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EXPERIMENTAL MODELS TO STUDY TUMOUR ANGIOGENESIS – *IN-VITRO, EX-VIVO* AND *IN-VIVO* APPROACH

K. A. Janani [1], T. K. Praveen [2] and Ashish Wadhwani [*1]

Department of Pharmaceutical Biotechnology [1], Department of Pharmacology [2], JSS Academy of Higher Education & Research - JSS College of Pharmacy, Ooty - 643001, Tamil Nadu, India.

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Correspondence to Author:
Dr. Ashish D. Wadhwani

Assistant Professor and Head, Department of Pharmaceutical Biotechnology, JSS Academy of Higher Education & Research - JSS College of Pharmacy, Ooty - 643001, Tamil Nadu, India.

E-mail: adwadhwaniad@gmail.com

ABSTRACT: Angiogenesis is the formation of new blood vessels from preexisting vasculature. It is a key process in some physiological conditions such as wound healing, growth and reproduction. Any disturbance in mechanisms of angiogenesis plays a key role in the pathogenesis of some diseases through the over-proliferation of blood vessels such as cancers, psoriasis, arthritis, retinopathies, obesity, asthma, and atherosclerosis and cardiovascular diseases, which have the highest rates of morbidity and mortality worldwide. Anti-angiogenic therapy has been proved to be a potential cure in the past decades for a number of cancers. Still, antiangiogenic therapy has to be realized to its fullest potential to turn cancer into a dormant disease. Many angiogenic inhibitors have been developed till date, and the main problem is the multiple angiogenic molecules produced by tumors and tumors at different stages of development. Therefore, blocking a single angiogenic molecule has less effect or no effect. Selection of a suitable model for angiogenesis research remains a challenge. Thus, in recent years, several models for evaluation of angiogenesis have been introduced for determining the target molecule. In this article, we briefly reviewed *in-vitro*, *ex-vivo* and *in-vivo* models to evaluate tumor angiogenesis and determine specific targets.

INTRODUCTION: Angiogenesis is the normal and vital physiological process which is described as the origin of new blood vessels from pre-existing vasculature [1]. It is an integral part of both general developmental processes; acts as a support in the healing process of wounds and the formation of granulation tissue and also numerous pathological conditions like tumor growth and ocular disease.

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Angiogenesis involves a variety of coordinated events; including degradation of the extracellular matrix surrounding the parent vessel, migration, and proliferation of the endothelial cells and mural cells to assemble the new vessel, lumen formation, and construction of the mural cell layer of the vessel wall with associated pericytes and smooth muscle cells [2].

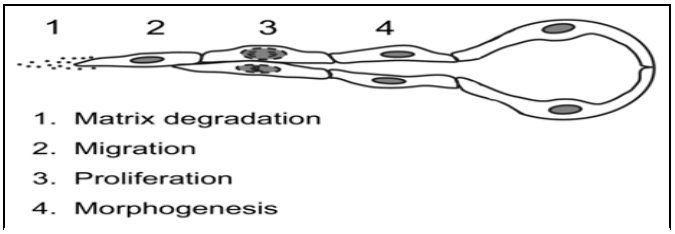

FIG. 1: ENDOTHELIAL CELL FUNCTIONS INVOLVED IN ANGIOGENESIS [2]

The importance of angiogenesis in tumor growth was first proposed by Judah Folkamanin 1971, and he described tumors as "hot and bloody". The body controls angiogenesis to maintain natural equilibrium between inducing agents and inhibitory factors, disturbance in this balance results in either accelerated growth in the vascular network or too much reduction of blood vessel development. Angiogenesis is regulated by both activator and inhibitor molecules.

Classification of Angiogenesis:

Sprouting Angiogenesis: It occurs in well-characterized stages. Sprouting angiogenesis involves the activation of the endothelial growth factors which stimulate the receptors from the pre-existing vasculature to release proteolytic enzymes such as protease causes the degradation of basement membrane allowing the endothelial cells to escape from the parent cells. They proliferate in the surrounding matrix forming solid sprouts around the neighboring vessels those sprouts extend towards the stimulus through the adhesion molecules such as integrins to form loops to develop as a full-fledged vessel [3].

Intussusceptive Angiogenesis: It is also known as the splitting angiogenesis, where the capillary wall extends into the lumen to split a single vessel in two. There are four phases of intussusceptive angiogenesis. First, the two opposing capillary walls establish a zone of contact. Second, the endothelial cell junctions are reorganized and the vessel bilayer is perforated to allow growth factors and cells to penetrate the lumen. Third, a core is formed between the 2 new vessels at the zone of contact that is filled with pericytes and myofibroblasts. These cells begin laying collagen fibers into the core to provide an extracellular matrix for growth of the vessel lumen. Finally, the core is fleshed out with no alterations to the basic structure. Intussusception is important because it is a reorganization of existing cells and allows a vast increase in the number of capillaries without a corresponding increase in the number of endothelial cells [4].

Tumour Angiogenesis: Cancer cells divide in an uncontrolled manner leading to tumor progression which mainly depends on an adequate supply of oxygen and nutrients and the removal of waste products. Hypoxia is a critical process for tumor angiogenesis and is carried out primarily by the transcription of hypoxia-sensitive genes and HIF [5]. Several mechanisms are involved in vascularization of tumors including endothelial sprouting and bone marrow-derived endothelial cells. Endothelial sprouting is a process that is controlled by a balance between ″pro- and anti-angiogenic″ factors and is a basic mechanism for tumor vascularization. During sprouting, pericytes detach, and blood vessels dilate, and the process is under control of VEGF and angiopoietins [6]. During bone marrow-derived endothelial cells process, circulating cells in the peripheral blood may participate in vessel formation [7].

Tumour angiogenesis involves the production and release of growth factors –Vascular endothelial growth factor (VEGF), angiopoietin; migration stimulating factors, proteolytic enzymes like protease, Extracellular matrix and adhesion molecules such as integrins; Non-specific factors *i.e.* Platelet-derived growth Factors, Transforming growth Factor (TGF- the parent vessel, migration and proliferation of the endothelial cells$^{\beta}$), fibroblast growth factor, Tumor necrosis factor TNF-$^{\alpha}$. [8]

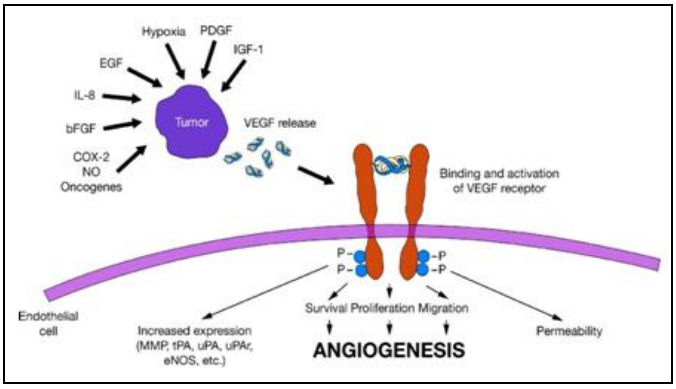

FIG. 2: TUMOR CHARACTERISTICS AND ENVIRONMENT PROMOTE VEGF EXPRESSION [8]

Experimental Models to Study Angiogenesis: Earlier, Literature on tumor angiogenesis was descriptive and many investigators like Sandison (1928), Algire (1945), Greenblatt and Shubik (1968), Peterson (1979) used several models like rabbit, hamster to study angiogenesis and demonstrated that the tumor was still induced by capillary proliferation even after the tumour was separated from host's vasculature by a micropore filter. In 1970, Folkman and his associates

developed new techniques for quantifying capillary proliferation and for testing of angiogenic activity of multiple fractions of tumor extracts. Knowledge of molecular mediators of angiogenesis is fundamental in understanding the mechanisms that control its pathways and may ultimately be useful in developing therapies for angiogenesis-related diseases [9].

In a pathological condition such as cancer, angiogenesis is required for tumor survival and proliferation. Folkman's intuition (1971) demonstrated that tumor growth and metastasis strictly depend on angiogenesis led to the idea that blocking tumor nourishment could be one of the ways to avoid its spread. Some of the models are:

***In-vitro* Models:** Basement membrane disruption, cell migration, cell proliferation, and tube formation, can be a target for intervention, and each can be tested *in-vitro*. *In-vitro* assays can be used to assess both angiogenic and anti-angiogenic factors, though the choice of assays and exact assay conditions may vary depending on which type of factor is being tested. *In-vitro* angiogenesis assays are quick and easy. The flexibility of using different cell types, staining, and observation methods, and quantitative analysis, enhances the experimental design and provides deep understanding of the morphology and mechanism of angiogenesis [10]. The point to be considered when setting up an *in-vitro* angiogenesis model is the choice of Endothelial Cells. (*i.e.* EC monoculture or the choice to co-culture ECs with other cells types, such as fibroblasts, smooth muscle cells, myoblasts, cardiomyocytes, hepatocytes, and cancer cells) depends on the angiogenesis process being studied. Now *in-vitro* models for angiogenesis research include 2D models and 3D spherical- and plate-shaped models.

Endothelial Cells: Endothelial Cells are the most important tool for *in-vitro* studies of angiogenesis. Human umbilical vein endothelial cells (HUVEC) are commonly used [7]. The limitations of using with endothelial cells are heterogeneity, the difference in endothelial shape, size and complexity of junctions. Currently, 19 different types of endothelial cells are available as primary cell cultures, including pulmonary, uterine, cardiac, bladder, dermal and lymphatic microvascular cells **Fig. 3**. A majority of endothelial cells within the body are naturally dormant, whereas cultured endothelial cells adapt to proliferate *in-vitro*, which may be a potential problem when assessing pro-angiogenic factors, as these cells are already exhibiting an angiogenic phenotype.

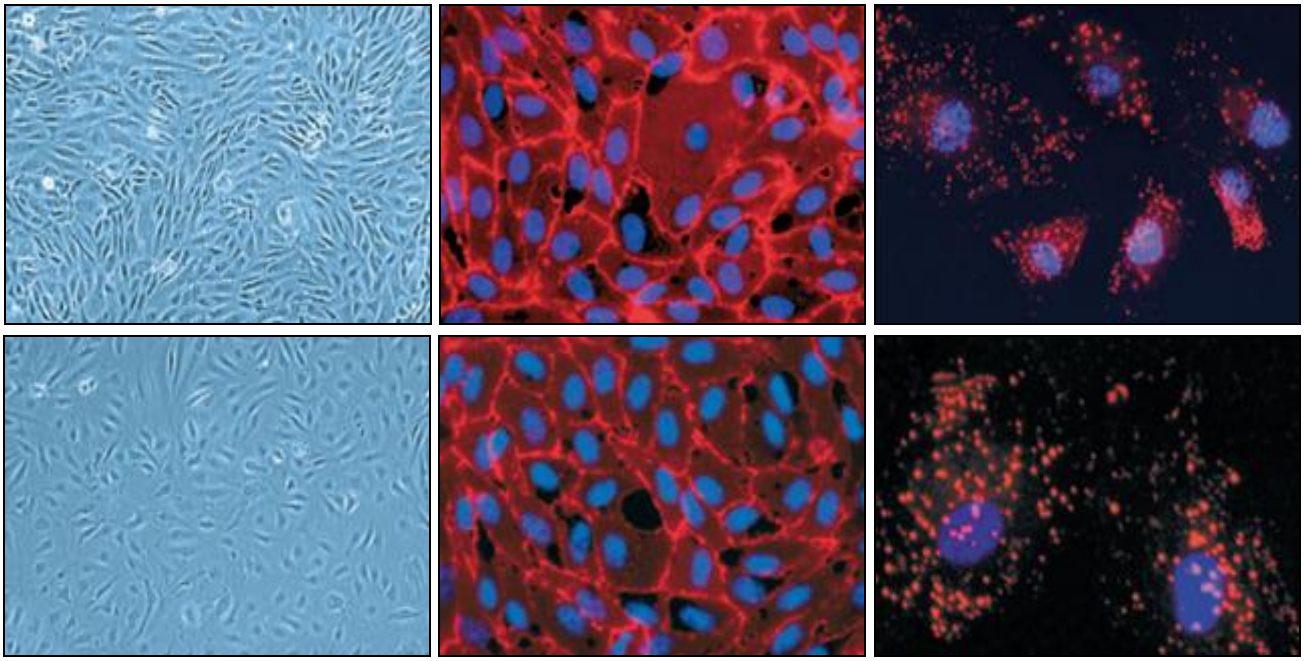

FIG. 3: ENDOTHELIAL CELLS. REPRESENTATIVE PHOTOGRAPHS OF CONFLUENT HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC; a-c) AND HUMAN DERMAL MICROVASCULAR ENDOTHELIAL CELLS (HuDMEC; d-f) AS SEEN WITH PHASE-CONTRAST MICROSCOPY (a, d), OR STAINED WITH CD31 (b, e) ORVONWILLEBRAND'SFACTOR (c, f) [11, 12]

In the fluorescent photographs, the nuclei are counterstained with DAPI. The morphology of both cell types is very similar although the HUVEC form a more classical cobblestone pattern. Photographs courtesy of Promo cell GmbH, Heidelberg, Germany.

Cell Proliferation Assays: Cell proliferation can be assayed by measuring the incorporation of [3H] Thymidine into the DNA of the cell using scintillation counting where the amount of radioactivity is proportional to the DNA synthesis [13]. Cell-cycle kinetics can be studied by using BrdU (Bromodeoxyuridine) which competes with thymidine for participating in the DNA in the S-phase of the cell cycle. BrdU can be determined by immunocytochemistry for individual cell level or by Elisa for the cell population.

Alternative methods for analyzing proliferation are direct cell-cycle examination using DNA-binding molecules and flow cytometric analysis. In this method, at first, BrdU goes into cellular DNA, and then saturation staining of propidium iodide is carried out. Apoptosis is measured by determining the correlation between the BrdU content of the cells with propidium iodide, and it shows the cell-cycle distribution, proliferation state.

Direct cell-cycle analysis using DNA-binding molecules with flow cytometric analysis is an alternative method for analyzing proliferation. Again, in this method, BrdU is commonly used. BrdU incorporation into cellular DNA followed by saturation staining with propidium iodide (PI), which provides a measure of the total DNA, per cell. A correlation between the BrdU content of the cells with that of PI measured generates cell cycle information using the fluorescence-activated cell sorter (FACs) [14].

The most reliable results with proliferation assays are achieved by the combined use of two or more methods; such as counting (MTT or direct cell count) combined with a measure of DNA synthesis and cell death analysis [12].

MTT Assay: The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely when metabolic events leading to apoptosis or necrosis, the reduction in cell viability. The principle is based on the reduction of the MTT to formazan by enzyme Succinyl dehydrogenase. Only the viable cells take up the dye, and it can be quantified using a Spectophotometry. The cells with a confluence of 90-95% are trypsinized and seeded in a 96 well microtitre plate (10000cells /well) and incubated for 24 h till the formation of a partial monolayer. The cells are then exposed to various concentrations of the drugs in triplicate. Control wells are seeded with only maintenance medium. The plates are incubated at 37 °C in a CO_2 incubator for 72 h. At the end of 72 h, MTT dye is added and incubated for 2-4 h. Then MTT dye is removed and the purple formazan crystals formed are dissolved by addition of 50 µl of Propan-2-ol to each well. The absorbance can be monitored at 540 nm. Data is collected for each in triplicates and used to calculate the means and the standard deviations [7]. Optical density of cells incubated in the presence and absence of the different concentrations of drugs will be compared together and the percentage of surviving cells was determined using the following formula:

$$\% \text{ Cell Viability} = \frac{\text{Mean OD of the individual test group}}{\text{Mean OD of the control group}} \times 100$$

Scratch Wound Assay: The *in-vitro* scratch assay is an easy, low-cost and well-developed method to measure cell migration.

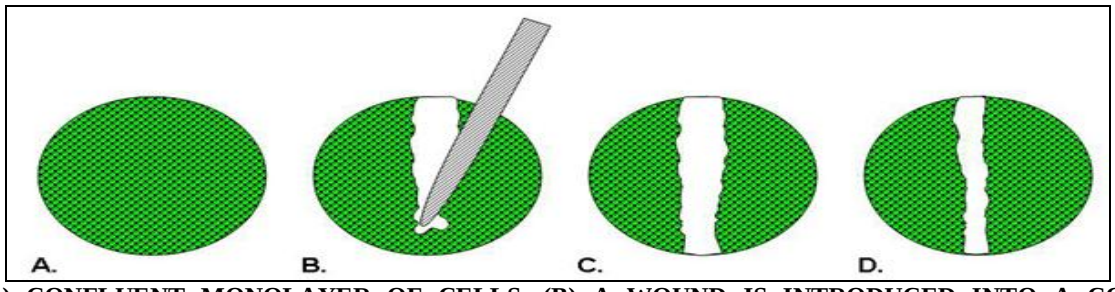

FIG. 4: (A) CONFLUENT MONOLAYER OF CELLS. (B) A WOUND IS INTRODUCED INTO A CONFLUENT MONOLAYER OF CELLS BY DRAWING A TIP/CELL SCRAPER ACROSS THE CELL LAYER. (C) THE DENUDED AREA. (D) CELLS HAVE MIGRATED INWARD TO FILL. THE VOIDS ARE IMAGED TO MEASURE THE BOUNDARY OF THE WOUND AT PRE-MIGRATION AND AFTER THE CELLS MIGRATE TO FILL THE VOID [16]

The endothelial cells are grown to confluence, and a wound is introduced by clearing an area of the monolayer using a pipette tip, needle or cell scraper. Quantification involves measuring the distance moved by the endothelial cells, the area covered by the endothelial cells, or the amount of time required to close the wound area to know whether a treatment causes chemokinesis or chemotaxis [15].

Transwell Assay / Boyden Chamber Assay: Endothelial cells during the process of angiogenesis break down the basement membrane with release of proteolytic enzymes such as matrix metalloproteinase (MMPS) and migrate in response to a gradient of angiogenic factors including VEGF. The porous filters used to assess migration and therefore are called as transwell assay or Boyden Chamber assay or Modified Boyden chamber assay [17]. With this technique, endothelial cells are plated on one side of a porous membrane (3 μm) coated with fibronectin or collagen before plating the cells to facilitate adherence, and a solution containing the potential migratory factor is placed on the opposite side of the membrane from the cells. After an incubation period of 3-18 h, the migrated cells are stained and counted manually after removal of the non-migrated cells from the upper surface of the membrane or by newer Fluorescent Staining techniques and Light-blocking membranes (automated cell counting).

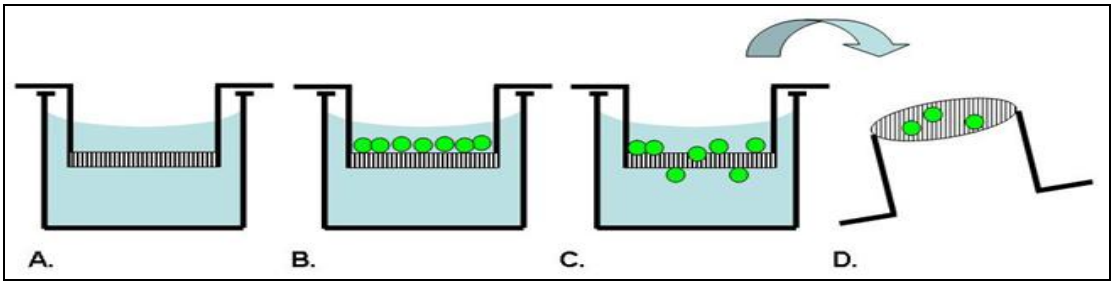

FIG. 5: (A) TWO COMPARTMENTS ESTABLISHED IN A WELL USING A MEMBRANE INSERT. (B) CELLS ARE ADDED TO THE UPPER COMPARTMENT. (C) CELLS MIGRATE THROUGH THE MEMBRANE, AND THE CELL MIGRATION IS MEASURED BY COUNTING THE NUMBER OF CELLS ON THE UNDERSIDE OF THE MEMBRANE. (D) COATING OF THE MEMBRANE WITH A MATRIX PROTEIN AND ADDING A CHEMOATTRACTANT TO THE LOWER COMPARTMENT FOR QUANTIFICATION [16]

Endothelial Tube Formation Assay: The principle is to determine the ability of endothelial cells to divide and migrate rapidly in response to angiogenic signals [18]. The tube formation forms tube-like structures when cultured on a matrix of basement membrane extract (BME). These tubes contain a lumen surrounded by endothelial cells linked together through junctional complexes. Tube formation occurs quickly with most tubes forming in this assay within 2-6 h depending on quantity and type of angiogenic stimuli. The BME must be thawed overnight in a refrigerator at 4 ºC temperature. The thawed BME solution can be stored up to 10 days; it should be allowed to refreeze. Add 150 µl of thawed BME to each well of a pre-chilled 6-well sterile plate. As the undiluted BME solution is very viscous and quickly gels at room temperature, so it must be kept on ice all the time. Pipetting must be done using a pre-chilled pipette tip to prevent any BME solution, gel formation during transfer. The plate has to be incubated for 30 min to 1 h at 37 ºC to allow the BME solution to form a gel. Cells can be harvested and re-suspended in Minimum Essential Medium (MEM) containing 10% serum and 150 µl of cell suspension should be added to each well of a 6 well microtitre plate maintaining a cell count of (1×10^6 cells/well) onto the solidified BME solution. The plate has to be incubated for 4 hours at 37 ºC. After that 150 µl of the drugs of different concentrations should be added to each well. The plate should be incubated at 37 ºC for 4 to 18 h. The endothelial tubes were examined using a light microscope in high magnification field [2].

Co-Culture Assays: The co-culture assay of angiogenesis was first described by Bishope (1999), who developed the assay to screen both inhibitors and activators of angiogenesis. In the original assay, primary Human Umbilical Vein Endothelial cells (HUVEC) are mixed with Normal Human Dermal Fibroblasts (NHDF) in equal ratio and plated on fibronectin-coated dishes [19]. This assay is a modified *in vitro* angiogenesis assay to

include co-cultured cell aggregates of Endothelial Cells and Smooth Muscle Cells. When dual labeling techniques are used, smooth muscle cells are seen in close opposition to the endothelial cells forming the sprouts. This model will provide new perception into the processes involved in angiogenesis to study synthetically active smooth muscle cells in the presence of endothelial cells, and it provides for selective manipulation of these cells *via* both intra and extra-gel delivery of adenovirus genes or growth factors [20].

Organ Culture Assays (*ex-vivo* Models): In angiogenesis, not only endothelial cells are involved but also their surrounding cells/organs participate, and this fact leads to the development of more recent methods of angiogenesis assessment which are called organ culture methods [11].

CAM Assay (Chick Chorioallantoic Membrane Assay): The chick embryo Chorioallantoic membrane (CAM) assay facilitates the testing of multiple samples and the generation of dose - dilution curves, and has been used to identify almost all of the known angiogenic factors. It is easy to perform, cost effective and time-saving model as well as provides easy observations to study angiogenesis [10].

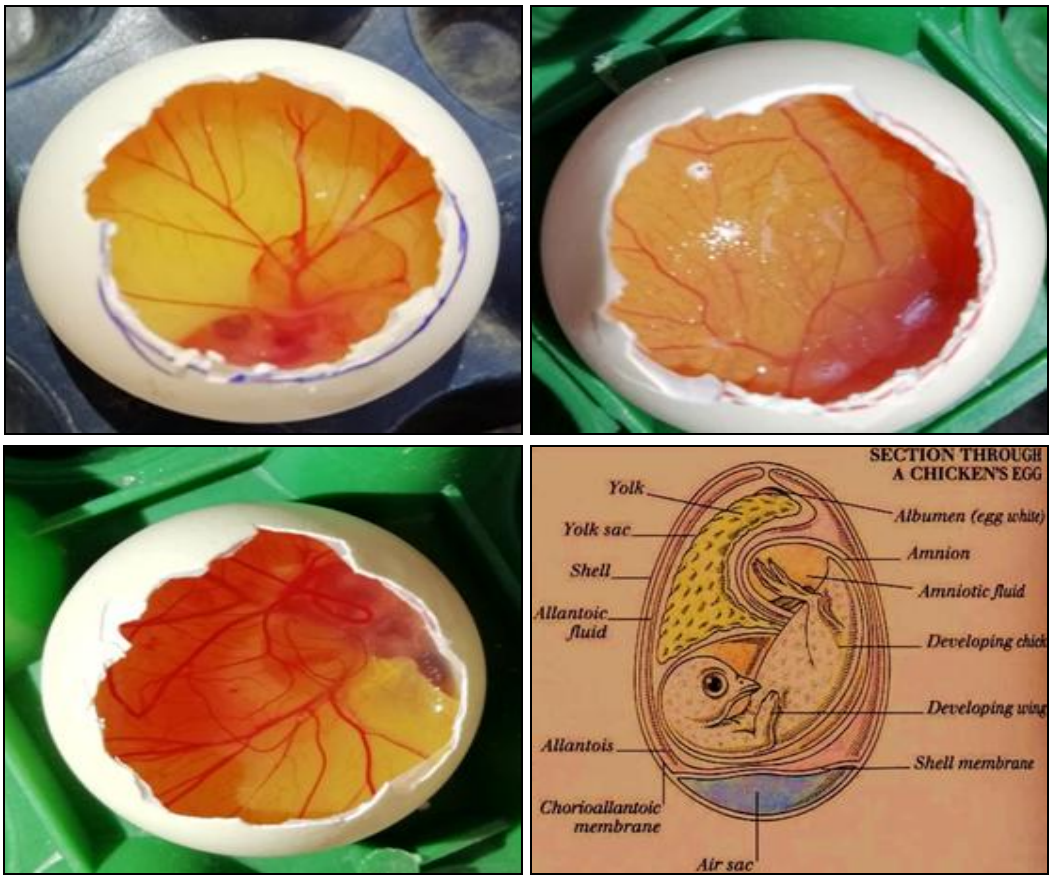

FIG. 6: THE DEVELOPMENT OF CAM. (A) CAM DEVELOPMENT ON DAY 7. (B) CAM DEVELOPMENT ON DAY 8. (C) CAM DEVELOPMENT ON DAY 10. (D) SCHEMATIC DIAGRAM OF A DAY 9/10 CHICK EMBRYO *IN OVO*

CAM assay is carried between 8 to 10 days as the central portion of CAM gets fully developed by 8 to 10 days. Different concentrations of drugs and 50µl of DMSO and normal saline are entrapped in 1.5% agarose discs. The egg shell should be cut from the upper side with a sterilized scissor, and egg air sac should be exposed. To make a clear transparent appearance of the egg's vascular system some light liquid paraffin can be applied over the Chorioallantoic membrane by using cotton buds. Care should be taken not to apply more force while applying paraffin because it may injure the inside vascular network of the egg. The agarose discs which contain drugs are implanted on the Chorioallantoic membrane of the chick embryo. After application of agarose discs, the hole in the egg was sealed with micropore tape. The eggs shall be placed back into the incubator for 1-2 days. The angiogenesis can be quantified via image analysis [7, 21].

Rat Aortic Ring Assay: The aortic ring assay is an angiogenesis model that is based on organ culture from a segment of the aorta. The rat and mouse aortic ring assays have been used to assess both angiogenic and anti-angiogenic agents. Among the organ culture assays, the rat aortic ring assay has become the most widely used [21]. Briefly, Rat thoracic aorta is excised, the fat layer and adventitia are removed, and rings approximately 1 mm in length are prepared. Individual rings are then embedded in a small solid dome of basement matrix extract (BME), and then placed inside individual wells. Angiogenic factors and inhibitors of angiogenesis can be directly added to the rings, and a mixed co-culture of aortic rings and other cell types can be employed for the study of paracrine angiogenic effects. Sprouting is observed by inspection under a Stereomicroscope over 6-12 days. Due to the large variation caused by the irregularities in the aortic segments, experimentation in 6-plicates is strongly advised. New vessel outgrowth is monitored throughout the experiment and imaged using a phase microscope.

Chick Aortic Arch Assay: The Chick aortic arch assay represents a major modification of the rat aortic ring assay. Initially, it was developed for the specific purpose of testing thalidomide (which had previously been shown to have limited effects in rodents, but strong effects in chick embryos). The assay minimizes the utilization of laboratory animals, is rapid with a time of 1-3 days, and can be conducted in serum-free medium [22]. Aortic arches are separated from day 12-14 chick embryos and cut into rings analogous to those of the rat aorta. When the rings are placed on Matrigel, considerable development of cells occurs within 48 h with the formation of vessel-like structures readily superficial. If the aortic arch is everted before explanting, the time of the procedure can be minimized up to 24 h. Both growth stimulating and inhibiting factors such as FGF-2 and endostatin respectively can be added to the medium, and their measurement of effect can be done easily [23]. Quantification can be done with the help of fluorescein-labeled lectins such as BSL-I and BSL-B4 or by the use of the process of staining of the cultures with labeled antibodies to CD31. Labeling with CD31 enables the measurement of length and abundance of vessel-like extensions from the explants [1].

***In-vivo* Models:** *In-vivo* assays may better mimic angiogenesis as it occurs in normal and pathologic states, *in-vivo* tests are not easy to perform and take more time than *in-vitro* assays. The process of quantification is also more complicated. However, these *in-vivo* assays are vital because of the complex nature of vascular responses to test reagents [3], that no *in-vitro* model can fully achieve. Different types of *in-vivo* angiogenesis assays include Sponge Implantation Assay, Matrigel Plug Assay, The Corneal Angiogenesis Assay, Wound Healing Assay, and Dorsal Air Sac Model.

The Sponge Implant Model: The technique involves the implantation of sterile sponge disks into subcutaneous pockets of the dorsum of mice, and compounds of interest are injected directly into the sponge [24]. Some different sponge matrices have been used, including polyvinyl alcohol, cellulose acetate, polyester, polyether, polyurethane, and gelatin sponge alone or in combination [25]. Sponge implantation model has been optimized and adapted to characterize essential components and their roles in blood vessel formation in a variety of physiological and pathological conditions. Differences in shape, size, and composition of these sponges make it difficult to compare. Furthermore, implantation of these sponges can cause nonspecific immune responses that may themselves lead to an angiogenic response [24].

In this method, the sponge is prepared by using sterile absorbable gel foam. The gel foam is cut and treated with sterile agarose along with the test substance which is used for angiogenesis study. The animals are anesthetized, and an incision is given at midline, and the gel piece is inserted at the subcutaneous layer. Animals are endorsed to improve, and at the 14th day the animals are sacrificed with an excessive dose of sodium phenobarbitone and gel foams are harvested from the mice carefully without any remaining of the peritoneum. Then these sponges are weighed by placing them into pre-weighed 1 ml tube of double distilled water and for homogenization kept on ice for 5 to 10 min. The supernatant of the sample is collected by centrifugation with a speed of 10,000 revolutions per minute (rpm) on a microcentrifuge for 6 min. The resultant supernatant is used to measure the hemoglobin level by filtering it through a 0.22 μm filter. Fifty microliters of

supernatant are mixed with Drabkin's reagent and kept at room temperature for 15-30 min. Hemoglobin (Hb) in the samples is then quantified [26].

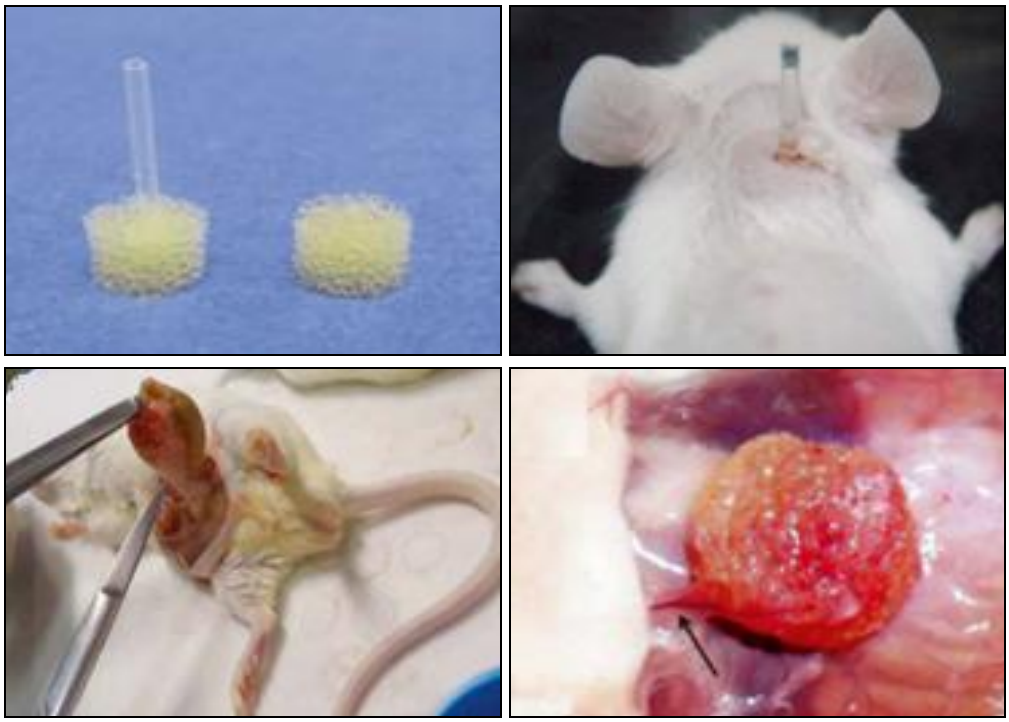

FIG. 7: (A) IMPLANT SPONGE DISK WITH AND WITHOUT THE CANNULA AND (B) THE SUBCUTANEOUS ARRANGEMENT OF THE IMPLANT IN A MOUSE, (C) INTRAPERITONEAL IMPLANTATION OF SYNTHETIC MATRIX IN MICE SHOWING AN ADHESION-LIKE TISSUE, (D) A VASCULARIZED SPONGE 14 DAYS POST IMPLANTATION IS SHOWN IN BLOOD VESSELS OF VARIOUS SIZES CAN BE SEEN INFILTRATING THE SUBCUTANEOUS SPONGE IMPLANT MODEL [27]

Quantification is done by a variety of methods including immune histological staining (*e.g.*, the CD31/34 or integrin status of vessels in the sponge), the levels of a radioactive tracer in blood and the blood hemoglobin content of the sponge. Hemoglobin (Hb) from the samples is quantified calorimetrically at 540 nm in a spectrophotometer. The resultant level of hemoglobin in the sample is then compared with a known amount of hemoglobin assayed in parallel, and the results are expressed as Hb µg/mg [26].

Matrigel Plug Assay: The Matrigel plug angiogenesis assay is a simple *in-vivo* technique to detect the formation of new blood vessels (precise visualization of the angiogenesis process) in the transplanted gel plugs in nude mice. These implant models were developed to trap a putative angiogenic compound into a suitable carrier, mostly an avascular sponge-like structure, which can slowly release the factor at the site of implant [28]. The Matrigel matrix is mainly derived from the engelbroth-holm-swarm of mouse sarcoma, and its composition is similar to the basement membrane proteins. The Matrigel can induce differentiation of a variety of cell types such as mammary epithelial cells, hepatocytes, and endothelial cells. Matrigel plug assay has become the method of choice for many studies involving *in-vivo* testing for angiogenesis [11]. Reconstituted basement membranes have been extensively used to study specific steps of the angiogenic process *in-vitro* and assess angiogenesis *in-vivo* [29].

Matrigel is thawed at 4 °C water bath, and an equal amount of a concentrated solution (10 mg/mL) was carefully pipetted into 14 ml tubes on ice without bubbling and volume equalized using PBS. Tubes should be included for the positive and negative controls. The negative control is Matrigel alone; positive controls contain 10 nanogram/mL and 100 nanogram/mL ECGS or 150 nanogram/mlb FGF added to Matrigel is injected 0.5-1 ml into the subcutaneous space of the animals. After 7-10 days, sacrifice the animals and remove the plugs. Plugs appear as bumps on the ventral side of the animal and are removed using a sharp pair of scissors or a scalpel.

Remove the plug and place plugs in labeled scintillation or specimen vials filled with 10% formalin. Allow plugs to fix overnight before embedding in paraffin. Section and stain with Masson's Trichrome stain, which stains the endothelial cells/vessels dark red and the Matrigel blue Visually inspect the sections to determine if the test substance is stimulatory and if further quantitation is necessary, Quantification of angiogenesis in this assay is achieved either by measuring hemoglobin or by scoring selected regions of histological sections for vascular density [30]. The assays are photographed and monitored with an inverted microscope, a modification using tetrazolium dye (MTT) colorimetric assay has been recently described [7]. Human umbilical vein endothelial cells suspended in 10% fetal calf serum (FCS)-containing medium with heparin and endothelial cell growth supplement (ECGS) organize into capillary-like networks within 6 h.

Corneal Assays: Corneal assays allow the linear measurement of individual growing capillaries.

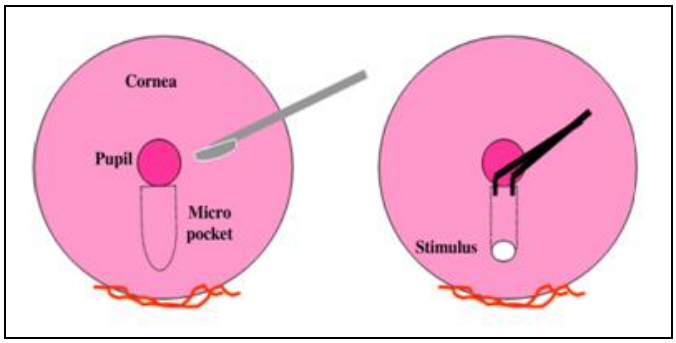

FIG. 8: SCHEMATIC REPRESENTATION OF THE CORNEAL MICRO POCKET ASSAY. (A) A MICRO POCKET IS SURGICALLY PRODUCED IN THE CORNEAL STROMA OF ANAESTHETIZED ANIMALS BY A SURGICAL SCALPEL AND A PLIABLE SPATULA. (B) THE TEST SUBSTANCE IS INSERTED IN THE MICRO POCKET [31].

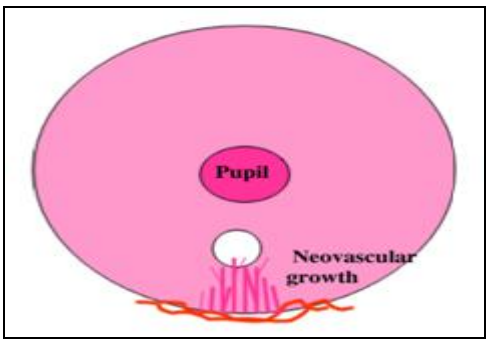

FIG. 9: THE NEWLY FORMED VESSELS START FROM THE LIMBIC VASCULATURE AND PROGRESS TOWARD THE IMPLANTED STIMULUS [31]

Rabbit Corneal Assay: The animals are anesthetized with methoxyflurane, and a pocket is made in the cornea of the animal (rabbit/mouse) at a distance of 1-2 mm from the edges, then tumor implants ($1mm^3$) are inserted. New capillaries grow at right angles from the edge of the cornea and elongate about 0.2mm/day. The length can be measured with a slit-lamp stereoscope [32].

Mouse Corneal Assay: The mouse corneal micro pocket angiogenesis assay has capitalized upon this property of neovascularization in cornea and is often considered the gold standard to determine whether signaling peptides, drugs, etc. function as proangiogenic or anti-angiogenic factors *in-vivo*. The animals are anesthetized with methoxyflurane, and a pocket is made in the cornea at 1 mm from edge and pellets containing substances to be tested coated with hydron are implanted. When peptides are tested, Hydron should be used as a casting solution, and prepared by dissolving the polymer in absolute alcohol at 37 °C. When peptides are tested, sucralfate is added to stabilize the molecule and to slow its release from Hydron [33]. The vascular response is measured as the maximal vessel length and the number of clock hours of neovascularization is measured at fixed time (usually on postoperative 5 and 7 days) using a slit-lamp biomicroscopy and photographed [31].

Rat Corneal Assay: Purified growth factors are combined 1:1 with Hydron Pellets are implanted 1.5 mm from the edge of the cornea of anesthetized rats. Neovascularization is assessed at 3, 5, and 7 days; animals are perfused with colloidal carbon solution to label vessels; eyes are enucleated and fixed in 10% neutral buffered formalin overnight. The following day, corneas are excised, flattened, and photographed. A positive neovascularization response is recorded only if sustained directional in the growth of capillary sprouts and hairpin loops toward the implant is observed. Negative responses are recorded when either no growth is observed or when only an occasional sprout or hairpin loop showing no evidence of sustained growth is detected [34].

Dorsal Air Sac Model: The dorsal air-sac model was developed by Selye (1953) as a means of monitoring the vascularization of tumor grafts [35]. Air is injected under the dorsal skin of rats (or

mice); the skin is lifted from an area of white fascia, permitting the introduction of cells or tissue fragments temporarily creating a thin, isolated vascularized membrane for cells or tissues to establish a new blood supply.

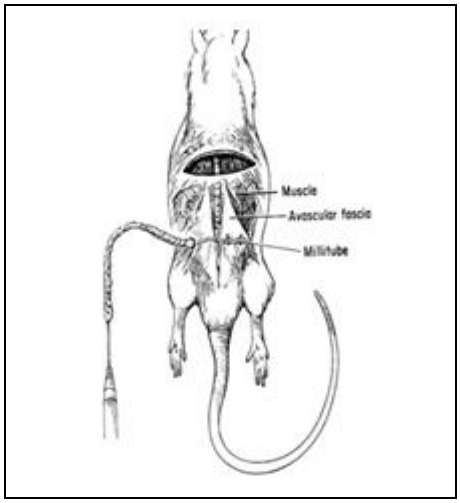

FIG. 10: SCHEMATIC DIAGRAM OF THE RAT DORSAL AIR-SAC ASSAY [36]

Both sides of a Millipore ring are covered by filters and the resultant chamber filled with a tumor cell suspension and then it is implanted into the preformed dorsal air sac of an anesthetized mouse. The vascular response, which was apparent within 48 h after tumor implantation, consisted of vasodilation and an increase in the number of visible vascular channels. Following treatment with the compound of interest, the chamber is carefully removed, and rings of the same diameters are placed directly upon the sites that were exposed to direct contact with the chamber. The number of newly formed blood vessels that lie within the area marked by the ring is counted using a dissecting microscope. Care must be taken not to irritate the surface upon which the chamber is placed, as this may itself induce angiogenesis [37].

The results of angiogenesis can be evaluated by using a dissecting microscope and calculating the blood vessels which are newly formed within the observational area highlighted using the ring. Still, it is difficult to differentiate between the pre-existing and new blood vessels. In recent practices, Evans blue is injected into the mice, which leaks out of the angiogenic vessels and accumulates in interstitial spaces whereas this dye is retained within the pre-existing vessels. The accumulation of this dye in the interstitial spaces is then considered as a semi-quantitative measure of angiogenesis. This quantification technique is not very much accurate; it only gives a relative measurement [11].

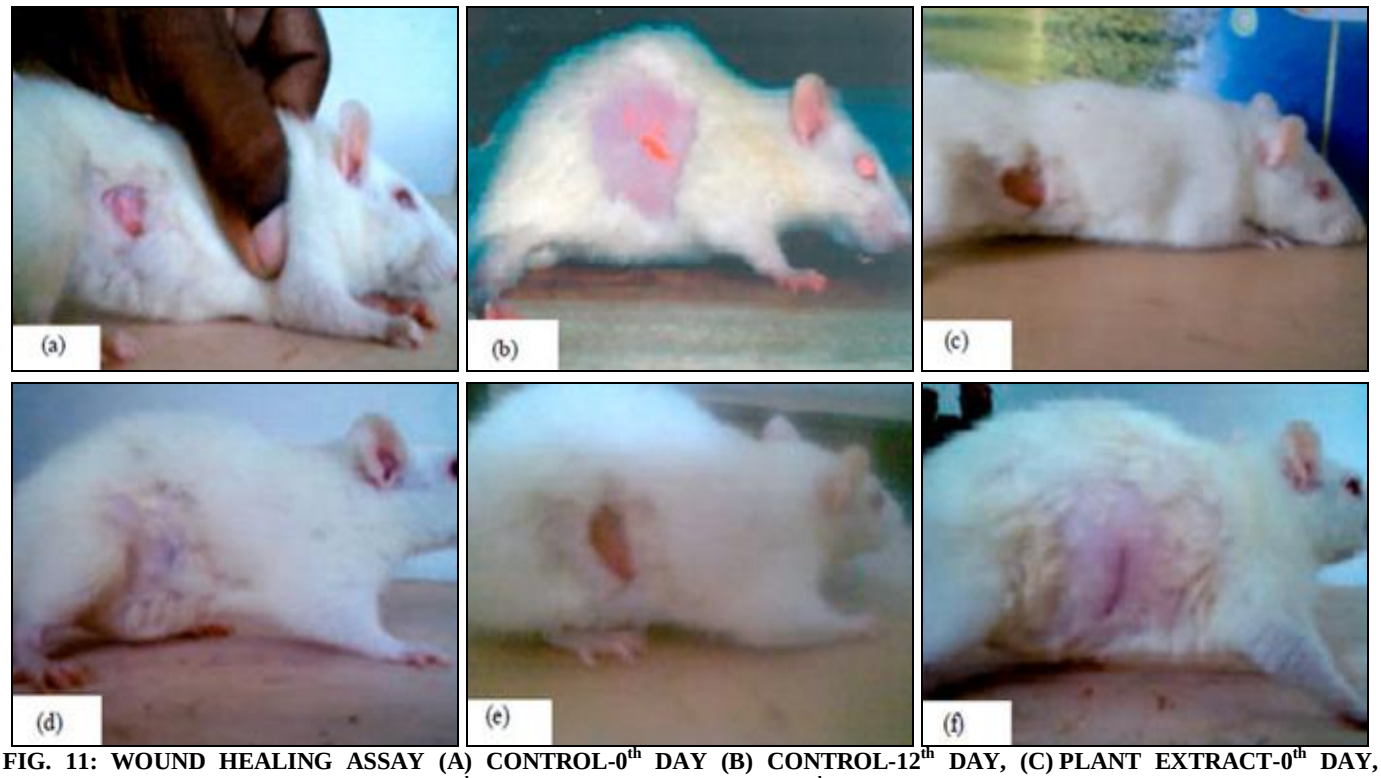

FIG. 11: WOUND HEALING ASSAY (A) CONTROL-0th DAY (B) CONTROL-12th DAY, (C) PLANT EXTRACT-0th DAY, (D) PLANT EXTRACT TREATED RAT-12th DAY, (E) STANDARD DRUG 0th DAY AND (F) STANDARD DRUG TREATED RAT 12th DAY [38]

Wound Healing Assay: It is simple, less expensive, and an earliest proposed method. It is based on the opinion of cell migration into a wound which is formed on cell monolayer [10]. Two circular holes of approximately 5 mm in diameter are punched with a tissue puncher through the dorsal skin of an anesthetized mouse. Wound size, scar formation and re-epithelization of the wounds should be recorded daily by photography and by measuring the wound area with calipers. Treatment can consist of pro-or anti-angiogenic compounds, and their effects on angiogenesis are determined to post mortem after the regenerated tissue has been excised, fixed and stained. Transgenic or knock-out mice can be used for the study of the specific effects of specific genes.

The impact of different culture conditions can be assessed by spotting the change in the size of the wound area. Cellular behavior is measured for further detailed examination. Spontaneous cell tracking in phase contrast microscopy images to wound healing assay has also been recently introduced. In which the performance of cell under three different culture conditions is investigated [11].

CONCLUSION: During the last decade, it has become standard care for a number of cancers. Therefore a thorough understanding of angiogenesis, with more relevant *in-vitro*, *ex-vivo* and *in-vivo* models may lead to significant discoveries in the molecular, cellular, and genetic biology of neovascularization. This may be an aid in developing effective anti-angiogenic treatment strategies in the future.

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