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Chapter 7

Hybridoma Technology for the Generation of Monoclonal Antibodies

Chonghui Zhang

Abstract

Hybridoma technology has long been a remarkable and indispensable platform for generating high-quality monoclonal antibodies (mAbs). Hybridoma-derived mAbs have not only served as powerful tool reagents but also have emerged as the most rapidly expanding class of therapeutic biologics. With the establishment of mAb humanization and with the development of transgenic-humanized mice, hybridoma technology has opened new avenues for effectively generating humanized or fully human mAbs as therapeutics. In this chapter, an overview of hybridoma technology and the laboratory procedures used routinely for hybridoma generation are discussed and detailed in the following sections: cell fusion for hybridoma generation, antibody screening and characterization, hybridoma subcloning and mAb isotyping, as well as production of mAbs from hybridoma cells.

Key words: Cell fusion, ELISA, Flow cytometry, Hybridoma technology, Immunohistochemistry, Immunization, Monoclonal antibody, Myeloma cells, Therapeutic antibody, Screening

1. Introduction

The invention of hybridoma technology by Georges Köhler and César Milstein in 1975 is a significant milestone in immunology and biomedicine (1). This technology has enabled scientists for the first time to generate unlimited quantities of pure, monospecific antibodies directed against virtually any antigen. A monoclonal antibody (mAb) is a highly specific and homogeneous species of immunoglobulin molecule produced by a single hybridoma clone that has been generated by the fusion of a myeloma cell with a B lymphocyte from a donor or from an immunized animal. Hybridoma technology has thus revolutionized discovery research and therapeutic development in such diverse fields as immunology, biology, oncology, and infectious diseases (2–4). The mAbs generated from

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this technology have served as reagents for the identification and characterization of cell surface antigens (5, 6), for classification and isolation of hematopoietic cell subsets (7–9), and for the development of biomarkers to distinguish aberrant or cancerous cells from normal cells (10–13). Hybridoma technology has long been a powerful tool for investigators to make discoveries in the biological sciences and has led to many important advances in medicine.

With the breakthrough in molecular engineering and antibody humanization (14, 15), mAbs have emerged as the most rapidly expanding category of biopharmaceuticals for a large variety of clinical scenarios. For example, mAbs have been used to aid in successful organ transplantation (16, 17), as well as being used to treat inflammatory diseases (18, 19), cancer, and infectious diseases (20–22). Based on published data, nearly 30 FDA-approved antibody drugs are on the US market today (Fig. 1) and it is estimated that hundreds of mAbs are currently in various phases of clinical trials worldwide (23).

mAbs can be produced from an immune or nonimmune resource using a range of recently developed antibody technologies, including methods such as display technologies (24, 25) or memory B-cell immortalization and cloning (26, 27). However, since hybridoma technology is so well established, it will continue to provide a powerful and indispensable platform for generating high-quality mAbs to meet unmet needs. It is important to note that mAbs generated from immune hosts by the hybridoma approach often exhibit good binding affinity due to in vivo secondary immune responses. These mAbs routinely obviate the requirements for subsequent in vitro affinity maturation or other modifications to improve antibody potency by additional technologies (28, 29). Furthermore, the primary production of the whole Ig molecule from hybridomas allows investigators to screen directly for the desired biological function of mAbs from the very beginning. Therefore, it is not surprising that 26 out of the 28 therapeutic mAbs that have been approved by the FDA in the United States today have originated from hybridomas, with or without chimerization or humanization (Fig. 1). With the recent development of transgenic humanized mouse strains that are capable of natural recombination and affinity maturation in vivo and which have a large repertoire of high-affinity antibodies to any antigen (30–32), the "old-fashioned" hybridoma technology will open up new avenues for more effectively generating large panels of high-quality and fully human mAbs. These fully human mAbs generated from transgenic humanized mice will accelerate the development and application of mAbs as therapeutics for human cancer and disease (29, 33).

Hybridoma technology is composed of several technical aspects, including antigen preparation, animal immunization, cell fusion, hybridoma screening and subcloning, as well as characterization and production of specific antibodies (Fig. 2). mAb generation by

Hybridoma Origin (26 mAbs)

Murine mAb (-omab) 3
- ORTHOCLONE OKT3® *muromonab-CD3* (1986)
- ZEVALIN® *ibritumomab tiuxetan* (2002)
- BEXXAR® *tositumomab* (2003)

Chimeric mAb (-ximab) 5
- REOPRO® *abciximab* (1994)
- RITUXAN®/ MABTHERA® *rituximab* (1997)
- SIMULECT® *basiliximab* (1998)
- REMICADE® *infliximab* (1998)
- ERBITUX® *cetuximab* (2004)

Humanized mAb (-zumab) 11
- ZENAPAX® *daclizumab* (1997)
- SYNAGIS® *palivizumab* (1998)
- HERCEPTIN® *trastuzumab* (1998)
- CAMPATH® MABCAMPAT® *alemtuzumab* (2001)
- XOLAIR® *omalizumab* (2003)
- AVASTIN® *bevacizumab* (2004)
- TYSABRI®/ ANTEGREN® *natalizumab* (2004)
- LUCENTIS® *ranibizumab* (2006)
- SOLIRIS ® *eculizumab* (2007)
- CIMZIA® *certolizumab pegol* (2008)
- Actemra® *Tocilizumab* (2010)

Human mAb (-umab) 7
- VECTIBIX® *panitumumab* (2006)
- SIMPONI® *golimumab* (2009)
- STELARA® *ustekinumab* (2009)
- ARZERRA® *ofatumumab* (*2009*)
- ILARIS® *canakinumab* (2009)
- PROLIA®/ XGEVA® *Denosumab* (2010)
- YERVOY® *ipilimumab* (2011)

Other Approach (2 mAbs)

Human mAb (-umab) 2
- HUMIRA®/ TRUDEXA® *adalimumab* (2002)
- BENLYSTA® *belimumab* (2011)

Fig. 1. A list of FDA-approved therapeutic mAbs currently on the market. Over 30 therapeutic mAbs have been approved by the FDA for marketing in the United States to date, whereas a small number of the mAb drugs, such as Mylotarg (*Gumtuzumab ozogamicin*) and Raptiva (*Efalizumab*), have been withdrawn from the market due to their side effects and/or poor clinical benefits. Most of the FDA-approved therapeutic mAbs currently on the market have originated from hybridomas and are in the full-length antibody molecular format, including the murine (suffixed with -*omab*), chimeric (-*ximab*), humanized (-*zumab*), and human (-*umab*) antibody category. All human mAbs of hybridoma origin are generated from the XenoMouse® or HuMAb-Mouse® transgenic strain, both of which have nearly the entire human Ig loci introduced into the germ line with inactivation of the mouse Ig machinery. For each antibody drug, its trade name, generic mAb name and the year of FDA approval are indicated in the figure. The digit shown represents the number of therapeutic mAbs in the antibody category.

the hybridoma approach requires knowledge of multiple disciplines and practice of versatile technical skills, ranging from animal handling (immunization and sample collection), immunology (immunoassays and antibody characterization) to cellular and molecular biology (cell fusion for hybridoma generation, protein sequencing analysis for antigen preparation, and flow cytometry or other cell-based assays for screening hybridomas). Generation and identification of high-quality hybridoma clones is a comprehensive and labor-intensive process, and requires months of work during the time frame from immunization to specific hybridoma identification. The key aspect of hybridoma generation is the screening procedure used to identify and select the desired hybridoma clones from the fusion plates. As shown in Fig. 3, cell fusion

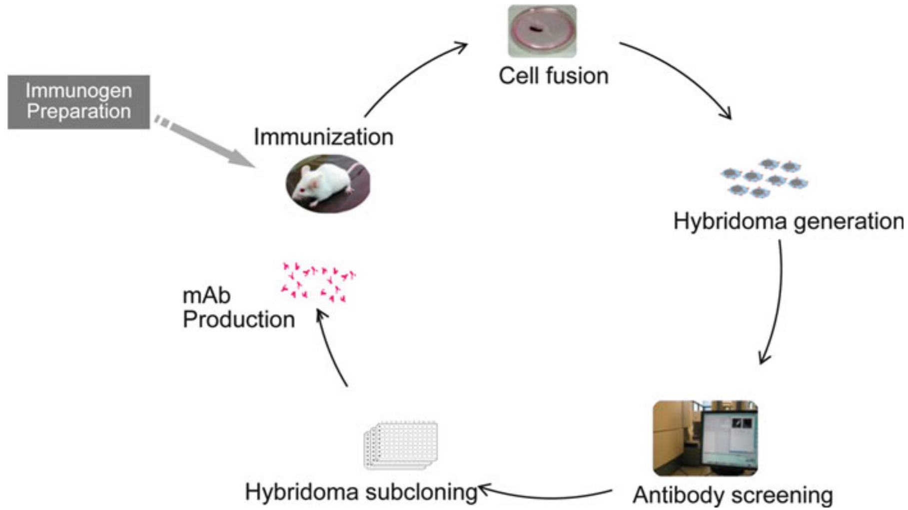

Fig. 2. A diagram of mAb generation by the hybridoma approach. Generation and identification of high-quality mAbs by the hybridoma approach requires months of work during the time frame from immunization to establishment of specific hybridoma clones. The work involves stages of antigen preparation, animal immunization, cell fusion for hybridoma generation, hybridoma screening and subcloning, as well as characterization and production of specific mAbs.

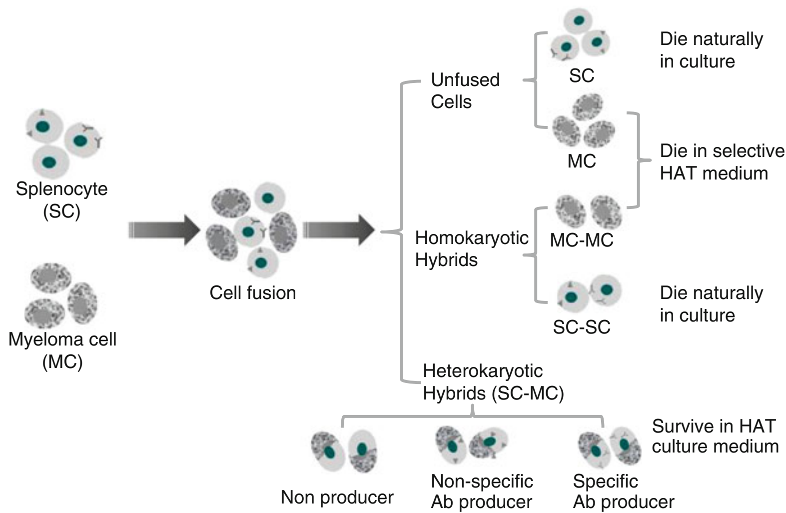

Fig. 3. Multiple cell types generated from fusion of splenocytes (SC) and myeloma cells (MC). PEG-mediated cell fusion is likely to result in a mixed population of cells consisting of nonproducing hybridomas, antibody-producing hybridomas and unfused cells. In the presence of aminopterin in HAT-selective medium, cells are dependent on another pathway that needs the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT) for survival. Under this culture condition, unfused myeloma cells or hybrids of myeloma cells with myeloma cells will die because of the absence of HGPRT, whereas unfused splenocytes or hybrids of splenocytes with splenocytes also die because of their lack of immortal growth potential. Only hybridomas from fusion of splenocytes with myeloma cells will inherit the HGPRT gene from splenocytes and the immortal growth property from myeloma cells, and can thus grow in HAT medium. By hybridoma screening and subcloning, specific hybridoma clones will be identified and isolated from nonspecific antibody producers or nonproducers of myeloma-splenocyte hybridomas.

mediated either by PEG or electrofusion typically generates a mixture of cells within the culture, which is composed of unfused splenocytes or myeloma cells, heterokaryotic hybrids (hybridomas) of splenocytes and myelomas with or without the secretion of specific antibodies, and the homokaryotic hybrids of either myeloma–myeloma cells or splenocytes–splenocytes. However, only the hybridomas from the fusion between splenocytes and myeloma cells are able to survive in the HAT medium. It is important to note that the myeloma–splenocyte hybridoma cells may turn out to be a specific antibody producer, nonspecific antibody producer or nonproducer. Development of appropriate antibody screening assays is thus required to efficiently identify the subpopulation of hybridoma cells in the fusion plates. The screening assays of choice should be specific, reliable, and effective. In general, the identification and selection process of antibody-secreting hybridomas comprises an initial screening of antibodies in polyclonal cultures and a secondary, more sophisticated characterization of mAbs afterwards. With the initial screening, antibody-secreting hybridomas are identified from the well of fusion plates, of which positive hybridomas are selected and then subcloned into monoclonals. A more sophisticated characterization of the mAbs generated will further determine their specificity, binding affinity, molecular features, and the functional activity of the mAbs, if any. Culture supernatants from the fusion plates are initially screened for positive hybridoma clones by a number of different immunoassays. While immunofluorescence flow cytometry is often applied to particulate antigens such as whole cells, an enzyme-linked immunosorbent assay (ELISA) is used for soluble antigens such as proteins or polypeptides, and immunohistochemistry (IHC) is developed for tissue antigens. Lastly, the hybridoma clones selected from the initial screens often require more testing for biochemical features of the mAb by immunoprecipitation and/or immunoblots, and further testing for biological activity by in vitro functional assays, such as blocking of the ligand binding to its receptor, detection of protein phosphorylation or signaling pathway, mediating agonistic or antagonistic activity, inhibiting cell proliferation, or interfering with the potency to mediate cell killing (34–36). In general, the functional screening assays are complex to perform and construe, and therefore are only carried out as necessary.

In this chapter, the strategy and laboratory methods for hybridoma generation are described and detailed in the following sections: cell fusion for hybridoma generation, antibody screening and characterization, hybridoma subcloning, cryopreservation and antibody isotyping, as well as production and purification of mAbs from hybridoma cells.

2. Materials

2.1. Preparation of Splenocytes

1. Spleens from immunized mice.
2. RPMI-1640 medium.
3. Petri dishes.
4. Sterile surgical instruments, including microdissecting scissors and forceps, for collecting animal samples.
5. Sterile microscope glass slides with frosted ends.
6. 15-mL conical tubes.

2.2. Preparation of Myeloma Cells as the Fusion Partner

1. Murine myeloma P3X63Ag8.653 cell or other fusion partner (ATCC, Manassas, Virginia, USA).
2. RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS).
3. RPMI-1640 medium supplemented with 10% FBS and 130 μM 8-azaguanine.
4. 50-mL conical tube.

2.3. Cell Fusion

1. Splenocytes from immunized mice.
2. Myeloma cells.
3. Sterile polyethylene glycol-1500 (PEG-1500), i.e., 50% PEG-1500 solution (w/v) in 75 mM HEPES buffer, pH 8.0 as fusogen.
4. Serum-free RPMI-1640 medium.
5. Hybridoma culture medium: RPMI-1640 medium supplemented with 20% FBS, 1× MEM nonessential amino acid solution, 2 mM L-glutamine, 0.5 mM sodium pyruvate, 50 μM beta-mercaptoethanol, penicillin (100 U/mL), and streptomycin (100 μg/mL).
6. Hypoxanthine–aminopterin–thymidine (HAT) medium: hybridoma culture medium (above) supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine.
7. HT medium: hybridoma culture medium (above) supplemented with 100 μM hypoxanthine, and 16 μM thymidine.
8. 96-Well flat-bottom culture plates.

2.4. Hybridoma Screening by FACS

1. Phosphate buffered saline (PBS), pH 7.4.
2. F buffer: PBS containing 0.1% bovine serum albumin and 0.01% sodium azide.
3. Fixation buffer: PBS containing 1% formalin (i.e., 37% formaldehyde solution).

4. Fluorescein-conjugated anti-mouse IgG antibody.
5. 96-Well microtest U-bottom plates.
6. Centrifuge with plate carriers.
7. Flow cytometer.

2.5. Hybridoma Screening by ELISA

1. 96-Well ELISA plates, e.g., Immulon 2HB plates.
2. 0.2 M carbonate–bicarbonate buffer, pH 9.4.
3. Tris buffered saline (TBS): 25 mM Tris and 0.15 M sodium chloride, pH 7.2.
4. Wash buffer: 0.05% Tween-20 in TBS.
5. Sealing tape for 96-well plates.
6. Blocking buffer: 3% normal goat serum and 0.05% Tween-20 in TBS.
7. TMB (3,3′,5,5′-tetramethylbenzidine) or other substrate solution.
8. Stop solution: 2.5 M sulfuric acid in H_2O.
9. Purified protein or polypeptides as antigen.
10. Hybridoma culture supernatants.
11. Peroxidase-conjugated anti-mouse IgG antibody.
12. ELISA plate reader with an appropriate analysis software.

2.6. Hybridoma Screening by IHC

1. Tissue section slides.
2. Tissue-Tek* OCT Compound (Sakura* Finetek).
3. Antibody supernatant samples.
4. 50 mM Tris–HCl buffer saline (TBS-50), pH 7.6.
5. 1% Hydrogen peroxide in 50% methanol solution.
6. 10% Normal goat serum in TBS-50.
7. Peroxidase-conjugated anti-mouse IgG antibody.
8. AEC substrate solution consisting of 5 mg of 3-amino-9-ethylcarbazole dissolved in 3 mL dimethyl sulfoxide, 2.5 mL of 200 mM acetate buffer, pH 5.5, 22.5 mL of 150 mM NaCl, and 200 μL of 0.3% hydrogen peroxide.
9. Gill’s hematoxylin solution.
10. Scott’s water: Tap water containing 0.2% sodium carbonate and 1% magnesium sulfate.
11. Glycergel mounting medium (Dako, Denmark).
12. Humid chamber.
13. Microscope slides and cover.
14. Microscope.

2.7. Hybridoma Subcloning

1. Hybridoma culture medium (see Subheading 2.3).
2. HT medium (see Subheading 2.3).
3. 96-Well flat-bottom culture plates.
4. Cell counter.
5. Sterile cryogenic vials.
6. Multichannel pipettes.

2.8. Hybridoma Cryopreservation

1. Cells to be frozen.
2. Centrifuge tubes.
3. Freezing medium: RPMI-1640 medium supplemented with 20% FBS, 2 mM L-glutamine, 0.5 mM sodium pyruvate, penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% dimethyl sulfoxide (DMSO).
4. Cryogenic vials.
5. Liquid nitrogen freezer.

2.9. Antibody Isotyping

1. Isotyping strips.
2. Hybridoma supernatant.

2.10. Thawing and Growth of Hybridoma Cells

1. Water bath at 37°C.
2. 70% Ethanol.
3. 15-mL conical tube.
4. Hybridoma culture medium (see Subheading 2.3).
5. Tissue culture flasks, e.g., T-25 or T-75.
6. 5% CO_2 tissue culture incubator at 37°C.

3. Methods

The following protocol describes the method by which splenocytes from the immunized mouse are fused with a BALB/c mouse myeloma line using polyethylene glycol-1500 (PEG-1500) as a fusogen to generate hybridoma cells. Upon fusion, cells are suspended in an HAT-selective medium and then cultured in 96-well flat-bottom plates for the growth of hybridoma clones.

3.1. Preparation of Splenocytes from the Immunized Mouse

1. Three days before cell fusion, boost mice with the antigen. On the day of the fusion, sacrifice mice for spleen collection according to the IACUC-approved standard animal use protocol.
2. Autoclave surgical instruments and perform all experiments under sterile conditions.
3. Prepare and warm serum-free RPMI-1640 medium in a waterbath at 37°C.

4. Collect spleen from the euthanized mouse under tissue culture conditions and place the spleen in a conical tube containing approximately 10 mL RPMI-1640 medium.
5. Rinse spleen twice in sterile Petri dishes with RPMI-1640 medium.
6. In a Petri dish with 10 mL of RPMI-1640 medium, grind the spleen tissue between the frosted ends of two sterile microscope glass slides.
7. Pipette cell clumps vigorously and transfer the suspension into a 15 mL conical tube.
8. Allow debris to settle for 5 min onto the bottom of the tube and gently transfer the cell suspension into another conical tube, leaving the debris behind.
9. Spin down cells at 300 × *g* for 5 min and discard the supernatant.
10. Suspend cells in RPMI-1640 medium and count the lymphocytes using a cell counter.
11. Wash cells twice in RPMI-1640 medium by centrifugation.

3.2. Preparation of Myeloma Cells

1. Mouse myeloma cell P3X63Ag8.653 as the fusion partner (37), is thawed from a stock stored in liquid nitrogen a week prior to the fusion.
2. The myeloma cells are first cultured in medium containing 10% FBS and 130 μM 8-azaguanine to select for clones that are HAT-sensitive and thus unable to survive in the presence of aminopterin (see Note 1).
3. After a few days, transfer and culture the myeloma cells in RPMI-1640 medium supplemented with 10% FBS.
4. Check culture microscopically to assess the status of the cells, and harvest the cells from culture flasks in the logarithmic phase of growth.
5. Spin down cells at 300 × *g* for 5 min and discard the supernatant.
6. Suspend myeloma cell pellets in serum-free RPMI-1640 medium.
7. Count and calculate the number of cells needed for fusion. Typically, splenocytes from the immunized mouse are fused with the myeloma cells at a ratio of 2:1 or 3:1.
8. Wash myeloma cells once more with RPMI-1640 medium by centrifugation and discard the supernatant.
9. Suspend cell pellet in a small volume of RPMI-1640 medium and then mix the myeloma cells with splenocytes in a 50-mL conical tube for cell fusion.

3.3. Cell Fusion

1. Spin down the mixture of splenocytes and myeloma cells at 300 × *g* for 5 min, and aspirate all supernatant from the cell pellet.
2. Suspend cell pellet by running the bottom of the conical tube over the air-grill of the biosafety cabinet.
3. Keep fusion tube in a beaker of warm water at 37°C for all of the following steps during cell fusion.
4. Using a 2-mL pipette, gradually add 1.5 mL of PEG-1500 over 90 s to the mixture of splenocytes and myeloma cells in the bottom of the fusion tube, and then allow the cells to stand for 1 min with occasional stirring.
5. Add 10 mL of warm RPMI-1640 medium gradually over 3 min to dilute the PEG-1500 fusogen (see Note 2).
6. Fill the fusion tube up to 45 mL with warm RPMI-1640 medium and allow the suspension to incubate in a water-bath at 37°C for 5 min.
7. Spin down cells at 300 × *g* for 5 min and discard the supernatant.
8. Suspend cell pellets in warm HAT medium at a concentration of approximately 5×10^5 splenocytes/mL.
9. Plate out cells in 96-well flat-bottom culture plates by adding 200 μL of cell suspension per well (see Note 3).
10. Incubate fusion plates in a 5% CO_2, 37°C incubator to grow hybridomas (see Note 4).
11. On day 5 post-fusion, remove half the volume (100 μL/well) of HAT medium from the fusion plates and replace with 100 μL of HT medium (i.e., HAT medium without aminopterin).
12. Sample culture supernatants from the fusion plates for antibody screening between days 10 and 14 when hybridomas have grown to be half-confluent and the medium has changed to an orange color.

3.4. Hybridoma Screening Using Flow Cytometry

Flow cytometry is one of the most powerful techniques for screening antibodies against cell surface antigens. It not only enables one to determine the presence of a specific mAb in the hybridoma culture supernatant but also allows one to measure the binding profile of the mAb. Flow cytometry in a high-throughput mode is much more rapid and suitable for the quantitative screening of a large number of samples in a short amount of time. The flow cytometric procedure includes cell labeling with antibodies, acquiring data with a flow cytometer, and analyzing the data with the appropriate software (see Notes 5 and 6).

1. Harvest antigen–expressing cells from culture flasks or isolate cells from tissue samples according to the standard cell isolation protocol.

2. Wash cells twice in PBS, followed by F buffer and by centrifugation at $300 \times g$ for 5 min.
3. Count and calculate the number of cells needed, which is usually $0.25–0.5 \times 10^6$ cells per labeling sample.
4. Place 50–100 μL of hybridoma culture supernatant or control antibody in each well of a 96-well microtest U-bottom plate.
5. Suspend cell pellets in F buffer at a concentration of 1×10^7 cells/mL, and add 25–50 μL of cell suspension to each plate well containing the appropriate antibody supernatant.
6. Mix reaction plate by shaking gently and incubate at 4°C for 30–45 min.
7. Wash plate three times in 250 μL of F buffer per well by centrifugation at $400 \times g$ for 5 min using plate carriers. Flick supernatant off the cell pellet in each of the plates between washes.
8. Suspend and incubate cells in 100 μL of fluorescein-conjugated anti-mouse IgG antibody at an appropriate dilution in F buffer.
9. Incubate plate at 4°C for 30–45 min.
10. Wash labeling plate twice with F buffer as above, and then once with fixation buffer.
11. Suspend cell pellets in an appropriate volume of fixation buffer. Typically, 30–50 μL sample volumes are required for high-throughput flow cytometric analysis.
12. Analyze samples by flow cytometry immediately, or store the samples at 4°C covered with foil to analyze at a later time.

3.5. Hybridoma Screening by ELISA

To detect protein- or polypeptide-reactive antibodies in the hybridoma supernatant, a standard ELISA protocol is commonly used. The ELISA procedure consists of an antigen pre-coating onto ELISA plates, an incubation with the antibody supernatant followed by another incubation with the enzyme-conjugated antibody, which is an enzymatic reaction for color development, and a final reading and subsequent analysis of the ELISA data.

1. On day 1, coat plates with purified protein or polypeptides as antigen. Dilute the antigen to a concentration of 0.5–1 μg/mL in carbonate–bicarbonate buffer, pH 9.4.
2. Add 100 μL of antigen solution (0.05–0.1 μg antigen) to each well of ELISA plates.
3. Seal plates with sealing tape and incubate the plates at 4°C overnight.
4. On day 2 after incubation overnight, empty all solution from the antigen-coated plates.
5. Rinse plates once with 250 μL of wash buffer.

6. Add 250 μL of blocking buffer to each well on all the plates and incubate at room temperature for 20 min to block nonspecific-binding sites.
7. Empty plates and rinse once with 250 μL of wash buffer.
8. Add 25–50 μL of hybridoma supernatant to each well of the antigen-coated plates, together with the appropriate negative and positive controls. Cover plates with sealing tape and incubate by rocking at room temperature for 45–60 min.
9. Empty and then rinse plates with 250 μL of wash buffer three times.
10. Fill plate wells with 250 μL of blocking buffer, and incubate at room temperature for 15–20 min.
11. Empty liquid from plates and add 50 μL of peroxidase-conjugated anti-mouse IgG antibody diluted in blocking buffer, and incubate plates at room temperature for 45–60 min.
12. Empty and rinse plates with wash buffer three times, followed by one wash with TBS.
13. Remove all liquid from ELISA plates.
14. Prepare substrate solution immediately before use. Add 100 μL of TMB substrate solution to each well of all the plates, and incubate at room temperature for 15–30 min or until the desired color develops. Peroxidase substrate solution is prepared according to the manufacturer's instructions.
15. Measure the absorbance (optical density) of each well at 450 nm immediately with an ELISA plate reader, or add 50 μL of stop solution to each plate well before reading.
16. Analysis of data (optional): a standard curve from the serial dilutions is prepared with concentration on the *X*-axis (log scale) versus absorbance on the *Y*-axis (linear). Interpolate the concentration of the hybridoma supernatant sample from this standard curve.

3.6. Hybridoma Screening by IHC

The reactivity of antibodies to the tissue antigen is often tested by IHC assays on slides of freshly frozen tissues or renatured paraffin-embedded sections. For the latter, specific antigen-retrieval techniques are commonly used to improve staining by modifying the molecular conformation of target antigens through an exposure of sectioned tissue to a heated buffer solution (38, 39). In general, mAbs selected by IHC assay may recognize denatured epitopes or intracellular antigens in addition to the native antigens on the cell surface.

Fresh tissue specimens are either snap-frozen in OCT medium in liquid nitrogen or embedded in paraffin. Frozen tissue sections are usually cut 4–8 μm thick on a cryostat and coated onto microscope slides. Tissue sections are fixed in acetone at 4°C and stored

at −80°C until use. Paraffin-embedded sections in the form of tissue microarrays have become commercially available in recent years for antibody screening and characterization. The IHC assay is a very valuable tool for localization of the antigen defined by mAbs. A detailed protocol is described below to screen antibodies in the hybridoma supernatant.

1. For frozen tissue sections, thaw tissue slides at room temperature and then place the samples in a humid chamber.
2. Apply 50–100 μL of hybridoma culture supernatant to the section slide and incubate at room temperature for 30 min.
3. Rinse slides with TBS-50 three times for 5 min each.
4. For tissue sections with highly endogenous peroxidase activity (e.g., thymus tissue sections), slides are submerged in 1% hydrogen peroxide in 50% methanol solution for 20 min on an ice bath to inactivate the endogenous enzyme after the first incubation with antibody supernatant.
5. Incubate slides with 10% normal goat serum in TBS-50 for 15 min to block nonspecific binding.
6. After draining the slides, apply 300 μL of peroxidase-conjugated anti-mouse IgG antibody at an appropriate dilution to each slide and incubate for 30 min at room temperature. The antibody conjugate reagent used at this concentration should yield an optimal reaction based upon previous titrations.
7. Following three washes in TBS-50, the color reaction is developed by incubating the slides for 40 min with AEC substrate solution.
8. After rinsing, counterstain the sections with Gill's hematoxylin for 1 min.
9. Wash slides and then submerge them in tap water or Scott's water for 2 min for better background staining.
10. Mount the slides with heated glycergel and examine the slides microscopically.

3.7. Hybridoma Subcloning

Hybridoma cells from fusion plates require subcloning to achieve a truly monoclonal population that produces a monospecific antibody. Under the initial plating conditions of cells in the fusion plates, a plate well probably contains no hybridoma clone or more than one hybridoma with or without the ability to produce antibodies (Fig. 3). While some hybridomas may be genetically unstable at an early stage, the stable clones must be identified and selected as soon as possible. Hybridoma cloning is a time-consuming step in the generation of mAbs; however, single-cell cloning can be accelerated by limiting dilution and microscopically picking single colonies, as described in Fig. 4 and discussed in this section.

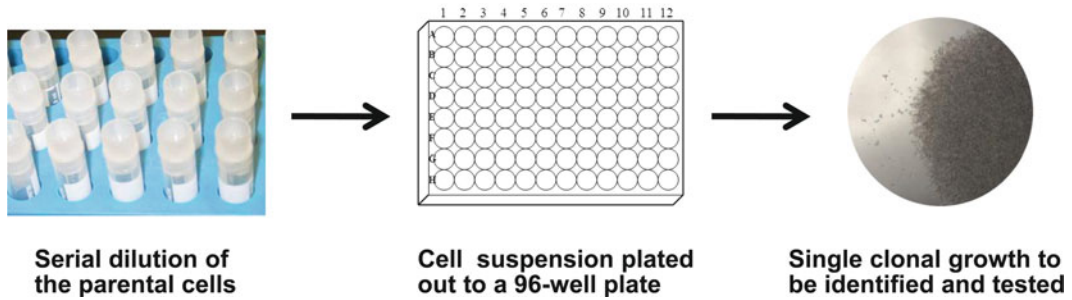

Fig. 4. Hybridoma subcloning by limiting dilution followed by microscopic selection of single cell colony. Parental hybridoma cells are diluted in culture medium by serial dilution to a final concentration of 10 cells/mL and 200 μL of the cell suspension plated into each well of a 96-well plate (2 cells/well). After incubation for 12–14 days, single clonal growths in the subcloning well are microscopically identified and tested for production of a specific antibody using hybridoma-screening protocols.

1. For subcloning, hybridoma cells are cultured either in hybridoma culture medium or HT medium, depending on the previously selected medium (see Note 7).
2. With a pipette, gently suspend hybridoma cells in culture. Transfer approximately 100 μL of cell suspension to a sterile vial labeled with the hybridoma clone name.
3. Count cells using a cell counter, and determine the initial concentration of cells.
4. Make serial dilutions of each hybridoma clone to a final concentration of 10 cells/mL in culture medium.
5. Using a multichannel pipette, plate out 200 μL of cell suspension (theoretically, 2 cells per well) into each well of a 96-well culture plate, which may have been pre-seeded with feeder cells as necessary.
6. Incubate plates in a 5% CO_2 incubator at 37°C, and microscopically examine the plates regularly for colony growth after the first week.
7. Examine and record the wells containing a single colony.
8. Sample supernatant from the single colony culture for antibody testing when the culture medium has turned an orange color.
9. Screen for specific antibodies from the subclone supernatant as described in Subheadings 3.4–3.6.
10. Based on antibody screening results, the desired subclones are selected, expanded for antibody production, and/or frozen for further studies.

3.8. Hybridoma Cryopreservation

1. Harvest hybridoma cells from culture plates or flasks. Transfer cell suspension to a centrifuge tube and spin for 5 min at 300 × *g*.
2. Remove supernatant and suspend cell pellets in a prechilled freezing medium at a density of $0.5–1 \times 10^7$ cells/mL.

3. Transfer 1-mL aliquots of cell suspension to a cryogenic vial.
4. Store cells in liquid nitrogen (see Note 8).
5. Record identity and location of cells in the liquid nitrogen freezer.

3.9. Antibody Isotyping

Identification of the antibody isotype not only provides information about the basic structure of an antibody and the isotype-related functions but also aids in selecting effective methods for antibody purification. The isotype of murine mAbs was traditionally determined by solid-phase ELISA or by immunodiffusion on agar plates, but both assays are less efficient and at times yield inconsistent results. In contrast, characterization of the antibody isotype with recently developed isotyping strips has made the process much easier, and as a result, the mAb isotype can be determined in minutes, especially for rat or murine antibodies. Since the isotyping strip bears immobilized bands of anti-rodent antibodies corresponding to each of the common antibody classes or subclasses and the κ or λ light chain, the strip reacts with any rodent antibody regardless of its isotype or purity. The detailed procedure to characterize the antibody isotype using isotyping strips is available in the manufacturer's instruction manual.

3.10. Thawing and Growth of Hybridoma Cells

Once the desired hybridoma clones have been identified and selected, the cells should be tested to ensure the absence of mycoplasma contamination in culture, and then frozen in several vials for long-term storage in a liquid nitrogen freezer. For production of mAbs from hybridoma cells, a vial of frozen hybridomas is thawed and grown in hybridoma culture medium to collect supernatant for antibody purification.

1. Locate and retrieve the cryovial of cells from a liquid nitrogen freezer.
2. Thaw cells immediately by placing the cryovial in a clean water-bath at 37°C. Agitate the cryovial in the water-bath gently until the frozen medium is completely thawed (see Note 9).
3. Submerge vial in 70% ethanol in a small beaker for 1–2 min and leave it to air-dry within the biosafety cabinet prior to opening.
4. Transfer thawed cell suspension from the cryovial into a 15-mL conical tube.
5. Add 10 mL of hybridoma culture medium gradually to the tube.
6. Centrifuge at $300 \times g$ for 5 min and aspirate supernatant from the tube.
7. Suspend the cell pellet in hybridoma culture medium and transfer the cell suspension to a culture flask.

8. Add more medium to the culture as necessary (usually 8 mL of medium for a T-25 flask or 30 mL of medium for a T-75 flask).
9. Incubate the cell culture in a 5% CO_2 incubator at 37°C.
10. Examine growth of hybridomas regularly and expand the cells for mAb production (see Notes 10 and 11).

4. Notes

1. Normal lymphocytes and other cells from the mouse spleen die naturally after a few days in culture. Only hybridoma cells are able to survive in HAT medium and grow indefinitely because they are supplied from both the parental B-lymphocytes, from which the X-chromosome encodes the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and from the parental myeloma cells which have the ability to grow immortally in vitro. With the exception of hybridomas, almost all of the cells undergo death in selective HAT medium in the first couple of weeks after fusion. The HAT medium is gradually replaced by hypoxanthine-thymidine (HT) medium, followed by a routine hybridoma culture medium without either HAT or HT supplement. For fusion plates, when the hybridoma cells have grown to be half-confluent in the culture wells and the color of the medium has changed to an orange color (usually 10–15 days post-fusion), the culture supernatant is sampled for antibody screening by various immunoassays.
2. To enhance the cell fusion efficiency, different fusion partners or other fusion methods should be considered besides the PEG-mediated fusion. For example, electrofusion-based protocols have reportedly been established and optimized for generating hybridoma clones (40, 41). However, the PEG-mediated fusion is a conventional and convenient technique for hybridoma generation, and has remained one of the best approaches for cell fusion.
3. To promote hybridoma growth, mouse peritoneal exudate cells or fibroblasts are often seeded on the fusion plate as a feeder layer, if necessary (42).
4. To maintain good culture conditions, such as having a stable CO_2 concentration and constant temperature, do not open the incubator for the first 2 days post-fusion if possible.
5. The choice of which hybridoma screening assay to use depends largely on the nature of the antigen that is available and the prospective application of the mAbs that is being generated. However, it should be noted that the outcome of the antibody

reactivity usually varies depending on multiple factors, such as the binding antigen used to coat the plastic plates, the direct reactivity with the targeting antigen in cells, or the inhibition of binding of a ligand to its receptor. Moreover, the conformation of peptides and even proteins bound to plastic can be affected by charges present on the plastic surface.

6. Some antibodies from unfused B-lymphocytes in the early culture may yield a misleading positive reactivity in the initial screening of hybridomas from fusion plates. The false positives can be diminished by changing the culture medium in fusion plates twice with fresh medium.
7. In order to promote the growth of a single hybridoma cell during the subcloning stage, the culture plate may be pre-seeded with mouse peritoneal exudate cells or fibroblast lines as a feeder layer, or enhanced with commercially available hybridoma cloning supplements.
8. During the hybridoma freezing stage, cells in a cryovial can be directly placed into a liquid nitrogen freezer. This may appear contrary to the general recommendation to gradually lower the temperature by placing cells in a –80°C freezer or in the freezing chamber of liquid nitrogen before transferring the cells to a liquid nitrogen freezer; however, cells that have been frozen by the direct-freezing method routinely show no difference in the loss of cell viability on recovery.
9. It is critical that frozen hybridomas are thawed as quickly as possible when the cells are removed from the liquid nitrogen freezer. It is also important to assess the viability of the recovered cells upon thawing.
10. Most hybridoma cells in a conventional culture media, such as complete RPMI-1640, should steadily be adapted to either a medium with low-IgG serum or a serum-free medium by lowering the serum level in the culture medium gradually.
11. Traditional cell culture medium supplemented with serum contains a considerable level of immunoglobulins of animal origin, which are difficult to separate from the murine mAbs during purification. Therefore, a medium containing low-IgG serum or serum-free medium must be used to grow hybridoma cells for in vitro production of mAbs. The culture conditions in the absence of animal immunoglobulins and the low level of other proteins present make the purification of mAbs from the hybridoma supernatant much more effective and expedient. In order to maximize the yield of mAbs in the culture supernatant, hybridoma cells are allowed to grow until the medium is depleted of nutrients. When the cells reach saturated density and the medium has turned a yellow color, the culture supernatant is collected for mAb purification.

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Chapter 8

The Application of Transgenic Mice for Therapeutic Antibody Discovery

E-Chiang Lee and Michael Owen

Abstract

In 2006, panitumumab, the first fully human antibody generated from transgenic mice, was approved for clinical use by the US Food and Drug Administration (FDA). Since then, a further seven such antibodies have been approved. In this chapter, we discuss how transgenic mice technologies can provide a powerful platform for creating human therapeutic antibodies.

Key words: ES cells, Homologous recombination, Human antibody, Humanized mice, Ig locus, Immunoglobulin, Isotype, Phage display, Transgenic mice, Therapeutic antibody

1. Introduction

The B cell arm of the immune system has evolved to produce high affinity, antigen-specific antibodies in response to antigenic challenge. Antibodies are generated in B lymphocytes by a process of gene rearrangement in which variable (V), diversity (D; for the IgH locus), and joining (J) gene segments are recombined, transcribed, and spliced to a Cμ (for IgH) or a Cκ or Cλ (for Igκ or Igλ) constant region gene segment to form an IgM antibody. Depending on the stage of B cell development, IgM is either located on the cell surface or secreted. The recombination process generates a primary antibody repertoire with sufficient germ line diversity ($\sim 10^{11}$) to bind a wide range of antigens. However, it is usually not large enough to provide the high affinity antibodies that are required for an effective immune response to an antigen such as an infectious agent. The primary response is limited further by the number of B cells circulating in the lymphoid organs and tissues at any particular time. This number (10^{8}–10^{10} depending on the organism) is orders of magnitude less than the encoded

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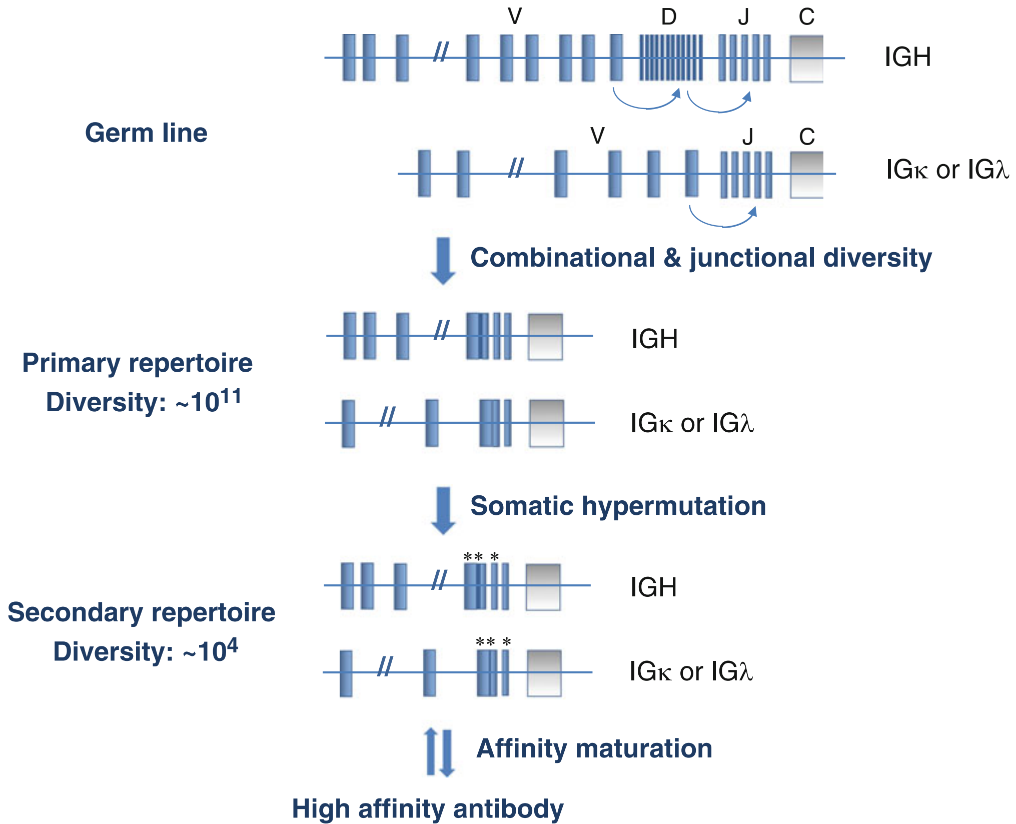

Fig. 1. Antibody diversification. The primary repertoire is formed by combination of germ line V, D, J segments for the heavy chain (IgH) and V, J segments for the light chains (Igκ or Igλ). During recombination, diversity is further increased by random insertions and deletions at the junctions between segments. Upon antigen challenge, engaged low-affinity antibodies are leads to somatic hypermutation to form the secondary repertoire. With intense competition for antigens, high-affinity antibodies are eventually selected out from the secondary repertoire. *Asterisks* indicate point mutations resulted from somatic hypermutation.

germ line diversity. Therefore, the immune system adopts a two-stage diversification process to increase diversity further (1) (Fig. 1). When challenged with antigens, B cells undergo selection and maturation by a process called somatic mutation. B cells expressing antibodies which bind to antigen undergo multiple rounds of diversification, clonal expansion, and antigen selection in the germinal centers (GCs) of the secondary lymphoid organs. During this process, the rearranged variable regions of the immunoglobulin genes acquire somatic hypermutation through nucleotide substitution. This stepwise process creates a secondary repertoire from the weak binders selected originally from the primary repertoire (2, 3) and combines rapid proliferation of antigen-reactive B cells with intense selection for quality of binding, eventually giving rise to high affinity antibodies with broad epitope coverage. During this process, antibodies undergo class switching in which the Cm constant region is replaced by Cγ, Cα, or Cε to produce IgG, A, or E classes of antibody with different effector functions.

The safety, specificity, and potency of antibodies have made them ideal candidates for pharmacological intervention in disease. Clearly, when used in a therapeutic setting, particularly during repeated administration, antibodies should contain no sequences that induce an immune response in patients which could produce an adverse reaction or alter the pharmacokinetic profile of the drug; ideally, they should be fully human. This requirement has resulted in a number of different strategies for "humanizing" monoclonal antibodies. Perhaps the most attractive of these approaches is the use of in vivo strategies such as transgenic mice that have harnessed the natural beauty of the two-stage diversification process and antigen-mediated selection for obtaining high affinity antibodies. The availability of these humanized mice has resulted in the development of powerful therapeutic agents against a number of human diseases.

2. Development of Human Immunoglobulin Transgenic Mice

Since the early 1980s, the introduction of rearranged immunoglobulin genes into the mouse germ line has been successfully exploited to study the mechanisms of assembly, expression, allelic exclusion, and somatic hypermutation of immunoglobulin (Ig) genes (4, 5). In 1985, Alt et al. noted that the transgenic mouse approach could not only enhance basic studies of immunology but also evolve to have a practical value in generating human antibodies through in vivo rearrangement, V_H and V_L assembly, and somatic mutation processes (4). There were four major challenges to the generation of such transgenic mice. Firstly, human Ig transgenes would be required to function as well as endogenous mouse Ig genes, properly utilizing the mouse machinery of the immune system, including gene rearrangement, heavy chain–light chain assembly, expression, allelic exclusion, hypermutation, class switch, and affinity maturation. Secondly, endogenous Ig genes would need to be inactivated to avoid expression of hybrid mouse–human antibodies. Thirdly, human sequence encoding sufficiently large repertoires would need to be inserted into mouse germ line. A limited repertoire may reduce the diversity of antibodies and constrain the application of the system. For the full human repertoire, this would necessitate the insertion of around 1 Mb of human DNA into the mouse germ line for each of the three Ig loci (Fig. 3). Finally, physiological levels of human Ig transgene products, equivalent to those in wild type mice, would need to be expressed. All of these challenges would need to be overcome to generate an uncompromised and fully functional immune system.

Four years after Alt's prediction, Bruggemann and her colleagues reported the generation of transgenic mice by pronuclear injection with a limited human repertoire of unrearranged V_H

and D_H segments, six human J_H segments, and a chimeric human/mouse μ constant region gene (6). In this process, the transgene randomly integrates into the mouse genome. This was the first study to demonstrate that human immunoglobulin gene segments are able to be rearranged and expressed in mice, suggesting that human cis-elements including IgH promoters, the intronic enhancer (iEμ), and recombination signal sequences (RSS) have at least some activity in transgenic mice. In 1992, Lonberg's group further showed that transgenic mice carrying an IgH minilocus including one human V_H, ten D_Hs, six J_Hs, Cμ, and Cγ1 genes together with their respective switch regions had detectable serum human IgM and IgG1 antibodies at levels ranging from 0.1 to 1 μg/ml, indicating that proper class switching of the human IgH transgenes had occurred (7). In addition, allelic exclusion was unimpaired in these mice since human and mouse IgM were never expressed on the surface of the same B cell. Both allelic exclusion and class switching require functional signaling from the transmembrane (cell surface-associated) form of human IgM. Therefore, the demonstration of that these functions were intact implies that the hybrid B-cell receptor (BCR) consisting of human IgM, mouse Igα, Igβ (essential co-receptors for B cell signaling), and other mouse signaling molecules can induce signaling during B cell development and activation.

The transgenic mouse lines generated by Bruggemann's and Lonberg's groups had a background of endogenous mouse Ig genes, which may have caused of the observed low expression of human antibodies. To overcome this problem, two groups reported the generation of human Ig gene-transgenic mice in a mouse IgH and IgK knockout background (8, 9). Lonberg's group created transgenic mice with a human V_H minilocus having two more human V_H segments than in their previous version, and a human V_κ minilocus with four V_κ, five J_κ segments and Cκ. Sequence analysis showed that the IgH minilocus did not only undergo VDJ rearrangement but also underwent somatic hypermutation following antigen challenge. In the absence of endogenous IgH and Igκ products, the levels of serum human IgM (~100 μg/ml) and human IgG1 (~0.1 to 10 μg/ml) were significantly higher than in transgenic mice harboring endogenous Ig genes (7, 8). The majority of human heavy chains were shown to be associated with human κ chains. A small portion (~1 to 5%) was complexed with mouse λ chains because the endogenous Igλ locus was not inactivated in these transgenic mice. Green's group used yeast artificial chromosomes (YACs) with human IgH or Igκ genes to generate transgenic mice through yeast protoplast fusion to embryonic stem (ES) cells (9). These YACs carried a much bigger fragment of genomic DNA (220 kb for IgH; 170 kb for Igκ) than the minilocus vectors used by Lonberg (80 kb for IgH; 40 kb for Igκ). Since YACs are able to accommodate more than 1 Mb of genomic DNA, this technology

can be used as a vehicle to introduce a larger human Ig repertoire than with other types of vector.

Initial studies on the B cell immune response to the CD4 antigen in Lonberg's transgenic mice generated only low affinity antibodies ($8–9 \times 10^7$/M) (8). It was thus difficult to determine whether these antibodies underwent affinity maturation. His group subsequently reported the generation of new YAC transgenic mice carrying the same IgH minilocus but with a much larger Igκ locus comprising nearly half of the germ line human Vκ region (10). High-affinity antibodies with 5×10^9 to 1×10^{10}/M affinity to the CD4 antigen, were generated in these mice. In 1997, Mendaz et al. generated YAC transgenic mice carrying approximately 66 human V_Hs and 32 V_κs using modified YACs with sizes of 1,020 kb and 800 kb, respectively (11). With such diverse repertoires, they also generated specific antibodies with high affinities around 1×10^9 to 1×10^{10}/M to a variety of antigens including IL-8, EGFR, and TNFα. With persuasive evidence to support effective in vivo affinity maturation for human Ig transgenes in their transgenic mice, both groups provided a vital breakthrough in the use of transgenic strategies for therapeutic antibody discovery.

In 1999, Bruggemann and her colleagues reported the generation of YAC transgenic mice carrying all three human Ig loci, IgH (240 kb), Igκ (1.3 Mb), and Igλ (410 kb), in a strain with inactivated endogenous IgH and Igκ (12). Human IgM was detected in serum at levels of 50–400 μg/ml and was elevated after immunization. These levels are similar to those observed in wild-type mice. However, it was impossible to determine whether the IgG response was normal since the IgH locus in these mice only contained Cμ and Cδ genes and did not include any Cγ gene segments.

In addition to transgenic technologies using pronuclear microinjection of minilocus vectors and protoplast fusion of YACs, microcell-mediated chromosome transfer (MMCT) has been used to transfer large chromosome fragments carrying the Ig genes into mice. This approach enables transfer of single human chromosome or chromosome fragments with a centromere and two telomeres into pluripotent mouse ES cells by fusion of human primary fibroblast-derived microcells with mouse ES cells (13, 14). Although this technology offers the advantage of transfer of the complete Ig repertoire, the unpredictable transmission rates due to chromosome instability and somatic mosaicism significantly limit its application.

3. Is the Full Human Primary Repertoire Necessary?

The successful isolation of high-affinity antibodies from transgenic mice with a limited repertoire raises the question of whether the full human repertoire is necessary to identify clinically relevant

monoclonal antibodies (10, 11). Studies on antibody binding to antigen have revealed that the CDRH3 region (complementarity determining region 3 of the IgH chain; the region spanning the VDJ junction) is responsible for most of the diversity of the primary repertoire. The additional diversity derived from CDRH1 and CDRH2 encoded by the V_H segments in the primary repertoire seems less important. It has been proposed that the highly diverse CDRH3 sequences are the primary determinants of specificity of antigen recognition (15). In this context, Davis and colleagues demonstrated that transgenic mice with one V_H segment are able to generate high affinity antibodies to variety of antigens through affinity maturation (16). However, it is possible that these antibodies bind to only limited epitopes because they were derived from one V_H segment. Unlike the primary repertoire, the diversity of the secondary repertoire created by hypermutation involves all three CDRH regions. The limited diversity observed in germ line CDRH1 and CDRH2 V_H sequences is thus amplified through second-phase diversification in GCs. From an antibody drug discovery perspective, broad epitope coverage is crucial for identifying clinical candidates. Therefore, the full primary repertoire may be required.

4. Compromised Immune Response from Transgenic Mice

Although the transgenic mouse approaches described above result in fully human antibodies, the level of antibody expression is lower than that found in wild-type mice. While the levels of serum human IgM in transgenic mice are usually around 10–400 μg/ml (8, 9, 12, 14) which are close to the serum IgM levels of wild-type mice (~500 μg/ml), the serum IgG concentration, around 10–600 μg/ml in transgenic mice, is much lower than the normal range of IgG levels (~2,000 μg/ml) (8, 11, 14). In addition, splenic B cell populations in these human Ig transgenic mice are usually only 5–40% of the number found in wild-type mice (8, 9, 12). Taken together, these results suggest that the immune response in these mice may be compromised with the consequence that obtaining high-affinity neutralizing antibodies usually requires more transgenic mice, a more intensive immunization schedule and more hybridoma screening when compared to wild-type mice (17).

This compromised immune response may be due to suboptimal use of human cis-regulatory elements within the immunoglobulin loci. Two critical long-range regulatory elements, the iEμ and the enhancer downstream of constant gene segments (the “3′ IgH regulatory region”), have been described in the IgH locus (18, 19). In both YAC and mini-locus transgenic mice, the human IgH

	EMPD	TMD	CYT
IgM_H. sapien	GEVSADEEGFENL	WATASTFIVLFLLSLFYSTTVTLF	KVK
IgM_M. musculus	GEVNAEEEGFENL	WTTASTFIVLFLLSLFYSTTVTLF	KVK
IgG1_H. sapiens	LQLEESCAEAQDGELDGL	WTTITIFITLFLLSVCYSATVTFF	KVKWIFSSVVDLKQTIIPDYRNMIGQGA
IgG2_H. sapiens	LQLEESCAEAQDGELDGL	WTTITIFITLFLLSVCYSATITFF	KVKWIFSSVVDLKQTIVPDYRNMIRQGA
IgG3_H. sapiens	LQLEESCAEAQDGELDGL	WTTITIFITLFLLSVCYSATVTFF	KVKWIFSSVVDLKQTIIPDYRNMIGQGA
IgG4_H. sapiens	LQLEESCAEAQDGELDGL	WTTITIFITLFLLSVCYSATVTFF	KVKWIFSSVVDLKQTIVPDYRNMIRQGA
IgG1_M. musculus	LQLDETCAEAQDGELDGL	WTTITIFISLFLLSVCYSAAVTLF	KVKWIFSSVVELKQTLVPEYKNMIGQAP
IgG2a_M. musculus	LDLDDVCAEAQDGELDGL	WTTITIFISLFLLSVCYSASVTLF	KVKWIFSSVVELKQTISPDYRNMIGQGA
IgG2b_M. musculus	LDLDDICAEAKDGELDGL	WTTITIFISLFLLSVCYSASVTLF	KVKWIFSSVVELKQKISPDYRNMIGQGA
IgG3_M. musculus	LELNETCAEAQDGELDGL	WTTITIFISLFLLSVCYSASVTLF	KVKWIFSSVVQVKQTAIPDYRNMIGQGA

Fig. 2. Alignment of amino acid residues comprising the transmembrane forms of human and mouse Igs. *EMPD* extracellular membrane proximal domain, *TMD* transmembrane domain, *CYT* cytoplasmic tail (45). The divergent residues are *underlined*.

transgenes use human instead of mouse regulatory elements. In addition, the low level of antibody expression can be due to the inefficient signaling of a hybrid BCR composed of human immunoglobulin, mouse Iga, Igβ, and other mouse signaling molecules. The Ig transmembrane region and cytoplasmic tail are crucial for BCR-mediated signaling (20–22). The mouse and human IgM transmembrane region and cytoplasmic tail are more similar than the equivalent mouse and human IgG regions (Fig. 2). Human IgM may, therefore, interact better with mouse Igα and Igβ than human IgG does, which could result in a normal range of human IgM but much lower levels of human IgG in transgenic mice (8, 11, 14). In this context, Rajewsky and colleagues generated mice in which mouse κ and γ1 constant regions were replaced with the corresponding human regions. The whole mouse Cκ region was replaced with the human Cκ region, whereas only the secreted exons of the mouse Cγ1 region were replaced in order to minimize the danger of disturbing membrane expression and signaling of the humanized IgG1 in the mouse. Interestingly, the serum levels of humanized IgG1 in transgenic mice were similar to those of mouse IgG1 in wild-type mice of either naïve or immunized status (23). This result suggests that mouse components of the transmembrane region and cytoplasmic tail or cis-regulatory elements are important to maintain antibody expression levels, particularly for IgG isotypes.

5. Learning the Lessons from Previous Studies

The pioneering studies described above have pointed the way to the creation of the second generation of transgenic mice expressing human immunoglobulin diversity. They have revealed the importance of maintaining the myriad of control elements within the Ig

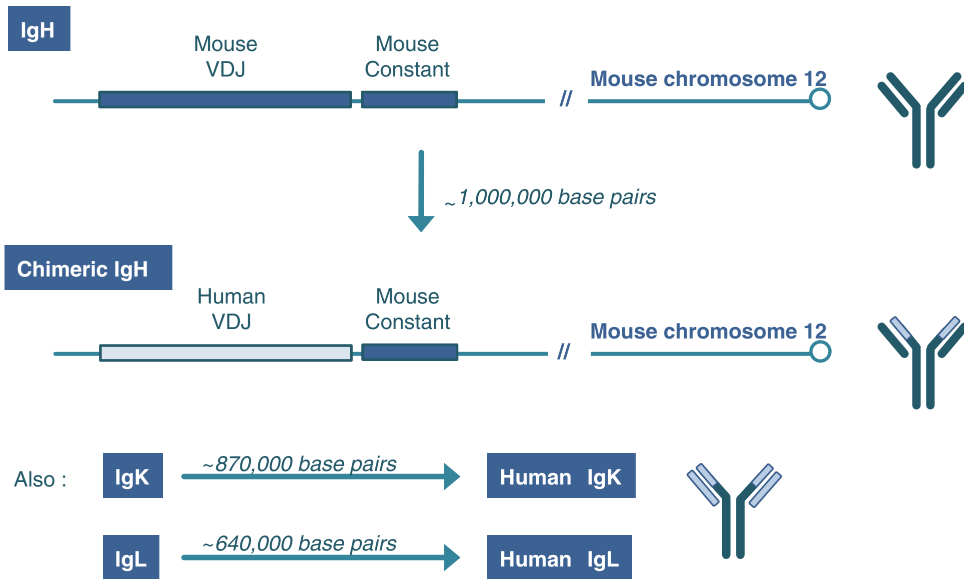

Fig. 3. Generation of a chimeric IgH locus with endogenous transcriptional control cis-elements and signaling machinery. The human variable region (V_H, D_H, and J_H) segments are inserted into the 5′ end of the endogenous mouse constant region to maintain the function of mouse cis-regulatory elements (Eμ and 3′ regulatory elements), and BCR signaling.

loci that regulate the processes of rearrangement, class switching, and somatic hypermutation and also the requirement of efficient signaling via the BCR for the optimal generation of the B cell compartment and the maturation of the B cell immune response. A new generation of transgenic mice has been constructed by precise replacement of mouse variable region genes with human counterparts in the Ig loci including the endogenous transcriptional control cis-elements and signaling machinery (17). These animals encode the mouse transmembrane domains and cytoplasmic tails and contain endogenous mouse iEμ and the 3′ regulatory elements (Fig. 3). The details of this transgenic mice technology have not been published, although it is likely that bacterial artificial chromosome (BAC) recombineering and homologous recombination of modified BACs in ES cells were applied to generate these transgenic mice (17, 24) and see also Chapter 9. Transgenic mice produced using this strategy generate chimeric antibodies with human variable regions and mouse constant regions and apparently possess normal serum levels of all isotype antibodies, and normal mature B cell numbers in blood, spleen, and lymph nodes (http://www.regeneron.com/velocimmune.html). Several antibodies produced from these transgenic lines are now in various stages of clinical development (25). The success of this new generation of transgenic mice in producing therapeutic grade human antibodies underpins the importance of the species-specific elements in the regulation of antibody signaling and expression.

6 Phage Display Technology: In Vitro Versus In Vivo

Phage display and other in vitro display technologies provide an alternative platform for generating human antibodies. In vitro technologies can be advantageous over transgenic approaches when applied to auto-antigens or when tailoring both affinities and cross-reactivities (26, 27) and see Chapters 3–5. However, antibodies against antigens that are similar between human and mouse can be generated in mice with a genetic knockout of the antigen-coding gene (28, 29). In addition, a wide range of adjuvants is available to modulate the immune response including breaking immune tolerance in mice (30, 31). These adjuvants, particularly agonists of Toll-like receptors (TLRs), will likely be useful in raising antibodies against auto-antigens.

Phage display technology is also amenable to high-throughput screening which relies critically on the quality of the libraries with sufficiently large effective diversity (26). One of the major drawbacks for phage display is that its diversity is generally limited to the range between 10^6 and 10^{11} unique antibody molecules whether it is derived from naïve or synthetic repertoires (27). The diversity of phage display is constrained by the transformation efficiency practically achievable in *E. coli* (32). At its best, phage display captures only 0.01% of the potential diversity of in vivo antibody structures (i.e., 10^{15} B cell somatic diversity). A high affinity antibody usually cannot be directly isolated from such a library. Thus, for the phage display approach, the initially identified antibodies require further optimization or maturation, a manual step which takes time and can be tedious and problematic. While the phage display technology is limited by its overall efficiency, in vivo transgenic technologies can generate fully human antibodies that can be directly moved into clinical development without further optimization (33).

While transgenic technology generates fully human antibodies through in vivo affinity maturation in a normal physiological context, phage display technology usually requires optimization or in vitro affinity maturation. In vivo high affinity antibodies are generated by somatic hypermutation which is dependent on activation-induced cytosine deaminase (AID), an enzyme that induces mutation in DNA by error-prone repair of G:U lesions (34). Somatic hypermutation is not a random process that generates mutations distributed along the variable region (35, 36). Rather, the mutation spectrum of antibodies relies on the intrinsic substrate specificity of AID which exhibits a clear preference for certain major, strategically targeted hot spots within V regions (37–39). In contrast, the in vitro-introduced or synthetic sequences used during the library construction or affinity maturation for phage display may contain mutations not within the natural spectrum of AID, these less natural sequences potentially act as immunogenic

human T-cell epitopes. Immunogenicity is an important issue for application of a therapeutic antibody. The development of an anti-therapeutic antibody response in antibody-treated patients can limit efficacy and reduce the safety of antibody treatments. Adalimumab, the first fully human antibody approved in the clinic, was developed from the phage display platform. It binds to TNFα with high affinity and effectively blocks TNFα activity. Clinical studies, however, revealed that up to 89% human anti-human antibody (HAHA) incidence in adalimumab-treated patients (40–42). Although it is not clear what causes the immunogenicity in adalimumab, golimumab, a human anti-TNFα antibody generated from a transgenic platform, did not show detectable neutralizing antibody in treated patients in clinical studies (43, 44). It is important to continuously monitor the immunogenicity of human antibodies derived from different platforms in clinical trials. The outcome may further change the trend of human antibody development.

7. Future Perspectives

In early 1990s, both phage display and transgenic mice technologies were developed to generate fully human monoclonal antibodies. By 2011, there were nine human antibodies approved by FDA for clinical uses. Two (adalimumab and belimumab) were generated from phage display platforms and seven (panitumumab, golimumob, canakinumab, ustekinumab, ofatumumab, denosumab, and ipilimumab) from transgenic platforms. The advantage of transgenic technology over phage display is clearly revealed by the numbers of approved therapeutic human antibodies generated from each technology. In particular, human antibodies generated from the transgenic technology have relatively higher phase II to III and Phase III approval transition rates than those from the phage display technology (25).

Although the utility of transgenic mice for human antibody development is already apparent, it is likely that we have only scratched the surface of its full potential. In our view, it is difficult for any in vitro technology to compete against the huge diversity that in vivo somatic mutation and selection provides. New technology development would further help us to explore this powerful in vivo system. Several new in vivo antibody formats including heavy-chain only and bispecifics are currently being explored. Advances in our understanding of what constitutes useful diversity should allow the generation of transgenic mice with increased levels of germ line diversity. With our ever increasing understanding of the basic mechanisms of immune regulation, we may be able to

tune up the immune response by improving the cellular signaling and somatic hypermutation machinery, thus optimizing affinity maturation. The availability of these next-generation humanized mice will undoubtedly result in an ever increasing number of fully human therapeutic antibodies developed for diseases of unmet medical need.

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