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Research paper

Production of rabbit monoclonal antibodies against mouse embryonic stem cells and identification of pluripotency-associated surface antigens

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ABSTRACT

Embryonic stem (ES) cells are pluripotent stem cells derived from the inner cell mass of the blastocyst. ES cell surface molecules are important for the identification, labeling, sorting, quality control and functional studies of ES cells. Currently, knowledge of ES surface molecules is limited. To identify new surface molecules, we generated a panel of rabbit monoclonal antibodies (rMabs) against mouse ES (mES) cells. We identified three monoclonal antibodies that interact with molecules on the mES cell surface and found that the expression of their respective antigens decreased upon mES cell differentiation. The antigen of the rMab ZjuESrMab29 was identified as granulocyte macrophage colony-stimulating factor receptor α (GM-CSFR α) by liquid chromatography–tandem mass spectrometry (LC–MS/MS). This study demonstrated that rabbit monoclonal antibody production via whole-cell immunization could be a practical method for the discovery of stem cell surface antigens.

1. Introduction

Embryonic stem (ES) cells are widely used in basic research and hold great potential for regenerative medicine (Keller, 2005). The availability of specific antibodies against ES surface antigens will greatly benefit the study and application of ES cells because antibody labeling is the only non-invasive method available for labeling and sorting ES cells prior to their use. The specific antibodies against ES surface antigens can also be used to negatively select undifferentiated ES cells out of *in vitro* differentiated populations in animal models or clinical trials in order to avoid teratomas.

There are three general approaches used to discover new ES cell surface molecules: a genomics-based approach, a proteomics-based approach and an antibody-based approach (Solter and Knowles, 1978; Tanaka et al., 2002; Nunomura et al., 2005). The first two methods take a forward genetics approach in which candidate surface molecules are identified through high-throughput methods and confirmed in antibody-based assays. These high-throughput methods tend to provide hundreds of candidate molecules with relatively low accuracy; thus, the cost of this type of approach could be high and the discovery phase could be long. Another disadvantage of high-throughput approaches is that they only take primary sequence information into account, while they disregard information regarding the conformation and modification of the candidate molecules, which are of special importance for stem cell surface molecules. The antibody-based method takes a reverse approach in which a panel of monoclonal antibodies is generated against whole cells, and specific antigens for stem cells are selected from this panel. This

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method has the potential to generate monoclonal antibodies with great diversity in a single immunization process, and it has the added benefit of utilizing native proteins, which possess physiologically relevant conformations and modifications. Thus, the antibody-based approach is an efficient method for surface marker identification and is affordable for most researchers.

The mouse is the most widely used animal for monoclonal antibody production because of its breeding capabilities and its highly efficient hybridoma production. Mouse monoclonal antibodies have been used in the identification of ES cell surface markers (Son et al., 2005; Choo et al., 2008). Two major shortcomings of mouse antibody production method hamper its use in ES surface antigen identification via the whole-cell immunization approach. First, mice have a relatively smaller antibody repertoire, which limits their antibody diversity. Second, mice are generally immunotolerant to cell surface antigens on mouse ES (mES) cells.

Rabbit monoclonal antibody production is a recently developed technology. Rabbits possess a larger antibody repertoire than mice, and their antibodies exhibit a higher affinity and specificity in recognizing conformational and modified epitopes than do mouse antibodies (Spieker-Polet et al., 1995; Huang et al., 2007). Thus, rabbits are more suitable for the large-scale production of antibodies specific for mES cell surface antigens.

To identify new surface molecules for mES cells, we immunized rabbits with mES cells and produced a panel of monoclonal antibodies. From 240 rabbit monoclonal antibodies (rMabs), we identified three mES surface molecule antibody candidates. The antigen for one antibody candidate was purified by immunoprecipitation and was identified as granulocyte-macrophage colony-stimulating factor receptor α (GM-CSFR α), the expression of which was further shown to be highly restrictive to undifferentiated ES cells.

2. Materials and methods

2.1. Cell lines and cell culture

The mES cell lines D3 (ATCC, http://www.atcc.org/) and J2 (Jiang et al., 2005) were cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs) as previously described (Huang et al., 2007). The mouse embryonic carcinoma (EC) cell line F9 (ATCC, http://www.atcc.org/) was cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 12% fetal bovine serum (FBS, Hyclone) at 37 °C in 5% CO_2. Human ES (hES) H9 cells were obtained from WiCell Research Institute (Madison, WI) and cultured on mitomycin C-treated MEFs as previously described (Thomson et al., 1998).

2.2. Commercial antibodies

The following commercially available antibodies were used in this study: anti-Oct4 rat IgG monoclonal antibody (MAB1759, R&D, USA), anti-SSEA-1 mouse IgM monoclonal antibody (MAB2155, R&D, USA), rabbit anti-GMCSFR α polyclonal antibody (0804-8, Huabio, China), Alexa Fluor 488 conjugated goat anti-rabbit IgG (H + L) (A11008, Invitrogen, USA), Alexa Fluor 488 conjugated goat anti-mouse IgM (A21042, Invitrogen, USA), Alexa Fluor 488 conjugated goat anti-rat IgG (H + L) (A11006, Invitrogen, USA) and R-PE-conjugated goat anti-rabbit IgG (H + L) (00008-2, Proteintec, USA).

2.3. Immunization and hybridoma production

mES D3 cells (10^8) were injected into three-month old New Zealand rabbits (purchased from the Animal Experiment Center of Zhejiang University) for each immunization. For the initial immunization, mES cells were harvested and resuspended in 1 ml of PBS. The suspension was emulsified with Complete Freund's Adjuvant (CFA) in a 1:1 (v/v) ratio and subcutaneously injected at 10 points on the rabbit. Subsequent boosts were done using mES cell suspension emulsified in Incomplete Freud's Adjuvant (IFA) instead of CFA, with an interval of two weeks between each immunization. One week after the fourth immunization, serum from the immunized rabbits was collected and tested for immunocytochemical staining of mES cells. Two weeks after the fourth immunization, the rabbit (ZjuESR2) was injected intravenously with 10^8 mES cells suspended in 1 ml of PBS to boost the immunization. The rabbit was sacrificed three days after the last boost, and spleen cells were isolated and fused with 240E-1 cells (Huang et al., 2007) to produce hybridomas.

2.4. Hybridoma selection

Supernatants from the hybridoma cultures were collected. mES cells were cultured on mitomycin C-inactivated MEF feeders in 96-well culture plates for two days. Cells were fixed with 4% paraformaldehyde, blocked with 10% goat serum and then stained with hybridoma supernatants and a FITC-conjugated goat anti-rabbit secondary antibody. Positive clones were subcloned by limiting dilution in 96-well plates. Two weeks later, the remaining positive clones were propagated, and their culture supernatants were collected.

2.5. Immunoaffinity purification of antigens

2.5.1. Classic immunoprecipitation (IP) protocol

A cell lysate was prepared from 5×10^8 F9 EC cells by ultrasonication in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 1 mM PMSF). Culture supernatant from the hybridomas (1 ml) was incubated with the cell lysate overnight at 4 °C. The supernatant was then incubated with Protein A-conjugated resin (GE) at 37 °C for 1 h. The resin was loaded onto a 5 ml column and washed with 10 column volumes of lysis buffer. Putative antigens were eluted with a 2.5 mM citrate solution (pH 3.0).

2.5.2. Live cell IP protocol

Cultured F9 cells (5×10^8) were separated into single cells using 0.05 mM EDTA, resuspended in 1 ml of the hybridoma culture supernatant and further incubated while rotating gently for 1 h at 37 °C. The cells were washed three times with PBS and lysed with lysis buffer. The cell lysate was clarified by centrifugation, and the supernatant was incubated with 20 μl of a 50% (w/v) Protein A-Sepharose bead slurry (GE) overnight at 4 °C. Putative antigens were eluted with a 2.5 mM citrate solution (pH 3.0).