Chapter 19
Applications of Animal Cell Culture-Based Assays

Pallavi Shah, Anil Kumar, and Rajkumar James Singh

19.1 Introduction

In vivo models offer a better prospective of the activity of substances when tested in animals as compared to in vitro models (for example in cells), but their use has been banned in numerous countries because of several ethical and economic concerns. This has led to a tremendous increase in the use of in vitro cell-based models for various applications starting from biomarker identification, genetic manipulation, drug discovery programs, stem cell research to toxicity analysis. Often these in vitro tests are defined as bioassays. With the increase in the understanding of the underlying biochemical processes involved in cellular functions and different disease pathologies, there has been a tremendous increase in the development of a number of various cell-based assays.

19.2 Biomarker Identification

A biomarker can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Strimbu & Tavel, 2010)". Classification of biomarkers is governed by several parameters, which include

P. Shah · A. Kumar (✉) R. J. Singh
Division of Veterinary Biotechnology, ICAR – Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India

Rani Lakshmi Bai Central Agricultural University, Jhansi, Uttar Pradesh, India

S. Mani et al., *Animal Cell Culture: Principles and Practice*, Techniques in Life Science and Biomedicine for the Non-Expert,
https://doi.org/10.1007/978-3-031-19485-6_19

characteristics such as biomarkers based on imaging (computed tomography, magnetic resonance imaging) or molecular biomarkers. Molecular biomarkers include non-imaging biomarkers which possess biophysical properties, allowing them to be determined in biological samples. These include biomarkers based on nucleic acids such as gene polymorphisms or mutations and gene expression analysis and other small molecules such as peptides, proteins, lipids and metabolites. Further classification of biomarkers can be based on their application, such as biomarkers used in disease diagnosis, biomarkers for assessing disease stages, biomarkers used in disease prognosis such as cancer biomarkers and biomarkers used for monitoring the clinical response to a potential novel treatment. Biomarkers are also utilized during early drug development such as pharmacodynamic biomarkers to monitor a certain pharmacological response and help in dose optimization studies (Huss, 2015). Modern day advances in the fields of genomics, proteomics and molecular pathology have helped characterize numerous candidate biomarkers with potential clinical value.

19.2.1 Cell Culture-Based Cancer Biomarker Identification

Cancer causes millions of deaths round the globe every year. Present cancer biomarkers have low diagnostic specificity and sensitivity and have not yet made any deep impact on reducing the cancer burden (Kulasingam & Diamandis, 2008). Limited understanding of cancer initiation and progression is one of the major reasons for the lack of effective diagnostic tools and therapeutics. Carcinogenesis is a complex process and involves alterations at the genomic, proteomic and metabolomic levels. Alterations at the genome level include gene mutations and alterations in the expression of protooncogenes, tumour suppressor genes and DNA repair genes. Alterations at the proteome level include protein alterations in the serum, plasma (secretome) and cell surface protein expression. This is further accompanied by the alterations in metabolite composition at the metabolome level. These alterations are often targeted as potential biomarkers for diagnostic, prognostic and therapeutic biomarkers. High-throughput genetics, genomics, proteomics, many non-invasive imaging techniques and other technologies allow measurement of several biomarkers (Bhatt et al., 2010).

Advances in studying cancer pathobiology heavily depend on different experimental model systems to decipher disease biology (van Staveren et al., 2009). Cancer cell line models are important in biomedical research and are critical gene discovery tools in human cancer research. Even though animal models aid in understanding the progression of cancer in vivo, they do not illustrate the molecular mechanism causing the initiation of carcinogenesis. Cancer cell lines act as a valuable model for replicating the various stages of initiation and progression of carcinogenesis in vitro. The cell line models are useful in the identification of biomarkers and potential therapeutic targets (Raju et al., 2017). Cell lines prove to be invaluable

Table 19.1 Details of various cell lines used for cancer biomarker identification

Cell line (s)	Cancer Studied	Marker (s)	Method Used for Study	Conclusion	References
DOK, NOK, KB, HN5, HN13, FaDu, Hep-2, CAL27, SCC-4, Tca8113	Oral cancer	Expression of miRNA-451, c-myc expression	Real-time PCR	miRNA 451 has tumour suppressor role by down-regulating c-myc expression	Wang et al. (2015)
HMEC, HCC1599, HCC1143, HCC1937, HCC202, HCC2218, MCF10a, HMT-3522-S1, MDA-MB-453, MFM223, MCF7	Breast cancer	Levels of IDH2, CRABP2 and SEC14L2 proteins	SILAC labelling, 2D-PAGE, LC -MS	High levels of IDH2 and CRABP2 and low levels of SEC14L2 have been used as prognostic markers for breast cancer	Geiger et al. (2012)
LCCLs, HepG2 and Huh6	Hepatocellular carcinoma	*FGF19, CK19, TP53, TSC-1, TSC-2, MET, NQO1* gene (s) expression	RNA sequencing, analysis of the transcriptome, miRNA profiling, reverse phase protein assay	LCCLs can act as a valuable resource for discovery of drug-biomarkers	Caruso et al. (2019)
ALST, CAOV3, DOV13, OVCA3, OVCA420, OVCA429, OVCA432, OVCA433, OVCA633, and SKOV3	Ovarian cancer	Osteopontin gene and protein expression	Real-time PCR, immunohistochemistry and ELISA	The findings suggest an association between plasma levels of osteopontin and ovarian cancer and hence its potential for use as a diagnostic marker	Kim et al. (2002)
SW480, SW620, and HT-29 and T84	Colorectal cancer	Akt, STAT3, AMPKα and bad activation	Angiogenesis-related antibody array, intracellular signalling arrays, activated receptor tyrosine kinase arrays, Western blot analyses	IL-6-induced activation of Akt, STAT3, AMPKα and bad- and down-regulation of EGFR, HER2 receptor, insulin R and IGF-1R can be utilized as biomarkers for CRC	Chung et al. (2015)

experimental models for studying cancer and simplify the task of molecular characterization and genetic manipulation. Cell line-based studies have elucidated several signalling pathways involved in cancer, which have been targeted to test and develop drugs and therapeutic interventions (Table 19.1).

19.3 Genetic Manipulation

The development of methods to isolate and transfer DNA from one species to another has given rise to numerous possibilities to determine the functional and regulatory mechanisms of gene expression. Genetic engineering has been employed to genetically manipulate cells to knockout genes or overexpress specific genes. Overexpression usually involves the introduction of recombinant DNA present in a vector into the cells where it transcribes and expresses recombinant protein. This is utilized for the large-scale production of recombinant proteins of biopharmaceutical importance.

19.3.1 General Scheme for Genetic Engineering of Animal Cells in Culture for Protein Production

1. Requirement of mammalian cells – The common mammalian host cell lines for recombinant protein expression by genetic manipulation include the Chinese hamster ovary (CHO), mouse myeloma-derived NS0 and Sp2/0 cells, human embryonic kidney cells (HEK293) and human embryonic retinoblast-derived PER. C6 cells. Mammalian cells are preferred as they provide proper folding and necessary post-translational modification of the expressed proteins.
2. Expression vector – An expression vector contains signal sequences (promoter, ribosome binding site, terminator) that are needed to ensure gene expression in mammalian cells. Further it also carries a selective marker (e.g., the dihydrofolate reductase gene (DHFR)) for selection and amplification purposes. Transfection of mammalian cells with expression vector is carried out for either transient or stable expression of gene (s). Vectors used may be plasmid-based vectors or viral vectors (adeno-associated viral (AAV) vectors, retroviral vectors, baculovirus vectors and vaccinia virus-based vectors).
3. Transfection – Often two general methods are used for the transfection of mammalian cells. In one method the direct transfer of DNA into the cells is carried out by utilizing liposomes, chemicals such as calcium phosphate, polybrene and DEAE-dextran, physical methods such as electroporation and microinjection and other methods by virus infection. Calcium-phosphate and electroporation-mediated transfection is useful for large-scale transient gene expression. For recombinant protein expression, most often CHO cells are used for transfection with the expression vector and incubated in low-nucleotide media containing appropriate selective agent based on the marker used.
4. Expression and regulation of gene expression – Cells that have incorporated the foreign gene along with the selectable marker will survive under selection pressure. By increasing selective pressure, the expression level of protein can be increased. For example- the gene of interest is co-amplified with the DHFR gene by increasing the selective pressure with high concentrations of methotrexate

(Mtx). More high yielding clones are selected by further increasing the selection pressure and clones are cryopreserved for future use. These candidates are adapted for growth in serum-free, suspension culture and evaluated for stable product yield and growth characteristics. Expression can be transient, for a short period of time or stable with cells capable of indefinite production of proteins.

19.3.2 Applications of Genetic Manipulation of Animal Cells in Culture

19.3.2.1 Recombinant Therapeutic Protein Production

Mammalian cells are the most preferred platforms for the expression of complex glycoproteins from recombinant genes such as growth factors, cytokines, hormones, enzymes, blood products and antibodies (Table 19.2). Majority of the proteins have complex structures and require complex modifications for full biological function. Glycosylation is one of the most widely recognized and complex form of post-translational modification which involves extensive processing and trimming of the protein sequence in the Golgi apparatus and endoplasmic reticulum. Such modifications can be carried out in eukaryotic cells and hence are preferable for biopharmaceutical processes. Baby hamster kidney (BHK) and Chinese hamster ovary (CHO) cells are often used as the host cells of preference as they produce human-like glycosylation patterns (Verma et al., 2020).

19.3.2.2 Gene Therapy

The deletion, insertion or alteration of a working gene copy for curing a disease/defect or to slow the progression of a disease is known as gene therapy. It involves the identification of the malfunctioning gene followed by isolation and generation of a correct gene construct for faultless expression. Correct delivery of the genetic material in vivo or ex vivo followed by integration of gene is pivotal for successful gene therapy. In vivo therapy involves direct introduction of the genetic material into the individual at a specific site, whereas the target cells are treated outside the patient's body in ex vivo treatment. The ex vivo technique involves gene therapy in the cultured cells, which are multiplied and ultimately introduced to the targeted tissue. Gendicine is the first product designed for gene therapy used as a medication produced by Shenzhen Sibiono Genetech, China. It is used for head and neck carcinoma treatment. The tumour suppressing gene p53 is placed in recombinant adenovirus which leads to tumour control and elimination. SBN-cel is a cell line subcloned from the human embryonic kidney (HEK) cell line 293 and is utilized for Gendicine production.

Table 19.2 List of some important approved recombinant therapeutic proteins produced in animal cell lines

Cell line	Recombinant therapeutic protein	Available as	Manufacturer	Mode of action	Disease used for
CHO	Antibody, humanized IgG1/k	Avastin	Roche	Anti- VEGF	Colorectal cancer
Sp2/0	Antibody, chimeric IgG1/k	Remicade	JNJ & Merck & Mitsubishi	TNFα inhibitor	Rheumatoid arthritis, Crohn's disease
BHK	Factor VIIa	Novo seven	Novo Nordisk	Blood clotting factor VIIa	Hemophilia A + B
CHO	Humanized IgG1; DM1	Kadcyla	Genentech	By inhibiting HER2 receptor signalling	Breast cancer
CHO	Humanized IgG4κ	Keytruda	Merck, Sharpe and Dohme	Blocks PD1 pathway	Cancer
CHO	Erythropoietin (Epoetin alfa)	Epogen	Amgen, JNJ and Kyowa	Proliferation and terminal differentiation of erythroid precursor cells	Anaemia caused by chemotherapy or chronic kidney disease
CHO	Glycosylated IgG1	Ocrevus	Genentech	Anti-CD20 antibody that depletes circulating immature and mature B cells	Multiple sclerosis
CHO	Interferon beta-1a	Rebif	Merck KGaA	Acts as interferon beta-reduction of neuron inflammation	Multiple sclerosis
CHO	Human IgG1λ	Tremfya	Janssen Biotech, Inc.	Binds to the p19 subunit of interleukin 23 (IL-23) and inhibits its interaction with the IL-23 receptor	Psoriasis
CHO	Recombinant enzyme	Brineura (Cerliponase alpha)	BioMarin Pharmaceutical	Recombinant form of human tripeptidyl peptidase (TPP-1)	Batten disease

Source: Verma et al. (2020) and Zhu et al. (2017)

19.3.3 Advanced Editing Tools

With the advent of modern gene-editing tools, mammalian host cells can be easily manipulated for the development of novel, potential and cost-effective recombinant products. Even though gene editing tools such as Transcription Activator-Like

Effector Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs) are useful, the discovery of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas system has made gene editing more precise and easy for the study and treatment of human diseases like muscular dystrophy, haemophilia and cystic fibrosis. Recently, the dystrophic gene in Duchenne muscular dystrophy (DMD) has been corrected in patient-derived induced pluripotent stem cells by the use of CRISPR-Cas system that led to the restoration of the dystrophin protein in the cells (Li et al., 2015). CRISPR-Cas-based gene editing is being utilized for the development of animal models, which can mimic human diseases. These models can provide ample opportunities for the prediction of possible clinical trial outcomes as well as ensure the safety and efficacy of drugs against the particular disease.

19.4 Pathological Studies

Pathological Studies can be defined as the branch of science which deals with the study of causes and effects of diseases. It involves the laboratory examination of body tissue samples for diagnostic or forensic purposes. Pathology deals with four components of disease i.e., cause, development mechanisms (pathogenesis), morphologic changes like structural alterations of cells and lastly the clinical manifestations like consequences of changes. General pathology largely deals with analysis of clinical abnormalities that can be used as markers or precursors for both infectious and non-infectious disease. Cell culture can play an important role in pathological studies of a disease or infection under in vitro conditions.

One of the best examples for pathological studies is the use of cell culture for virology. Viruses are infectious agents that cause widespread infections in humans, animals and plants alike. Cell-based pathological studies include visual structural / morphologic changes of cells, change in gene and protein expression of cells and change in composition of spent medium, for example, release of specific enzymes, metabolites or proteins. Rapid detection of viruses in samples taken from patients, natural samples and further study of the properties of the isolates are of immense importance. At present, numerous approaches for virus detection are used which include PCR, CRISPR/Cas technology, NGS (next generation sequencing), immunoassays and cell-based assays. Of these, cell-based assays are the only ones that can detect viable virus particles (Dolskiy et al., 2020). Cell-based detection methods provide the added advantage of isolation and characterization of virus as well. Electron microscopy can be used for the morphological examination of viral particles isolated from cell culture. The first method utilizes the cytopathic effect (CPE) of viral infection during which dead cells after virus release and can be detected and quantified under a microscope. CPE-based detection methods suffer from certain drawbacks such as they are labour-intensive with low sensitivity and they fail to detect non-cytopathic viruses. Various cell lines are used for virus detection, such as NSK, UMNSAH/DF1, BS-C-1, Vero, MDCK, A549, HEK293 or HeLa cells. They serve as substrates for the virus culture and further aid in their differential detection

based on differences in efficiency and specificity (Parker et al., 2018; Lombardo et al., 2012; Liu et al., 2019).

The second method for detection and identification of viral particles is based on the development of a reporter cell line, having specific cells that are modified to make a reporter protein when infected with a virus. The reporter must possess characteristics highly specific to the particular virus to be detected. When infected by a virus, the reporter construct is recognized as a viral genome or a chimeric protein with a specific cleavage site, which generates a quantifiable signal that can be detected. Different principles for the detection of different viruses depend on their life cycle and genome structure. Infection with virus proteins or cleavage of proteins and release of reporters by viral proteases can cause the transactivation of viral structures in the vectors of some RNA viruses. Furthermore, minigenome mimic viruses can be used for the detection of negative-strand RNA viruses with nuclear replication cycles (Kainulainen et al., 2017).

19.5 Pharmaceutical Studies

Earlier studies utilized animal models for testing drugs for toxicity, corrosion, bioavailability and activity, but recent times have seen the emergence of cell culture-based assays for drug testing and development. The reason being animal models do not effectively mimic or provide human in vivo conditions as well as unexpected results are obtained during animal trials. Cell culture-based studies have made significant contributions in this regard. Contrary to animal models, the requirement of low drug quantity and a short response time are characteristic of in vitro cell culture-based assays. Well-designed in vitro cell culture studies render a huge reduction in costs spent on animal experiments. FDA has recommended the use of human cell lines to identify metabolic pathways for drugs and have mentioned their applicability in in vitro testing in their guidelines published in the year 2004 (Şahin et al., 2017).

Cancer cell lines are used extensively for the screening and testing of drugs during drug development studies. Some commonly used cell lines for drug testing are given in Table 19.3.

19.5.1 Drug Screening in Cell Lines

Drug development begins with drug testing in cancer cell lines. Drug testing in cell culture / cell lines have both advantages and disadvantages (Table 19.4). Drug cytotoxicity is often determined in cell lines and data so obtained has found relevance in clinical value prediction. A diversity of responses to drugs are displayed by different cancer cell lines and cell line panels are useful for drug tests. The first such panel was NCI-60 which utilizes 60 cancer cell lines. It was developed to reduce animal experiments for testing of the drugs (Shoemaker, 2006). Similarly, Japanese

Table 19.3 Extensively used cell lines for drug testing and development

Cell line	Species and Disease
HeLa	Human cervix adenocarcinoma
Caco2	Human colorectal adenocarcinoma
HepG2	Human hepatocellular carcinoma
HEK 293	Human embryonic kidney 293 cells
K562	Human chronic myeloid leukaemia
A549	Human lung carcinoma
MCF7	Human breast adenocarcinoma
PC3	Human prostrate adenocarcinoma
A375	Human malignant myeloma
ND-E	Human oesophageal adenocarcinoma
CHO	Chinese hamster ovary cell line
Vero	African green monkey kidney epithelial cells

Table 19.4 Advantages and disadvantages of using cell lines for drug testing

Advantages	Disadvantages
(i) Ease of handling and manipulation.	1. HeLa cells cross-contamination
(ii) High homogeneity.	2. Loss of heterogeneity and genomic stability during testing
(iii) Similarity with initial tumour.	3. Susceptibility to contamination with bacteria and mycoplasma
(iv) Unlimited infinite source of cells.	4. Challenging maintenance of cultures for long periods
(v) High reproducibility of results.	

Foundation for Cancer Research (JFCR) developed a panel consisting of 30 tumour lines derived from the NCI-60 panel, plus nine tumour cells lines specific to the Japanese population, specifically gastric cancer cells and breast cancer cells. The panel with 39 cell lines was hence named JFCR39 (Nakatsu et al., 2005).

The release assays to analyse the release of molecules from carriers, drug diffusion tests, and toxicity testing can be used in cell lines to study the mode of action, impact on physiological processes, and therapeutic treatment of disorders. Toxicity and efficacy studies are of utmost importance as an alternative to animal testing (Michelini et al., 2010). Cell lines are mostly utilized to study toxicity of drug, effect on cell viability and to carry out permeability/bioavailability studies. Present day advances in cell culture such as co-culture with normal cells, 3D matrices for cell culture and control of levels of specific growth factors and additives by microfluidic systems have enabled better mimicking of in vivo systems and thus better screening of drugs (Kitaeva et al., 2020).

Cell viability assays utilize a number of viable cell markers to determine metabolically active cells (Riss et al., 2004; Sittampalam et al., 2016). Commonly used

markers include assessing the ability to either reduce a substrate, measuring enzymaticactivityoflivecellsandmeasuringATPlevels.Theseassayscanbebroadlyclassifiedinto-

(a) *Real-Time Cell Viability Assays* – These include ATP cell viability assays whereby ATP can be measured using reagents which contain a detergent, luciferase enzyme and the substrate luciferin. Viable cells are lysed by the detergent lyses, which release ATP into the medium. Luciferase utilizes this ATP to convert luciferin into oxyluciferin to generate luminescence, which can be detected by using a luminometer. Another example of real-time cell viability assay is live cell protease activity assay. In this assay, a cell-permeable fluorogenic protease substrate (GF-AFC) is used to detect the live-cell protease activity. On entering the live cells, the substrate is cleaved by live-cell protease and generates a fluorescent signal which is directly proportional to the number of viable cells.
(b) *Tetrazolium Reduction Cell Viability Assays* – Positively charged compounds such as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT readily penetrate viable cells. Viable cells are able to convert MTT into a purple-coloured formazan product by dehydrogenase activity in the mitochondria. This method usually has a long incubation time of 4 hours and the formazan crystals formed are insoluble, hence a solubilizing reagent like DMSO must be added before measuring absorbance. Another category of compounds include negatively charged compounds such as MTS, XTT and WST-1 that do not penetrate the cells readily and require assistance of intermediate electron coupling reagents. These can enter cells, get reduced and then exit the cell to convert tetrazolium to the soluble formazan product (Fig. 19.1).
(c) *Resazurin Reduction Cell Viability Assay* – A dark blue-coloured cell permeable indicator dye resazurin possesses little intrinsic fluorescence. Viable cells reduce resazurin into resorufin, which is pink and fluorescent. Similar to MTT-based reduction assay after hours of incubation, the pink product is measured using a microplate fluorometer.

Cell cytotoxicity assays involve measuring of cell death. Loss of membrane integrity is a major event that occurs during cell death. This makes the cells easily permeable to chemicals or proteins or molecules. Cell death can thus be monitored by detecting efflux of particular proteins like lactate dehydrogenase or influx of chemicals like DNA binding dyes. Some common cell cytotoxicity assays are

(a) *Lactate Dehydrogenase (LDH) Release Assays* – Lactate dehydrogenase (LDH) leakage occurs from dead cells that have lost membrane integrity. LDH converts lactate to pyruvate with the concomitant production of NADH. LDH activity is measured by providing the substrates lactate and NAD+ to generate NADH. Different assay chemistries are used for measuring the NADH.
(b) *DNA Dye Cytotoxicity Assay* – DNA-binding dyes cannot enter live cells, but they enter and stain the DNA of dead cells with permeable membranes. A number of fluorescent DNA binding dyes such as propidium iodide, Hoechst 33342, SYTOX Green and CellTox Green are available for determining cell cytotoxicity.

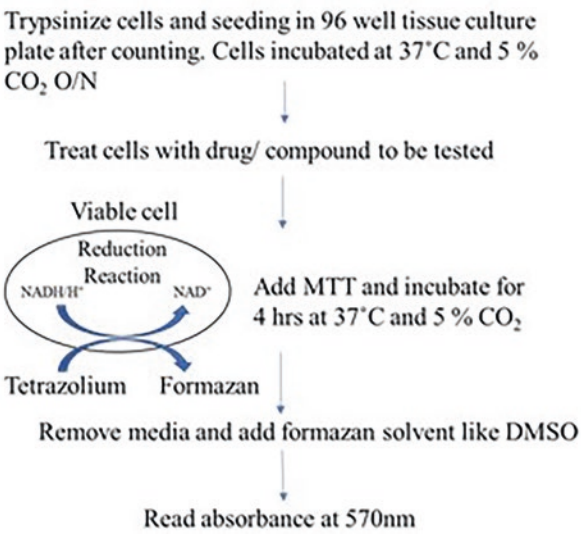

Fig. 19.1 MTT reduction assay

Intestinal cell permeability studies can also be carried out in cell culture models to determine the bioavailability of the tested compounds. Cell culture models such as the Caco2 cells have been developed and used extensively for permeability studies (Rubas et al., 1996). Caco-2 is a human colon adenocarcinoma cell line with well-established tight junctions and capable of undergoing spontaneous enterocytic differentiation. It is further utilized for prediction of absorption of drugs in humans because of its similarity with permeation characteristics of human intestinal mucosa. There is an increased use of Caco-2 cells as a screening tool in the pharmaceutical industry at present. Lewis lung carcinoma-porcine kidney 1 (LLC-PK1) cells and Madin–Darby canine kidney (MDCK) are other cell line models used in permeability studies.

19.6 Stem Cell Research

Animal body is made up of cells, and there are three major types of cells viz germ cells, somatic cells and stem cells. Among these three types of cells, somatic cells constitute the majority of the cells in an adult animal. All somatic cells in their differentiated stage carry their own copy, or copies, of the genome; the red blood cells which are without nuclei are the only exception. Germ cells are gamete producing cells. These cells give rise to either egg or sperm, in case of females and males, respectively (Bongso & Richards, 2004). Whereas, undifferentiated or "blank" cells are regarded as stem cells. This means that stem cells have the ability to differentiate into different varieties of cells that serve different functions in various parts and

activities in the body. This ability of stem cells to differentiate into cell types beyond those of the tissues in which they normally reside is sometimes called stem cell plasticity (Biehl & Russell, 2009). The stem cells capable of not only developing into various cell types but also retaining their proliferative capacity throughout life. Therefore, stem cells are those cells that are capable of dividing indefinitely in culture and possessing the potential to differentiate into mature specialized cell types.

Depending upon the two hallmarks of stem cells, perpetual self-renewal and the ability to differentiate, they can have four possible fates. First, and most often, stem cells remain quiescent (without diving or differentiating), which ensures the maintenance of the stem cell pool. Second, they undergo symmetric self-renewal by cell division and producing daughter stem cells that are identical to their parent cells, thus increasing the number of stem cells. Third, there is asymmetric self-renewal characterized by production of two non-identical daughter cells, one identical copy of the parent and the other which is usually a more specialized somatic or progenitor cell. Fourth, there is production of two daughter cells both of which are different from the parent stem cell. As a result of stem cells taking this (fourth) fate, there is a net loss in stem cell numbers; however, there is an increase in proliferation of differentiated progenies (Biehl & Russell, 2009). With the advancements in stem cells biology, researchers have proposed some of the important attributes of "stemness" as well as the possible fates of stem cells. Some of the important attributes include (i) active Janus kinase signal transducers and activators of transcription, TGFβ and Notch signalling; (ii) sensitivity to growth factors, and integrins-mediated interaction with extracellular matrix; (iii) engagement in the cell cycle, which can be either arrested in G1 or cycling; (iv) remarkable resistance to stress with up-regulated DNA repair, protein folding, ubiquitination and detoxifier systems; (v) DNA helicases, DNA methylases and histone deacetylases regulated chromatin remodelling and modification and (vi) regulation of translation by Vasa type RNA helicases, thus regulating the Germ Cell Development and Reproductive Aging (Bongso & Richards, 2004).

There are several types of stem cells, and they are classified according to their tissue of origin viz embryonic, adult, cord blood and amniotic fluid stem cells. Embryonic stem (ES) cells are derived from 3 to 5 days old embryos. ES cells can give rise to virtually any other type of cell in the body. These pluripotent embryonic stem cells are isolated from inner cell masses (ICM) of mammalian blastocysts and cultured in a flask over a layer of "feeder" cells, which provide various factors essential for proliferation and sustaining their pluripotency. ES cell lines are developed by continuous in vitro subculture and expansion of an isolated ICM on an embryonic fibroblast feeder layer. Adult stem cells are derived from developed tissues and organs of the body. They are also known as somatic stem cells owing to the fact that they are derived from somatic cells/tissues. Unlike embryonic stem cells, adult stem cells are multipotent (not pluripotent). However, the term "adult" stem does not necessarily imply that it is originated from an adult; it can also be found in newborns and children. These cells have been extensively studied for over 50 years

and have been found to play a major role in the repair and replacement of damaged tissue in the same area where they are present. A popularly studied adult stem cell is the haematopoietic stem cell (HSC), which forms the basis of successful bone marrow transplantation. Cord blood stem cells are derived from the umbilical cord. These cells are harvested and cryopreserved in cell banks for use in the future. These cells have been utilized to successfully treat children with blood cancers, such as leukaemia, and certain genetic blood abnormalities. Stem cells have also been discovered and isolated from amniotic fluid. Amniotic fluid surrounds the developing foetus inside the pregnant female's uterus. Presently, the study and application of amniotic fluid stem cells is only at its nascent stage; further research is required to understand its potential use.

With recent advancements in stem cell biology and biotechnological methods scientists are now able to transform adult stem cells into pluripotent stem cells, which are otherwise only multipotent. This transformed pluripotent adult stem cells are known as induced pluripotent stem cells (iPSCs) (Bacakova et al., 2018). iPSCs are created by genetic reprogramming of adult stem cells (known as de-differentiation) so that they behave like embryonic stem cells. Thus, iPSCs can differentiate into all types of specialized cells in the body, and potentially produce new cells for any organ or tissue.

The practice of medicine as well as other biological studies has been revolutionized by the growing knowledge of regenerative medicine and emerging biotechnologies. Developments in stem cell biology, both embryonic and adult stem cells, have transformed the prospect of tissue regeneration into an implicit reality (Sylvester & Longaker, 2004). The ability of stem cells to differentiate into various other types of cells has been exploited by scientists to devise various methods for treating and understanding diseases as well as in a variety of clinical applications. Using the perpetual potential of self-renewal, proliferation and differentiation combined with advanced targeted gene transfer technology, stem cells have been used to address numerous heritable gene defects as well as acquired diseases (Biehl & Russell, 2009). Embryonic stem cells treated with specific growth factors can be made to differentiate into specialized cells under suitable conditions. For instance, embryonic stem cells appropriately treated with growth factors, when injected into the brain can be transformed and serve as progenitors for glial cells. Likewise, new myelinated neurons or retinal epithelial cells have been created with the help of pluripotent stem cells for the treatment of animals with acute spinal cord injury or visual impairment, respectively. Similarly, diabetic animals have been treated with stem cells that produce insulin-producing cells which are responsive to blood glucose levels.

As early as the 1960s, multipotent stem cells harvested from bone marrow have been used to treat leukaemia, myeloma and lymphoma. Bone marrow-derived stem cells have been subject of great interest for the treatment of blood cancers because of their potential to differentiate and give rise to lymphocytes, megakaryocytes and erythrocytes. Also, the application of stem cells in the treatment of other diseases

has been extensively explored (Kimbrel & Lanza, 2020). For example, mesenchymal stem cells that develop into bone and cartilage have been successfully used for the development of whole joints in murine models. Besides these, there have been remarkable research and application of stem cell research, some of which can be listed as below:

(i) Correction of disorders that are associated with loss of normal cells (functions) like Alzheimer's disease, Parkinson's disease and diabetes
(ii) Engineering, in vitro culture and replacement of damaged tissues or organs
(iii) Study model for developmental biology
(iv) Studying the causes of genetic defects in cells
(v) Elucidating disease pathogenesis as well as in cancer biology in understanding mechanism of normal cells developing into cancer cells
(vi) Discovery of new drug(s) as well as drugs safety and efficacy testing.

Besides having tremendous applicability and potential, the use of pluripotent and multipotent stem cells has their specific limitations. Pluripotent stem cells have therapeutic advantages over multipotent stem cells provided the fact that the former can become any cell type and have higher cell prolificacy (Liu et al., 2019). However, the requirement of immune-suppressive therapy entails the use of pluripotent stem cells, as these cells are not from the host and therefore trigger the host's immune response (graft-rejection). On the other hand, such rejection immune response is (theoretically) non-evident in the case of host-derived multipotent stem cells. In this context, the possibility to utilizing host's own cells has a great advantage; the immune system recognizes specific surface proteins/markers on these own multipotent stem cells and no graft-rejection immune response is developed. Contamination with animal-derived culture medium also causes serious issues leading to immunological complications. Besides these, there are various other practical and technical limitations like hurdles in manipulation and differentiation, which are also associated with stem cells culture and their therapeutic and clinical applications.

One of the major concerns in stem cell research is ethical, religious, legal and political issues. With the rapid growth and advancements in stem cell research, there is also parallel growth in various agendas and debates against it (Bacakova et al., 2018). Many people oppose the use of embryos for therapeutic purposes based on their beliefs and genuine ethical concerns. Governments in various countries banned the allocation of public money for research on human embryonic stem cell. The present rules and regulations governing study and research on stem cell also vary in different countries or societies. For clear ethical reasons, studies and experimentations involving embryonic cells, blastocyst manipulation, foetal material, foetal stem cells, ectopic grafting, etc. have generated a great deal of public interest (Bongso & Richards, 2004). Consequently, a majority of the population are still not ready to accept this cell-based therapy. In this context, it may take few more years before a scientifically-, legally- and ethically approved stem cell research application for the welfare of both humans and animals becomes a reality.

19.7 Cellular Development and Differentiation

Stem cells possess the remarkable property of self-renewal, and can simultaneously transform into more lineage-committed cells. Pluripotent stem cells (PSCs) being multipotent can give rise to all mature body cells. Human PSCs (hPSCs) were first obtained from human blastocysts and named as embryonic stem cells (ESCs) (Thompson, 1998). Further, the creation of "induced pluripotent stem cells", or iPSCs, from human fibroblasts in 2007, gave rise to the possibility of developing any cell type for regenerative medicine. In addition to regenerative medicine, the availability of human ESCs (hESCs) and iPSCs contributes to a better understanding of human development and the creation of models for the study of human disorders. Cellular differentiation especially of hESCs, which are multipotent with potential to generate any differentiated progeny, is an alluring area of study for regenerative medicine, transplantation therapy and tissue engineering. In order to reach this potential, it is imperative to control ESC differentiation and direct their development along specific pathways.

For induction of PSCs to differentiate, scientists have utilized the information available for development. Initially, differentiation comprises of the development of embryoid bodies, which undergo spontaneous differentiation as a result of signals arising from the various cell populations. For better control of the differentiation process researchers have utilized the step-wise addition of compounds such as growth factors and cytokines, or inhibitors. This controlled differentiation has given rise to a number of cell types like cells resembling cardiac muscle (Burridge et al., 2014), neural subpopulations (Shaltouki et al., 2013), or hepatocytes (Roelandt et al., 2013) and several others. Although, these cells often resemble foetal tissue rather than adult tissue in majority of cases.

Incomplete understanding of complex events that occur during development processes often make the complete differentiation of PSCs difficult. Modern day approaches to ensure lineage-specific committed differentiation of PSCs include genome editing, chemical engineering of the culture medium, recreating niche for stem cell, precursor, and mature cell growth and differentiation by using microfluidics, 3D culture, using mechanical and electrical stimulation and finally the use of vascular networks in 3D cultures for better access and availability of nutrients, oxygen and endogenous factors.

Present day advances like single-cell RNAseq data in combination with novel genome editing tools, especially the CRISPR technology, have enabled the scientists to program PSC progeny toward specific lineages. Furthermore, libraries of small molecules to identify factors that enhance differentiation have been developed by researchers. An example is differentiation of pancreatic-beta cell from PSCs, by utilizing 20 different molecules for creating insulin-responsive cells (Pagliuca et al., 2014). Recently, researchers are searching for methods such as 3D culture systems with co-culture of different cell types or PSC-derived organoid cultures to develop a "niche", in which committed cell differentiation occurs during development and ultimately giving rise to mature cells as in organs. Tailored hydrogels in 3D culture

along with appropriate addition of growth factors and small molecules can copy the physicochemical properties of the in vivo environment. Apart from these, even electromechanical forces can influence the growth of developing cells similar to forces exerted by organs on their respective cells. Therefore, in numerous studies additional influence of electrical or mechanical stimulation on differentiation of cardiac and neural cells has been studied (Park et al., 2011; Eng et al., 2016).

19.8 Hybridoma Technology

A major technological breakthrough in the field of immunology is the development of monoclonal antibodies (mAb) by hybridoma technology. It is the first, most basic and successful methodology for mAb isolation. This technology has influenced numerous areas of research such as cell biology, medical microbiology, parasitology, biochemistry and physiology. Furthermore, many diagnostic and therapeutic applications of mAbs, as well as their use in the biotechnology industry, are becoming apparent. Köhler and Milstein (1975) opened up new vistas in immunology by demonstrating that somatic cell hybridization could be used to generate hybrid cells capable of growing in culture and secreting antibody molecules of predefined specificities. This noble discovery has been awarded the "Nobel" prize in 1984 for physiology and medicine. The hybridoma technology has already fulfilled the long-lasting desire of immunologists i.e., the routine production of large number of homogeneous antibodies against a wide variety of antigens.

Hybridoma technique involves the immortalization of antibody producing lymphocytes but with liming growth characteristics. The lymphocytes are fused with continuously growing tumour cell line (myeloma) cells so that hybrids produced are immortal like parent myeloma cells and continue to secrete antibodies. This technology involves selection and isolation of a hybridoma producing antibodies with predefined properties, and the mAbs so produced by a clone or family of cells derived from a single progenitor are thus homogeneous in composition (Fig. 19.2). This technology is quite robust and useful for production of mAbs for various applications such as research tools for the identification of specific epitopes of polypeptides, in vitro and in vivo identification and localization of disorders, purification of antigens and therapeutics for either prevention or treatment of diseases (Smith & Crowe Jr., 2015). Easy and efficient production of mAbs is ensured once the hybridoma clones are obtained.

The majority of antibodies in the market today are generated in cell cultures (van Dijk & van de Winkel, 2001). Preference to animal cells is given as they can carry out glycosylation and protein folding, which is essential for a protein to be effective. The CHO cell line is the most commonly used for mAb production. Apart from CHO other cell lines used include murine myelomas NSO, Sp2/0, HEK-93 and BHK. Large-scale industrial production of mAbs is possible in cell suspension culture. Anchorage free cell lines with the capacity offer an easy scale-up option. Sp2/0 and NSO cells can normally grow in suspension, whereas the CHO cells can be easily adapted for the process.

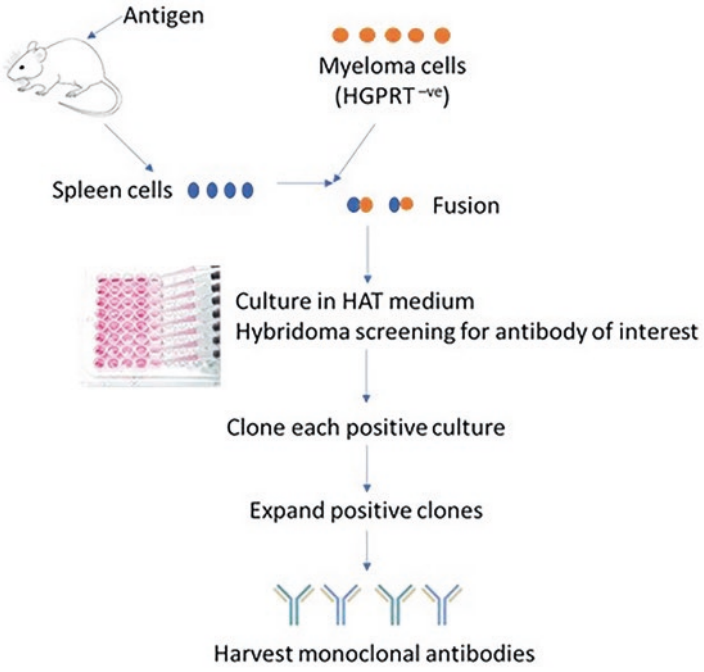

Fig. 19.2 Monoclonal antibody (mAb) production by hybridoma technology

References

Bacakova, L., Zarubova, J., Travnickova, M., Musilkova, J., Pajorova, J., Slepicka, P., et al. (2018). Stem cells: Their source, potency and use in regenerative therapies with focus on adipose-derived stem cells–a review. *Biotechnology Advances, 36*(4), 1111–1126.

Bhatt, A. N., Mathur, R., Farooque, A., et al. (2010). Cancer biomarkers – current perspectives. *The Indian Journal of Medical Research, 132*, 129–149.

Biehl, J. K., & Russell, B. (2009). Introduction to stem cell therapy. *The Journal of Cardiovascular Nursing, 24*(2), 98.

Bongso, A., & Richards, M. (2004). History and perspective of stem cell research. *Best Practice & Research. Clinical Obstetrics & Gynaecology, 18*(6), 827–842.

Burridge, P. W., Matsa, E., Shukla, P., et al. (2014). Chemically defined generation of humancardiomyocytes. *Nature Methods, 11*(8), 855–860.

Caruso, S., Calatayud, A. L., Pilet, J., et al. (2019). Analysis of liver cancer cell lines identifies agents with likely efficacy against hepatocellular carcinoma and markers of response. *Gastroenterology, 157*, 760–776. https://doi.org/10.1053/j.gastro.2019.05.001

Chung, S., Dwabe, S., Elshimali, Y., et al. (2015). Identification of novel biomarkers for metastatic colorectal cancer using angiogenesis-antibody Array and intracellular signaling Array. 1–14. https://doi.org/10.1371/journal.pone.0134948

Dolskiy, A. A., Grishchenko, I. V., Yudkin, D. V. (2020). Cell Cultures for Virology: Usability, Advantages, and Prospects. *International Journal of Molecular Sciences, 21*(21), 7978. https://doi.org/10.3390/ijms21217978

Eng, G., Lee, B. W., Protas, L., et al. (2016). Autonomous beating rate adaptation inhuman stem cell-derived cardiomyocytes. *Nature Communications, 7*, 10312.

Geiger, T., Madden, S. F., Gallagher, W. M., et al. (2012). Proteomic portrait of human breast cancer progression identifies novel prognostic markers. *Cancer Research, 72*, 2428–2440. https://doi.org/10.1158/0008-5472.CAN-11-3711

Huss, R. (2015). Biomarkers. In A. Atala & J. G. Allickson (Eds.), *Translational Regenerative Medicine 21* (pp. 235–241). Academic.

Kainulainen, M. H., Nichol, S. T., Albariño, C. G., & Spiropoulou, C. F. (2017). Rapid determination of ebolavirus infectivity in clinical samples using a novel reporter cell line. *The Journal of Infectious Diseases, 216*, 1380–1385.

Kim, J. H., Skates, S. J., Uede, T., et al. (2002). Osteopontin as a potential diagnostic biomarker for ovarian cancer. *JAMA. 287*(13), 1671–1679. https://doi.org/10.1001/jama.287.13.1671

Kimbrel, E. A., & Lanza, R. (2020). Next-generation stem cells—Ushering in a new era of cell-based therapies. *Nature Reviews. Drug Discovery, 19*(7), 463–479.

Kitaeva, K. V., Rutland, C. S., Rizvanov, A. A., & Solovyeva, V. V. (2020). Cell culture based in vitro test systems for anticancer drug screening. *Frontiers in Bioengineering and Biotechnology, 8*, 1–9. https://doi.org/10.3389/fbioe.2020.00322

Köhler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*, 495–497. https://doi.org/10.1038/256495a0

Kulasingam, V., & Diamandis, E. P. (2008). Tissue culture-based breast cancer biomarker discovery platform. *International Journal of Cancer, 123*, 2007–2012. https://doi.org/10.1002/ijc.23844

Li, H. L., Fujimoto, N., Sasakawa, N., et al. (2015). Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. *Stem Cell Reports, 4*, 143–154. https://doi.org/10.1016/j.stemcr.2014.10.013

Liu, H., Liao, H. M., Li, B., Tsai, S., Hung, G. C., & Lo, S. C. (2019). Comparative genomics, infectivity and Cytopathogenicity of American isolates of Zika virus that developed persistent infections in Human Embryonic kidney (HEK293) cells. *International Journal of Molecular Sciences, 20*, 3035.

Lombardo, T., Dotti, S., Renzi, S., & Ferrari, M. (2012). Susceptibility of different cell lines to avian and swine influenzaviruses. *Journal of Virological Methods, 185*, 82–88.

Michelini, E., Cevenini, L., Mezzanotte, L., & CoppaA, R. A. (2010). Cell based assays: Fuelling drug discovery. *Analytical Biochemistry, 397*, 1–10.

Nakatsu, N., Yoshida, Y., Yamazaki, K., Nakamura, T., Dan, S., Fukui, Y., & Yamori, T. (2005). Chemosensitivity profile of cancer cell lines and identification of genes determiningchemosensitivity by an integrated bioinformatical approach using cDNA arrays. *Molecular Cancer Therapeutics, 4*(3), 399–412.

Pagliuca, F. W., Millman, J. R., Gürtler, M., et al. (2014). Generation of functional human pancreatic β cells *in vitro*. *Cell, 159*(2), 428–439.

Park, S. Y., Park, J., Sim, S. H., et al. (2011). Enhanced differentiation of human neural stemcells into neurons on graphene. *Advanced Materials, 23*(36), H263–H267.

Parker, S., de Oliveira, L. C., Lefkowitz, E. J., Hendrickson, R. C., Bonjardim, C. A., WSM, W., Hartzler, H., Crump, R., & Buller, R. M. (2018). The virology of taterapox virus in vitro. *Viruses, 10*, 463.

Raju, K. L., Augustine, D., Rao, R. S., et al. (2017). Biomarkers in tumorigenesis using cancer cell lines: A systematic review. *Asian Pacific Journal of Cancer Prevention, 18*, 2329–2337. https://doi.org/10.22034/APJCP.2017.18.9.2329

Riss, T. L., Moravec, R. A., Niles, A. L., et al. (2004). Cell viability assays. *Assay Guid Man*, 1–25.

Roelandt, P., Vanhove, J., & Verfaillie, C. (2013). Directed differentiation of pluripotent stemcells to functional hepatocytes. *Methods in Molecular Biology, 997*, 141–147.

Rubas, W., Cromwell, M. E., Shahrokh, Z., Villagran, J., Nguyen, T. N., Wellton, M., Nguyen, T. H., & Mrsny, R. J. (1996). Flux measurements across Caco-2 monolayers may predict transport in human large intestinal tissue. *Journal of Pharmaceutical Sciences, 85*, 165–169.

Şahin, Ş. H. T., Mesut, B., & Özsoy, Y. (2017). Applications of cell culture studies in Pharmaceutical technology. *ACTA Pharmaceutica Sciencia, 55*(3), 63.

Shaltouki, A., Peng, J., Liu, Q., et al. (2013). Efficient generation of astrocytes from human pluripotent stem cells in defined conditions. *Stem Cells, 31*(5), 941–952.

Shoemaker, R. H. (2006). The NCI60 human tumour cell line anticancer drug screen. *Nature Reviews. Cancer, 6*(10), 813–823.

Sittampalam, G., Coussens, N., Arkin, M., et al. (2016). Assay guidance manual. *Assay Guid Man, 11*, 305–336.

Smith, S. A., & Crowe, J. E., Jr. (2015). Use of human hybridoma technology to isolate human monoclonal antibodies. *Microbiology Spectrum, 3*(1), AID-0027-2014. https://doi.org/10.1128/microbiolspec.AID-0027-2014. PMID: 26104564; PMCID: PMC8162739.

Strimbu, K., & Tavel, J. A. (2010). What are biomarkers? *Current Opinion in HIV and AIDS, 5*(6), 463–466. https://doi.org/10.1097/COH.0b013e32833ed177. PMID: 20978388; PMCID: PMC3078627.

Sylvester, K. G., & Longaker, M. T. (2004). Stem cells: Review and update. *Archives of Surgery, 139*(1), 93–99.

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145–1147.

van Dijk, M. A., & van de Winkel, J. G. J. (2001). Human antibodies as next generation therapeutics. *Current Opinion in Chemical Biology, 5*, 368–374. https://doi.org/10.1016/S1367-5931(00)00216-7

van Staveren, W. C. G., Solís, D. Y. W., Hébrant, A., et al. (2009). Human cancer cell lines: Experimental models for cancer cells in situ? For cancer stem cells? *Biochimica et Biophysica Acta, 1795*, 92–103.

Verma A, Verma M,Singh A. (2020). Animal tissue culture principles and applications. In *Animal biotechnology* (pp. 269–293). Academic

Wang, H., Zhang, G., Wu, Z., et al. (2015). MicoRNA-451 is a novel tumor suppressor via targeting c-myc in head and neck squamous cell carcinomas. *Journal of Cancer Research and Therapeutics, 11*, 216.

Zhu, M. M., Mollet, M., Hubert, R. S., et al. (2017). *Handbook of industrial chemistry and biotechnology*. Springer.