reporting expression of nestin at only gene level in PC3 cell line⁽¹⁴⁾, we did not detect expression of nestin in this cell line (in contrast to our data).

The NES-4G10G8 antibody detects nestin from other species. This might imply recognition of a common epitope. Amino acid sequence alignment of the immunogenic peptide of human, bovine, mouse, hamster, pig, horse, dog and rat showed that the consensus sequence of XEXEXQEXXRPL in which glutamic acid E (2), arginine R (10), and leucine L (11) might represent the potential epitope recognized by anti-nestin clone 4G10G8 (Figure 5).

If this is true, then any nestin protein from other species containing such epitope can potentially be recognized by this antibody. So, hypothetically the horse, dog, and rat's nestin might be recognized by this antibody which needs further investigations. This antibody could be used as a powerful tool in stem cell studies as it is reacting in a wide variety of applications like western blot, ELISA, flow cytometry, and immunocytochemistry.

Our results show that this antibody is capable of recognizing nestin in both normal

and pathological conditions as well as with possible structural diversities.

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