Delineating the role of oncology research models for the development of diagnostic and therapeutic approaches for cancer.

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Abstract

Cancer causes the second highest mortality rate after the cardiac diseases in humans. In addition to death rates, cancer morbidity paints a rather grim picture. Despite the advances in treatment modalities, cancer management still remains as a challenging task. In vitro biological evaluations use cytotoxicity as one of the most essential indications. In vitro, cytotoxic drugs cause cell membrane disintegration, protein synthesis inhibition, and irreversible receptor binding, among other things. To identify cell death caused by these insults, a number of short-term cytotoxic effects and cell proliferation assays have been developed and employed. Chemical carcinogenesis and xenograft models for imitating human malignancies are among the in vivo models that have been developed. This article discusses the current in-vitro and animal models used in the discovery and therapeutic efficacy assessment of anti-cancer drugs.

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Abstract: Cancer causes the second highest mortality rate after the cardiac diseases in humans. In addition to death rates, cancer morbidity paints a rather grim picture. Despite the advances in treatment modalities, cancer management still remains as a challenging task. In vitro biological evaluations use cytotoxicity as one of the most essential indications. In vitro, cytotoxic drugs cause cell membrane disintegration, protein synthesis inhibition, and irreversible receptor binding, among other things. To identify cell death caused by these insults, a number of short-term cytotoxic effects and cell proliferation assays have been developed and employed. Chemical carcinogenesis and xenograft models for imitating human malignancies are among the in vivo models that have been developed. This article discusses the current in-vitro and animal models used in the discovery and therapeutic efficacy assessment of anti-cancer drugs.

Key words: - Anticancer, cancer, cell viability, cytotoxicity,*in vitro* , *in vivo*

Contents

Introduction

Cancer causes the second highest mortality rate after the cardiac diseases globally[1]; rose to 19.4 million new cases and 10.0 million deaths in 2020. Deaths from cancer worldwide are projected to reach over 13 million in 2030[2]. There are 36 different types of cancer to affect men in the form of colorectal, liver, lung, prostate, and stomach cancer and women in the form of breast, cervix, colorectal, lung, and thyroid cancer [3]. Cancer treatment is a challenging task due to the intrinsic mechanisms of resistance development towards therapeutic approaches.

Conventional treatment modalities for cancer management include chemotherapy, radiation therapy, and surgery[4].The Radiation therapy (usually called radiotherapy) is a type of cancer therapy intended to kill cancer cells and to decrease tumours by using intense radiation doses. Radiotherapy not only kills or slows the growth of cancer cells, but it can also harm healthy cells nearby. Damage to healthy cells can have unintended consequences. Adverse effects of radiotherapy include fatigue, nausea, abdominal discomfort etc[5].

Surgery is the commonest treatment modality used for localized tumors. This approach is associated with several side effects such as pain, discomfort, bleeding and surgical-wound complications[5]. Chemotherapy (often called chemotherapy) is a cancer therapy which uses drugs to kill cancer cells. Chemotherapy does not only kill or reduce cancer cell growth, it also kills or slows healthy cell growth. The side effects of cell damage include mouth sores, nausea and hair loss[6].

Major risk factors contributing to the carcinogenesis process include genetic predisposition, alcohol and tobacco consumption, sedentary lifestyle/lack of physical activity, dietary habits, environmental influence, exposure to biological carcinogenic agents (EBV, HPV etc.) and exposure to various physical and chemical carcinogenic molecules [7].

Carcinogens may directly or indirectly (metabolic activation) be converted to electrophilic molecules that interact with DNA. Depending of the cell injury level (formation of DNA-adducts), different ways of cellular response may occur; with low damage level, cells can be reverted back to normal by DNA repair, while with excessive damage, cells can undergo programmed cell death. However, under cellular replicative pressure, damaged DNA allows cell mutation, thus initiated cells carry permanent and heritable DNA changes that may predispose to cancer development(Figure 1).

Figure 1: Pathogenesis of cancer progression

The carcinogenesis progression includes three stages i.e.initiation, promotion and progression. The genotoxic carcinogen produces initiated cells with a mutant genotype;during promotion these cells stimulate to proliferate by tumor promoting carcinogens to form clusters of initiated cells, the formed lesion is further predisposed to progress into a cancer, but, additional exposition to genotoxic and tumor promoting substances accelerates progression stage by increasing genomic instability and cell proliferation rate to convert a preneoplastic lesion into cancer.

In vitro and in vivo animal models are significant tools in cancer research as they allow to identify carcinogens, screen chemotherapeutic agents help to trace and molecular pathways of tumour growth and spread. Cancer cells migrate or flow across a variety of micro environments throughout the metastatic process, including the stroma, blood vessel endothelium, the vascular system, and tissue at a secondary location. Thus, in this current review we have focused on the advanced models which can be cancer implanted research.

Cell viability assays & cytotoxicity study

The different types of cytotoxic and cell viability assay include (a) dye exclusion assay such as Trypan blue, erythrosine B assay, (b) colorimetric assay such as MTT assay, MTS assay, LDH assay, SRB assay, NRU assay & crystal violet assay, (c)fluorometric assay such as Alamar Blue assay and CFDA-AM assay, and (d) ***luminometric assay*** such as ATP assay &areal-time feasibility assay.

2.1 Dye exclusion assays

Multiple methods can be implemented to determine the fraction of viable cells in a cell population. The dye exclusion method is the simplest and most often utilised method. This method excludes living cells, but it does not exclude dead cells. The staining procedure isconvenient, and though it is difficult and time consuming to perform the test on a large number of samples[8]. The dye exclusion method can be used to determine membrane integrity. eosin, Congo Red, Erythrosine B, and Trypan Blue are among the dyes that have been use[9]. Trypan blue has been used the most frequently of the dyes described[10].

Here in the following issues need to be considered while utilising dye exclusion testing that includes; (a) cells harmed by cytotoxic chemicals may lose membrane integrity over several days, (b)survivor cells can continue to develop during this period & (c) at the end of the growing phase, certain deadly damaged cells may not be labelled as dye since they dissolve too fast.

For chemo sensitivity testing, dye exclusion tests have distinct advantages. They are quite basic, use a minimal number of cells, are fast, and can detect cell viability in nondividing cells.More investigation into the assays' potential value in chemosensitivity assessment is needed[11]. Next, these dyes are designed for use on cells in suspension rather than monolayer cell cultures, hence monolayer cells should be trypsinized before dye exclusion experiments may be performed[9].

2.1.1 Trypan blue dye exclusion assay

To assess the percentage of living and/or dead cells in a cell suspension, the trypan blue dye exclusion experiment is commonly employed. trypan blue, molecular weight of 872.88, is a large and negatively charged. This is predicated on the notion that living cells have intact cell membranes. In this test, adherent or non-adherent cells are cultured for varying amounts of time with varying dilutions of the test substance. The cells are washed and resuspended in medium following the chemical treatment. Dye is added to the cell suspension, loaded onto cell counters (haemocytometers/automated counters) and then microscopically examined to determine whether cells take up or exclude dye. Viable cells possess a clear cytoplasm, whereas dead cells have a blue cytoplasm, as a results of dye permeating the compromised cell membrane and entering into the cytoplasm[12][13]. The amount of active & dead cells/ unit volume is compared to untreated control cells using light microscopy

This method is advantageous since it is simple to apply, inexpensive, and an excellent indicator of membrane integrity. If deceased cells are exposed to the dye, they become blue in seconds[14].The drawbacks of this technique include counting mistakes, as cell counting is often performed with a haemocytometer[20,]. Poor cell dispersion, cell loss during cell dispersion, inaccurate cell dilution, insufficient chamber filling, and the presence of air bubbles in the chamber could all contribute to counting errors[14].However, the staining technique is simple anda large number of samples can be handled if it is regained to assess cytotoxic effects escalate. In addition, the black trypan of differentiate the healthy and impaired cells[8]. Hence it is not sufficiently sensitive to measure *in vitro* cytotoxicity. Another drawback of trypan blueis that, if exposed to prolonged durations, is that it has harmful side effects on mammalian cells[16].

2.1.2 Erythrosine B dye exclusion assay

Erythrosine B is largely used an food colouring additive. It is also known as erythrosine or Red No. 3. Erythrosine B is an essential dye used to count live cells[17]. The basis of this colour exclusion test is similar to the trypan blue colour exclusion test. Although erythrosin B is a bio-safe vital dye for cell counts, calculating the amount of live and dead cells is not a frequent practise. Low cost, adaptability, and bio-safety are just a few of the benefits of the test[16].The time and labour-intensive nature of this method are its drawbacks. Furthermore, impurity of the reusable cell counting chamber, differences in haemocytometer filling rates, andinter-user variances are also potential drawbacks[16].

2.2 Colorimetric assays

The colorimetric tests are based on the detection of a biochemical marker to evaluate cell metabolic activity. Colorimetric test reagents change colour in response to cell viability, letting for colorimetric cell viability assessment with a spectrophotometer. Colorimetric tests can be used with adherent or suspension cultures,

are simple to perform, and are relatively inexpensive [18]. Colorimetric test kits are available from a variety of suppliers, and most experimental protocols for these assays are included in kit packages.

2.2.1 Methyl Thiazolyl Tetrazolium (MTT) assay:

One of the most popular and widely used colorimetric techniques for assessing cell viability or cytotoxicity is the (3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide) MTT assay. This assay primarily measures cell viability by measuring the activity of succinate dehydrogenase in cells and determining mitochondrial function[11]. In this test, NADH reduces MTT to a purple formazan, which is solubilized in DMSO and measured by light absorbance at 570 nm. The cytotoxicity of the test substances is calculated in comparison with untreated cell control[14]. Since it is simple, safe, and reproducible, this method outperforms the dye exclusion methods listed above.

The insolubility of formazan in water results in the production of crystals of purple shaped needles in the cells; is one of the principal drawbacks of the MTT assay. The crystals are solubilized by an organic solvent such as dimethyl sulfoxide (DMSO) or isopropanol prior to absorbance measure. The cytotoxicity of formazan makes it tough to remove cell culture fluid from platform wells because of the presence of floating cells with formazan crystals, leading to major errors. Further control tests should be conducted in order to avoid false positive or false negative results from background interference induced by particle inclusion. The cell vitality could be underestimated as a result of this impact.

2.2.2 Methyl ThiazolylSulfophenyl (MTS) assay

The MTS assay (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium, inner salt assay) is a colorimetric test that uses mitochondrial enzymes to convert a tetrazolium salt into a coloured formazan. The amount of formazan produced is proportional to the number of live cells in the culture and can be determined using a colorimetric method at 492 nm. This test effective in terms of ease of use, precision, and rapid toxicity indication. The MTS assay is a rapid, sensitive, low-cost, and selective in vitro cytotoxicity assay. As a result, it can be utilised for on-site toxicological evaluations. The absorbance reported at 492 nm is affected by the incubation period, cell type, and cell counts, as well as the proportion of MTS detection reagents to cells in culture, which is one of the assay's limitations. Previous investigation reports have a linear association between incubation time and absorbance for up to 5 hours, with 1-3 hours being the optimal length [19].

2.2.3 Sulforhodamine B (SRB) assay

This assay is a colorimetric approach for evaluating drug-induced cytotoxicity in adherent and suspension cell cultures that is quick and accurate. Skehan and colleagues developed this assay for use in the National Cancer Institute's (NCI) disease-oriented, large-scale anticancer drug development initiative, which started in 1985. SRB is a vivid pink amino xanthene dye containing two sulfonic groups. The SRB binds to basic amino acid residues in trichloroaceticacid fixed cells under slightly acidic circumstances, providing a sensitive assessment of cellular protein. The SRB assay is used to assess colony formation and extinction when certain test substances are supplied[19]. The SRB test can be used easily, quickly and accurately. It has strong cell numbers linearity, saturates dye levels, is less susceptible to environmental variation, is independent of intermediate metabolism, is stationary, requires no time-consuming initial reaction speed measurements, and is less sensitive to environmental fluctuations[19].This assay has certain limitations in terms of the formation of cell clumps/clusters affecting the homogeneity of the cultures, leading to inappropriate endpoint measurements.

2.2.4 Lactate dehydrogenase (LDH) assay

The LDH cytotoxicity assay is a colorimetric method for determining cellular cytotoxicity that may be used with a variety of cell types to analyse cytotoxicity caused by harmful chemicals and other test substances as well as cell-mediated cytotoxicity. The assay quantifies the stable, cytosolic LDH enzyme, which is released from injured cells and evaluated via a linked enzymatic reaction that culminates in the diaphorase conversion of iodonitrotetrazolium (INT) into a red-colored formazan. LDH catalyses the conversion of lactate to

pyruvate in the first phase of the process, reducing NAD to NADH/H+. The catalyst (diaphorase) then transfers H/H+ from NADH/H+ to the tetrazolium salt w2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), which is reduced to red formazan in the second. Over a set time period, LDH activity is measured as NADH oxidation or INT reduction. Red fluorescence generated from this can be measured via 490 nm. In the LDH experiment, the detergent Triton X-100 is usually used as a positive control to measure the maximum LDH release from the cells. Positive controls can also be utilised, such as crystalline silica, which is a well-known membranolytic particle[20]. This test has several advantages, including reliability, rapidity, and ease of evaluation.

The fact that blood and several other medium components have inherent LDH activity is a major drawback of this test. The background measurements in foetal calf serum, for example, are extraordinarily high. As a result, this assay is limited to serum-free or low-serum conditions, limiting the assay's culture length and breadth because it can no longer detect cell death generated under usual growth conditions with 10% foetal calf serum in the media. Readings from additive-free media, such as incomplete DMEM, should always be compared to readings from an unused section of the study medium[21].

2.2.5 Crystal violet (CV) assay

The crystal violet assay is described as the separation of adhering cells during apoptosis and can be used to indirectly detect cell death as well as toanalyse changes in proliferation rate in response to cytotoxic medications. In this experiment, crystal violet dye binds to the proteins and DNA of living cells, colouring the cells that are joined. During cell death, cells lose their adherence and are removed from the population, reducing the quantity of crystal violet staining in a culture. The crystal violet test is a quick and easy way to see how chemotherapeutics and other chemicals affect cell survival and growth inhibition[22].

Crystal violet staining is a simple and adaptable approach for determining cell viability in response to a variety of stimuli[23]. Proliferative responses that occur concurrently with cell death responses, on the other hand, may compromise it. Chemical caspase inhibitors and/or necroptosis inhibitors could be used in the experiment. Alternatively, to understand more about the reasons of cell death, molecular investigations (e.g., overexpression or knockdown) can be used[24].

The insensitivity of the crystal violet assay to vary in cell metabolic activity is a source of concern. As a result, this test is not suitable for research involving chemicals that influence cell metabolism. The crystal violet assay can be used to investigate the effects of chemotherapeutics and other medications on cell survival and growth inhibition, however it cannot be used to assess cell reproduction speed[24].

2.3 Fluorometric assays

The fluorometric studies for measuring cell viability and cytotoxicity are simple to execute and offer significant advantages over traditional dye exclusion and colorimetric investigations when performed with a fluorescence microscope, fluorimeter, fluorescence microplate reader, or flow cytometer. For adherent or suspension cultures, fluorometric tests can be used. Colorimetric tests are less sensitive than these assays(38). Several manufacturers offer commercial fluorometric test kits, and most experimental techniques for these tests are included in kit packages.

2.3.1 Alamar Blue (AB) assay

The resazurin reduction assay is also termed as alamar Blue assay. The mitochondrial enzymes convert the blue nonfluorescent dye resazurin into the pink fluorescent resoruin in this experiment[27]. A resazurin is a cell permeable redox indicator and an aphenoxazin-3-one dye that may be used to determine viable cell numbers. It is well understood that using molecular oxygen as an electron acceptor in the electron transport chain acts as an intermediate electron acceptor between the final reduction of oxygen and cytochrome oxidase[28]. This material is non-fluorescent and blue in colour. Resazurin is converted to resorufin, a red-colored and highly luminous molecule, when it penetrates cells[25]. Resazurin is converted to resorufin by viable cells, which increases the fluorescence and colour of the cell culture medium. The production of resorufin is proportional to the number of viable cells. A microplate reader fluorimeter and a 560 nm excitation/590 nm emission

filter combination can be used to calculate the ratio of living cells.The changes in absorbance can also be used to detect resorufin, but this method is less sensitive than fluorescence detection. The amount of time it takes to obtain a meaningful fluorescence signal above the background depends on the metabolic activity of the cells, the cell density per well, and other factors including the type of growth media[26]. Compared to tetrazolium tests, the alamar blue (resazurin reduction) assay has multiple advantages, including lower cost and higher sensitivity. It can also be used with other approaches to understand more about the cytotoxicity process, such as identifying caspase activity. Fluorescence interfering from the test substances, as well as the commonly missed direct harmful effects on the cells generated by this fluorescence interference, are also drawbacks of the assay[26].

2.3.2 5-carboxyluorescein diacetate, acetoxymethyl ester (CFDA-AM) assay

5-carboxyluorescein diacetate, acetoxymethyl ester (CFDA-AM) is a fluorogenic dye used to assess cytotoxicity. It is a measure of the integrity of the plasma membrane. As a harmless esterase substrate, the dye CFDA-AM can be transformed from a membrane permeable, nonpolar, nonfluorescent material to a polar, fluorescent dye, carboxyfluorescein, by nonspecific esterases found in live cells (CF). Because the cytoplasmic environment required for esterase activity can only be maintained by an intact membrane, the cells' conversion of CFDA-AM to CF implies plasma membrane integrity[29].This assay has the advantage of being relatively harmless to cells, requiring minimal incubation time, and being detectable at different wavelengths without interference.The main constraint of this assay is fluorescent interference from test chemicals, which affects the endpoint readings.

2.4 Luminometric assays

Cytotoxicity and cell proliferation in mammalian cells may be determined quickly and easily using luminometric techniques. These tests can be carried out in a 96-well or 384-well microplate format & detected using a luminometric microplate reader. The persistent and steady glow-type signal produced following reagent addition is a distinguishing feature of luminometric tests. This property can be used to generate both cytotoxicity & viability values from the same well[30]. The commercial luminometric assay kits are sold by a number of businesses, and the experimental methods for these assays are usually included with the kit packages.

2.4.1 Adenosine triphosphate (ATP) assay

The ATP is the most important chemical energy store in cells and it is used for biological production, signalling, transport, and movement. As a result, one of the most sensitive biomarkers of cell viability is cellular ATP[31]. If the integrity of their membranes is compromised, their ability to synthesise ATP is hindered, and cell ATP levels decrease. The ATP assay is based on the activation of luciferin to oxyluciferin. The enzyme luciferase catalyses this activity in the presence of Mg^{2+} ions and ATP, resulting in the bright signal. The bright signal's strength is related to the amount of ATP[32] or the number of cells[33]. The ATP test chemical is often used in the 1536-well plate format since it can detect fewer than 10 cells per well.

Comparedto other viability assays, the ATP assay is the quickest, most sensitive, and least susceptible to artefacts. Within 10 minutes of adding the reagent, the luminous signal reaches a steady state and stabilises. The substrate is converted into a colourful compound without the need for an incubation stage. This also avoids the need to handle plates[26].The sensitivity of the ATP assay is typically limited by the reproducibility of pipetting duplicate samples rather than the test's chemistry[26]

2.4.2 Real-time viability assay

A new approach for counting viable cells in real time has just been developed. This test uses a modified luciferase from a marine shrimp, as well as a small molecule pro-substrate. The pro-substrate and luciferase are immediately combined in the cell culture medium. The pro-substrate is converted to a substrate by viable cells with active metabolism, which is then utilised by luciferase to generate a strong signal. The test has two options i.e. continuous reading and endpoint measurement. The bright signal from the sample wells

can be repeatedly recorded over a long period of time in the continuous read format to measure the number of cells in "*real time* ".

This assay is advantageous since it is the only one that can detect cell viability and cytotoxicity in real time. Since this luminous signal declines quickly after cell death, this test can be multiplexed with other luminous assays that include a lysis step that disrupts the cells. To avoid interference in future luminous experiments, the fall in brightness following cell death is crucial.

The drawback of the real-time test is that metabolically active cells would eventually deplete the pro-substrate. The total number of metabolically active cells determines the amount of strong signal produced. The length of time the brilliant signal is linear with cell number is determined by the number of cells per well as well as their metabolic activity. As a result, for each cell type and seeding density, the maximum incubation period required to maintain linearity should be determined empirically.

Apoptosis assay (Annexin V-FITC)

This assay is performed by seeding the appropriate cell type and treatment with the test compounds (in serum free media) in their IC_{50} concentration for the predetermined time-points at 5% CO_2 and 37 *C in a humidified atmosphere. Untreated cells will be used as a negative control. Post-treatment with the test compounds, cells are harvested by trypsinization followed by PBS washing to clear off the cell debris. This step is followed by the addition of Annexin V-FITC, mixing and incubation in dark. The cells are then resuspended in binding buffer and incubated with propidium iodide (PI). The cells are analysed by flow cytometry[34]

Brine Shrimp Lethality Bioassay

The brine shrimp lethality assays are used to measure the cytotoxicity of the test compounds[35]. Brine shrimp eggs (*Artemia salina*) are used in this experiment, which are incubated for 48 hours in a tank containing sterile artificial seawater at 28–30^0 C with good aeration (using an air pump) and continuous light (60 W lamp). Brine shrimps are placed in each well containing seawater after nauplii are collected with a Pasteur pipette. Individual vials are filled with 5 mL of seawater containing 10 nauplii shrimps, and test solutions of varied concentrations are generated using the serial dilution technique with seawater. The number of nauplii that survived in each vial is counted after the vials have been viewed with a magnifying glass for 24 hours. The data is used to calculate brine shrimp nauplii % mortality for control and increasing concentrations, as well as LC_{50} values. Potassium dichromate is used as a reference standard[36].

Although patient-derived xenograft (PDX) models can preserve the integrity of original tumours after quick transfer, in vitro and in vivo approaches frequently include deconstruction and, in some circumstances, reconstruction of the original tumours for subsequent medication response assessment. Ex vivo approaches, such as patient-derived explant (PDE) models, are used to assess therapy efficacy in fresh tumour tissues collected without deconstruction or reconstruction. In each model system, the cell types available for derivation and application are schematically illustrated. The use of mouse models is very much beneficial in the discovery of cancer drugs. To find and verify novel cancer pathways and therapeutic targets, characterise in vivo drug pharmacokinetics and pharmacodynamics (PK/PD), and assess in vivo anti-cancer efficacy of proposed treatments, preclinical research in mice cancer models is required. Phase I–III clinical studies are done to examine the safety and anti-cancer effectiveness of these medications in human patients when promising preclinical results are acquired. Due to inherent or resistant mechanisms, a small percentage of patients will have a poor response, which can be examined mechanistically in preclinical mice models to uncover response biomarkers and combination therapies to avoid or overcome resistance. Improved patient classification, identification of novel biomarkers, and development of appropriate combination medicines will arise from the close alignment of mice studies with human clinical trials, resulting in better cancer patient care (figure 2).

Fig-2: Preclinical Cancer models

In vivo **models of cancer activity**

Spontaneous tumour models, virus-induced tumour models, radiation-induced tumour models, chemically induced tumour models, transplantable tumour models, syngenic models, and xenogenic models are among the *in vivo* models used to assess anticancer activity.

5.1 Spontaneous tumor models: It includes animals with a high natural cancer rate, such as mice from specific inbred strains, which are more prone to developing cancers such as breast cancer, leukaemia, hepatomas and pulmonary adenomas[37]. The endometrial cancer kills more than 60% of female rats in the DA/Han strain. Endometrial cancer kills 87 to 90 percent of BDII/Han rats[38]. These models most closely match the clinical condition, as they have kinetics and antigenicity that are similar to human malignancies.

A significant number of tumours of equal size cannot be obtained at a time for screening purposes. The tumours usually become detectable only later in their course, and the metastatic distribution is rarely consistent, making proper staging difficult. These models are rarely replicable, and the vast majority are found to be viral in nature. However, while such tumours provide a thorough test of anticancer efficacy, they are rarely used for primary screening. They are crucial in cancer and carcinogenesis research at the molecular level[38].

5.2 Virus induced Tumor Models: The Friend leukaemia and Rous sarcoma are the widely and most prevalent virus-induced cancers. However, these models are rarely used in drug development[38]

5.2.1 Friend leukaemia: The Friend found this tumour in adult Swiss mice for the first time. By injecting cell-free filtrates of leukemic –spleen homogenates into other mice, it can be spread to other mice. Various evaluation metrics include preventing splenic weight increase, lowering the titer of live virus (as determined by bioassay), and extending survival time. The 2 to 4 months' time interval between virus inoculation and the beginning of leukaemia makes evaluation difficult and time-consuming, preventing these models from being employed in antitumor screening studies.

5.2.2 Rous sarcoma: Rous was the first to report this tumour in young chicks. It can be spread by implanting tumour pieces or inoculating tumour homogenates with cell-free material. The most often used evaluation measures are tumour growth inhibition and survival time. The tumour growth can be more easily assessed because it is a localised tumour. However, because it is insensitive to a wide range of agents, it is possible that it will overlook the action of essential molecules.

5.3 Radiation-induced tumors: UV radiation is known to cause cancer. This fact is utilised to generate tumor in laboratory animals by exposing them to specific quantities of radiation, especially in case of skin cancers. Radiation is sometimes employed in conjunction with other chemical agents such as TPA or DMBA. The UV-induced skin carcinogenesis in the SKH-1 hairless mouse and two-stage skin tumorigenesis models are thetwo examples of radiation-induced tumour models. One advantage of these models is that tumours develop on the skin, making them plainly visible. The researcher may be exposed to radiation if radiation is used, which is a serious limitation. Furthermore, the disadvantages of this sort of model include the extended tumour induction period and the time-consuming evaluation parameters. However, this type of model can be used to forecast general activity of anticancer drugbased on the evaluation criteria applied. These models are not in standard programmes for screening.

5.3.1 UV induced skin tumorigenesis in SKH-1 hairless mouse

Over a total dose of 74.85 J/cm^2 UVA and 2.44 J/cm^2 UVB in the 22 weeks, the hairless Inbred (SKH-1) mouse if exposed to UVA and UVB by week 23, radiated mouse acquires on average 16 mouse tumours, with an average of 2.1 mouse carcinomas. The incidence of skin tumours, tumour multiplicity, the decrease of skin papillomas, the onset of early tumour appearance, histological examination of the tumour, and the expression of tumor-related proteins involved in cell cycle regulation are all investigated[39].

5.3.2 Two stage models for skin tumorigenesis:

Tumor induction is performed in this model in two stages: initiation and promotion. A single topical injection of dimethyl benzanthrazene (DMBA) (50 nMol) is used to initiate the treatment, which is then

followed by two weekly UV light treatments (250 mJ/cm^2) for a total of 25 weeks. These models have been used in studies to assess the process of carcinogenesis. Certain test medicines' modes of action can also be investigated. For example, whether the drug can stop or stop the process of start or promotion[40].

5.4 Chemically induced tumors:

The chemical carcinogen-induced tumours originate from the host's own cells, therefore closer than other transplantable neoplasms, closer to the human clinical tumours. The potential effects of the carcinogen on tumour physiology, as well as the risks to other animals and persons posed by the carcinogen and its metabolites released in the chemically induced animal's faeces and urine, are all the boundaries of chemically produced tumours. There are two types of chemical carcinogens:[41]

i. Direct acting agents—carcinogenicity is induced without the need for a chemical transition.

i. Indirect acting agents—agents that only become active after undergoing metabolic conversion. Procarcinogens are another name for them, and ultimate carcinogens are the active end products.

The electrophiles (atoms with a lack of electrons) react with electron-rich atoms in RNA, DNA, and cellular proteins to form both direct-acting and ultimate carcinogens. While RAS gene mutations and TP53 genes have been the most common aims, chemical carcinogen\soutes can impact every gene. In rats, for example, DMBA caused breast cancer and DMAB caused colon cancer. 3,4,9,10 dibenzopyrene induced fibrosarcoma in mice, 3,4 benzopyrene generated spindle cell sarcomas in mice, and 20-methylcholanthrene caused leukaemia and sarcomas in mice.

5.4.1 DMBA induced mammary tumors

The wistar rats aged 50 days were given a single dose of DMBA. The first malignant tumour can be identified after 20 to 30 days of DMBA treatment. Palpation and comparison of a tumor's volume to that of prefabricated plasticine models are used to determine its weight. The tumour weight is determined by multiplying the model weight by a formula that accounts for the weights of plasticine and tumour tissue. The pharmacological treatment begins once the overall tumour mass in the animal has reached around 1 gm. The mitotic index, as well as tumour histopathology, may provide information about the drug's mode of action[42].

5.4.2 DMAB (3, 2-dimethyl-4-aminobiphenyl) induced colon tumors: Male F344 rats are given a DMAB 50mg/kg s.c. injection once a week for 20 weeks to produce tumours. Around 26-30% of animals were fed a low-fat diet, while 74% of animals were fed a high-fat diet, it can induce multiple colon cancers. Adenomas (benign tumours) and adenocarcinomas (cancerous tumours) develop in the large bowel as a result of this (malignant tumors). The variables studied include tumour incidence, tumour size, and histology. The model's limitations include the need for numerous DMAB injections to develop colon cancers. Furthermore, breast adenocarcinomas in female rats, salivary sarcomas, squamous cell carcinomas of the ear duct and epidermis, stomach papillomas, sarcomas and lymphomas, and urinary bladder carcinomas are all inducible. Comparing pharmaceutical reactions becomes more difficult as a result of this[42].

5.4.3 3, 4, 9, 10 Dibenzopyrene induced fibrosarcoma in mice:

The C57BL/6 mice develop tumours after receiving a single subcutaneous injection of 500g of 3, 4, 9, 10 Dibenzopyrene in peanut oil. Within 4 to 5 weeks of treatment, all injected animals develop consistent subcutaneous fibro-sarcomas at the injection site. Tumor weight, histological examination of the tumour, including mitotic index, and other evaluation criteria are included. The model has the benefit of being able to produce tumours with a single dosage of carcinogen. Furthermore, carcinogens do not pass through the intestines or urine and remain in the created tumours, making the animals safer to handle[42].

5.5 Transplantable tumors: These models involve cancer cell lines or tissues that can be produced in mice or rats. Transplantation can be done in two ways.

A. Heterotopic transplantation

B. Orthotopic transplantation

A. Heterotopic transplantation: It includes the transplanting of tumour cells or tissue to a location different than the one where they originated. This procedure usually includes i.p. or s.c. transplantation, depending on whether the tumour proliferates as ascites or a solid tumour. This inoculation method is straightforward and quick. As a result, it is now possible to vaccinate a huge number of animals at once. It also demands a restricted skill set and knowledge.

B . Orthotopic transplantation: It is the process of transplanting tumor cells to the anatomic region or tissue from where a tumour originated. A lung tumour, for example, is transplanted into the lungs. In terms of histology, vascularity, gene expression, chemotherapeutic responsiveness, and spreading behaviour, this technique has produced tumour models that are more similar to human tumours. Orthotopic malignancies are preferable over standard flank (s.c. transplant) models as more is learned about host-microenvironment interaction. The cancer cells can be transplanted orthotopically by (ii) direct injection of tumour cells or (ii) surgical orthotopic implantation (SOI), which is the surgical implantation of entire tumour pieces orthotopically[43].The use of SOI increases the model's repeatability and metastatic result. Furthermore, depending on the origin of the tumour and the host employed, transplantable models can be split into two groups:

5.5.1 Syngenic models: Cancer cell lines or tissues derived from mice or rats (murine) are transplanted into inbred animals with the same genetic background as the cell line or tissue in these models. For example, the L1210 leukemic cell line was derived from a DBA/2 mouse and grown in the same conditions. These are cancer cell lines that have grown on their own or as a result of exposure to toxins. Mice with the same genetic background as the tumours can be implanted with these cell lines or fresh tumour samples. In syngeneic models, the transplanted tissues, tumour microenvironment, and host are all from the same species. This is especially crucial given the tumor's tight relationship with the host. These model systems, on the other hand, lack many of the core features of real tumours. They lack the genetic complexity of human tumours since they are often produced from homozygously inbred mice. Furthermore, they may not have the same constellation of mutations as human patients due to species-specific differences in oncogenesis[44].

5.5.1.1 Leukemia 1210 (L1210): **T** he DBA/2 mouse served as the implantation host. Following the injection of 0.2 percent 20-methylcholanthrene to the epidermis of a female DBA/2 mouse, the tumour developed, which was then transplanted subcutaneously/intramuscularly and obtained in ascitic form[6].(

Intraperitoneal injection of leukemic cells and subcutaneous transplantation of solid tumour fragments into the flank region are two methods of transplantation. Following i.p. inoculation, the peritoneum experiences a period of fast development. Animals are killed in 9-12 days after disseminating in both solid and ascitic forms. The size of the inoculum affects the mean survival time (MST) of the ascitic form. In the pre-screening of test substances, this approach is extremely useful. This model is low-cost and enables for the processing of a large number of compounds. However, it was later discovered that screening for fast increasing leukemic cells skewed selection in favour of drugs that are preferentially active against rapidly growing malignancies. The development of effective medications for solid tumours would almost definitely necessitate a different strategy[6].

5.5.1.2 Lewis Lung Carcinoma model: Dr. Margaret R. Lewis isolated in 1951 from a spontaneous epidermoid cancer of the lung in a C57BL/6 mouse. The subcutaneous transplantation of tumour tissue fragments and anaesthesia-induced injection of tumour cells in suspension into the right main stem bronchus of the lungs are common transplantation procedures. Both of these strategies have resulted in lower metastatic rates[45]. The tumor's metastatic potential is greatly improved by surgical orthotopic transplanting of tumour fragments or injection of tumour cell suspension into the tail vein. It has been used as a tumour model for metastasis and angiogenesis research. The FP gene, which codes for fluorescent protein, is put into the cells to make distant metastases visible. For this, a retroviral vector is used, which then transfects lung cancer cells. [46].

5.5.1.3 Ehrlich Ascites carcinoma

The intraperitoneal injection of $2x10^5$ tumour cells in animal on day 0 causes tumours in experimental animals. The pharmacological treatment begins after the tumour has been inoculated for 24 hours. After the predetermined time intervals, the animals are slaughtered and the peritoneal fluid is collected. Repeated saline washes are used to harvest tumour cells from the peritoneal cavity. For the survival time assay, additional groups of animals might be used. For evaluation, parameters such as peritoneal fluid volume, tumour cell viability in peritoneal fluid, packed cell volume (PCV) in peritoneal fluid, and percent increase in survival time of drug-treated mice are used. In addition, haematological, biochemical and tumor cell morphology are studied in order to assess the tumor response to test drugs[47].

The cancer develops in the peritoneal fluid and can reach a cell density of 25 to 100 million per ml of ascitic fluid. This is a popular model for primary screening since it can predict broad antitumor activity. The injection of $4x10^6$ tumour cells s.c. in the animal's flank results in the development of a modified solid tumour model. In 14 days, the tumour was develop to a diameter of 12 mm. The primary methods of evaluation are tumour volume and histological inspection of tumours[48].

5.5.2 Xenograft models: The transplantable tumours of human origin should be employed for tumour models that are more closely related to the clinical illness. However, transplanting such human malignancies into mice could lead to severe immunological rejection. Athymic (nude) mice or severe combined immunodeficiency (NOD-SCID) animals are employed in this study.These animals have no immunological reaction to the transplanted foreign material. Mice immunocompromised by irradiation, thymectomy, or steroids were utilised for transplantation before athymic mice became available.

The first nude mice appeared spontaneously in a closed colony of albino mice in a laboratory in Glasgow, Scotland, and were characterised as without fur by Isaacson and Cattanach. A mutant gene (*nu* , meaning nude) is found on chromosome 11 as an autosomal recessive gene that causes hair loss, slow growth, a short lifespan, and low fertility.*Nu/nu* mice do not have a thymus, but *nu* /1 mice do from their heterozygous mother, the *nu/nu* athymic mice have a low quantity of T cells. B cell function, on the other hand, is normal in such animals, and natural killer cell activity is increased[49].

The ability to maintain the histologic and biologic identity of tumours in vivo over multiple passages, as well as the success of human tumour xenografting into nude mice, have revolutionised cancer research in several ways. Subcutaneous, intraperitoneal, intravenous, intracranial, intrasplenic, renal subcapsular, and a new orthotopic model using site-specific organ injection can be used to transplant tumour cell lines into nude mice.

The human tumour cells show kinetic alterations when implanted into nude mice. Most of the time, the doubling time is faster than the original tumour, and it gets faster in successive passes. Many xenografted human cancers retain their original morphologic and metabolic properties despite this. As a result, human tumour xenografts are the backbone of cancer therapy development[50].

5.5.2.1 Subcutaneous implantation: This route is considered as the most accessible one for the transplantation of human tumors into the nude mouse due to the simplicity of the procedure, this method has been chosen by the National Cancer Institute as the principal *in vivo*test for its drug research and screening programme. Typically, a tumour cell suspension (about 10^6 to 10^7cells per animal) is injected into the animal's flank region. Tumors can grow in as little as a few days or as long as a few months, depending on the cell line employed. With subcutaneous xenografts, invasion of neighbouring tissues and metastases are uncommon[51].

5.5.2.2 Renal Subcapsular (RSC) Assay: Bogden and colleagues first reported this approach in 1978. The cells are implanted under the capsule of the kidney in a nude mouse as a 1 mm tumour fragment. The advantage of these tumours is that they retain the original tumor's genuine morphologic, functional, and growth properties, such as cell-cell contact and tumour spatial connection. As a result, they are a better representation of human tumour metastatic features. The appropriate evaluation methods are growth assays, clonogenic assays, and animal survival assays.

Unlike the subcutaneous xenograft assay, the renal subcapsular assay has a relatively short and constant time between tumour injection and the appearance of a graphically palpable mass. Tumors are routinely inspected over a course of six days. As a result, when a short-term in vivo experiment is necessary, this model comes in handy. Despite its many benefits, it is notanideal model since the subcapsular region of the kidney is not immune-privileged. Various numbers of lymphocytes have infiltrated the tumour in this place, which could be attributable to it or for another reason unrelated to the initial tumour. However, it could be a useful orthotopic model for renal cell carcinoma[51].

5.5.2.3 Intraperitoneal Microencapsulated Tumor Assay: The Alternative short-term in vivo assays have been developed due to the RSC's limitations and limited adaptation to slow-growing malignancies. One of these uses microencapsulation technology: the microencapsulated tumour assay. The semi-permeable gels that can be manufactured into 0.05 to 1 mm in size microcapsules enclose tumour cells. These microcapsules are inoculable in experimental animals' peritoneal area. Around 600 microcapsules are injected into the peritoneum in normal test settings using the mouse. The capsule half-permeability protects the tumour cells from immune cytotoxicity through the host cells and does not necessarily require anthymical (nude) mice. It also enhances the circulation and reaching of tumour cells by nutritional and systemic cytotoxic substances. The efficacy of the anti-cancer effect is evaluated by rehabilitating and counting live tumour cells in treated vs. control animals. The microencapsulation assay is straightforward, quick, and affordable. When compared to the subcutaneous implanted tumour experiment, it utilizes less mice. Tumor cells are tested after being exposed to medication quantities that would be found in vivo. Furthermore, unlike the subcutaneous transplanted tumour experiment, the technique is adaptable to most solid tumours and uses immunocompetent mice. In the same mouse, many tumours can be assessed at the same time. The NCI screening programme uses the microencapsulated tumour assay as an in vivo second-line screen to follow up on first therapy leads that pass the in vitro screening method for these reasons[51].

5.5.2.4 Orthotopic Xenograft Model: Transgenic tumour models and subcutaneously developing human tumours in immunodefficient mice do not accurately replicate human clinical cancers because they lose metastatic potential and change medication sensitivity when implanted heterotopically [52].

A system of tumour cells transplanted at the site of the organ of genesis is the orthotopic xenograft model. When the SOI (surgical orthhotopic implantation) models were compared to transgenic mouse cancer models, the SOI models were found to be more relevant to clinical metastatic cancer[53]. This organ-specific location is thought to offer the tumour cells with the best conditions for growth and development. This methodology has yet to be widely adopted by the NCI drug-screening programme due to its high cost and novelty[54].

5.6 Genetically Engineered Mouse Models (GEMs)

Cancer in genetically altered animals is more similar to human cancer than the other models listed because the tumour develops naturally in its original site, unlike xenograft tumours, which are frequently implanted in an area other than an orthotropic site. The tumours have a natural growth rate and metastatic characteristics that are identical to those seen in humans. Because these tumours are nonimmunogenic in their natural host, the immunocompromised animal is not required to grow[51].

The GEMs are classified into two types: transgenic mice and knockout mice.

5.6.1 Transgenic Mice: The transgenic mouse is a product of injecting a foreign gene into the pronucleus of a fertilised egg. This progeny then carries and expresses the foreign gene, passing it along through the generations. Microinjection, retroviral infection, and embryonal stem cell (ESC) transfer are all possible ways to get genes into the pronucleus. Transgenic animals are the ideal models for investigating the oncogenic phenotype caused by a known gene's deregulation.

Oncogene-expressing transgenic mice, which develop spontaneous tumours as a result of a known biochemical pathway defect, are excellent models for testing thedrugs that target a specific biochemical route. NF1 gene in neurofibromatosis, c-fos, N-myc, and erb B2 gene in transgenic mice are examples. Ras inactivation,

for example, has been linked to the development of numerous malignancies, including breast cancer. The effectiveness of new breast cancer-specific chemotherapy therapies was tested using transgenic mice with ras mutations that generate mammary tumours[51].

5.6.1.1 TRAMP transgenic mice: The expression of SV40 tumour antigens is controlled by a modest probasin promoter. Within 12 weeks of birth, these mice acquire prostate cancer, which progresses to metastases by 30 weeks. Many important characteristics of human prostate cancer are replicated in TRAMP mice[55].

5.6.1.2 p53+/- Wnt-1transgenic mice: To generate the model of mammary carcinogenesis, *p53+/- mice* were crossed with MMTV-Wnt-1 transgenic mice. MMTV stands for mouse mammary tumour virus promoter[56].

5.6.1.3Apcdeficient mice: A dominant mutation in the*Apc* (adenomatous polyposis coli) gene causes these mice to spontaneously grow preneoplastic intestinal polyps. Most human colon tumours have this gene mutated[56].

5.6.2 Knockout Mice

A knockout animal is one in which both alleles of a certain gene are missing.

5.6.2.1 Nkx 3.1 knockout mice: *Nkx 3.* 1 is a tumour suppressor gene that is only found in the prostate. It is required for the differentiation and function of the prostate. Inactivation of this gene causes histopathological abnormalities that are similar to human prostate cancer. This model can be used to investigate the causes of prostate cancer as well as the disease's tissue-specific characteristics[54].

5.6.2.2 Homozygous *p53 knockout* mice: The most common genetic lesion in human cancer is a mutation in the *p53* tumour suppressor gene. Identifiable *p53* gene point mutations or deletions are seen in more than half of all human malignancies. These mice are predisposed to spontaneous carcinogenesis, notably lymphomas[55].

5.6.2.1 *Brca1*conditional knockout model: The CreIoxp method is used to induce *Brca1* deletion by expressing Cre under the direction of MMTV-LTR or WAP. Mammary tumours appear in animals between the ages of 10 and 13[57].

6. In vitro models for angiogenesis research

Angiogenesis, defined as the production of new capillaries from pre-existing vessels[58], has attracted researchers' interest since it is involved in embryonic development, reproductive function, adult wound healing, and a variety of diseases, including cancer.(Carmeliet, 2005; Muñoz-Chápuli et al., 2004; Folkman, 1995; Simons, 2005).We discuss and highlight some of the current, popular in vitro models for angiogenesis research in this article including 2D models and 3D spherical- and plate-shaped models, particularly for investigation into the morphological differentiation process of endothelial cells.

6.1 Two-Dimensional Angiogenesis Models

6.1.1 Boyden Chamber Assay for Endothelial Cell Migration

The Boyden chamber is a widely used experiment for evaluating the endothelial cells (EC) movement in response to chemotaxis. A polycarbonate filter with a particle size of 5-12 m separates the two compartments[63]. Extracellular matrix (ECM) protein, such as gelatin, collagen, fibronectin, or complex matrices like matrigel, can be coated on the filter. The upper chamber is seeded with(Albini et al., 2004; Albini and Benelli, 2007).ECs, while the lower chamber is supplied with culture media containing pro- or anti-angiogenic agents. Anti-angiogenic chemicals can be incorporated into the coatings(Kuzuya et al., 1998; Harvey et al., 2002). As a non-contact experiment, a cancer cell layer can be employed under the filter to produce a gradient of angiogenic agents[68]. The Image processing of labelled cell nuclei makes it simple to analyse EC migration via the filter[69].Enhanced sensitivity, high repeatability, and quick duration are all advantages of

this assay (4-6 hrs)[70]. The difficulty in observing cell vertical movement through a 'foreign' matrix is one of the disadvantages (polycarbonate or polypropylene filter)[70].

6.1.2 Scratch Wound Assay for Endothelial Cell Migration

The scratch wound test is a straightforward way to measure EC migration. To depict an artificial wound, a confluent monolayer of ECs is mechanically scratched in a specific location of the well, using a cell scraping instrument or a p200 tip[70]. To explore the influence of shear stress on cell migration, an enhanced microfluidic variant of the scratch wound test was recently devised, in which the fake wound is accurately generated utilising a laminar flow of trypsin solution[71].After creating a scratch wound, the rate and extent of EC migration from the remaining monolayer into the "wound area" is observed and quantitatively analyzed using image processing(Bahramsoltani et al., 2009; Wang et al., 2009; Weis et al., 2002; Pepper et al., 1990; Glen et al., 2012). Using this assay, the migration rate can be monitored easily in a 2D horizontal X-Y plane. However, it has been proven that cell migration in this model also involves cell spreading and proliferation, leading to an inaccurate determination of the net migration effect(Staton et al., 2009; Coomber and Gotlieb, 1990).

6.2 Three-Dimensional Spherical-Shaped Angiogenesis Models

To fabricate 3D spherical-shaped models, several methods exist, including scaffold-based and scaffold-free techniques.

6.2.1 Microcarrier Assay

Using this method, cytodex-3 microcarrier scaffolds can be coated with gelatin and suspended with ECs[77]. After 2-4 days' incubation, ECs are attached to and cover the microcarriers, which are then embedded in a fibrin matrix. Consequently, the migration of endothelial cells (ECs) generating capillary-like structures was observed and quantified. Microcarriers with an appropriate diameter (usually 100-400 m) for cell attachment can be manufactured of plastic, glass, dextran, cellulose, collagen[59], and fibrin[78]. The type of ECM used to insert the microcarriers has a significant impact on endothelial cell activity. For example, hyaluronic acid promotes endothelial cell migration, but not capillary formation.[27] The ECM density also strongly regulates capillary formation[79][80].The benefit of this model is that it avoids the EC detachment problem that occurs with 2D models. This model's 3D matrix better simulates the in vivo environment and improves endothelial tube development. Because of the wide range of microcarrier coatings available, this is a useful tool for researching the role of cell-cell and cell surface contacts for angiogenesis since it controls several aspects such as surface charge and integrin binding[81]; Hall and Hubbell, 2004). However, like with 2D models, one limitation of this test is that EC migration is largely detected in a 2D horizontal X-Y plane.

6.2.2 Three-Dimensional Plate-Shaped Angiogenesis Models

3D plate-shaped models can also be fabricated using scaffold-based and scaffold-free techniques.

6.2.3 Three-Dimensional Basement Membrane Assay

The 3D ECM plays an important role as a critical framework and cytokine immobilization scaffold for EC migration, connection, lumen formation and tube stabilization[83].By increasing the thickness of the basement membrane or adding an additional layer, the 2D basement membrane assay described above can simply be converted to a 3D scaffold model[84].Single cells or monolayers of ECs can be created and placed at the bottom, middle, or top of the 3D scaffold. ECs attach, spread and proliferate to form a monolayer on a fibronectin, laminin or gelatin-coated dish, or at the surface of a collagen gel. After covering with a collagen layer as a 3D matrix, If the collagen layer is removed, ECs gradually organise into a network of tube-like structures, which then regress[84].Collagen type I and fibrin matrix appear to be particularly conductive to EC tube morphogenesis and sprouting[85]. Fibroblasts cultured in the same conditions do not form a network, indicating that the 3D basement membrane is important for network formation as the specific behavior of ECs[84].When ECs are mixed into the 3D matrix, most of them participate in the morphogenic response. In contrast, in the case of the EC monolayer, only a subset of ECs participates in sprouting formation[83].

The formation of an EC lumen in the 3D matrix has been observed and found to be dependent on the formation and coalescence of pinocytic intracellular vacuoles[86]. Compared to the 2D test, the 3D basement membrane inspection offers several advantages and is now one of the most used models for quantitative 3D angiogenesis in vitro[63]. It allows ECs to build structures that are not only capillaries, but lumens also. The movement of ECs in both horizontal and vertical aspects can be detected and analysed readily[86].Fluidic flow is also applicable to understand the effect of shear stress on EC behaviour [87].However, limitations exist regarding the 3D basement membrane assay. For example, these assays take considerably longer to run (5-15 days)[70].Moreover, the 3D matrix thickness must be relatively small to allow oxygen and nutrients to diffuse and to avoid excessive mortality of the cells.

7. Metastasis

The cancer metastasis, or the spread of the main lesion cell into the distal organs, is the leading cause of cancer mortality[88]. The diffusion of cells from a primary tumour involves a plethora of cell mechanisms. These include stromal invasion or collusion, immune avoidance by inhibiting or co-opting anti-tumorigenic processes, avoidance and modification of the tissue microenvironment, and the development of therapeutic intervention resistance[89].

Figure 3 : An Overview of the Metastasis Routes that Have Been Observed

Metastasis is a multiscale, complex process that entails numerous sub processes running in parallel along partially overlapping routes. According to recent studies, premalignant lesions can cause distant, latent metastasis and are thus not exclusively connected with late-stage primary tumours. When microenvironmental stressors drive cellular reprogramming processes, it is still widely thought that metastasis occurs predominantly as a result of cellular reprogramming events that accelerate cell migration and invasion toward more nutrient-rich niches. Cancer cells can undergo phenotypic alterations when various stressors are combined with metabolic reprogramming, enabling them to adopt more mesenchymal-like states that are malleable rather than binary, allowing the cells to sample these dynamic states throughout the metastatic process. While this may increase a cell's ability to propagate, it is no longer thought to be the primary mechanism. Cancer cells accomplish their objectives in a variety of ways. Although cancer has no preference for specific organs, the most common routes for cell seeding into metastatic organs are lymphatics and blood arteries (lymph nodes, liver, lung, bone marrow, and brain). Several genetic and epigenetic changes, as well as interactions with the complex milieu of cells in the host microenvironment, influence cancer cell survival and expansion [88,89].

8. Conclusion and Future perspectives: Spontaneous tumor models have provided considerable insight into analysing natural illness progression, despite not being especially beneficial in medication research. Virus-induced tumours are no longer often used. Tumors caused by chemicals or radiation have their own position in drug testing and evaluation. However, due to some of their drawbacks (particularly the protracted induction period), they are not suitable for use in large-scale screening programmes. Transplantable tumours are the greatest alternative in this situation, which demands short-term, repeatable, and less expensive procedures due to which they have been widely used in drug testing. Evaluationofthe activity of the test drug on a specific form of cancer is another significant component of cancer research. The use of cell lines (in vitro or in transplantable malignancies) gives this sort of screening a lot of versatility. The use of genetically altered mice models for disease-oriented screening is potentially a possibility. However, they are mostly used in the study of carcinogenesis mechanisms. The application of these models has yielded a number of targeted therapeutic compounds that have shown promising results in clinical trials. As a result, each model has its unique set of advantages and disadvantages. Comprehensive and appropriate use of these assays is warranted in the area of drug discovery and therapy response assessment.

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