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Hybridoma technology a versatile method for isolation of monoclonal antibodies, its applicability across species, limitations, advancement and future perspectives

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ABSTRACT

The advancements in technology and manufacturing processes have allowed the development of new derivatives, biosimilar or advanced improved versions for approved antibodies each year for treatment regimen. There are more than 700 antibody-based molecules that are in different stages of phase I/II/ III clinical trials targeting new unique targets. To date, approximately more than 80 monoclonal antibodies (mAbs) have been approved. A total of 7 novel antibody therapeutics had been granted the first approval either in the United States or European Union in the year 2019, representing approximately 20% of the total number of approved drugs. Most of these licenced mAbs or their derivatives are either of hybridoma origin or their improvised engineered versions. Even with the recent development of high throughput mAb generation technologies, hybridoma is the most favoured method due to its indigenous nature to preserve natural cognate antibody pairing information and preserves innate functions of immune cells. The recent advent of antibody engineering technology has superseded the species level barriers and has shown success in isolation of hybridoma across phylogenetically distinct species. This has led to the isolation of monoclonal antibodies against human targets that are conserved and non-immunogenic in the rodent. In this review, we have discussed in detail about hybridoma technology, its expansion towards different animal species, the importance of antibodies isolated from different animal sources that are useful in biological applications, advantages, and limitations. This review also summarizes the challenges and recent progress associated with hybridoma development, and how it has been overcome in these years to provide new insights for the isolation of mAbs.

1. Introduction

Antibodies are the glycoproteins produced by the B-cells also known as immunoglobulins, which are present in higher eukaryotes. Immunoglobulins are present in either as a soluble form (blood or plasma) or as membrane-bound form (B cell receptors). Antibodies are the major component of the humoral immune system that provides protection against the invading pathogens i.e. viruses and bacteria [1].

An antibody is made up of two structural unit's i.e. heavy and light chain. Generally, each heavy chain has one variable and three constant regions whereas the light chain has one variable and one constant region. The variable region of antibodies is mainly responsible for its interactions with the invading pathogen and antigen recognition. The antigen-antibody recognition mechanism works like a lock and key fashion. Each antibody has a particular paratope (i.e. lock) that binds to a particular antigen (i.e. key). One type of B cell produces one type of antibody against a particular antigen. There are five different types of heavy chains based on the structure of crystallizable fragments (Fc) that is attached to the antigen-binding fragments. On the basis of different Fc region, antibodies are grouped into five different isotypes i.e; IgM, IgG, IgA, IgD, and IgE. Among all the isotypes IgG is the smallest and the most common isotype with the highest therapeutic potential. It makes 70–80% of the total antibodies. IgGs have a longer half-life and are permeable to extravascular spaces [2,3].

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Antibodies are potentially used for various applications as extraordinary tools in biomedical research for many years. High specificity and selective binding have expanded the scope of antibodies to various applications such as flow cytometry, magnetic cell sorting, immunoassays, therapeutic approaches etc. [4]. Antibodies have developed about 40 years ago and have expanded the scope of antibodies to various applications due to their specificity and selective binding ability. These antibodies are classified into two primary subtypes, monoclonal and polyclonal on the ~~means they are~~ basis of their origin from the lymphocytes [5,6]. Both polyclonal and mAbs have their advantages and limitations which make them equally suitable for different applications.

Polyclonal antibodies (pAbs) are a pool of immunoglobulin molecules that are secreted by different B cell lineages and react against multiple epitopes of a specific antigen. The pAbs are generated by injecting an immunogen into an animal using a prime-boost immunization strategy to produce high titres of antibodies against the particular antigen. After immunization, pAbs can be used directly or in the purified form (through affinity column chromatography to remove other serum protein components). Polyclonal sera display multiple epitope binding properties which make them an attractive reagent for various purposes, like its use as research or therapeutic reagent either directly or in purified form. The polyclonal serum is widely used for several decades for the treatment of toxin-mediated bacterial and viral diseases [7]. Emil Adolf von Behring was awarded Nobel Prize in Physiology and Medicine in 1901 for his work on serum therapy, especially its application against diphtheria, through which he opened doors for new ways of treatment in medical sciences [8,9]. Animal serum-derived therapy has been successfully applied for different medical aspects like overdosing of medication, viral disease (like rabies) and as antitoxins in snakebite envenoming [10]. The beneficial effects of pAbs come from its polyclonal nature and biophysical diversity. The poly clonality nature allows targeting multiple sites in a single window of the application and biophysical diversity provides greater stability in environmental changes [11,12]. Despite having beneficial effects of serum-derived pAbs therapy has several limitations, which need to be evaluated before introducing new interventions. As blood-derived products, intravenous polyclonal immunoglobulins (IVIG) have limited availability, batch-to-batch variability; carry the risk of blood-borne disease transmission and only a small fraction of antibodies from the pool of antibodies binds to the target of interest to exert the desired effect. This sometimes results in low specific activity and relatively needs high doses to observe a desired beneficial clinical effect [13,14]. The other limitation of polyclonal serum is that it cannot be used for the treatment of chronic diseases [15]. Due to some of these potential drawbacks of pAbs, the way for mAb isolation, need, and urgency comes into the light. However, mAbs are most suitable and frequently used because of their high sensitivity, specificity, affinity and homogenous nature [16].

Monoclonal antibodies are monospecific in nature and produced by identical B cells having high affinity and specificity towards a single epitope of an antigen. In 1975 hybridoma based technology was used to generate the mAbs showing very minimal and acceptable batch to batch variation, produced in an indefinite amount continuously. The mAbs can be produced against any given epitope present on an antigen or immunogen. Moreover, they can be used to detect, purify and characterize the substance of interest. Since the development of mAb, the scope of antibodies has expanded to various further applications due to their target specificity [17]. This has made mAbs a powerful tool in the fields of biochemistry, molecular biology, and medicine.

Antibody-based biologics are one of the best-selling classes of biomolecules in today's market. Advancement in mAb generation technologies in recent years has made ease in identification of new target antigens to be explored in diagnostic and therapeutic approaches [18,19]. Several mAb generation technologies had been developed over the years, to isolate mAbs from immune and non-immune sources using hybridoma, display methods, and more advanced novel mAb development technologies like single B cell amplification and culturing methods. Unlike the hybridoma method, other methods rely mostly on recombinant production of mAbs. Each of these technology platforms has their respective advantage, limitations, and applicability [18,20,21].

Hybridoma technology is the primitive, most fundamental and successful methodology in the field of mAb isolation [19]. This technology is quite robust and useful in discovering thousands of antibodies for different applications [22]. The basic practical advantage associated with hybridoma technology is, once the hybridoma clones are established, the production of mAb becomes simple and efficient. The antibodies isolated through hybridoma methodology preserves the native pairing of variable and constant regions gene combination, which further supports studies on both direct and indirect functions of a mAb. Hybridoma technology relies on B cells that are matured in secondary lymphatic organs in response to an antigen. These B cells undergo natural antibody maturation process where the variable region of antibodies diversified by accumulating somatic hypermutations which further results in the selection of high-affinity tight binders [18,20]. Resulting antibodies possess the natural pairing of variable heavy and light chain genes with naturally class-switched matured constant region gene through class switch recombination (CSR). Such freedom of natural CSR is not possible in other mAb isolation method that makes hybridoma a unique way to produce naturally matured in vivo antibodies in the laboratory [19].

Nevertheless, hybridoma technology is the most preferred technology for mAb discovery for in vivo applications. Presently there are more than 90% of the antibodies approved by the United States Food and Drug Administration (US FDA) are generated by traditional hybridoma technology and are used either directly or in chimeric or humanized versions [19,23,24]. A list of these FDA approved antibodies has been listed in Table 1. The dominance of this technology has been continued with the recent development of transgenic humanized animals that has opened new avenues for more effective generation of high-quality human antibodies in the modern biotherapeutic era.

In this review, we have discussed in detail about hybridoma technology, how it has been expanded to different animal species and their associated biological applications which determine their usage for certain intends. We have also discussed the advancement and challenges associated with hybridoma development, and how it has been overcome in years.

2. Hybridoma technology overview

Georges Kohler and Cesar Milstein in 1975 invented the hybridoma technology, for which they received the Nobel Prize in 1984 in physiology and medicine [25]. During the same period, Herve Bazin in Louvain-la-nerve Belgium created a rat myeloma cell line IR983, allowing the generation of first rat mAbs (https://www.synabs.be/2019/11/25/hybridoma-vs-display-the-fight-of-the-century/). Hybridoma cells are generated via fusion between a short-lived antibody-producing B cell and an immortal myeloma cell. Each hybridoma cell constitutively expresses a large amount of one purely specific mAb, and favoured hybridoma clones can be cryopreserved for continuous mAb production for a long period. Hybridoma generation process takes advantage of a host animal's natural ability to generate functional, highly specific and high-affinity mAbs [19]. To date, several mAbs are developed using this technology and are presently used for diagnosis, prevention, and treatment of different diseases Tables 1, 2 And 3.

Initially, hybridoma technology was limited to murine antigens but with advancement in this field, it is a well-established technology to develop mAbs against a vast range of antigens and from different species like rabbits [26], humans [27,28], chickens [29], goats, sheep [30], cows [31], mice [32], guinea pigs, and rats [33]. Choice of animal species especially for mAb isolation depends on several factors such as the presence of a homologous protein in the immunized species,

Table 1
A list of hybridoma derived mAbs or their engineered versions.

	Approved mAbs	Antibody isotype	Antibody origin	First FDA /EU Approval year	Target Antigen	Therapeutic application
1.	[fam-]trastuzumab deruxtecan (Enhertu)	IgG1	Humanized	2019	human epidermal growth factor receptor 2 (HER2)	HER2 positive metastatic breast cancer
2.	Enfortumab vedotin (Padcev)	IgG1	Human (Xenomice)	2019	Nectin-4	Urothelial cancer
3.	Crizanlizumab (Adakveo)	IgG2	Humanized	2019	P-selectin	vaso-occlusive crisis in patients with Sickle cell anemia
4.	Romosozumab (Evenity)	IgG2	Humanized	2019	Sclerostin	postmenopausal osteoporosis
5.	Polatuzumab vedotin (Polivy)	IgG1	Humanized	2019	CD79b (B cell)	large B-cell lymphoma
6.	Risankizumab (Skyrizi)	IgG1	Humanized	2019	interleukin 23A (IL-23A)	treatment of multiple inflammatory diseases like psoriasis
7.	Ibalizumab (Trogarzo)	IgG4	Humanized	2018	CD4	Treatment of Multidrug-Resistant HIV-1 Infection
8.	Ravulizumab (Ultomiris)	IgG2/IgG4	Humanized	2018	Complement component 5	Treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome.
9.	Fremanezumab (Ajovy)	IgG2	Humanized	2018	Calcitonin Gene-Related Peptide (CGRP)	preventive treatment of migraine in adults.
10.	Tildrakizumab (Ilumya)	IgG1	Humanized	2018	interleukin-23	treatment of immunologically mediated inflammatory disorders (plaque psoriasis.)
11.	Mogamulizumab (Poteligeo)	IgG1	Humanized	2018	chemokine receptor 4 (CCR4)	treatment of relapsed or refractory mycosis fungoides and Sézary disease
12.	Galcanezumab (Emgality)	IgG4	Humanized	2018	calcitonin gene-related peptide (CGRP)	prevention of migraine
13.	Gemtuzumab ozogamicin (Mylotarg)	IgG4	Humanized	2018	CD33	acute myeloid leukemia
14.	Benralizumab (Fasenra)	IgG1	Humanized	2017	alpha-chain of the interleukin-5 receptor (CD125)	treatment of asthma
15.	Ocrelizumab (Ocrevus)	IgG1	Humanized	2017	CD20 (B cells)	treatment for multiple sclerosis
16.	Emicizumab (Hemlibra)	IgG4	Humanized (bispecific)	2017	Activated factor IX, factor X	treatment of haemophilia A,
17.	Inotuzumab ozogamicin (Besponsa)	IgG4	Humanized	2017	CD22	treat relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL)
18.	Obiltoxaximab (Anthim)	IgG1	Chimeric	2016	protective antigen (PA)	treatment of exposure to Bacillus anthracis spores
19.	Atezolizumab (Tecentriq)	IgG1	Humanized	2016	programmed cell death-ligand 1 (PD-L1)	bladder cancer treatment
20.	Reslizumab (Cinqaero, Cinqair)	IgG4	Humanized	2016	interleukin-5 (IL-5)	asthma
21.	Ixekizumab (Taltz)	IgG4	Humanized	2016	IL-17a	Multiple myeloma
22.	Elotuzumab (Empliciti)	IgG1	Humanized	2016	SLAMF7 (CD319)	Multiple myeloma‘
23.	Mepolizumab (Nucala)	IgG1	Humanized	2015	IL-5	Severe eosinophilic asthma
24.	Dinutuximab (Unituxin)	IgG1	Chimeric	2015	glycolipid GD2	Neuroblastoma
25.	Alemtuzumab (Lemtrada; MabCampath, Campath-1H)	IgG1	Humanized	2014	CD52	lymphocytic leukemia (CLL) and multiple sclerosis
26.	Pembrolizumab (Keytruda)	IgG4	Humanized	2014	programmed cell death protein 1 (PD-1) receptor	treat melanoma, lung cancer, head and neck cancer, Hodgkin lymphoma, and stomach cancer
27.	Vedolizumab (Entyvio)	IgG1	Humanized	2014	α4β7 integrin	Ulcerative colitis, Crohn disease
28.	Siltuximab (Sylvant)	IgG1	Chimeric	2014	interleukin-6	treatment of idiopathic multicentric Castleman disease (iMCD)
29.	Obinutuzumab (Gazyva, Gazyvaro)	IgG1	Humanized	2013	CD-20	first-line treatment for chronic lymphocytic leukemia
30.	Ado-trastuzumab emtansine (Kadcyla)	IgG1	Humanized	2012	HER-2	Breast cancer
31.	Pertuzumab (Perjeta)	IgG1	Humanized	2012	HER-2	Breast cancer
32.	Brentuximab vedotin (Adcetris)	IgG1	Chimeric	2011	CD-30	Hodgkin lymphoma, systemic anaplastic large cell lymphoma, cutaneous T-cell lymphoma (CTCL)
33.	Tocilizumab (RoActemra, Actemra)	IgG1	Humanized	2010	interleukin-6 receptor (IL-6R)	treatment of rheumatoid arthritis (RA) and systemic juvenile idiopathic arthritis
34.	Eculizumab (Soliris)	IgG2/4	Humanized	2007	Complement protein C5	treatment of paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), and neuromyelitis optica
35.	Natalizumab (Tysabri)	IgG4	Humanized	2004	alpha-4 integrin	multiple sclerosis and Crohn's disease
36.	Bevacizumab (Avastin)	IgG1	Humanized	2004	vascular endothelial growth factor A (VEGF-A)	colon cancer, lung cancer, glioblastoma, renal-cell carcinoma and age-related macular degeneration
37.	Cetuximab (Erbitux)	IgG1	Chimeric	2004	epidermal growth factor receptor (EGFR)	treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer
38.	Omalizumab (Xolair)	IgG1	Humanized	2003	immunoglobulin E (IgE)	allergic asthma
39.	Efalizumab (Raptiva, Genentech, Merck Serono)	IgG1	Humanized	2003	CD11a	treat paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), and neuromyelitis optica.

(*continued on next page*)

Table 1 (*continued*)

	Approved mAbs	Antibody isotype	Antibody origin	First FDA /EU Approval year	Target Antigen	Therapeutic application
40.	Trastuzumab (Herceptin)	IgG1	Humanized	1998	HER2 receptor	Breast cancer
41.	Infliximab (Remicade)	IgG1	Chimeric	1998	TNF-α	Crohn's disease, ulcerative colitis, rheumatoid arthritis, ankylosing spondylitis, psoriasis, psoriatic arthritis, and Behçet's disease.
42.	Palivizumab (Synagis)	IgG1	Humanized	1998	A antigenic site of the F protein of RSV	respiratory syncytial virus (RSV) infections
43.	Basiliximab (Simulect)	IgG1	Humanized	1998	α chain (CD25) of the IL-2 receptor	prevent rejection in organ transplantation, especially in kidney transplants
44.	Rituximab (MabThera, Rituxan)	IgG1	Chimeric	1997	CD20	non-Hodgkin's lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis, granulomatosis with polyangiitis, idiopathic thrombocytopenic purpura, pemphigus vulgaris, myasthenia gravis and Epstein-Barr virus-positive mucocutaneous ulcers
45.	Daclizumab (Zinbryta; Zenapax)	IgG1	Humanized	1997 2016≠	α chain (CD25) of the IL-2 receptor	prevent acute rejection of kidney transplant ≠multiple sclerosis (MS)

Table 2
List of FDA approved diagnostic and therapeutic mouse monoclonal antibodies.

	Trade Name	Target	Isotype	Company	FDA / EU EMA Approval year	Application	Target
	Besilesomab (Scintimun)	NCA-95	Murine IgG1	CIS Bio	2010	Diagnostics	Inflammatory lesions and metastases
	NeutroSpec® (Fanolesomab)	CD15	Mouse labelled with radioisotope, technetium-99m (99mTc)	Palatin	2004	Diagnostics	appendicitis
3.	Ibritumomab tiuxetan (Zevalin)	CD20	Murine IgG1	Biogen Idec	2002	Therapeutics	Treatment for relapsed or refractory, low grade or transformed B cell non-Hodgkin's lymphoma,
4.	Tositumomab and iodine 131 tositumomab (Bexxar)	CD20	Murine IgG2a	Corixa and GSK	2003	Therapeutics	Non-Hodgkin lymphoma
5.	Arcitumomab (CEA-scan)	carcinoembryonic antigen	Fab' fragment of a murine monoclonal antibody	Immunomedics	1996	Diagnostics	Imaging of colorectal cancers
6.	Capromab (ProstaScint)	Prostate Specific Membrane Antigen (PSMA)	Murine IgG1κ	Cytogen	1996	Diagnostics	Prostate adenocarcinoma
7.	Nofetumomab (Verluma)	Carcinoma associated antigen	Fab fragment of murine IgG2b	Boehringer Ingelheim, NeoRx	1996	Diagnostics	Small cell lung cancer
8.	Satumomab (OncoScint)	tumor-associated glycoprotein (TAG-72)	Murine IgG 1	Cytogen	1992	Diagnostics	Colorectal or ovarian cancer
9.	Muromonab-CD3 (Orthoclone OKT3)	CD3	Murine IgG2a	Centocor Ortho Biotech (Johnson & Johnson)	1986	Therapeutics	Reduce acute rejection in patients with organ transplants

Table 3
A list of FDA approved rabbit mAbs that are used in diagnostics.

	Device Name	FDA Approval Year	Company	Target	Diagnosis
1.	FLEX Monoclonal Rabbit Anti-Human Estrogen Receptor A, Clone EPI, RTU (Dako Onmis)	2017	DAKO DENMARK	Anti-Human Estrogen Receptor α	human breast carcinomas
2.	VENTANA PD-L1 (SP263) ASSAY	2017	Ventana Medical Systems, Inc.	anti- Programmed Death-Ligand 1 (PD-L1)	in the assessment of the PD-L1 protein urothelial carcinoma tissue
3.	VENTANA PD-L1 (SP142) Assay	2016	Ventana Medical Systems, Inc.	anti- Programmed Death-Ligand 1 (PD-L1)	in the assessment of the PD-L1 protein urothelial carcinoma and non-small cell lung cancer (NSCLC) tissue
4.	VENTANA PD-L1(SP142) CDX ASSAY	2016	Ventana Medical Systems, Inc.	anti- Programmed Death-Ligand 1 (PD-L1)	in the assessment of the PD-L1 protein urothelial carcinoma tissue
5.	PD-L1 IHC 28-8 PharmDx	2016	Dakoako North America Inc.	anti- Programmed Death-Ligand 1 (PD-L1)	in the assessment of the PD-L1 protein non- squamous non-small cell lung cancer (NSCLC) tissue and melanoma tissue
6.	MONOCLONAL RABBIT ANTI-HUMAN ESTROGEN RECEPTOR (ER) A, CLONE EPI	2013	Dako North America Inc.	Human Estrogen Receptor α	human breast carcinomas
7.	CONFIRM ANTI-ESTROGEN RECEPTOR (SP1) RABBIT MONOCLONAL PRIMARY ANTIBODY	2012	Ventana Medical Systems, Inc	Anti-Human Estrogen Receptor α	human breast carcinomas
8.	CONFIRM ANTI-PROGESTORONE RECEPTOR (1E2) RABBIT MONOCLONAL PRIMARY ANTIBODY	2011	Ventana Medical Systems, Inc	Anti-Progesterone receptor	prognosis, and prediction of hormone therapy for breast carcinoma
9.	VENTANA ANTI-HELICOBACTER PYLORI (SP48) RABBIT MONOCLONAL PRIMARY ANTIBODY	2011	Ventana Medical Systems, Inc	anti-Helicobacter pyloni	in vitro diagnostic against H-. pylori organisms
10.	MONOCLONAL RABBIT ANTI HUMAN ESTROGEN RECEPTOR ALPHA ANTIBODY CLONE SP1, MODEL M3634	2009	Dako North America Inc.	Anti-Human Estrogen Receptor	Identification of estrogen receptor (ER) expression in normal and neoplastic tissues
11.	VENTANA MEDICAL SYSTEMS PATHWAY ANTI-C-KIT PRIMARY ANTIBODY	2004	Ventana Medical Systems, Inc	Anti-c-KIT	Gastrointestinal stromal tumors
12.	PATHWAY ANTI-HCR-2/NCU RABBIT MONOCLONAL PRIMARY ANTIBODY	2000	Ventana Medical Systems, Inc	ANTI-HCR-2/NCU	Assessment of Breast cancer patients for whom HERCEPTIN(R) treatment is being considered

availability of suitable fusion partner, the amount of protein or antigen available for immunization, the time required to obtain an antibody response and finally, a purpose for which these mAbs are needed. Most commonly used hosts for mAb production are the mice followed by rabbits. The inbred BALB/c strain of mice is usually the right choice and preferably suited for mAb isolation [34]. Chickens are also considered as a preferred host of choice due to its distinct phylogenetic relationship between the antigen donor and the antibody producer [16,35]. Hybridoma technology has not gained much attention because its success mainly depends on the availability of a suitable fusion partner. In the initial years of hybridoma discovery, technology was limited to mice but in progressive years researchers used this platform for human and rabbit hybridoma development. However major limitation associated with the production of mAbs from other species is the instability of hybridoma clones produced with fusion partners from heterologous-species [18]. This instability resulted in the hybridoma clones are due to the fusion of two cells from different species which leads to chromosomal instability. To overcome this, in the past few years many different strategies have been used to increase the fusion efficiency.

Generally, there are two types of hybridomas one is homo-hybridomas and second is hetero-hybridomas. In homo-hybridomas both, the IgG secreting B cells and fusion partners are from the same species. In hetero-hybridomas the antibody-secreting B cells and fusion partners are from two different species. Homo hybridomas are genetically more stable and secrete stable IgG as compared to hetero-hybridomas as it gradually lost the chromosomal recombinants during the clonal selection step due to their genetic instability.

3. Animal species used for hybridoma development over the years:

3.1. Mouse

Mouse polyclonal and mAbs held the largest market in 2019 as they are more specific and easier to produce in nature. The structural similarities between human and mice antibodies are the prime reason for their high acceptability rate. Upgradation and simplicity of mice hybridoma process have made it a more prime reason for their high adoption rate in research and therapeutics [36].

The mice hybridoma technology is a multi-step process that takes advantage of a host animal's natural ability to produce highly specific, high-affinity and fully functional mAbs. It involves the development and optimization of specific immunogenic antigen (Ag). Following the optimization, a host animal is immunized with the Ag along with adjuvant for several weeks. The sera from immunized animals are tested for their reactivity and specificity to the immunizing antigen while the animals with high titres of binding antibodies are selected further for splenocytes isolation [32]. The spleen cells are fused with the immortalised myeloma cells in the presence of fusogenic agents like viruses, chemicals and electric pulses. The most common myeloma fusion cell lines are X63-Ag 8.6539 [37] and Sp2/0-Ag 1410 [38], with the origin from BALB/c mouse. The fused cells are then selected on hypoxanthine-aminopterin-thymidine (HAT) medium. The myeloma cells are sensitive to HAT medium as they lack hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene required for nucleotide synthesis by the de novo or salvage pathways while the unfused B cells die as of short life span. In this process, only the hybrid (B cell-myeloma) survives, as they harbour the functional HGPRT gene from the B cells. However, hybrid cells retain the dual properties, antibody secreting property of B cells and continuously growing property (immortality) from myeloma cells. Fused or hybrid cells are then screened by “limited dilution cloning” method or with semi solid selective medium to select only those hybridoma that produce antibodies of appropriate specificity. A detailed schematic representation of steps involved in hybridoma production is shown in Fig. 1.

Production of antibodies by mouse hybridoma technology is quite robust and has been useful to discover thousands of antibodies for different applications. Academic and industrial research groups with expertise in the field of antibody isolation, hybridoma methodology continues being the methodology of the first choice, particularly if the goal is to obtain antibodies for analytical purposes. The main associated advantage is that, once the hybridoma clone is isolated, mAb production in mouse ascites is simple, efficient and reproducible. These hybridomas can be stored in liquid nitrogen for several years making them virtually immortal [39].

The mouse mAbs can be potentially used for diagnostic, therapeutic as well as for other research applications. The first therapeutic antibody that was approved by the Food and Drug Administration (FDA) in 1985, developed by murine hybridoma technology was potentially used to reduce the graft rejection in transplant patients [18]. OKT3, the first mAb to be used in organ transplantation and during the past one decade there has been an extensive experience of its use both for prevention and treatment of organ transplantation rejection. OKT3 blocks T cell function by modulating CD3 and the T cell receptor from the T cell surface, functions as an immunosuppressant [40]. Since its discovery, OKT3 was further improvised in progressive years from chimeric to humanized version, to further reduce adverse effects and to increase immunologic efficiency [41]. The use of murine derived mAbs has less therapeutic value as they are entirely from mouse origin and can cause immunogenic reactions in the target host. Such limitations have been addressed by chimerization and humanization of antibodies, potentially removing the mouse immunogenic content [42]. Several murine mAbs has been approved by the FDA for use in diagnostic and therapeutic purposes over the years as listed in Table 2.

3.2. Rabbit

Since the discovery of mouse mAbs, rabbit hybridoma has been potentially used as a dominant tool in the field of research, diagnostics and therapeutics from several decades [4]. However, a new technology for generating mAbs with improved affinity, specificity and having the ability to recognize non-immunogenic rodent’s epitopes, are in need as an alternative for the scientific community.

The rabbit immune system has been documented as a vehicle for developing antibodies with higher affinity and more diverse recognition of many molecules including phospho-peptides, carbohydrates and immunogens that are not otherwise immunogenic in mouse [24]. Antibodies produced in rabbits usually have about 10 to 100 fold greater affinity than those produced by mice. Rabbits generate more diverse and complex immune response towards target antigen as compared to human and mice because of gene conversion and somatic hypermutations phenomenon leads towards more mutations in rabbit antibody repertoire [43,44]. The gene conversion is responsible for introducing mutations and affinity maturation of variable antibody fragments which takes place in double-stranded rearranged V(D)J DNA segment of antibody gene via homologous recombination [19,45]. The rabbit IgGs are somewhat simpler than the mouse and human antibodies. Rabbit IgG has only one subclass i.e. Cγ gene and the majority (90–95%) of light chains are derived from isotype Cκ1. Only 5% to 10% of the total IgG light chains are isotype l. Fig. 2. Several efforts were made to generate rabbit mAbs after the development of mouse hybridoma technology in the 1970s. Due to the favourable properties of rabbit antibodies, many scientific groups tried to develop methods for the generation of rabbit hybridomas. This endeavour was significantly complicated by the absence of rabbit myeloma cell lines. Viral transformation of rabbit B cells to generate myeloma-like cell lines also proved to be difficult and rather inefficient [46]. For these reasons, substantial efforts are focused on generating rabbit–mouse hetero-hybridomas. Unfortunately, all hetero-hybridomas generated in the early days of hybridoma technology revealed poor fusion efficiency, genetic instability and impaired functional rabbit heavy- and light-chain pairings. In 1988, Raybould et al. generated the first stable rabbit–mouse

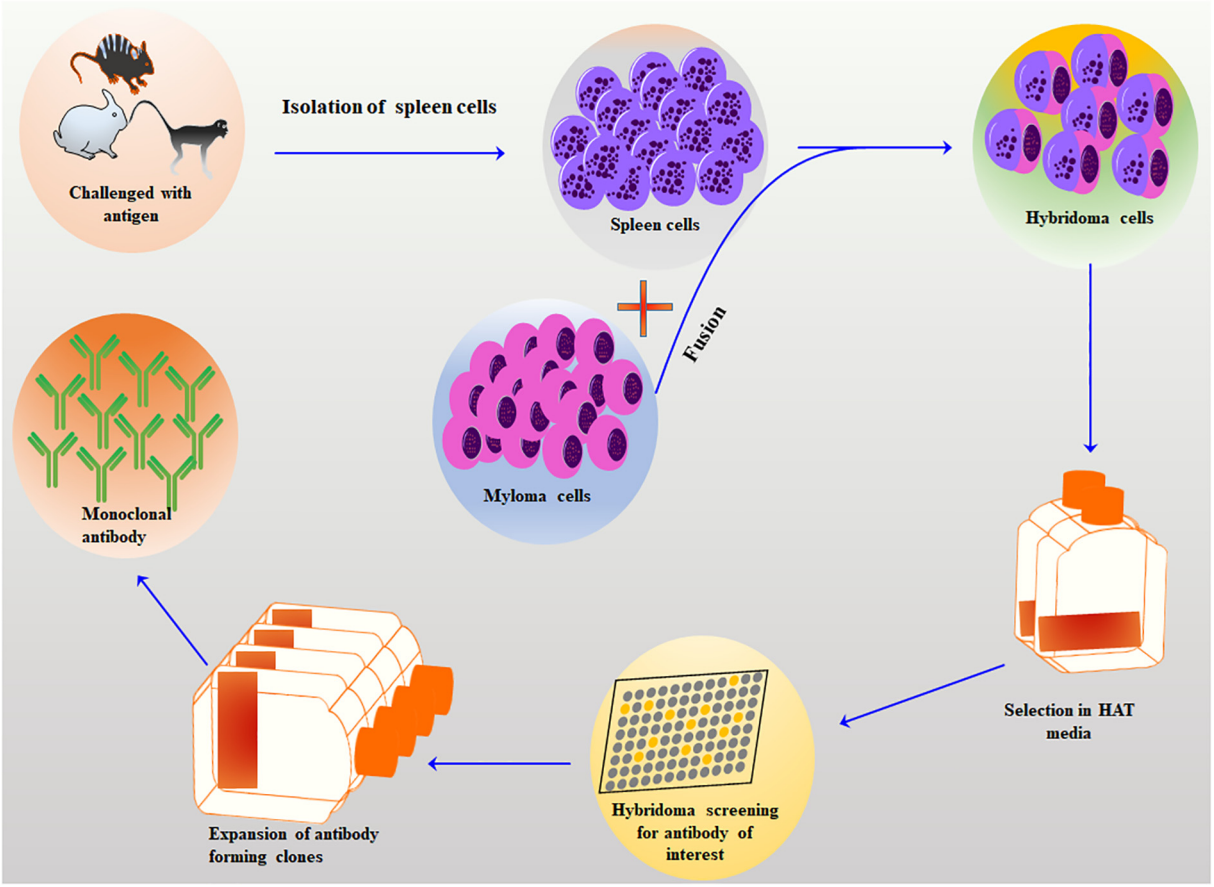

Fig. 1. Hybridoma technology used to produce mAbs: Generation of mAb by immunizing laboratory animals with any target antigen. Hybridoma cells generated by the fusion between B-cells from an immunized animal (generally a rat, mouse, rabbit or monkey) and the myeloma cells. Hybrid cells are selected in HAT media and finally, cells secreting desired antibodies are screened.

hetero-hybridoma by polyethylene glycol-mediated fusion of rabbit spleen B cells with the mouse myeloma cell line SP2/0-Ag14. Even though they observed stable rabbit IgG expression for several months, other groups observed genetic instability and concomitant decrease of mAb secretion [47]. These shortcomings could be partially addressed by extensive efforts to regularly subclone the rabbit–mouse hetero-hybridoma

In 1996, Weimin Zhu and Robert Pytela, at the University of California, developed an improved rabbit hybridoma fusion partner by repeatedly subcloning. After multiple rounds of subcloning, they selected high fusion efficiency clones based on characteristics like robust growth, morphological characteristics and named it as a new cell line 240E-W, with better fusion efficiency and stability. Since then this cell line 240E-W has been further developed and optimized to eliminate endogenous IgG and has been used for the production of rabbit mAbs for research and commercial applications [48]. The cell line 240E-W was further modified to a superior version 240E-W2. Abcam patented this technology to develop highly specific mAbs, under the name of RanMAb, which has been potentially explored for the production of diagnostic and research antibodies.

A large number of rabbit mAbs are used in basic laboratory research. Rabbit mAbs are preferred over the mouse and human mAbs in diagnostics and pharmaceuticals applications because of their high specificity and affinity. Various rabbit mAbs have been approved by the FDA to use as in vitro tumour diagnostic tool [49]. A list of FDA approved rabbit mAbs for diagnostics are listed in Table 3. Recently, Wei et al. developed an ultrasensitive test for Ebola virus diagnostics using carbon nanoparticle-labelled pad with rabbit anti-Ebola virus (EBOV)-VP40 IgG for rapid detection lateral flow test strip for Ebola virus [50].

Limited success has been gained in the development of rabbit mAbs as therapeutic agents but the potential use of rabbit pAbs for the prophylaxis and treatment of acute rejection against T cells in organ transplant opened venture to explore the use of humanized rabbit mAbs as a therapeutic agent [46]. A company name Epitomics has developed a humanized rabbit monoclonal drug candidate and demonstrated in vitro and in vivo efficacies [51]. Recent FDA approval of humanized rabbit monoclonal single-chain antibody fragment, Brolucizumab in 2019 for the treatment of wet age-related macular degeneration (AMD) has increased hope and venture of other humanized rabbit monoclonal for therapeutics in near future [52]. A detailed description of these antibodies has been reviewed by Mage et al. 2018 [53].

3.3. Human

Human hybridoma technology which allows the direct generation of human antibodies in a native form, is the most direct effective approach for the production of natural therapeutic and diagnostic antibodies with no additional modifications require [54]. It is believed to be the most promising and convenient technological platform for the isolation of therapeutic mAbs. However, success of human hybridoma technology for the therapeutic purposes has been limited since years due to several technical challenges like unavailability of human fusion partners, as most of the fusion partners available are from rodent origin or hetero-myelomas. A fusion of human B cells with different fusion partners limits the use of these mAbs for therapeutic applications. Several hetero-myelomas fusion has been successfully employed for the generation of mAbs of human origin for different diseases like HIV [28,55], Chikungunya [56,57], Dengue [58] etc. However, such hybridomas are

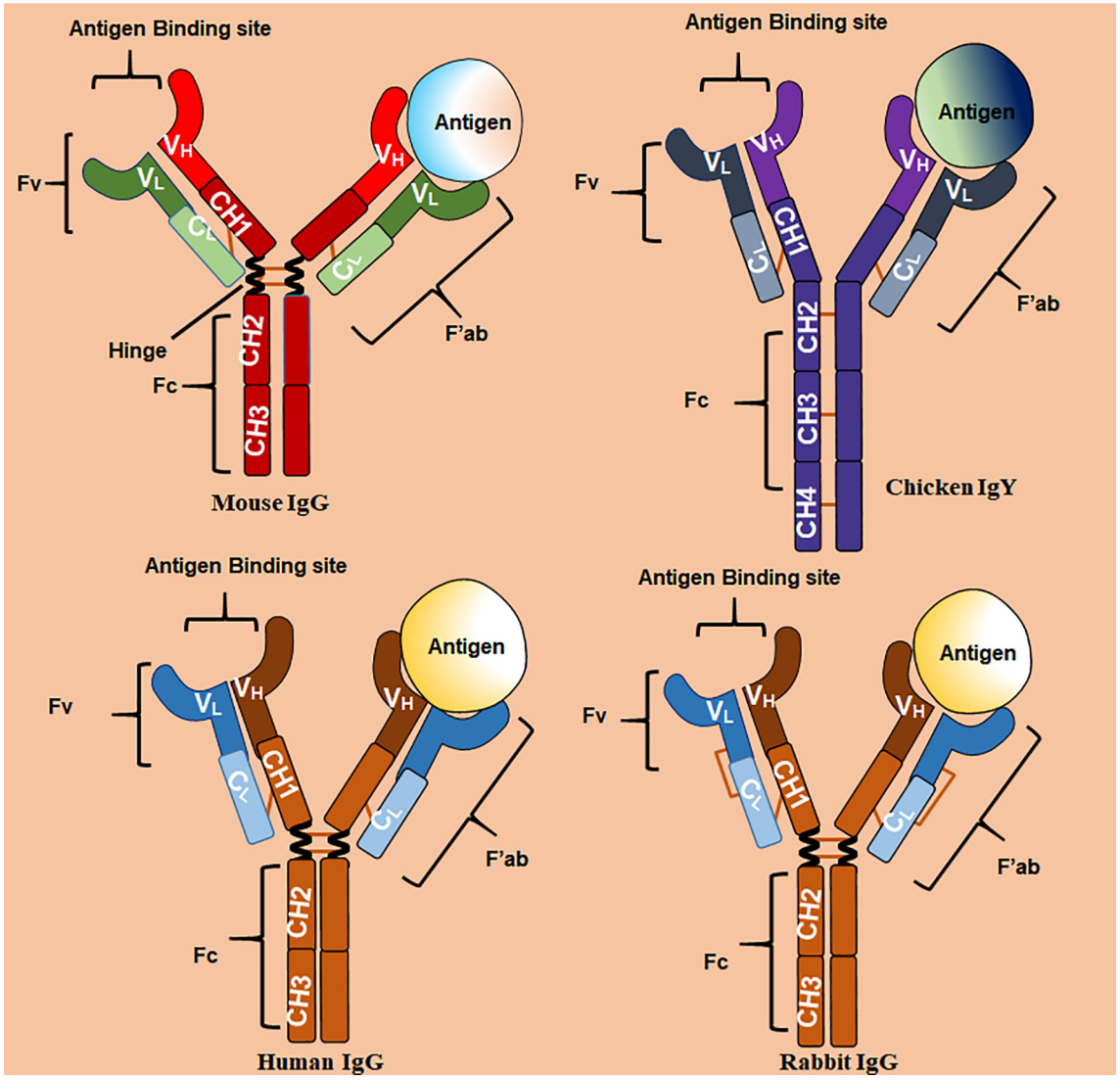

Fig. 2. Schematic drawing of natural rabbit, mouse, chicken and human IgG. Generally 150-KDa IgG comprises of two identical κ or λ light chains paired with two identical heavy chains. The light chain consists of an N-terminal variable domain (VL), followed by one constant domain (CL). The heavy chain consists of an N-terminal variable domain (VH), followed by three constant domains (CH1, CH2 and CH3) generally, however, the heavy chain of avian IgY contains four constant regions (CH1, CH2, CH3 and CH4). Schematic drawing of natural rabbit antibodies in IgG format. The ~150-kDa rabbit IgG molecule contains two identical κ (white) or λ (light grey) light chains paired with two identical heavy chains (dark grey). The light chain consists of an N-terminal variable domain (VL), shown with its three CDRs, followed by one constant domain (CL). The heavy chain consists of an N-terminal variable domain (VH), also shown with its three CDRs, followed by three constant domains (CH1, CH2 and CH3). CH1 and CH2 are linked through a flexible hinge region that has the amino-acid sequence APSTCSKPTCP (or APSTCSKPMCP in an allotypic variant) and anchors three disulphide bridges (orange) of the IgG molecule, one for each of the two light- and heavy-chain pairs, and one for the heavy-chain pair. Notably, rabbits have two κ light chains, K1 and K2. The more frequent κ light chain, K1, contains an additional disulfide bridge that links VL and CL. Rabbits of the commonly used New Zealand White strain have ~90% IgG-κ (K1), ~10% IgG-κ (K2) and < 1% IgG-λ antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unstable, leads to a loss in the ability of antibody secretion hence there is a challenge in achieving desired pharmacokinetic characteristics of natural human antibodies in terms of distribution, metabolism and excretion [54]. Several scientific groups have attempted to develop natural human fusion partner cell lines but limited success stories are reported using these fully human fusion partners. One such example of fusion cell is Human Karyochi cells which were successfully used to develop complete human stable hybridomas with IgG secreting properties for several months. The fusion efficiency of these Human Karyochi cells was in the range of 10^{-5} to 10^{-3} with no reports on the endogenous generation of immunoglobulin or chains that can interfere with subsequent synthesis, assembly and purification of mAbs.

The other major limiting step in the development of human hybridomas is low fusion efficiency of human B cells with the myeloma partner (0.001%) and secondly, the low percentage of circulating antigen-specific B cells in the peripheral blood (0.01%) also limits the use of this technology [59]. Frequency of antigen-specific B cells is very rare in circulation therefore, selection of a proper blood donor is critical for the success of this method. Acutely infected patients have a higher number of circulating B cells as compared to the convalescent patients. Donors showing a high titre of serum binding/neutralizing antibodies may have a higher frequency of peripheral B cells, and an indication of the greater chance of successful production of hybridomas. To overcome these challenges different groups have tried EBV(Epstein-Barr Virus) transformation approach to enrich the population of B cells [60]. The most commonly used cell line B95-8 is a continuous cell line releases high titres of transforming EBV in supernatants [61]. The B95-8 cell line was initiated by exposing marmoset blood leukocytes to EBV

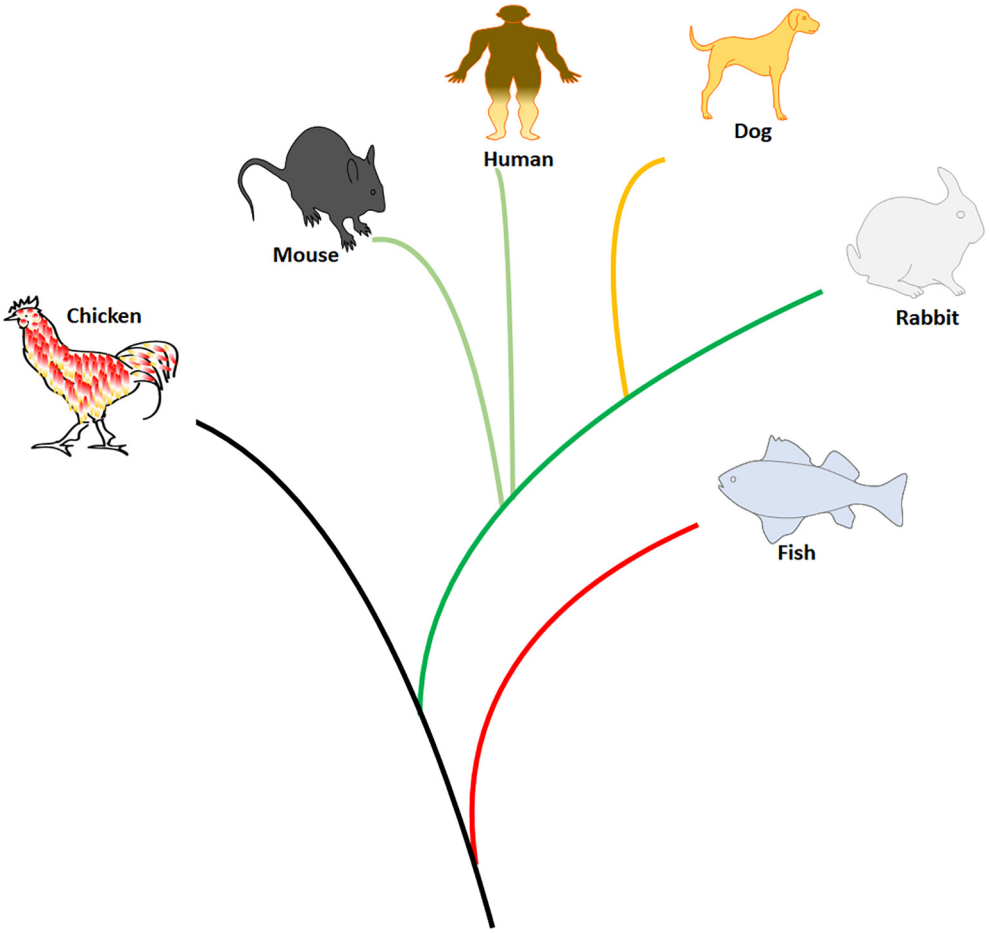

Fig. 3. Schematic representation of the phylogenetic lineage among different species.

extracted from a human leukocyte cell line. B95-8 provides a source for EBV to establish continuous lymphocytic cell lines from human donors. EBV mainly binds with the cells in peripheral blood that contains CD21 receptor and B cells in peripheral blood express these antigens on their surface and activates latent membrane protein (LMP) 2A and LMP1 [62,63]. Due to low antibody-secreting and chromosomal instability characteristics transformed B cells cannot be cultured for a long period. The transformation efficiency of EBV to B cells is low, ranges from 0.1 to 1%, however, the transformation efficiency of B cells in presence of EBV can be increased by the addition of CpG, which acts as toll-like receptor (TLR) 9 agonist [27]. CpG oligo-deoxynucleotides (or CpG ODN) are defined as short single-stranded synthetic DNA molecules where C refers for a cytosine triphosphate deoxynucleotide ("C") followed by a guanine triphosphate deoxynucleotide ("G"). The "p" denotes to the phosphodiester bond between consecutive nucleotides [64]. In unmethylated form, these CpG motifs act as immune stimulants and are recognized by the pattern recognition receptor (PRR) Toll-Like Receptor 9 (TLR9), which is constitutively expressed on immune system cells like B cells. These B cells undergo polyclonal response to CpG DNA stimulation by proliferation and differentiation to antibody-producing cells [65]. These transformed B cells are grown for a specific time for the emergence of immortalized cells, which further fused with the fusion partner to establish hybridomas. This CpG activation method has been successfully used to produce mAbs against severe acute respiratory syndrome coronavirus (SARS-CoV) [66] and HIV [27]. The main concern of developing hybridoma for therapeutic purpose is that the final hybrid cells should be free of EBV and other human viruses [67].

The two most common cell lines used for fusion are SHM-D3327 and HMMA 2.5. The SHM-D33; produced by fusing the human myeloma cell line FU-266, clone E-1 (HAT sensitive, 8-azaguanine resistant and resistant to G-418 - an antibiotic similar to gentamicin) with the murine myeloma P3X63Ag8.653 [68]. This cell line has been used as a fusion partner to stabilize the lymphoblastoid cell lines (LCL's) secreting immunoglobulins to produce mAbs against envelope proteins of HIV-1 and parvovirus B19 [55,69]. HMMA 2.5 is a human × mouse cell line that was generated by fusing mouse myeloma cell line P3x6Ag8.653 with bone marrow mononuclear cells of a patient with IgA myeloma [70]. Several mAbs have been produced using this cell line as a myeloma fusion partner [71].

The other limiting steps in human hybridoma development are fusion efficiency. Fusion is mainly performed by three methods (i) chemical agents like polyethylene glycol (PEG) (ii) Viral agent mediated methods and (iii) electric fusion methods.

PEG mediated fusion is the most common and traditional method of fusion due to its simplicity and most commonly convenient fusing agent of choice for hybridoma production. PEG fuses the plasma membranes of two adjacent mammalian cells by dehydrating the lipid head groups, leading to the asymmetry of the membrane bilayer, favouring fusion of two cells leading to a single cell with two or more nuclei. One major drawback of PEG mediated fusion is a generation of non-specific fusion between different kinds of cells [72]. Over the year's viral agents have also been used as a successful agent to perform fusion among two different cells. Sendai and vesicular stomatitis virus are the two most commonly viruses used as fusion partners. The most efficient and innovative method for cell fusion is electrical cytofusion. This mainly works on the principle of fusion of cells in the presence of high-intensity electric field pulse that causes transient membrane permeabilization. This method has higher fusion efficiency over chemical or viral-based fusion methods. However, electrofusion yields in low fusion efficiency

when fusion partner cells are different in size [73]. HMMA 2.5 myeloma cell line is found to be the most preferred cell line for electrofusion showing maximum fusion efficiency as compared to other myeloma cells [71]. Rems et al., developed a numerical calculation method to fuse cells with shorter, nanosecond (ns) pulses. The performance of this method works on the principle of contact areas between two fusion cells, regardless of their cell size [73]. Use of cephalin as fugenic reagent along with emetine and actinomycin D, Golestani as selection method dramatically increased the fusion efficiency and recovery (19–34%) [74].

The major advantage of human hybridoma technology is that antibodies produced by this technology are derived from human origin have more therapeutic applications than rodent derived counterparts due to attributable differences between the human and rodent immune systems.

3.4. Chicken

Due to evolutionary differences between mammalian (e.g. Human, mice) and avian species (chickens), the avian/chicken immune system recognizes more epitopes on mammalian proteins as foreign and generates a more vigorous and diverse immune antibody repertoire [35]. Phytogenic differences among different species have been illustrated in Fig. 3. As the phylogenetic difference between the immunizing antigen and the immunized animal increases, the immune response increases accordingly. It is, therefore, possible to produce antibodies in chicken that are difficult or impossible to produce in mammals such as G-protein-coupled receptors (GPCRs) against which producing antibodies in rodents is a challenge as of high sequence conservation (> 70%) at the protein level.

Chickens are emerging as valuable immunization hosts specifically for therapeutic antibody discovery for difficult targets having sequence conservation in mammals. The use of chicken egg yolk for antibody production represents a reduction in animal use (ethical issues) as chicken produces a larger amount of antibodies than laboratory rodents [75]. The advantages of chicken (Gallus domesticus) antibodies as diagnostic and therapeutic biomolecules are less characterized than their mammalian counterparts [16]. Initially, in 1989 a successful attempt was made to generate chicken hybridomas against Newcastle disease virus (NDV) by fusing the peripheral blood lymphocytes (PBL) and thymidine-kinase deficient (TK-) chicken myeloma cells [76]. Surprisingly, the secreted antibody hybridomas were initially obtained, but they lose the ability to produce antibody in the culture immediately [77]. To overcome this issue Nishinaka S et al., in 1991 developed a new improved fusion cell line R27H4, for the production of chicken mAbs [29]. The new cell line was efficient in the development of antibody-producing hybridomas with highly reactive IgG secretion ability of 6 months. These cell lines were further improved and several chicken hybridomas were successfully developed [29,78]. However, chicken hybridoma technology has been explored in limits and most of the investigators prefer to use phage display method over hybridoma for the development of chicken mAbs [79].

The main avian antibody isotype IgY shares structural and functional homology to mammalian IgG and IgE isotype counterparts but the difference in a constant region of antibodies, IgY has 4 constant regions whereas IgG has 3 constant regions [80–82] Fig. 2. IgY present in chicken sera gets passed to the embryo through the egg yolk [83]. Egg IgY antibodies have been used previously against bacterial and viral infections [84,85]. Humanization of these antibodies can have great potential for biopharmaceutical development [86,87]. The recent development of transgenic chicken with human immunoglobulin loci has expedited the use of transgenic chicken derived mAbs directly for human therapeutic use [88,89]. These engineered transgenic chickens express antibodies from immunoglobulin heavy and light chain loci containing human variable regions and exhibit normal B cell development raising immune responses to conserved human targets that are non-immunogenic in mice [90]. These transgenic chickens can be potentially used for the development of hybridoma secreting antibodies of human origin. However, like others, the unavailability of robust hybridoma fusion partner limits its potential utility [88]. In recent years different research groups have extensively explored the display method to overcome the limitations of chicken hybridoma.

4. Challenges and advancement in hybridoma technology over the years

Antibodies isolated through hybridoma methodologies have the advantage of being used directly and could be cryopreserved for future uses till an indefinite time, as the fusion partners are myelomas possessing remodelled transcriptional machinery to secrete a continuously large amount of antibodies [91]. Advancement in recombinant technology has overcome the challenges by cloning of variable heavy (VH) and variable light (VL) from unstable hybridoma; cloning in transient transfection vectors to produce antibodies in mammalian expression system [92]. Currently the development of stable cell lines has advanced the antibody production system by developing stable cell lines from unstable hybridomas to produce consistent antibody production. Chinese hamster ovary cell line (CHO) is the most preferred cells used for large scale production of mAbs. However, the development of stable cell lines is a tedious process that sometimes takes a month to year time where the success of this process depends on random genome integration of transgenes [93]. Development of CRISPR-Cas9 in recent years has overcome by immunogenomics reprogramming using plug and play technology, where CRISPR-Cas9 was used to engineer immunogenomics by homology-directed repair to replace endogenous immunoglobulin region by exogenous donor counterpart with the help of guide RNA. This technology platform has enabled the rapid generation of full-length antibody-secreting cell lines [94].

A major limitation of hybridoma technology is the lack of suitable fusion partners which limits the use of this technology and limits its applicability to other species. To overcome the problem of suitable fusion partners, the transgenic mice model *H-2K*^{b}-tsA58 has been developed. *H-2K*^{b}-tsA58 transgenic mice express the simian virus 40 (SV40) antigens (TAg) under the control of mouse major histocompatibility complex H-2k^{b} promotor. This promotor allows differential expression of SV40 antigen in different tissues at various levels. Expression of this antigen can be increased by simply increasing the levels of interferons (IFNs) [95]. The higher expression level of this antigen is responsible for tumorigenesis and aberrant development. In this approach transgenic mice *H-2K*^{b}-tsA58 were specifically used to isolate monoclonal against the filamentous phage. Transgenic mice were immunized with filamentous phage suspension. Splenocytes were recovered from the immunized animals and spleen cells were limited diluted from 6, 24-, 96- well plates. Cells from the positive wells were selected to develop monoclonal lines. The main advantage of this technology is that it eliminates the use of fusion partners and can parallelly be used for the development of monoclonal and polyclonal based therapies [96].

The other major challenge associated with hybridoma development is the requirement of purified antigen to generate a specific immune response. In some of the cases, it's a challenging task to purify the antigen. The lack of specific immune reagents for characterization and monitoring of these numerous proteins limits the overall time process for the production of hybridoma. Expression and purification of recombinant protein are time-consuming and sometimes not cost-effective. Additionally, immunization of animals with these purified recombinant proteins in formulation with adjuvants sometimes leads to alteration of native conformation of these proteins which finally leads to an undesired immune response in animals. In recent years, different approaches have been successfully implemented to overcome these challenges; A novel strategy has been developed to isolate mAbs against the native proteins [55]. In this strategy, animals were directly

immunized with transiently transfected HEK cells that express desire protein on the surface of these cells in a proper folded and glycosylated form. This modified technology is mainly useful for the isolation of mAbs against native antigens. The advantage of this technology is that expressed protein on transfected cells closely mimics as it expresses in naturally infected cells. A similar type of strategy was developed by Hazen M et al; where they had attempted to isolate monoclonal against the native conformation of extracellular loops & multi transmembrane proteins using DNA based immunization strategy in combination with immunomodulatory agents [97]. Use of immunomodulators in DNA immunization has positively enhanced the immune response and proved to be a successful approach to isolate desired specificity binding mAbs to native conformations. Kato et al., in 2019 developed a Cell-Based Immunization and Screening (CBIS) method for isolation of mAbs for podoplanin (PDPN) [98]. In this strategy, they immunized the mice with transfected cells overexpressing PDPN and screened the fused hybridoma cells using flow cytometry. These cell-based immunization methods are useful in the isolation of mAbs against various membrane proteins which is still difficult to achieve otherwise [56,57].

The other challenge in hybridoma technology is animal immunization. The overall efficiency of hybridoma depends upon the efficiency of immunization. Some factors that affect the efficiency of immunization are; route of administration of antigen, dose, choice of adjuvant, number of boosts and immunization protocol. DNA based immunizations are generally more preferred where it is difficult to express full-length proteins and immune response are mainly targeted towards native or conformational epitopes because the structural integrity of protein is critical for induction of functional mAbs. The major routes of DNA based delivery are intramuscular, intravenous and intrasplenic. Intrasplenic routes are considered to be the most efficient route. A single dose of DNA delivery is sufficient to induce antibody responses. Antibodies against different proteins can be produced at the same time by immunizing with several nucleic acids encoding for different proteins or single plasmid encoding different subunits of the protein. In such cases multiple booster doses are usually avoided, to reduce immune-dominance amongst antigens.

Another major challenge in the field of human hybridomas is the requirement of lymphocytes from actively infected patients or who have been exposed to antigen. If the active immune response is absent or not sufficient then the probability of circulating B cells in such cases is very poor or negligible. Due to ethical issues and considerations, it is generally not possible to immunize humans. To overcome these limitations Li *et al.*, in 2006 have developed a novel and rapid combined ex-vivo immunization strategy with morphogenics platform process for the isolation of therapeutic human mAbs. In this strategy, the group has purified B cells (CD19+) and CD4 positive T cells from healthy volunteer blood samples using magnetic bead-based sorting. These B/T cells were cultured in the presence of growth factor and antigen to activate B cells. The pool of these B and T cells were then fused with myeloma fusion partner using electric cyto plus. The fused cells were screened for antigen-specific antibody secreting properties by limited dilution method. The isolated mAbs had shown high specificity, biological activity and high affinity. This method avoids the collection and screening of a large number of patient samples which are normally the basic requirement for the generation of therapeutic human hybridomas. It also avoids the risk of potential viral transmission associated with conventional methods where PBMCs are sometimes used from viral infected patients. Screening of volunteers and blood cells can be performed before ex vivo immunization and after hybridoma development. This platform technology offers a rapid and cost-effective way for therapeutic human mAbs having natural pairing of immunoglobulin genes [99].

After cell fusions between B cells and myeloma cells, protocols of hybridoma technology include multistep screening and cloning processes to identify antigen-specific hybridomas, which is labour-intensive and time-consuming. However, recent advances in robotic screening methods have alleviated this to some extent [88]. The screening process on semi-solid selective medium has made it easy and reduces the overall time in hybridoma production by repeated selection and cloning steps. This screening technology has been used by companies to sale ready to use kit based systems for the development of murine hybridoma. It has also facilitated the use of murine hybridoma technology in a less cumbersome and user friendly. Methylcellulose-based semi-solid selective medium is preferentially used in hybridoma selection [56]. Technically it avoids the loss of rare clones from an overgrowth of faster-growing cells, which can occur during selection in a liquid medium. The selected clones are further dispersed into a liquid growth medium for screening and expansion. Similarly, Paul et al recently developed microarray-based screening technology for direct identification of high-affinity clones which avoids the loss of slow-growing clones. Additionally, this approach eliminates the enrichment, isolation, and purification of IgG for the characterization process. The crude culture supernatants can be directly used and thus avoids expensive and lengthy screening steps [100]. The screening process has advanced through flow cytometry-based methodology where single cells could be sorted from a bulk mixture of fused hybridoma cells. It has advanced by saving time and labour instead of the traditional multi-micro well plate seeding and limiting dilution sub-cloning [101]. In recent years tedious hybridoma screening and cloning processes are replaced with flow cytometry-based sorting methods. These methods avoid effort and time of tedious repeated screening processes. This method could also differentiate IgM and IgG secreting hybridoma. FACS based screening methodology can be applied in any laboratory easily setup as it doesn't require any special reagents [102–104]. The other alternative approach that some groups had used is the screening of hybridoma supernatant directly by Bio-Layer Interferometry based on disassociation rates to select clones containing high-affinity antibodies for further expansion and subsequent characterization. The main drawback of the ELISA based screening method is that clones that express high levels of a low-affinity antibody can give an equivalent signal to clones that express low levels of a high-affinity antibody. As a consequence, superior clones can be overshadowed by inferior clones because ELISA method score antibodies based on the binding signal strength and do not provide accurate affinities or dissociation rate constants [105,106].

5. Transgenic technology for isolation of fully human monoclonal antibodies

Current drug approval rates underline the revolutionary effect of fully human mAb therapeutics on drug development. Antibodies isolated for therapeutic applications from different species excluding human needs a multistep process of humanization and developability through rational sequence optimization [107]. Mouse is the most common and preferred progenitor used in mAb isolation. To avoid the multistep process of humanization, the concept of transgenic mice harbouring the human antibody repertoire has gain attention, where large human immunoglobulin loci are transferred into the mice germline using yeast artificial chromosome approach [108]. The XenoMouse and HuMAb Mouse are the first engineered transgenic mice that carry the majority of human VH & VL antibody repertoire [109].

XenoMouse transgenic technology was developed by Cell-Genesya a biotech company (now a part of Bristol Myers Squibb, New York, USA). The first therapeutic antibody developed by this transgenic technology was approved by the FDA in 2006 for the treatment of advanced colorectal cancer. The strength of transgenic technology can be evaluated by the recent data on approved mAbs. More than 18 fully human mAbs developed by transgenic animal-based technology are used for human therapy. A list of FDA approved mAbs developed by transgenic technology is listed in Table 4. Initially, this technology was limited to mice but over the years this technology has been established for other animal models like rabbits, rats, and cows. The success of this technology

Table 4
A list of FDA approved mAbs derived from transgenic technology.

	Trade Name	Target	Company	FDA Approval year	Application
1.	Cemiplimab (Libtayo)	PD-1	Regeneron	2018	Metastatic cutaneous squamous cell carcinoma (CSCC) or locally advanced CSCC and lung cancer
2.	Erenumab (Aimovig)	Calcitonin gene-related peptide receptor (CGRPR)	Novartis and Amgen	2018	Prevention of migraine
3.	Sarilumab (Kevzara)	Interleukin-6 receptor	Regeneron and Sanofi	2017	Rheumatoid arthritis (RA)
4.	Durvalumab (Imfinzi)	PD-L1	Medimmune/AstraZeneca	2017	Urothelial carcinoma
5.	Dupilumab (Dupixent)	IL4 receptor alpha	Regeneron and Sanofi Genzyme	2017	Allergic diseases
6.	Brodalumab (Siliq / Kyntheum)	Interleukin 17 receptor A	Valeant Pharmaceuticals	2017	Plaque psoriasis
7.	Olaratumab (Lartruvo)	Platelet-derived growth factor receptor alpha (PDGFRα)	Eli Lilly	2016	Solid tumors
8.	Secukinumab (Cosentyx)	interleukin (IL)-17A	Novartis	2015	Psoriasis, ankylosing spondylitis, and psoriatic arthritis
9.	Evolocumab (Repatha)	proprotein convertase subtilisin/kexin type 9 (PCSK9)	Amgen	2015	Treatment of hyperlipidemia
10.	Daratumuma (Darzalex)	CD38	Genmab/ Johnson & Johnson/Janssen Biotech	2015	Multiple myeloma
11.	Alirocumab (Praluent)	Proprotein convertase subtilisin/kexin type 9 (PCSK9)	Sanofi and Regeneron	2015	Treatment for high cholesterol
12.	Nivolumab (Opdivo)	extracellular domain of PD-1	Bristol-Myers Squibb	2014	Melanoma, lung cancer, renal cell carcinoma, Hodgkin lymphoma, head and neck cancer, colon cancer, and liver cancer
13.	Ipilimumab (Yervoy)	CTLA-4	Bristol-Myers Squibb	2011	Treatment of melanoma
14.	Denosumab (Prolia and Xgeva)	Receptor activator of nuclear factor kappa-B ligand (RANKL)	Amgen	2010	Treatment of osteoporosis
15.	Ofatumumab (Arzerra/ HuMax-CD20)	B-lymphocyte antigen CD20	GlaxoSmithKline (Genmab)	2009	Treatment of chronic lymphocytic leukemia
16.	Golimumab (Simponi /CNTO 148)	tumor necrosis factor alpha (TNF-alpha)	Johnson & Johnson/Merck	2009	Used as an immunosuppressive drug
17.	Canakinumab (Ilaris/ACZ885)	interleukin-1 beta	Novarti	2009	Treatment of cryopyrin-associated periodic syndromes (CAPS)
18.	Ustekinumab (Stelara)	IL-12 and IL-23	Johnson & Johnson	2009	Treatment of psoriasis, Crohn's disease, ulcerative colitis
19.	Panitumumab (INN/ABX-EGF)	Epidermal growth factor receptor	Amgen	2006	Treatment of colorectal cancer

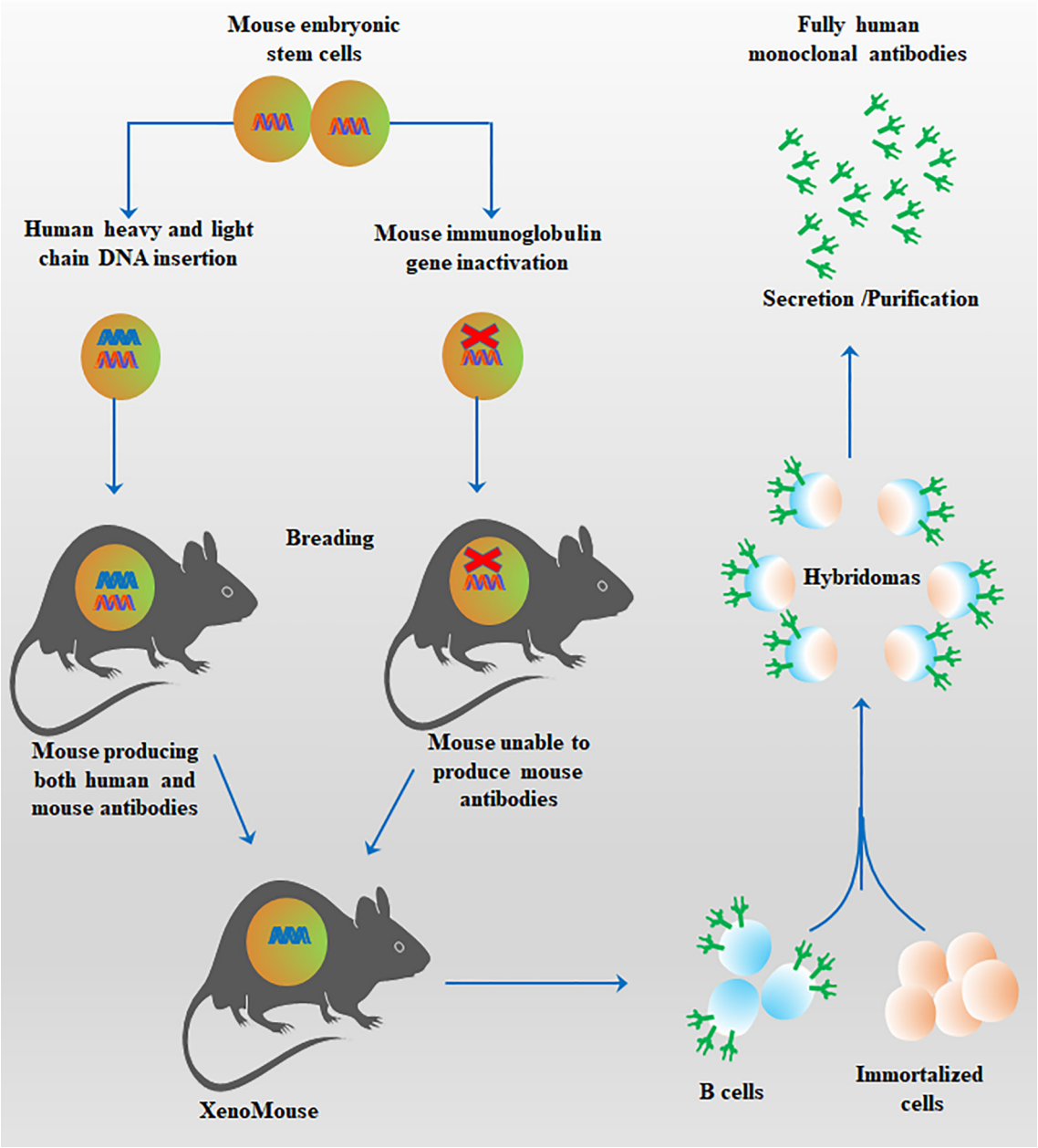

Fig. 4. Illustration of transgenic antibody technology shows the antibody production route: Mouse immunoglobulin gene loci were functionally inactivated in embryonic stem (ES) cells by targeted gene deletion used to generate mice homozygous for the necessary deletions. Crossbreeding between the transgenic mice (containing both human and mouse antibodies) with mice incapable of producing mouse immunoglobin, resulting in the XenoMouse strain which expresses human antibodies but not the mouse antibodies. B cells, isolated from immunized XenoMouse, are fused with myeloma cells to produce hybridomas producing human mAbs.

mainly depends on the proper representation of the target antigen to the immune system. In addition, designing a proper immunogen is very critical for success. To avoid the ambiguity of immunogen design in soluble form, genetic immunizations are more preferred over the traditional methods.

In contrast to human, mouse possesses very less antibody diversity represents the main limiting step in XenoMouse transgenic technology. Human antibody repertoire shows diversity more than 10^{11}, however, the number of B cells in a mouse is ~10^{8} only. A single mouse can harbour the only fraction of the antibody repertoire from human antibody [110,111]. Moreover, immunization of a large cohort of mice to increase the diverse response against antigens could be used to overcome the limited antibody diversity. The other transgenic mice technologies, where Kymouse & Trianni mouse models were developed to represent a more diverse human antibody repertoire that allows the selection of diverse human antibodies and overcomes the limitations of XenoMouse [112]. Fig. 4 represents an illustration of transgenic antibody technology showing the antibody production route.

Besides, the other limitation of this technology platform is immune tolerance when attempting to raise an immune response against human targets. A large number of human targets possesses a very high degree of sequence and structural homology, because of this homology the transgenic immune system recognizes these antigens as self-antigen. Different groups have tried to overcome the immune tolerance mechanism by adding T cell epitopes to the antigen [89,113]. The other similar approach tried by different groups to abolish the expression of murine orthologues gene [114]. But the major limitation with these defected mice models is that sometimes these mice suffer from health issues and some of these knockdown genes are necessary for the development of a foetus. Recent developments of transgenic rat and

chicken (OmniChicken) models have partially overcome these limitations.

The transgenic model-based technology harbours some advantages over the phage display derived antibodies. The antibody developed by transgenic technology requires less development or optimization and thus require a shorter time to reach the product development stage.

6. Perspectives of stereospecific monoclonal antibodies for future therapeutic medicine

Production of stereo-specific mAbs is still a very big challenge. There are hardly any practical technologies available for the generation of stereospecific antibodies, because of hurdles like how to immunize a mouse maintaining the antigen structure intact in the presence of adjuvant. In addition, adjuvants allow more effective sensitization, disrupting the native structure of proteins. However, if an adjuvant is not used, immunization efficiency could be very low. Another difficulty is strict selection of stereospecific mAb producing sensitized B lymphocytes. Although, if the immunization is successful with a native intact antigen, the number of desired sensitized B lymphocytes is extremely small, accounting for very less population of total spleen cells after repeated immunization [72].

Most of the established therapeutic mAbs have specificity for the antigen targets having primary structures. Generally mAbs can recognize two types of antigen epitopes which includes linear in the primary structures of proteins and the conformational, dependent on secondary and tertiary structures [115]. Stereo-specific mAbs recognizing conformational structures of target antigens may thus offer a markedly more versatile approach. Besides primary, secondary and tertiary structures, proteins may also exhibit quarterly structures. Which are formed by hetero or homo-subunits, providing unique interfacial geometric structures on their complexes. Native or conformation-specific mAbs are quite reasonable and attractive for future therapeutic purpose. There is need to replace the conventional mAbs with stereospecific mAbs in the near future for therapeutic medicine as they recognize the 3 dimensional conformation, which intrinsically determine their fundamental biological functions [116]. Stereospecific mAbs that recognize 3D molecular configuration has advantages over linear epitope-specific mAbs that consider only 2D configuration. In general, mAbs against primary structures, in target therapeutic antigens with the dominant secondary and tertiary folding are having affinity only with full antigens in restricted areas, as their linear epitope may be masked due to conformational folding. Alternatively, target antigens in the immune system can be identified by assuring indigenous structures via immunizing DNA of the target antigens expressed on the surface of the cell, allowing healthy and intact structural conformation. This can be linked to membranes, which mimics membrane protein, with a soluble protein harbouring sufficient signal peptides and membrane-penetrating areas that are genetically linked to the 5′ and/or 3′ terminals of genes of the desired soluble proteins as nucleic acid sequences to express as a fusion protein [117].

In the emerging diseases like human immunodeficiency viruses-1 (HIV-1), Coronavirus –19 (SARS-CoV2), Dengue, and Chikungunya, the immunogenic proteins of pathogens harbour complex structural glycoproteins, needs an urgent high-quality stereospecific mAbs to control their spread. Development of therapeutic antibodies against these pathogenic diseases is aimed to the structural antigens. The complex native structures of envelope proteins on viral surface facilitate the attachment of virus to the host cell, and subsequently entry inside the cell [118]. These native structural proteins induce predominantly cross reactive neutralizing antibodies. The neutralizing antibodies have shown promising results in the protection against pathogens however non-neutralizing antibodies help the virus in evading immune system [119]. In HIV-1, researchers are working to develop broadly neutralizing antibodies targeting conformational epitopes [120]. Similar approaches have been taken for other viral antigenic targets [121]. In many of these viral infections “conventional Abs” are generated in response to virus infection, but the virus adopts numerous evasion strategies like conformational masking of antigenic targets by glycosylation, high mutation rate etc. [122]. Generation of stereospecific mAbs are required to display impressive breadth and potency against the conformational proteins. These stereospecific mAb productions with promising therapeutic potential can be achieved by inclusion of critical steps like i. DNA or soluble protein immunization with native like targets or structural proteins ii. Selection of antigen specific myeloma cells and iii. Selective fusion of myeloma and B cells to generate hybridoma cells secreting stereospecific mAbs.

Different novel approaches and attempts are being taken to produce the soluble trimers which can display native-like conformational stable structure mimicking with virus surface. These conformational protein structures are promising targets for protein or DNA immunization and could subsequently require for the production of stereospecific mAbs. One major challenge associated with the use of native like antigens for the development of stereospecific antibodies is use of adjuvants in immunization process. Most of the adjuvants usually disrupts the original protein native structure and hence impede its ability to present relevant epitopes or occludes the trimer conformational epitope [115]. In recent years’ studies using ISCOM class of adjuvants in animal preceded by in vitro analyses showed that it has no adverse effect on native trimer conformation or antigenicity [123]. The other way to generate stereospecific antibodies is the direct immunization of mammalian cells expressing cell surface antigens in its native conformation [124]. A number of stereospecific antibodies has been generated against several targets like receptors [125], ligands [126], antagonist and chemical compounds [127]. A detailed schematic representation of different approaches used for isolation of stereospecific mAb is shown in Fig. 5.

The production of stereo-specific mAbs could be achieved by tweaking the conventional hybridoma fusion through stereo-specific targeting (SST) technique invented for the first time by Tsumoto et al [115]. Crucially, it involves a strict selection of the required sensitized B lymphocytes by intact antigens, expressed on myeloma cells, through B-cell receptors (BCRs) and their selective electrofusion (only attached cells can be fused among themselves) to generate a specific hybridoma [128]. SST technology offers selective production, against different protein types, of monoclonal stereospecific antibodies not only for membranous but also for soluble non-membranous antigens. Morshed et al recently showed efficiency in activation of the G-protein coupled receptor which holds seven-transmembrane domains by a stereo-specific mAb [117]. Furthermore, the new promising class of therapeutic mAbs, catalytic antibodies are capable of identifying and degrading antigens, has fundamentally demonstrated. Hifumi et al. have developed a catalytic antibody to degrade the active site for urease o Helicobacter pylori and eliminates the bacterial infection in the mouse [129]. Moreover, the catalytic antibodies have proven their utility in suppressing infection of the rabies virus [130] and the influenza virus [131] in vitro and in vivo using human antibody light chains. In addition, they have recently been noted in their ability to effectively reduce the accumulated ß-amyloid in the mice's brain [132,133]. MEDI9447 is a mAb that inhibits CD73 (ecto-5′nucleotidase) activity on a non-competitive basis and is considered a promising immuno-oncology target [134]. This mAb is antagonistic to CD73 employing dual inter-CD73 dimer cross-linking and/or steric blockage mechanisms that prevent the adoption of the CD73 catalytic active conformation [135]. Bispecific mAbs with stereo-detection may be especially effective for cancer cell detection of membranous antigens which have not been easily detected by conventional linear mAbs. In a nutshell, as proteins retain their native conformation in nature, development of stereospecific, alternate forms of bispecific and catalytic mAbs, for selective therapy is the founding factor in therapeutic future drugs.

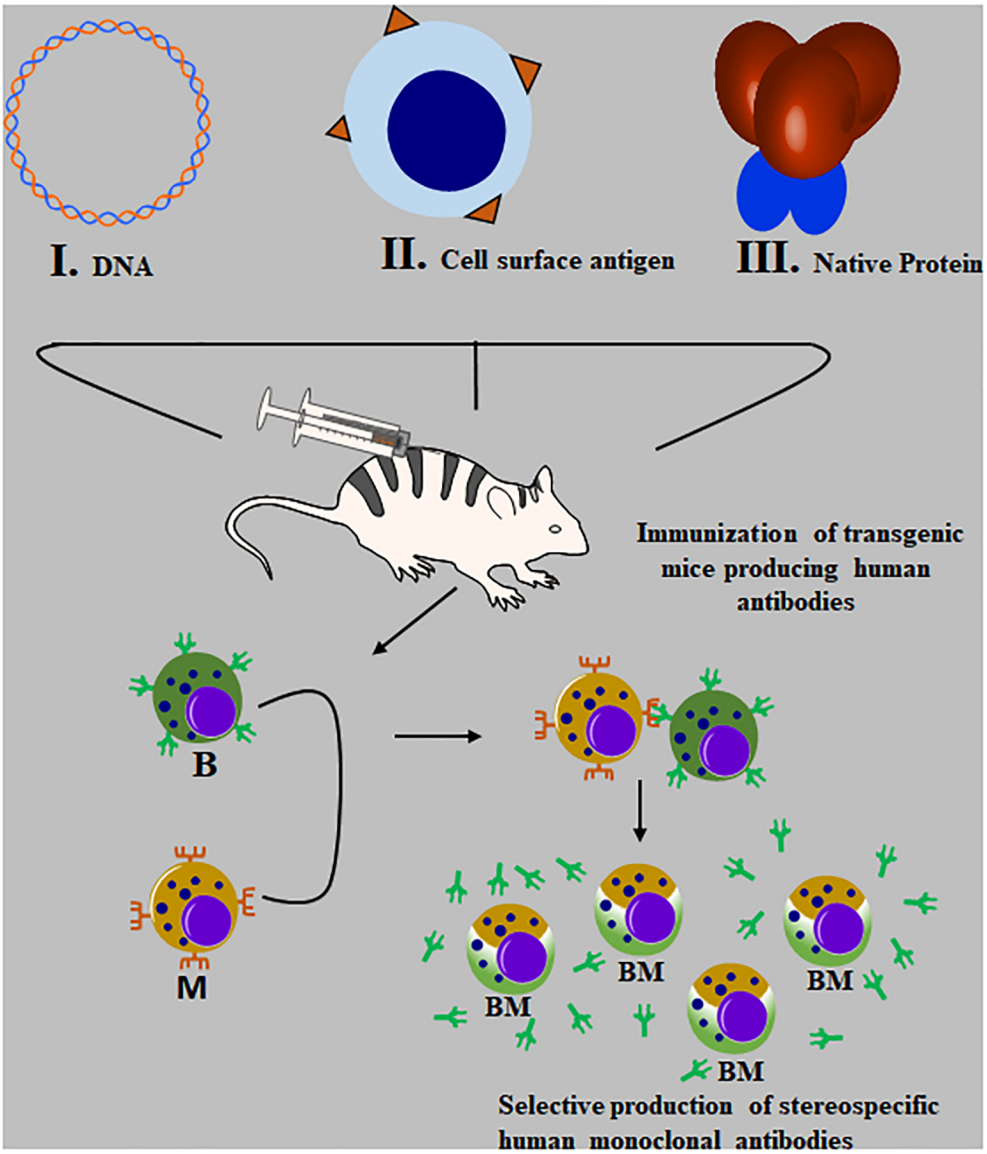

Fig. 5. Production of Stereospecific human mAbs. Transgenic mice B lymphocytes producing human antibodies were sensitized by DNA/cell surface expressed antigen/Native protein immunization are selected by antigen-expressing myeloma cells. Fusion of B lymphocyte and myeloma cell are performed by electrical pulses, according to the procedure based on stereospecific targeting. Hybridoma cells obtained by this new technology may secrete stereospecific 'human' mAbs.

7. Monoclonal antibodies as an alternative supporting arm: A new era in bio-therapeutics

The mAb has come a long way since the days when unmodified murine mAb was explored against the cancer-causing agents. From the last two decades, mAbs have been a standard element of cancer therapy, however, with still much room for further improvement in future [64]. The mAbs are always preferred over the chemical compound based therapies because of their high specific reactivity and affinity towards the target antigen recognition. They show minimal side effects with favourable pharmacotoxicity and pharmacokinetics properties. The high specificity of mAbs towards their targets presents an attractive and successful option for the development of medical treatment and molecular drug targets. [136].

Early clinical attempts exploring mAb-based therapeutics were very primitive and disappointing 20 years ago, some clinical experts considered the antibody-based therapy treatment for cancer as a failed hypothesis [137]. The first mAb which was clinically evaluated against cancer was the murine mAb. Although there were some fascinating hints that mAb therapy could be successful, however, the problems related to the administration of murine mAb to humans limited their clinical utility and applications [138]. Rise in immune response against the therapeutic mAb, very rapid clearance of the mAb from the system and suboptimal ability of the murine mAb to interact with the human immune system in a manner that led to immune destruction were the challenging tasks. However, some investigators tried continuously to explore the use of mAb as a possible cancer treatment. They also evaluated other strategies such as using; IgG to target cancer directly, alter the host immune response to cancer, provide cytotoxic substances to cancer, and retarget the cellular immune response towards cancer [64].

Over the last twenty years, the effectiveness of antibodies in the treatment of patients with cancer and other deadly diseases has been increasingly recognized, as mentioned in Tables 1 And 4. Many of these antibodies are specific for antigens expressed by the disease-causing agents itself [139]. In the case of viral targets, mAb-based therapeutics have shown limited success. However neutralizing antibodies play an essential part in antiviral immunity and human protection against viral diseases is primarily mediated by the humoral immune response [120,122]. It is well documented that early administration of mAbs in

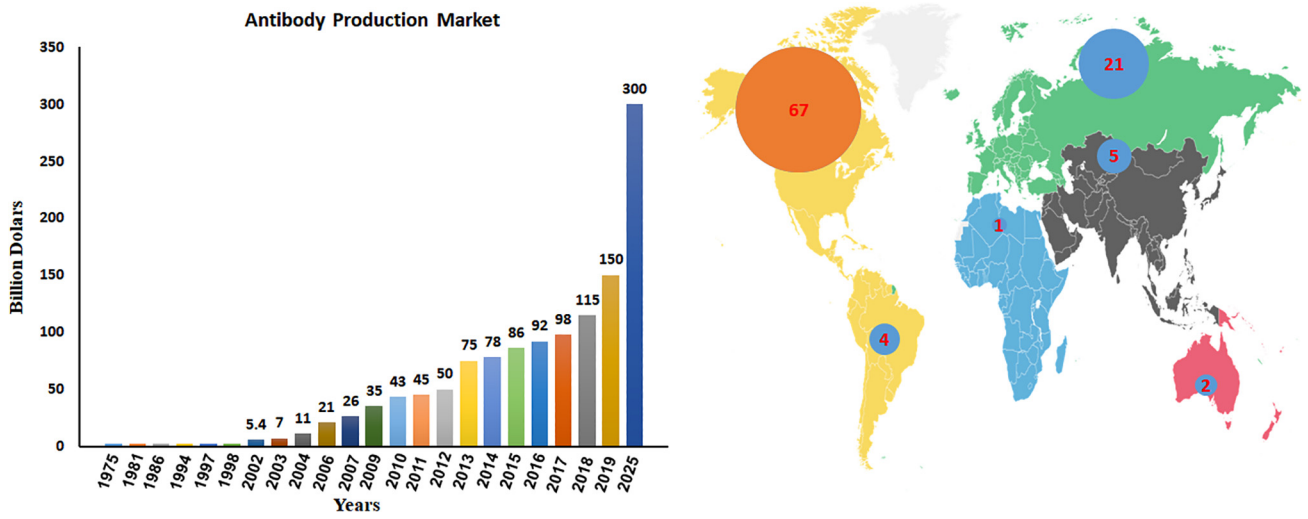

Fig. 6. Illustration showing the successful market production of therapeutic antibodies. Timeline from 1975 showing the significant increase in the antibody production market. Most of the biotech companies were launched from 1981 to 1986. The Bar height and numerical annotations represents the estimated production market value of antibody therapeutics in each indicated year (mentioned billions of US dollars). The global therapeutic mAb market is expected to generate the revenue of $300 billion by the end of 2025 as mentioned. Antibody production biotech companies generated antibodies against different disease-related immunogens.

the treatment regimen reduces mortality rate significantly up to 95% [140]. To date, only two mAb is licensed for viral infection i.e. Respiratory syncytial virus (RSV) and the other one is ibalizumab, which has been recently approved in 2018 for the treatment of HIV positive people with multidrug resistance towards anti-retroviral therapy (ART) [18,141]. Several other candidates are at the different stages of clinical trials e.g. Leronlimab, an anti-CCR5 IgG4 for HIV infection and REGN-EB3: a mixture of 3 IgG1 mAbs for Ebola virus infection (https://www.antibodysociety.org/antibodies-to-watch-in-2020-at-pegs-europe/).

Lack of vaccines against various deadly viral diseases necessitates the development of antibody therapeutics to save the loss of lives and control deadly diseases [142]. However, it is always easy to generate monoclonal to protect the population at the time of the outbreak in a much shorter time as compared to vaccine production. Though vaccines are one of the most cost-effective ways to manage infections, vaccines also require time to elicit protective immunity and depend on the host's ability to mount an immune response. A number of a prophylactic vaccine against pathogens such as; Herpes Simplex Virus Type-1 (HSV-1) and Human Immunodeficiency Type-1 (HIV-1) have shown protection in animal immunization studies, but, so far, no effective human vaccine against these diseases are available [58,59]. Antigenic drift and high diversity among the emerging pathogens; such as influenza virus and HIV have been reported [60,61] which further add to the complexity and may lead to vaccine mismatch drop in vaccine effectiveness against circulating serotypes and strains. In developing countries, it is not economically feasible to make a vaccine of every disease because of a lack of awareness of disease burden [62].

8. Conclusions and future prospective

The mAbs are widely used in the fields of diagnostic, therapeutic and biological applications due to their high specificity and affinity. At present, the majority of mAbs approved for therapeutics are humanized or the chimeric versions of mouse mAbs and were generated using hybridoma technology. In recent years, these engineered humanize and chimeric antibodies are potentially used to generate different forms of antibody fragments such as scFvs [143,144], diabodies [145], Tandom Abs [146], and domain antibodies [147], PEGylated Fabs [148] to target novel antigenic sites. Technical advancement in the applicability of hybridoma technology to other animal species (other than mice) of different phytogenic origin has led to the development of novel mAbs to conserve human antigens. It has opened a new path for therapeutic and diagnostic mAbs with high specificity and affinity to poorly immunogenic targets.

With the recent development of high throughput mAb generation technologies, hybridoma technology is the most favoured method due to its indigenous nature to preserve natural cognate pairing information of antibodies that is lost in other methodologies, reduces the specific diversity of antibodies [149]. Advancement in recombinant DNA technology methods like chimerization and humanization has increased the potential of hybridoma technology to a great extent. An antibody that undergoes the process of humanization preserves the natural specificity and limits risk of CDRS causing an immune response. The unnatural pairing of antibodies in terms of affinity maturation and recombination pairing in display methods sometimes results in high immunogenic response [19]. All these features have make hybridoma platform as first and most preferred mAb isolation technology. Recent advances in development of hybridoma cells secreting stereo-specific mAbs have opened new avenues of future therapeutics. In comparison with other anti-viral drug treatments, a stereospecific antibody-based therapy could offer potent anti-viral actions by more comprehensive target and potent neutralizing effect.

The mAb market has shown tremendous increase in the last five years as a diagnostic and therapeutic reagent. The commercial development of therapeutic mAbs commenced in the early 1980s, and by 1986 the first therapeutic mAb was FDA approved for the prevention of kidney transplant rejection. Over the years mAb market has changed rapidly as a major class of therapeutic agents for the treatment of many human diseases, in terms of global sales revenue for all mAb products was~$115.2 billion in 2018. A graphical representation of the global antibody-based therapeutic market trend is represented in Figs. 6 and 7. The Global mAb therapeutics market is expected to grow at a compound annual growth rate (CAGR) of 12.80% and is expected to reach market revenue of around USD 218.97 billion by the end of 2023 [150]. Humanized mAb accounts for the largest revenue-generating share among antibody-based therapeutics, showing to its widespread acceptance for numerous diseases including cancer, autoimmune diseases, inflammatory diseases, infectious diseases, haematological diseases, and others.

The other potential area where mAb has shown great success is diagnostics. The mAb -based diagnostic reagents potentially identify abnormal cell targets, infectious agents, or elements of the body's

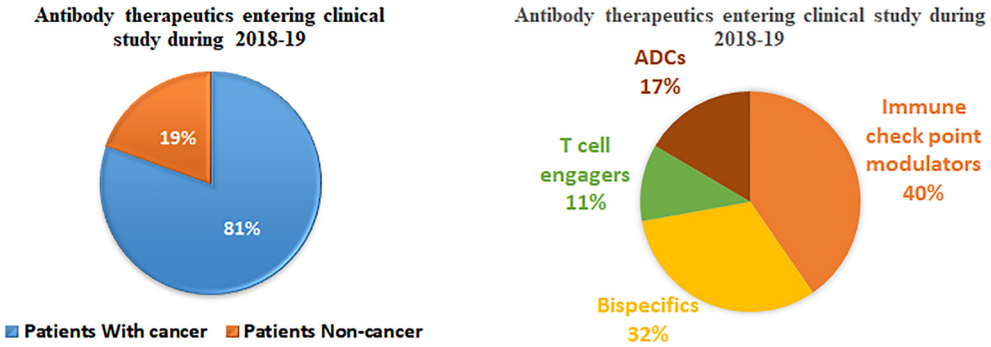

Fig. 7. Majority of the antibody therapeutics entering clinical study during 2018–19 were against cancer patients. Percentage of antibody therapeutics entering the clinical trials were bispecific 32%, ADCs 17%, T cell engagers 11% and immune check point modulators.

response to disease. The global antibodies based diagnostic market was valued at US$ 20,000 in 2017 and is projected to reach US$ 35,000 by the year 2026 at a CAGR of 5% from 2018 to 2026.

Ethical statement

This article does not contain any studies with animals performed by any of the authors.

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Author contributions

RK wrote the paper and finalized with the help of HAP & SSh, CS, SSa, SA &TS edited the manuscript; HAP designed the figures. RK & SS prepared the tables.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

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