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Chapter 2

Development of Antibody Reagents against Stem Cell Markers: Expression Pattern of Human Pluripotent Stem Cell Marker Nanog and Germ Cell Marker Fragilis by Rat Testis

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Abstract

The relative rarity of stem cells in embryonic or circulating bloods of the adult has human prompted the scientific community to search for markers that are biologically unique to stem cells and could be used for identification of cell type. Stem cell markers can be either on the cell surface or part of an intracellular signalling pathway associated with cell proliferation, differentiation or cell-cell interaction. Further a combination of markers facilitates identifying a particular cell type with consistency. Keeping this in view, attempts were made to develop antibodies against Germ cell marker Fragilis and embryonic stem cell marker Nanog. Antibodies were developed against synthetic peptides made from carefully chosen epitopic region from sequences available in NCBI. Antibodies were validated by Western Blot using various cell lines and tissues. Immuno histochemical staining of adult rat testis with anti-Nanog showed the presence of pluripotent stem cell factor in the tails of the spermatozoa and suggests that NANOG expression was markedly up regulated in the gonocytes shows stem cell potential in adult testis. This confirms that adult rat testis has a population of stem cells that can be act as biomarkers.

Also the strong expression of NANOG protein in adult rat gonocytes raises

the possibility of using the gonocytes to establish a cell line that could have germline characteristics. Together, these results suggest that PGCs maintain expression of pluripotent stem cell markers during and after sexual differentiation of the gonad.

Keywords: stem cell marker; testis; fragilis; nanog

1. Introduction

In the face of extraordinary advances in the prevention, diagnosis and treatment of human diseases, devastating illness such as heart disease, diabetes, cancer and disease of the nervous system, such as Parkinson's disease and Alzheimer's disease, continue to deprive people to health, independence and well-being. Research in human development biology has led to the discovery of human stem cells including embryonic stem (ES) cells, embryonic germ (EG) cells and adult stem cells. The stem cells need to be identified by the researchers to explore its significance and potential use in the field of medical research, and antibodies reagents are used to categorize the stem cells by identifying the marker proteins expressed by the stem cells.

Stem cells are originated from the inner cell mass of the embryo and are present in all stages of development from pre-implantation embryos through adulthood. They are characterized by the ability to renew themselves into a diverse range of specialized cell types. Pluripotent stem cells have the potential to develop into many different cell types in an animal which can only be isolated from the inner cell mass of embryos or from the gonadal ridge [7]. In contrast, multipotent stem cells have the ability to self-renew and to differentiate into two germ layers and can be isolated from adult animals [4]. Human stem cells hold out the potential to almost unimaginable medical breakthroughs and are prospective source of differentiated cells for a variety of therapeutic uses [15]. Thus they serve as a sort of repair system for the body. Transcription factors of embryonic stem cells play a central role in the regulation of pluripotency and self-renewal. These factors include the homeoprotein Nanog [6,16], Sox-2 [1] and the POU domain-containing protein Oct-4 [21-23], and are expressed in high levels in pluripotent cells and are considered markers of primitive stem cells. They regulate many genes in a cooperative fashion [3].

Nanog is a homeodomain-bearing transcription factor, which is reported to be transcribed in pluripotent stem cells in mouse preimplantation embryos, ES, embryonic germ and embryonal carcinoma cells [6,16,24] and in monkey and human ES cells [10]. Nanog is essential to maintain the pluripotency of cells as shown by the loss of pluripotency in Nanog-deficient ES cells and by the loss of epiblast cells in Nanog-null E5.5 embryos [16]. NANOG expression begins in the compacted morula [11] and quickly down regulated thereafter and only remains in the primordial germ cells (PGC) [11,25].

In recent years the male germ line stem cells have received a great deal of attention in

recent years, as it is possible to isolate and culture them in vitro [18,20]. These cells can be genetically modified and they further differentiate to spermatozoa following transplantation into a recipient testis, eventually producing transgenic offspring [17,19]. From all these reports it can be hypothesized that if expression of Nanog remains in PGCs then there must be some expression in the adult male germ cells which can be used to produce efficient transgenic breeds. There are no reports available showing the expression of Nanog in adult testes. The present study was carried out with the objective to check the expression pattern of Nanog in adult rat testis.

2. Material and Methods

2.1. Isolation of immunogens

The protein sequences deduced from NCBI database were analyzed using Clone Manager, a software program for selecting probable immunogenic peptide sequences. The peptides chosen were ES cell marker Nanog and Fragilis a germ cell marker. For synthesis of peptide; portion of amino acids 1-50 of human Nanog and portion of amino acids 1-50 of mouse Fragilis were used as the immunogen. Peptides were synthesized at Imgenex Sandiego, USA.

2.2. Immunization and collection of immune sera

New Zealand white rabbits were immunized with synthetic peptide conjugated to KLH. In general 200 µg of primary dose followed by three boosters containing half of the primary dose were given. Rabbits were bled; serum was separated by centrifugation at 5000 rpm for 10 min. Aliquots were stored at -20°C until purification.

2.3. Screening of sera by ELISA

Indirect ELISA was performed to check antibody titer in the sera. Free immunogens were coated in 96 well flat bottom plates with 100 ng /well, incubated for 2 hour at room temperature and kept at 4°C overnight. Rabbit serum was added at 1:1000 dilutions and anti-rabbit IgG conjugated to alkaline phosphatase (Jackson) were used at 1:5000 dilution. pNPP was used to develop the color. Absorbance was measured at 405 nm.

2.4. Purification of sera by affinity chromatography

For purification, 10ml of Western positive sera was taken and diluted with TBS buffer (100 mM Tris, 150 mM NaCl) at 1:1 before purification. Protein G column matrix was used for affinity purification. Bound antibodies were stripped off from the column by high Ph diethanolamine buffer (pH 11.5). The eluted sample was neutralized by 1M Tris pH 7. Finally a low pH Glycine buffer (100 mM Glycine, pH 2.7) was used to strip remaining antibodies followed by neutralization with 1M Tris pH 7.5. In both cases protein in the sample was measured by

Bio-Rad protein assay kit. All the fractions containing antibodies were dialyzed overnight with TBS containing 0.05% sodium azide. The samples were concentrated and stored at -70°C for longer duration.

2.5. Assessment of sera by Western Blot analysis

Mouse embryonic cell line (3T3) was obtained from ATCC and maintained by using standard protocol. Cell extracts of 3T3 were prepared by lysing the cell using Radioimmuno precipitation assay (RIPA) buffer and 200 µg of extracted protein mixed with SDS sample buffer (1:1) loaded onto a single well and resolved in SDS PAGE under reducing conditions [13]. Separated proteins were electro blotted onto a PVDF membrane by Bio-Rad electroblot apparatus. Electroblotting was performed for 90 min at a constant voltage of 50V. The membrane was stained with amidoblack (Sigma) and destained with 10% methanol and 10% acetic acid. The detection of immunogens in cell line was made by immunostaining using the antibody raised against that immunogen served as primary antibody at 1:5000 dilution and HRPO conjugated Goat anti-rabbit IgG (Jackson) at 1:10,000 dilution served as secondary antibody. Visualization of the antigen-antibody binding was made by using chemiluminiscencent solution (Pierce). Bands were depicted on the Hyper-Film (Kodak) by developing in the dark room.

2.6. Collection of rat testis and slide preparation

Testis tissues were collected from 2 months old rat in 4% Para-formaldehyde and preserved for 24-48 hrs. Then the tissues were washed with PBS with three changes of 5 minutes each and dehydrated by keeping in increasing percentages of alcohol (30% for 1 hour, 50% for 2 hours, 70% for 2 hours, 95% for 1 hour and 100% 2 hours) and Xylene with 2 changes 30 minutes each. After dehydration, infiltration was done by incubating the tissues in molten paraffin (Fisher Scientific) at 58-60°C, with 3 changes each for 30 min. Tissue embedded paraffin blocks were prepared by using molten paraffin in L shaped metal frames and left over night. The paraffin blocks were then fixed to fine plastic cassettes (Sakura). Tissue section ribbons (5 µm thickness) are cut using a microtome, kept in warm water maintained at 42-47°C for proper stretching and lifted with poly-L-lysine coated slides (Fisher Scientific) and dried overnight.

2.7. Peptide blocking

Antibody dilution conventionally taken as 1:2000 diluted the required amount of antibody (2.3µl for 5ml) in 100 µl TBST. This served as positive control. Antibody: Peptide: 1:25 or 1:40, taken depending on the observed band in the test bleed experiment. On an average our sera has a maximum of 10 mg/ml of antibody (data taken from Western Blotting and ELISA). If blocking is performed on purified protein then its concentration was estimated at 280 nm. For antibody concentration 2mg/ml, 50 µg of peptide Mixed antibody and peptide and kept

on a horizontal shaker for 2 hour at room temperature. Tubes containing antibody and peptide solution were incubated at 37°C for 2 hour. After incubation the tubes were centrifuged at 4°C for 15 minute at 8000 rpm. Supernatant was carefully removed leaving at least 20 µl at the bottom to avoid disturbing invisible immune complexes. Then routine Western Blotting was performed.

2.8. Immunohistochemistry

Slides were deparaffinized by immersing in Xylene with two changes for 5 min each and rehydrated by immersing in decreasing grades of ethanol and distilled water for 1 min each. Antigen retrieval was done by autoclave method and washed with Tris buffer saline having 0.1% Triton X100 three times for 15 minutes. Slides are kept in 1% H_2O_2 to quench the peroxidase. Blocking was done with 5% BSA (Sigma) and normal Goat serum in tris buffer saline with 0.1% Triton X100 (Sigma). Normal rabbit serum as negative control and experiment rabbit serum were diluted (1:1000) using the blocking solution, added to the tissue sections on the slides and incubated overnight at 4°C. Next day the slides were washed with TBST and Goat anti-rabbit IgG biotin conjugate (1:1000) was added and incubated for 30 minutes at room temperature. Avidin-Biotin complex (Vector Labs) was prepared as per the instructions of the manufacturer, added to the slides after washing and incubated for 30 minutes at room temperature. Then color development was done by using Nova red substrate (Vector Labs) and the slides were counterstained with hematoxylin for 5-10 seconds. Then slides were dehydrated with increasing grades of alcohol and Xylene and mounted with cover slip by use of DPX.

3. Results and Discussion

3.1. Results

ELISA results showed seropositivity against the synthetic peptides when incubated with the immunized rabbit sera. The expression level of anti-Nanog serum was more than anti-Fragilis serum (Fig.1). ELISA results gave preliminary confirmation of the presence of the antibody against the synthetic peptides in the sera.

Western Blot results showed that the antiserum developed against Nanog and Fragilis detected a 35 kDa and 16 kDa bands respectively in cell extract of 3T3 cell line which used as a positive control to confirm the presence of antibody against the immunized peptides (Fig.2). For Fragilis, a 16 KDa band was detected in the Mouse embryonic cell 3T3. It belongs to the highly conserved fragilis protein family. It is an interferon-inducible transmembrane protein consisting of 2 putative transmembrane domains. It is associated with germ cell specification and development. It is located on the cell surface and might be important in mediating interactions amongst germ cells and their surrounding neighbors. It is expressed in early embryos and

nascent primordial germ cells. These proteins might be involved in anti-proliferation signaling and homotypic cell adhesion [14]. Antibody development against a few embryonic stem cell markers will meet the need for identification of ES cells. Therefore in this study antibody raised against stem cell marker and the expression of the antibodies were validated by immunecyochemistry using mouse embryonic cell line 3T3. Antibodies were developed against synthetic peptides made from carefully chosen epitopic region from sequences available in the National Center for Biotechnology Information [https://www.ncbi.nlm.nih.gov].

In immunohistochemistry analysis, the expression of human NANOG in the tails of spermatozoa of rat testes was detected, which are easily distinguished by their size, topological position and morphology (Fig. 3a). NANOG staining was primarily detected in the nucleus of the stained germ cells and was also weakly detected in the cytoplasm of a few cells. NANOG was strongly expressed by differentiated germ cells (spermatocytes) and weak expression was detected in spermatogonia. NANOG expression was observed in the nuclei of most spermatocytes, and following their differentiation to spermatids, it was translocated to the perinuclear region and cytoplasm (Fig. 3b).

3.2. Discussion

Western blot analysis showed that the anti-NANOG antibodies could identify proteins of definite sizes in the adult testis. The smaller fragment in NANOG immunoblots indicate towards truncated protein that may have arisen from the cytoplasmic translocalized NANOG. Although amino acid sequences of NANOG (Accession Number(s): NP_079141.1; Human GeneID(s): 79923) show 88% homology to mouse NANOG (accession no. Q5TM83) our results show that NANOG is expressed in the primitive germ cells of adult rat testis. The pattern of expression was strikingly similar to that of the DBA binding in germ cells reported previously [9]. Strong NANOG expression, like DBA staining, was observed in undifferentiated germ cells of adult rat testis and it showed that it is not progressively lost with increasing age reported previously [8]. At adult age, many germ cells showed translocation of NANOG protein from the nucleus to the perinuclear region and cytoplasm.

NANOG expression was not only specific to germ cells but was also observed in Sertoli and interstitial cells of adult rat testes. NANOG expression was observed not only in PUC matrix cells but also in porcine fibroblast cell lines [5]. NANOG transcripts were detected not only in the ICM of porcine embryos but also in epiblast-derived somatic cell lines and adult organs [2]. Therefore, NANOG expression, unlike DBA binding, could not be used as a specific marker of pig undifferentiated germ cells in the testis or in culture. Nevertheless, the expression pattern of NANOG in the postnatal pig testis is a strong indication that it has a role in stem cell activity of undifferentiated germ cells. NANOG expression was localized mostly in germ cells in the adult rat testes, suggesting that rat gonocytes possess stem cell potential.

The cytoplasmic translocation of NANOG protein in the germ cells with increasing age is interesting because NANOG is a homeodomain-bearing transcription factor and nuclear localization of NANOG is essential for its function. Nuclear export of NANOG may result in the loss of function and quite possibly degradation by cytoplasmic machineries. Down-regulation of NANOG expression in goat blastocyst occurs by sequestration/degradation utilizing a nucleolar mechanism [12]. Although we failed to identify any such finding in this study, this discrepancy may be due to species difference or a cell-type-specific mechanism that exists in the testis. Interestingly NANOG expression was detected in somatic cells, such as Sertoli and interstitial cells in the adult testes. NANOG expression was detected not only in a few undifferentiated germ cells but also in differentiated germ cells such as spermatocytes in 10- and 20-week-old testis sections. In 20-week-old testis sections, the expression of NANOG protein was variable in the differentiated germ cells, suggesting that the expression pattern was much more dynamic. This is contrary to an earlier finding in mice, in which NANOG expression was restricted to PGCs and no germ cells stained positive in the adult testis or ovary [25]. This suggests that the expression pattern of NANOG varies among species. Intriguingly, NANOG protein localization changed from the nucleus to the cytoplasm in the differentiating germ cells in rat testes. Nevertheless, our finding that NANOG is expressed in the nuclei of spermatocytes and spermatids suggests that these proteins have some role in the meiotic stage of germ cells. Our finding suggests that NANOG expression was markedly upregulated in the gonocytes shows stem cell potential in adult testis. These results suggest that porcine gonocytes could proliferate in vivo and NANOG is essential for determining the stem cell potential of gonocytes. Nevertheless, these findings confirm that adult rat testis has a population of stem cells that can be act as biomarkers.

In conclusion, pluripotency-specific protein, such as NANOG, showed a unique expression pattern in developed adult rat testes. NANOG expression was abundant in primitive germ cells of adult testis such as gonocytes. NANOG protein strongly expressed in differentiated germ cells such as spermatocytes and weakly expressed in spermatogonia. The strong expression of NANOG protein in porcine gonocytes raises the possibility of using the gonocytes to establish a cell line that could have germline characteristics.

Furthermore, this study has provided a number of interesting findings regarding the development and differentiation of rat germ cells in the adult testis, including a very small population of PGCs with a molecular signature expressing NANOG. Being able to identify the molecular signature of PGC will provide valuable tools for isolating this distinct population of germ cells for cytological and molecular analysis. Of great interest is the period of development in germ cell fate studied here since it is the time at which human germ cells demonstrate the ability to form EGCs and ECCs. This information will be critical in future studies for identifying the progenitor cells of EGCs and ECCs, as well as the mechanisms involved in their

conversion.

4. Figures

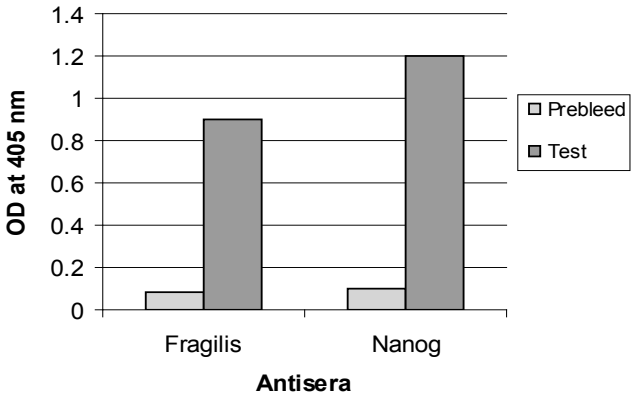

Figure 1: ELISA showing variation in absorbance at 405 nm to check the developed antibody titer in the sera. performed to check antibody titer in the sera. Data of ELISA showed the expression level of anti-Nanog serum was more than anti-Fragilis serum.

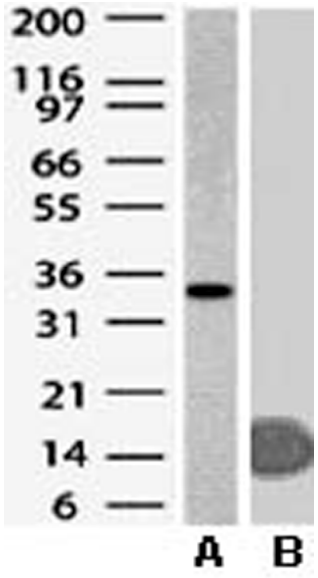

Figure 2:. Western blot analysis of developed antibody against the immunized peptides. SDS-PAGE separated proteins were blotted onto a PVDF membrane and incubated with the immunized rabbit sera. Lane A, Western Blot results against Nanog; Lane B, Western Blot results against Fragilis in cell extract of 3T3 cell line. Molecular weight of standard protein is indicated on left margin (kDa).

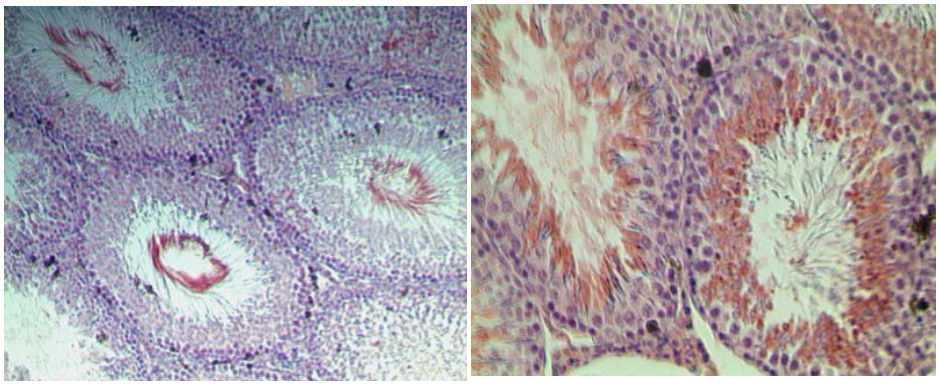

Figure 3: Immunohistochemistry analysis (a) Expression of NANOG in the tails of spermatozoa of rat testes, distinguished by their size, topological position and morphology; (b) NANOG expression in the nuclei of spermatocytes and their differentiation to spermatids.

4. References

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