

**DEVELOPMENT OF ADVANCED THREE-DIMENSIONAL TUMOUR MODELS  
FOR ANTI-CANCER DRUG TESTING**



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**A Thesis Submitted for the Degree of Doctor of Philosophy**

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**TRINITY TERM**

**2014**

## ABSTRACT

Animal testing is still the common method to test the efficacy of new drugs, but tissue engineered *in vitro* models are becoming more acceptable for replacing and reducing animal testing in anti-cancer drug screening by developing *in vitro* three-dimensional (3D) tumour models for anti-cancer drug testing. In this study, three-dimensional (3D) culture methods were developed to mimic the tumour microenvironment. 3D culturing is to seed, maintain and expand cultured cells in three-dimensional space, in contrast to the traditional two-dimensional (2D) method in which the cells attach to the bottom of culture containers as monolayers. To mimic the intercellular interplay for tumour study, cell co-culture was applied. In this thesis, perfusion culture showed a better homeostasis for 3D tumour model growth over 17 days, with a more controllable working platform and a more reliable response-dose correlation for data interpretation. In the Matrigel sandwich system, the co-culture of breast cancer cells and endothelial cells demonstrated the morphology featuring a vascular network and tumour structures, with the thickness of the three-dimensional structure around 100µm and tubule length 200-400 µm, and maintained for 10 days. The comparisons studies between Matrigel sandwich and other methods suggest that though not fully characterised, Matrigel is still a valuable scaffold choice for developing co-culture 3D tumour model. Finally, the combination of perfusion and co-culture showed the potential of applying this model in angiogenesis assay, with a drug response profile combining cell viability and morphology to mimic *in vivo* tumour physiology.

**Key words:** *Anti-cancer drug testing, breast cancer cells, co-culture tumour model, colorectal cancer cells, endothelial cells, perfusion, three-dimensional culture.*



## **ABBREVIATIONS**

|                |                                                                                               |
|----------------|-----------------------------------------------------------------------------------------------|
| 2D             | Two-dimensional                                                                               |
| 3D             | Three-dimensional                                                                             |
| 5-FU           | 5-fluorouracil                                                                                |
| AO             | Acridine orange                                                                               |
| BRCA-1         | Breast cancer type 1 susceptibility protein                                                   |
| Cdk            | Cyclin-dependent kinase                                                                       |
| CXCL12         | C-X-C motif ligand 12                                                                         |
| DCFH-DA        | 2',7'-dichlorofluorescein-diacetate                                                           |
| DLD-1          | Number one of two colorectal adenocarcinoma cell lines isolated by D.L. Dexter and associates |
| DMEM           | Dulbecco's Modified Eagle Medium                                                              |
| DMSO           | Dimethyl sulfoxide                                                                            |
| ECs            | Endothelial cells                                                                             |
| ECM            | Extracellular matrix                                                                          |
| ERK            | Extracellular signal-regulated kinases. Also see MAPK.                                        |
| EthD-1         | Ethidium homodimer-1                                                                          |
| FBS            | Fetal bovine serum                                                                            |
| GFP            | Green fluorescence protein                                                                    |
| HER-2/ErbB-2   | Human epithelial growth factor receptor 2                                                     |
| HIF-1 $\alpha$ | Hypoxia inducing factor - 1 $\alpha$                                                          |
| HSP90          | Heat shock protein 90                                                                         |
| HTS            | High throughput screening                                                                     |
| HUVECs         | Human umbilical vein endothelial cells                                                        |
| ICAM-1         | Intercellular adhesion molecule - 1                                                           |
| LDH            | Lactate dehydrogenase                                                                         |
| MAPK           | Mitogen-activated protein kinase                                                              |

|         |                                                                                       |
|---------|---------------------------------------------------------------------------------------|
| MCTS    | Multicellular tumour spheroids                                                        |
| MEK     | Mitogen-activated protein kinase kinase                                               |
| MMPs    | Metalloproteinases                                                                    |
| MPM     | Multiphoton microscope                                                                |
| NCI/ADR | A breast cancer cell line developed by National Cancer Institute/Adriamycin resistant |
| PBS     | Phosphate buffer saline                                                               |
| PDMS    | Polydimethylsiloxane                                                                  |
| PI3K    | Phosphoinositide 3-kinase                                                             |
| PKB/Akt | Protein kinase B                                                                      |
| PLLA    | Poly (L-lactic acid)                                                                  |
| PLGA    | Poly (lactic-co-glycolic acid)                                                        |
| pRB     | Retinoblastoma protein                                                                |
| ROS     | Reactive oxygen species                                                               |
| RTK     | Receptor tyrosine kinase                                                              |
| TCP     | Tissue culture Plastics                                                               |
| TPZ     | Tirapazamine                                                                          |
| TRITC   | Tetramethylrhodamine isothiocyanate                                                   |
| TSG     | Tumour suppressor gene                                                                |
| VEGF    | Vascular endothelial growth factor Contents                                           |
| vWF     | von Willebrand Factor                                                                 |

## ACKNOWLEDGEMENT

I would like to take this opportunity to thank my supervisors Professor Zhanfeng Cui and Dr Cathy Hua Ye. Every routine meeting I had with them has been efficient and targeted, and their professional knowledge and academic skills inspired me so much that I think coming to Oxford is one of my best choices in my life. They also give me countless help in my life throughout my DPhil studies, including helping me to settle down quickly in my first year in Oxford.

I would like to thank Dr Richard Callaghan from Nuffield Department of Clinical Laboratory Sciences, University of Oxford for kindly providing breast cancer cell line NCI/ADR and colorectal cancer cell line DLD-1. I am grateful for Zoe Zhaohui Li, and Dr Julian George for their help in bioreactor assembling and drug testing. My present and previous colleagues Shaoyang Yeh, Dr Jinnan Zhang, Dr Sammy Sambu, and Dr Xinhui Liu all gave me kind help in my work, from cell culture, image processing to data analysis. I would also like to thank China-Oxford Scholarship Fund (COSF), and my college Linacre College, for their generous financial support in the most difficult time of my DPhil study.

Finally, I would like to express deepest thanks to my family. My parents and grandmother generously covered all of my DPhil tuition fee, and a lot of living expenses. My husband helped me through the hardest time in my DPhil study, giving me valueless financial, language, and emotional support throughout my studies. Without my family, I can never imagine to finish this thesis, or to live in UK, which is my first time in a foreign country in my life.

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## Chapter 1. Introduction

Cancer biology is a consistently developing area with still many basic questions to be answered. In addition, the development of anti-cancer drugs witnessed rapid progress in the past decades. The understanding of cancer development process and further development of efficient anti-cancer therapeutics with relatively low toxicity both require relevant models to provide pre-clinical data. 2D *in vitro* models allow the high-throughput screening of anti-cancer drugs, however the important cell-cell and cell-ECM interactions are missing and testing results using these models often show large differences between their preclinical testing and clinical trials. Animal testing was used to confirm results from 2D *in vitro* models, but there are intrinsic financial and ethical problems in this method, and more importantly the relevance of animal testing results is still questionable due to the difference between humans and animals. The 3D *in vitro* cancer models aim to bridge the gap between conventional testing in animals and traditional 2D cancer cell line testing..

Tissue engineering provides a novel approach to solve the above problems. Using engineering methods, human-origin cells can be used to build up cancer tissue *in vitro*, to mimic the intercellular and cell-ECM interactions *in vivo*, to test drugs in a well-controlled testing environment, with lower cost and less stress experienced by researchers, and more clinically relevant data. The aim of this thesis is to develop advanced three-dimensional (3D) *in vitro* cancer models, to explore the potential of tissue engineering of *in vitro* tumour models and their applications in drug screening and mechanism study.

## Chapter 2. Literature Review

Cancer is responsible for 1/3 of deaths in the UK. A large amount is being spent on the treatment and life quality maintenance for cancer patients. Since 1975, clinicians and researchers are expanding the understanding of cancer pathology. Although there is still a lot unknown about this group of diseases characterized by the uncontrollable growth of cells and life threats caused by metastasis, there is now an effective treatment strategy combined of surgery, radiotherapy and chemotherapy. Chemotherapy means to use anti-cancer drugs to kill cancer cells or control the metastasis of cancer.

In this literature review, a brief history of the carcinogenesis and the microenvironment of cancer development will be given first, then chemotherapy and categories of anti-cancer drugs will be summarized. In the third section the development procedures for anti-cancer drugs will be discussed, and the 3D models for anti-cancer drug testing, especially the most recent advances, co-culture and perfusion for 3D *in vitro* tumour models will be discussed in detail. Finally, the challenges faced by 3D tumour model-related techniques will be described, and solutions to these challenges will be discussed.

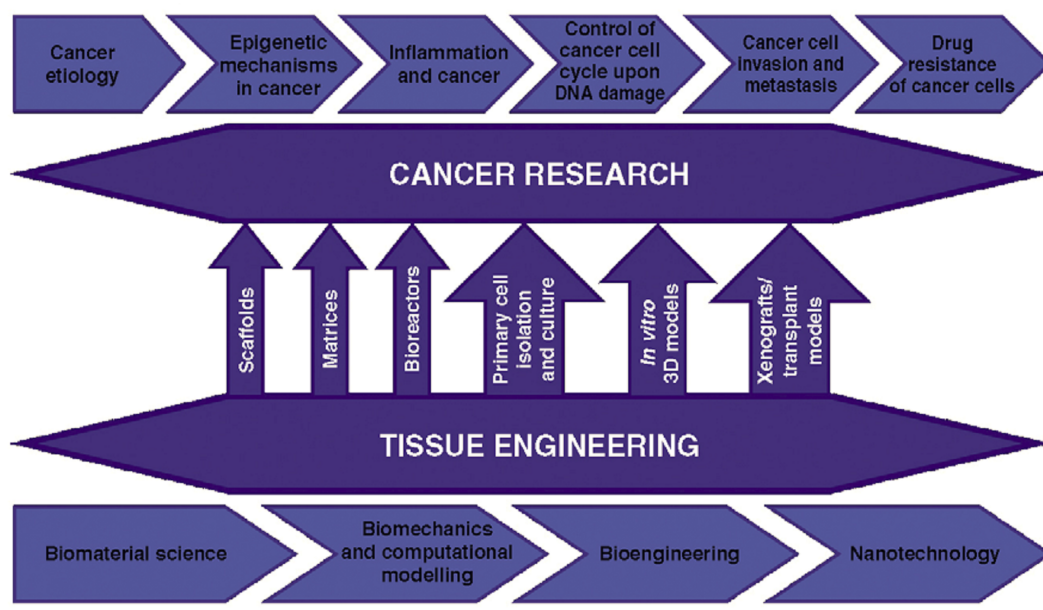
### *2.1 A Brief Introduction to Cancer Biology and Therapeutics*

#### *2.1.1 Classic theories of cancer biology – gene matters*

In modern days we all know cancer is a disease with the feature of uncontrolled growth of cells, due to the genetic malfunction of those cells, but in the 19th century, scientists were still arguing whether tumours were an external invader from the outside environment, like bacteria or virus, or an internal enemy developed inside human bodies. Both sides were trying to find evidences to support their hypotheses. The supporters of the invader-hypothesis



actually helped in discovering the risk factors of virus infection in certain types of cancer, like cervical cancer. The defenders of the internal-enemy hypothesis, however, finally laid the most well-developed genetic theory foundation for cancer – the genetic information with the potential to cause the cancer is already coded in our genes, but it keeps silent in normal cells. In the healthy body, there is an extremely strictly controlled balance of proliferation stimulation and death programme. Even if something goes wrong, like a mutation happens, there will be multiple levels of correction systems to eliminate those mistakes before any further problem occurs. Only if many external and internal factors accumulate for a years, then the cancer will finally happen, and cancer cells will invade, migrate, and disseminate to tissues other than the local primary tumour. This process of invasion and migration of cancer cells is called metastasis, and it is the metastasis of cancer which causes most of the mortality.



**Figure 2.1.** *Tissue engineering as a tool box interacting with cancer research. The development of cancer biology is shown on the upper panel of the figure, while the lower panel of this figure, showing the development of tissue engineering will be discussed in later sections. (Hutmacher et al., 2010)*

As early as 1890, David von Hanseemann noticed all of the 13 cancer tissue samples he collected showed abnormal chromosome morphology under the microscope (von Hanseemann, 1890). But only after Theodor Boveri systematically described the abnormal mitosis to support his genetic origin hypothesis in early twentieth century, the foundation of classic cancer genetic theories were finally founded, and cancer was viewed as a genetic disease afterwards. By using sea urchin eggs, he could introduce multi-polar mitoses into those cells and carried out his observation. He suggested that ‘incorrect combination of chromosomes will generate unlimited growth’ and this feature of unlimited growth can be passed from mother cell to daughter cells (Balmain, 2001). The significance of his hypothesis in cancer research history was further confirmed by the development of cell biology during that time. In his time, by doing experiments on fertilized eggs, scientists discovered that cells are the basic building blocks of all tissues, and all cells are divided from pre-existing cells. Those discoveries finally allowed scientists to deduce that all the cells in living organisms are originated from one cell. As a result, the malfunction of chromosomes in one cell can be passed to its daughter cells, and the accumulation of this disorganization of genetic information will finally cause out-of-control growth of dysfunctional tissues.

It was in 1902 that Theodor Boveri first suggested that tumours might be the result of abnormal behaviours of chromosomes. At that time there was still not enough experimental evidence supporting this hypothesis, however the observation obtained from on the morphology of fertilized eggs still inspired other researchers to think the malfunction of genetic materials is the origin of tumours. With the development of epidemiology which analyses large quantities of statistical data, and the development of cell and molecular biology to investigate the correlation of genes with cell functions, nowadays nearly no

researcher will believe that cancer is some living invading from the outside environment, like some scientists in the 19th century. Instead, we now know cancer cells are derived internally from previously normal tissues, though certain external environment factors might contribute to this triggering of cancer formation. By suggesting that cancer originates internally from genetic dysfunction of previous normal cells, Boveri is now widely recognized as the founder of cancer genetic theory (Weinberg, 2006). The development the cell molecular biology in 20 century helps us to understand the initialization and progression of cancer, and we can further combine the genetic knowledge with epidemiology to further predict the susceptibility of individuals to certain types of cancer and the response of cancer tissue to certain therapeutics.

Viewed from the perspective of modern but classic cancer genetic theories built up in the past decades, the development of cancer can be considered as the imbalance of two programmes of cell fates – cell death and cell proliferation. For either cell proliferation or cell death, there are genes coded by nucleic acids to be transcribed and translated into proteins, which then control important molecular pathways influencing cell functions. The accumulated effects caused by complicated molecular regulation in each cell will finally decide if normal cells can change into cancer cells, and if the mutated cancer cells can live, proliferate, migrate and eventually cause cancer. Compared with genes involved in cell death, genes in charge of cancer cell proliferation and growth are more widely studied, thus will be discussed in more detail as following. The newly developed stem-cell theory of cancer origination will also be briefly introduced.

Now from the molecular level, it is believed that carcinogenesis can be caused by genomic instability including mutational inactivation of tumour-suppressor genes or activation of

oncogenes (Markowitz et al, 2009). In other words, all the information needed to form cancer tissue, is already stored in normal cells, and the mutation of this information and accumulation of information ‘mistakes’ will eventually cause cancer. The normal genes with the potential to be mutated and then to cause over proliferation are called pro-oncogenes, and the pro-oncogenes that have been studied most are growth factor genes, which will be discussed with detailed example in the following section. The pro-oncogenes which obtain fatal mutations will become oncogenes. An oncogene is a gene causing cancer, by its high expression in cancer cells after mutation. To convert a pro-oncogene to an oncogene, there are different mechanisms to change the information carried by nucleic acids in DNA, including provirus integration and the defects of DNA repair system to cause gene amplification and change of copy number. Apart from information change in genes, the other most common genomic instabilities include chromosomal translocation, structure change of chromosomes, and methylation on DNA (Weinberg, 2006). Based on modern epidemiology supported by both epidemiologic studies and lab studies using animals, the external factors causing the above genetic instabilities are called carcinogens. Carcinogens include chemical and physical agents like tobacco or X-rays. In addition to the central rule of carcinogen-oncogene-oncoprotein, a more recent development of cancer genetics is the development of epigenetics, which studies functional and heritable genome change caused by mechanisms other than direct changes in DNA sequence, as suggested by the Greek-originated name (Greek of epi-: ἐπί- above, outer). Examples of such changes are DNA methylation and histone modification, both of which can alter gene expression without altering the underlying DNA sequence.

The basic theory that carcinogens facilitate the switch of pro-oncogene to oncogene, and then interfere with cell proliferation and survival is the basis of classic cancer genetic theories, and the 'internal carcinogenesis' idea of 19th century scholars such as Boveri is finally described in the terms of 'oncogenes'. The mutated and activated oncogenes will express oncoproteins, which will take part in crucial molecular pathways. The clinical significance of oncoproteins is they can be direct targets of anti-cancer medicine. There are many examples of oncoproteins, such as kinases HER2, cyclin-dependent kinases (Cdks), transcription factors like mutated p53, hypoxia-inducible factor 1 $\alpha$ , and some hormonal receptors, as well as catalytic subunits of telomerase. The most widely studied oncoproteins are probably growth factor receptors. Another clinical significance of studying oncoproteins is to find indirect way to inhibit their expression by targeting their upstream regulating proteins. Like all other proteins, the regulations of many oncoproteins are very complicated, but they share a similarity, which is their dependence on molecular chaperones like HSP90. These chaperons are usually proteins containing metal ions like iron or copper in their structures, and they play important roles in protein-protein interactions in growth-related molecular pathways (Rixe et al, 2007).

Functionally, many oncoproteins are tyrosine kinases, or their functions rely on tyrosine kinases. For example, two types of important carcinogenic proteins Src protein and EGF receptor both function as tyrosine kinases. By definition, tyrosine kinases are enzymes which can phosphorylate a tyrosine unit of a peptide sequence, and tyrosine phosphorylation is crucial in mitogenic signalling pathways. A good example to demonstrate the importance of those kinases is the pathway of growth factor receptor ErbB. ErbB is an oncoprotein that can stimulate cell proliferation by emitting growth-stimulating signals; its acting process is as

follows - N-terminal ectodomain of EGF receptor binds to EGF, then Src-like kinase present in the C-terminal cytoplasmic domain activated. This step is followed by kinase phosphorylates tyrosines on EGF itself, and the final effect is that cell proliferation is enhanced.

To describe the function of tyrosine kinases, the classic receptor-ligand model of molecular biochemistry also works. The first way to transduce signals through receptors of tyrosine kinase is to modulate signalling molecules. This can be fulfilled by forming non-covalent bonds, such as the binding of GTP binding to Ras, which is important in downstream signalling cascade acting as a G protein. Another way of transducing signal via tyrosine kinase receptor is to form covalent bonds such as phosphorylation of MEK by Raf. Besides those two ways, there are also other ways such as receptor dimerization. Now it is widely accepted that transphosphorylation is important for receptor signalling, and overexpression of EGF receptors facilitates dimerization of tyrosine kinase receptors.

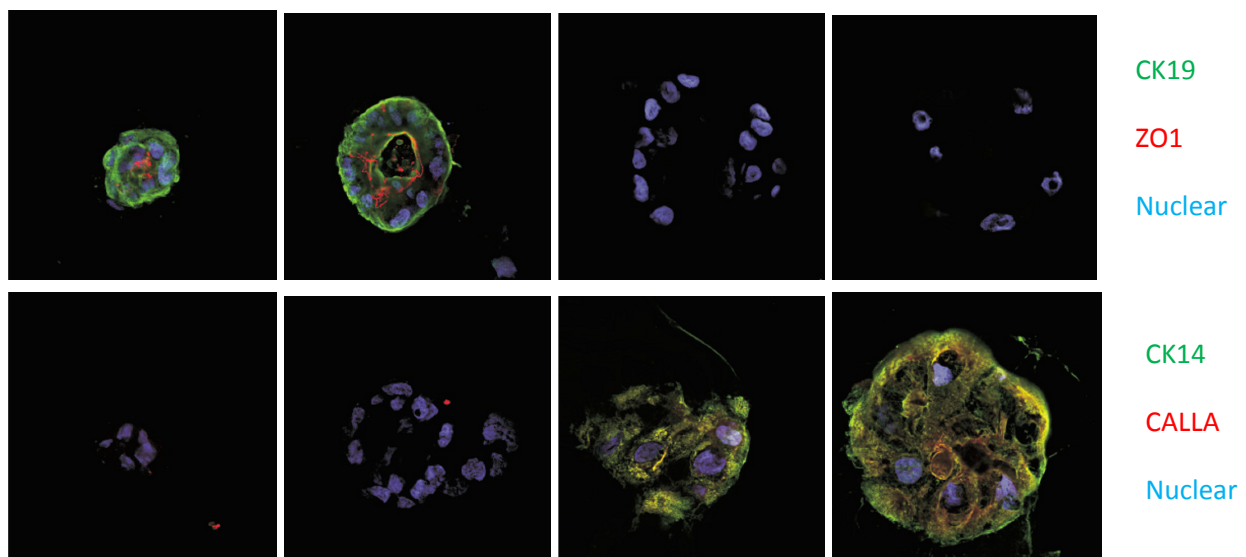
Other ways of passing signals through RTK include regulating the concentrations of signalling molecules, for example,  $\text{PIP}_3$  concentration increase can be caused by PI3K. In addition to changing structures of receptors or changing concentrations of signalling molecules, changing the locations of signalling molecules is also found in some RTK pathways, such as EGF pathway, which can be briefly summarized as  $\text{EGF-RTK} \rightarrow \text{Shc} \rightarrow \text{Grb2} \rightarrow \text{Ras} \rightarrow \text{PI3K} \rightarrow \text{Akt/PKB} \rightarrow \text{GSK} \rightarrow \beta\text{-catenin}$ . Translocations of cytoplasmic molecules to cell nucleus is commonly found in intercellular molecules location change. In summary, the phosphorylation process described above is not just important in cancer treatment by providing targets for anti-cancer pharmaceuticals development, but can also give

new clues in cancer diagnosis. The recent research revealed that phosphorylated HER2 can be a better reflection of HER2 protein itself, suggesting phosphorylation has potential to be a better indicator for clinical cancer diagnosis (Taniyama et al, 2008).

Besides the overexpression of growth signals, the low expression of cell growth suppressing genes can also cause carcinogenesis. The loss of tumour suppressor genes (TSGs) are more frequent than activation of pro-oncogenes into oncogenes, but the total loss of TSG functions can only happen when both copies are lost in a cell. The mechanisms of this function loss can be either genetic mutation or the epigenetic silencing via methylation. These growth suppressing genes are widely involved in the cell cycle model, in which the control of cell differentiation and the cell cycle are coupled by some regulatory proteins – hypophosphorylated pRB can promote cell differentiation and inhibit cell proliferation, while Myc has opposite effects.

In epithelial cells, cell-cell junctions form the special architectures, separating apical and basolateral membranes, and this apical-basal spatial distribution, or tissue polarity is essential for normal cell functions. The impaired polarity was observed in the malignant epithelial cells, represented by the disorganised laminin V and F-actin, in contrast to the normal apical-basal cell polarity in normal cells. This tissue polarity-loss process is considered crucial in tumourgenesis, supported by research in normal and cancerous breast cancer tissue (Ingthorsson et al, 2010) and a 3D culture model resembling colonic crypt (Tsunoda et al, 2010). The expansion of cell biology knowledge of signal cell proliferation, loss of cell death and tissue polarity-loss hypothesis give us a more dynamic picture of cancer development. Viewing cancer progress from tissue level, there will be mutations in a minority of cells, but

this mutation might be fixed by certain mechanisms. If the fixation mechanisms do not function, then these mutations will go irreversible, and cancer cells will be accumulated. Most of the fatality caused by cancer is due the migration of cancer cells and their penetration into other body tissues including locations of migration include lymph nodes, peripheral blood and bone marrow. This migration and penetration is called metastasis. Scientists used to believe that there was a clear sequence of metastasis events. Take breast cancer for example, the breast cancer cells will first move from local cancer tissue to lymph nodes, and then to peripheral blood, and finally to bone marrow. However, according to the recent research, it is found that these locations are actually involved in the first five years of 50% breast cancer patients (Lacroix, 2006).



**Figure 2.2.** *Structure disorganisation is a characteristic of malignant cells. Primary luminal epithelial (LEP) or myoepithelial cells (MEP) co-cultured with breast endothelial cells and embedded in Matrigel. For LEP co-culture, strong expression of tight junction related proteins were observed (ZO1), and the structure is more organized, while in MEP the structure is more disorganized with negative expression of ZO1. (Ingthorsson et al., 2010)*

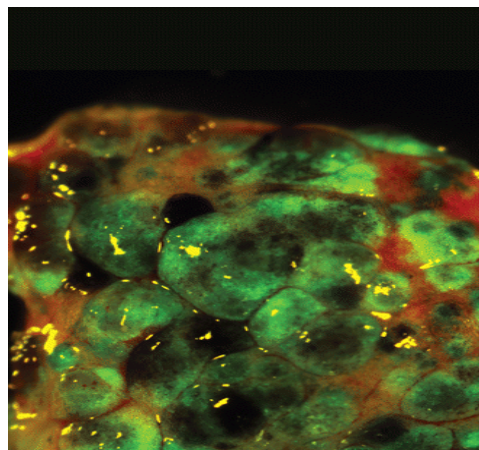


With the development of understanding molecular mechanisms behind tumour cell metastasis, medical authorities now divide tumours into either benign or malignant. Metastases of malignant tumours cause most death, and malignant cancer cells have high abilities of metastases. The morphology of malignant cancer tissue is featured with the missing boundary of basement membrane and loss of cell polarity. Tumours are categorized into four groups based on their origins – cancers are originated from epithelial cells, with squamous cell carcinomas arising from protective epithelia and adenocarcinomas originated from secretory epithelia; sarcomas originate from mesenchymal tissue; cancer originating from circulatory or immune systems is called hematopoietic cancer. Nowadays, the development of molecular biology theories and molecular techniques allow clinical researchers to correlate detection results with prognosis, and to track the origins of the cancer more effectively (Lacroix, 2006).

In summary, the classic theories of cancer genetics rely on the development of modern antibody-based and nucleic acid-based techniques, like ICC and PCR, and the understanding of carcinogens by studying epidemiology information helps us to correlate certain external and internal factors with the happening of cancer. The imbalance of cell growth and death is now widely accepted as the feature of cancer, and most cancer fatality is caused by metastasis. The important proteins and genes in cancer growth and metastasis, oncoproteins and oncogenes, can be potential targets of anti-cancer therapy.

### *2.1.2 New understanding of cancer – environment also matters*

In section 2.1.1, a brief history of cancer cell biology, especially the genetic aspects of this disease, has been given. However, even the cancer cells themselves are already complicated enough, an emerging and promising research trend is focusing more on cancer cells' communications with other types of cells, and the interactions between cancer cells and the tissue microenvironment around them. It is found out by animal and cell experiments that cancer cells in metastasis are like 'seeds', and without suitable 'soil' – tissue and other cells surrounding the cancer cells – the cancer cells will not be able to survive and grow. Thus, nowadays more and more researchers are focusing on the 'soil', which is the environment around cancer cells, including other cell types and extracellular matrix.



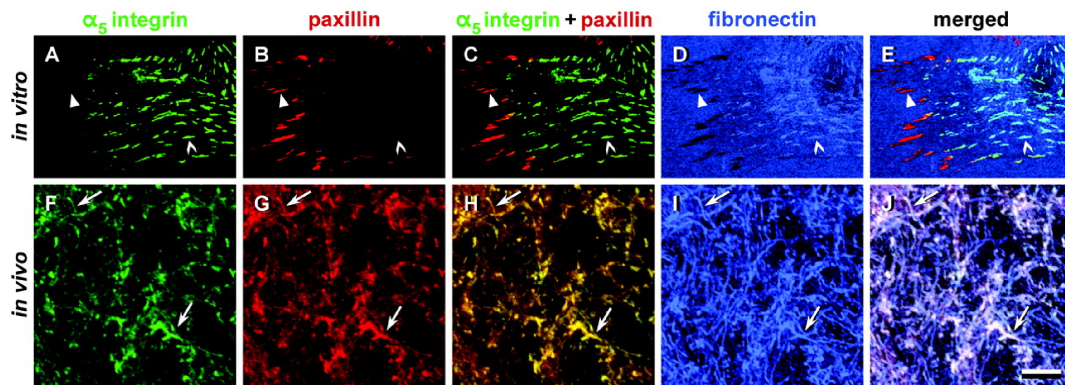
**Figure 2.3.** *Acidic microenvironment of cancer. Green fluorescence reveals the high accumulation of AO in lung cancer cells, and orange fluorescence beyond the boundary of tumour implies acidic environment of cancer. (Nishisho et al, 2011)*

This 'seed and soil' hypothesis is actually not a new story, and its development has relied on the observation of a specific step in cancer development – metastasis, which is the cancer cells' invasion and survival in tissues out of its origin place. Ever since 1889, an assistant surgeon in London called Stephen Paget already suggested the tissue to which the cancer

cells migrate to is crucial in the occurrence of final metastasis. By investigating 735 historical cases, he found out that compared with the spleen, the liver is more likely to form metastasis, even though spleen and liver share nearly the same blood flow exposure to cancer cells (Paget, 1889). He ironically stated that most of the excellent researchers during his time were like botanists, concentrating on the seeds, which are the cancer cells themselves, while he himself was like a ploughman focusing more on the soil, which is the growing environment for cancer. Unfortunately, his idea was not paid enough attention for many years, until in the 1980's researchers like Ian Hart and Isaiah Fidler provided more evidences in their lab, using mice as models (Hart et al, 1980). They transplanted kidney, liver and ovary tissues into mice, until those tissues developed their own blood flow, then they injected mice with melanoma cells. Interestingly, although radioactive labelled cancer cells were found in all of these tissues, only liver and ovary developed cancer metastasis, but not kidney. In other words, they proved that only 'landing' of cancer cells was not enough, there should also be a suitable environment to accept those cancer cells as the new residents of the tissue area.

The investigation on cancer cells' metastasis attract scientists' eyes from abnormal changes in cell nucleus to the unusual cytoskeleton structure alteration. To realize metastasis, which is the survival and growth of cancer cells out of the original birth place, cancer cells need to finish steps including invasion, migration, and dissemination. Although logically these processes can be finished in a random manner, in reality it is more often that the concentration gradients of certain chemicals in surrounding tissue will guide the directions of cell movements in those processes. This phenomenon in which cells move to a certain direction guided by concentration gradient of certain chemicals is called chemotaxis. Chemokines are chemicals who can act on cell movement by their chemical gradients

(Roussos et al, 2011). The gradual cytoskeleton morphology change inside a cancer cell starts from the polarization of actin directed by intracellular signals in response to the chemokines. Then the cell will adhere to the surface of the cell-substrate edge. This adherence is mediated by integrin, an important protein in surface interactions of cancer cells with the extracellular matrix (ECM). The asymmetrical movement of cytoskeleton is then repeated in the same direction and the cell can realize the movement into the substrate it wants to migrate into (Roussos et al, 2011). Integrin receptors in this process are essential because they sense cells' association with ECM and then act as the translators between cancer cells and their environments.



**Figure 2.4.** *Cell adhesion molecule localisations differences between 3D in vivo tissue and 2D in vitro culture. (A) to (E): Confocal images of indirect immunofluorescence staining of mouse fibroblast NIH-3T3 in 2D in vitro culture; (F) to (J): cryostat craniofacial mesenchyme sections of an E13.5 mouse embryo. It was noted that  $\alpha 5$  integrin ((A) and (F), green) and paxillin ((B) and (G), red) co-exist in a 3D mesenchymal tissue, which is shown as yellow in merged image (H), but not on a 2D substrate in vitro (C). Furthermore, merged image J shows co-expression of all three molecules including fibronectin ((D) and (I), blue), compared to (E). Focal adhesions, which are indicated by filled arrowheads) show differential localization of the  $\alpha 5$  integrin and paxillin markers on traditional flat 2D substrates in vitro. Scale bar, 5  $\mu$ m. (Cukierman et al, 2001)*

In ECM, cancer cells do not play their roles alone. There are also other cells supporting them in a successful metastasis. They are called stromal cells, and they are involved in important events such as chemotaxis. There was a new concept raised up to describe this whole community in cancer progression - Morphogenesis, which means the creation of shapes, is used to describe the remodelling of environment in cancer progression. The key factor of the morphogenesis hypothesis is morphogens. Different from chemokines mentioned above, which can guide cells' movement directions by their concentration gradients, the difference of morphogen concentration has direct effects on cell differentiation, which means the expression of gene profiles for cell functions. Different concentrations of morphogens can decide the fates of cells, leaving them to develop various behaviours. The molecular biology explanation for this differentiation-determining power of morphogens is that their concentration gradients can release the information on cell position, tissue orientation and the size of the morphogenesis structure which will then influence the cells. The theory of morphogenesis suggests an active, dynamic responses from surrounding environment in cancer cells' progression (Potter, 2007).

The chemotaxis and morphogenesis phenomenon can also be observed in other processes in normal physiological situations, like acute inflammation. Thus, a lot of comparisons have been made between inflammation and cancer. However, compared with acute inflammation, in carcinogenesis the events of inflammation happen in a very disorganized way, and in contrast to the final homeostasis formed in acute inflammation, in cancer tissue remodelling there will be no homeostasis reached, but the stimulus will carry on to promote the uncontrolled growth of cancer tissue (Albini et al, 2007). This comparison between cancer

cells and inflammation triggered a landmark in cancer biology research - one major event was recognized by scientists - angiogenesis, the new blood vessel formation process in the development of cancer. Without the formation of a new blood flow network in the metastasis area, the cancer cells will not go that far. The cancer- angiogenesis hypothesis was first proposed by Judah Folkman in 1971 (Folkman, 1971). It eventually took more than 30 years for the first drug based on anti-angiogenesis to be taken from bench work to finally being approved by the FDA (Ferrara et al, 2004). The interactions between cancer cells and the endothelium is crucial in cancer metastasis, and the importance of this interaction is confirmed in both static and dynamic testing systems (Orr et al, 2000). These experiments do not only confirm the significances of the relations between cancer cells and endothelial cells, but also reveal that many molecules are important in this process, including integrins, selectins, cadherins. Many of them are required in the adhesions of cancer cells to the endothelium. Among those molecules, integrins might be the family of proteins which are studied most systematically. Now it is already known that the  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  can support vascular endothelial growth factor signalling in the migration of endothelial cells, and thus influence angiogenesis in tumour (Senger et al, 2002).

The regulation of angiogenesis can be viewed from different perspectives – genetic and epigenetic. The epigenetic regulations of angiogenesis include pH and redox (reduction-oxidation). Many small epigenetic chemical molecules with redox characteristics, not only oxygen ( $O_2$ ), but also nitrogen monoxide (NO), and hydrogen peroxide ( $H_2O_2$ ) were studied (Miller et al, 2009). It is noticeable that in many cases the genetic and epigenetic regulations are not independent but closely related to each other. Vascular endothelial growth factor (VEGF) is a good example. The receptor for VEGF - VEGF receptor-2 (VEGFR2) is

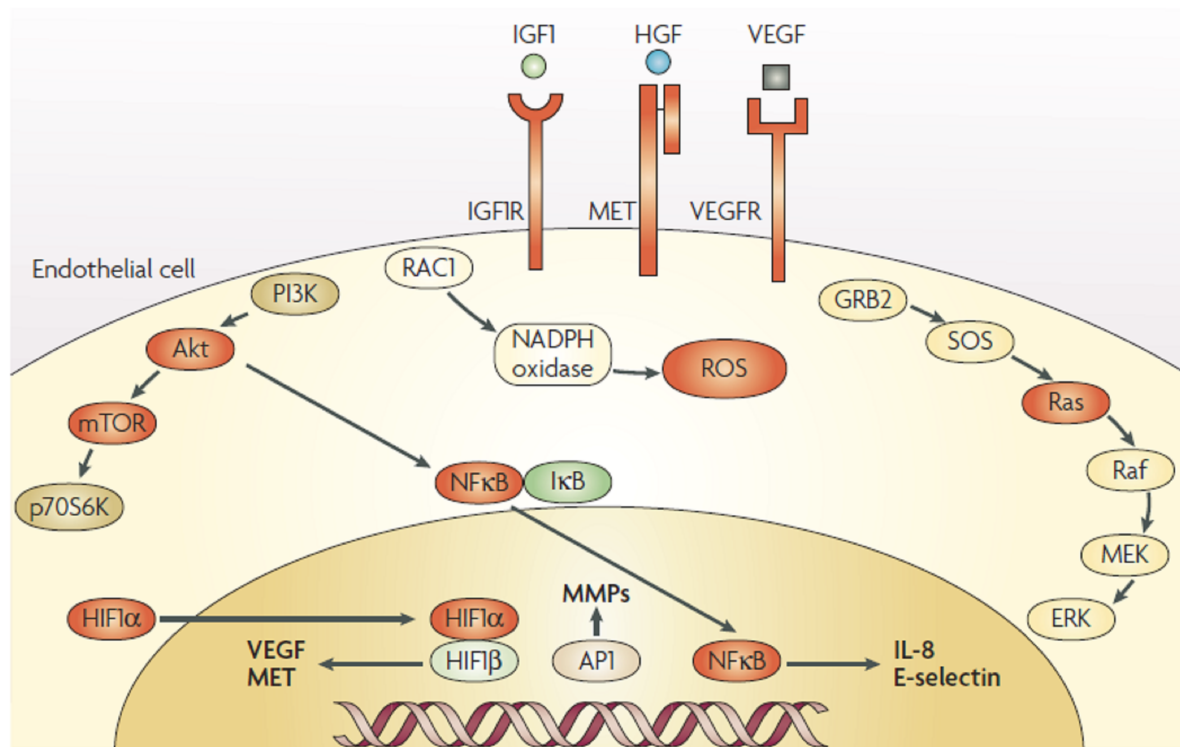
revealed to be regulated by oxidative stress, which is the physiological cause of some epigenetic factors like oxygen ( $O_2$ ), but also nitrogen monoxide (NO), and hydrogen peroxide ( $H_2O_2$ ). The VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF). VEGF can be produced by tumour cells, and the receptors of VEGF are overexpressed in endothelial cell. So for a long time people believed that there was only paracrine cooperation between endothelial cells and cancer cells involved VEGF pathways. However, this old theory is challenged by the latest clinical reports, and further explanation is given that VEGF can also be produced by other cell types, and there is an 'intracrine' pathway in cancer cells for VEGF to act on themselves. The binding of VEGF and VEGFR-2 can lead to downstream pathways including proliferation, survival and migration of endothelial cells. The proliferation pathway includes the activation of  $PLC\gamma$ -PKC-Raf-MEK-MAPK, the survival pathway includes the activation of PI3K-Akt, and the Src is involved in the actin cytoskeleton changes in the migration of endothelial cells (Kerbel, 2008).

The endothelium-tumour interactions might be involved in even more complicate interactions of cancer cells with cell types other than endothelial cells. Research has shown that the direct contact between cancer cells and endothelial cells is responsible for the failure of immune cell filtration in the tumour microcirculation vessel system. However, this effect is dependent on the subtypes of immune cells, with the lymphocytes not influenced and neutrophils are strongly dependent on CD44-triggered endothelium activation (Blaheta et al, 2009). Another evidence of the heterotypic intrinsic of endothelium-tumour interaction is the paradox that anti-angiogenesis therapy is synergistic with traditional chemotherapy and radiation therapy of cancer, while the fact has been accepted that those traditional therapies should be less effective in hypoxic environment (MBuess et al, 2009).

There are both theoretical and practical significances of studying the microenvironment of cancer. First, by viewing the cancer formation as a heterogeneous process, we might find out more targets for chemoprevention, not only anti-proliferative, but anti-inflammatory and anti-angiogenic therapeutics will be taken into consideration (Albini et al, 2007). Second, getting to know the heterotypic interaction making the cancer cells and their environment into an integral community allows the researchers to re-evaluate the traditional models we used to test anti-cancer drugs, and this finally leads the researchers' attention to the developing of three-dimensional models, which can bring those complicated interactions into the testing models (Cukierman et al, 2001). Overall, the study of angiogenesis does not only extend our understanding of the cancer mechanism, but also provides a more targeted therapy for cancer, compared with conventional chemotherapy.

As discussed above, now the cancer research community already accept the dependence of cancer development on microenvironment. However, that is still not enough to emphasize the importance of cancer environment. One more recent hypothesis is that the interruption of microarchitecture around cancer cells does not only help them in later stages, but also is the primary cause of these cancer cells (Potter, 2007). The cancer stem cell theory is closely related to this hypothesis, suggesting only a small group of cells with stem cell features contribute to the resistance and recurrence of cancer. This hypothesis emphasized that is the relation between cells that matters, not just the single cells. In summary, the recent decades witness the expansion of the picture of cancer development by two beliefs – not only the cell nucleus matters, but also cytoskeleton is involved; not only the cancer cells themselves matters, but the environment also matters. The recent stem cancer cell hypothesis will further provide new perspectives into this vivid research area.





**Figure 2.5** *Molecular targets for angioprevention. Main pathways are schemed, including some versatile pathways like Ras-Raf-MEK-ERK pathways, PI3K-Akt-NFκB pathways. Growth factor receptors are highlighted including IGF, HGF and VEGF. Anti-cancer drug targeting VEGF has been developed, such as Avastin, which is a recombinant humanised monoclonal antibody against VEGF. It is also noticeable reactive oxygen species are playing important roles in angiogenesis, because ROS is the upstream signals for many angiogenesis regulating pathways, including VEGF.*(Albini et al, 2007)

### *2.1.3 Clinical Treatment of cancer and chemotherapy – past and future*

The oldest and best established treatment for cancer is surgery. In general, surgery is firstly considered in cancer treatment when it is believed that the cancer tissue is still restricted in regional areas, and can be removed without harming the surrounding normal physiological structure. When surgery cannot remove the regional cancer or there is a higher risk of interrupting surrounding environment than the potential benefits for the patients, radiotherapy might be then applied. The outcome of radiotherapy depends on the types of cancer, due to the different sensitivities of different cancer cells to ionizing radiation.

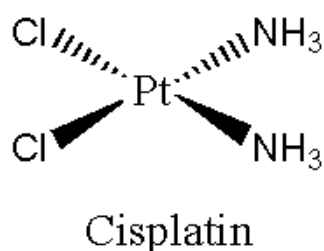
Besides the above two methods to treat cancer, chemotherapy - using anti-cancer drugs to inhibit cancer development - was traditionally used for cancer which is not regionally restricted, like leukemia. The old understanding was that cancer metastasis only occurs widely at a relatively late stage of development, so in the past for a while surgery and radiation therapies were on top of chemotherapy in the priority list of cancer treatment. However, more recent studies implied that cancer metastasis might happen in the early stage, so based on this new understanding chemotherapy might be used from the first time diagnosis of cancer (Skeel, 1999).

There are multiple mechanisms for anti-cancer drugs to act on cancer cells. Drugs can act on macromolecular synthesis or function, cytoplasm organization or environment of cancer cell survival and growth. For example, Cisplatin and Fluorouracil are classified as alkylating agents, which can interfere with the synthesis and function of DNA in cancer cells. Paclitaxel, which is a naturally originated anti-cancer compound discovered in tree bark, on the other hand, can block the normal process of mitotic division by interacting with

microtubule in cytoplasm. Some anti-cancer drugs rely highly on the cancer microenvironment, for instance, Tirapazamine (TPZ) is only effective in the hypoxic environment of cancer tissue. These anti-cancer drugs will be discussed further in categories as following.

### ***Alkylating agents***

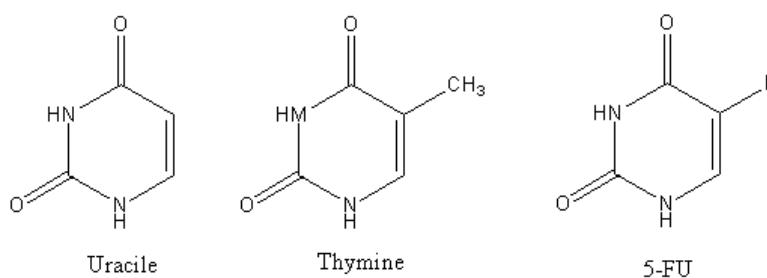
Cancer cells proliferate faster with less error correction, as a result they are sensitive to DNA damage. Alkylating agents add a methyl or other alkyl group to the double helix structure of DNA molecules, usually guanine base, thus interrupt the base-pair process in synthesis or transcription. Because of this characteristic, these drugs usually have toxicity for normal cells which are actively proliferating, which are also synthesizing actively the above macromolecules. Cisplatin, cis-Diamminedichloroplatinum (II), is an inorganic alkylating agent. The mechanism of Cisplatin is to bind and cross-link strands of DNA, and Cisplatin is usually applied in combination with other drugs to treat cancer (L'Azou et al, 2005).



**Figure 2.6** *An example of alkylating agents – Cisplatin. This platinum based anti-cancer agent is a classic example of inorganic anti-carcinogenesis chemicals. The configuration of Cisplatin structure allows it to insert into the double helix of DNA in cancer cells, thus cause cell death of these cells.*

### ***Antimetabolites***

This class of drugs are usually small molecule compounds, named by their abilities of mimicking the structures of natural metabolites and then interfering with these nucleic acids' metabolism (Roland T. Skeel, 1999). Types of antimetabolites include folic acid analogs, pyrimidine analogs, and purine analogs. Fluorouracil, 5-FU, is an example of a pyrimidine antimetabolite which can be converted to the active nucleotide and interfere with the DNA synthesis process by inhibiting the enzyme thymidylate synthetase (Zhao et al, 2008).

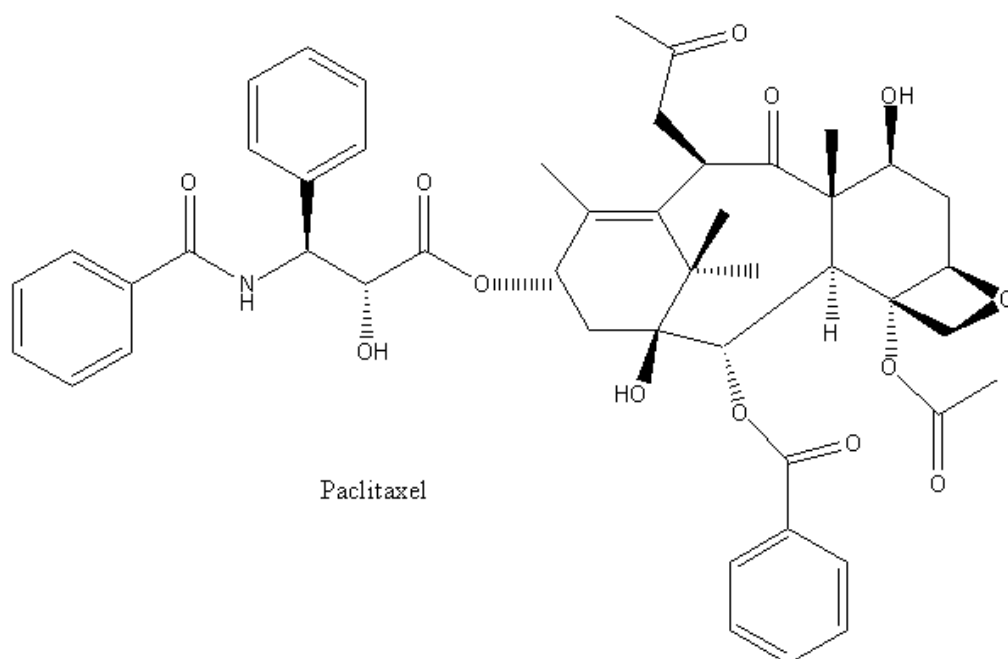


**Figure 2.7** *An example of antimetabolites – 5-FU. It has a similar structure to the materials for RNA and DNA synthesis in cancer cells, uracile and thymine, thus cause the inefficiency of proliferation system in those cells and inhibit cancer tissue growth.*

### ***Natural products***

As described by the name of this class, these drugs are categorized together mainly because of their origins - extracted from natural products, though they could have different acting mechanisms from each other. Popular members in this family are mitotic inhibitors and antibiotics with cancer inhibiting effect. Paclitaxel is a classic example of mitotic inhibitors.

Named by its natural resource, it can stabilize microtubules to arrest the mitotic proliferation process of cancer cells (Zhou et al, 2005).

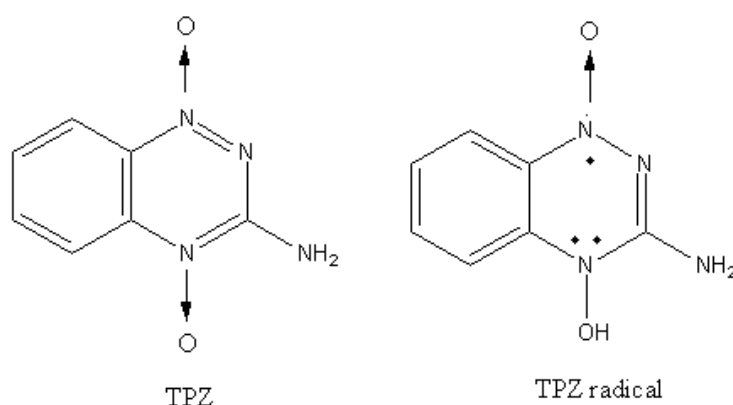


**Figure 2.8** *An example of anti-cancer natural products - Paclitaxel. This chemical shows the abilities to interfere mitosis of cancer cells, which leads to the inhibition of cancer development.*

### ***Other anti-cancer reagents***

In addition to the above three types of anti-cancer drugs, with the development of cancer molecular biology, some hormones and hormone antagonists have also been studied for their anti-cancer mechanisms. The development of cancer microenvironment theories lead to the invention of new drugs with higher selectivity to cancer tissue, such as redox-sensitive anti-cancer drug tirapazamine (TPZ). Tirapazamine is still under clinical investigation, but the

mechanism of its action is confirmed to be related to toxic radicals activated by reductases in cells, and only at relatively low concentration of oxygen, or hypoxia situations of cancer tissue this activation is possible. The free radicals will then cause DNA damage, and interfere with the DNA repair mechanisms in cancer cells. The hypoxia situation is common in the centre area of various types of solid tumour, and central areas happen to be resistant to traditional chemotherapy drugs mentioned above. Many researchers are trying to combine TPZ and other traditional chemotherapy and radiotherapy to further test its potential in cancer treatment.



**Figure 2.9** *A free-radical based anti-cancer reagent – Tirapazamine. The radical formed in low oxygen environment is the active formula to reveal the cytotoxic effect. This activity can only be observed in models with 3D structures, not in 2D conventional in vitro models.*

Due to the long and complicated history of cancer research, the above termination system has more than one standard of categorizing anti-cancer drugs, using mechanisms, sources and structures for standards at the same time. It is noted that there is consistently argument about the differences between cytotoxicity and cytostasis. Instead of killing cancer cells by

cytotoxicity, some cytostasis drugs such as Paclitaxel were considered to be only effective in stabilizing cancer cells but can still prolong survival of cancer patients. More recently, some evidence from clinical observations shows that there is no distinct difference between those two mechanisms (Rixe et al, 2007).

In addition to the understanding of the acting mechanisms on cell and molecular level, in order to develop a treatment of cancer for specific patients, the following factors should be taken into consideration – the growth distribution of cancer tissue, the total cell number in the cancer tissue, cell growth rate and cell death in cancer tissue. All of these factors will decide the growth kinetics of cancer tissue, which is how big the cancer tissue will grow, and how fast this process can be. One common challenge in determining a clinical therapy plan is the evaluation of potential benefits and toxicity of anti-cancer candidates, and the resistance cancer cell developed through the treatment to the original drug profile, which are the two main barriers in chemotherapy.

The mechanisms for anti-cancer drug toxicity are the interactions of those drugs with normal tissue and cells. Similar to cancer cells, normal cells also have macromolecules with similar structures such as DNA and important proteins. Especially for drugs acting on the synthesis of DNA, although cancer cells will be assumed to take in more medicine due to the higher proliferation activity than normal cells, normal cells especially those with active dividing states such as cells involved in hair growth and digestive system renewing will still be influenced significantly by those cytotoxic medicine.

Apart from the toxicity issue of chemotherapy, drug resistance is another concern to limit the application of chemotherapy. Drug resistance means the sensitivity of cancer cells to existing treatment drugs decreases over the treatment process. Mechanisms responsible for drug resistance of cancer cells include decreased absorption, increased excretion and metabolism of drugs by the cancer cells, and there is still considerable research going on in this area. It is noticeable that different cancer cell types have different sensitivities to the same anti-cancer drug, and possibilities of drug resistance developed by different cancer cell types can be different. To overcome the drug resistance and minimize drug toxicity, in modern chemotherapy, instead of using one drug all the time, physicians usually apply the combinations of drugs with different acting mechanisms, or combine one active drug with another inactive agent to enhance the absorption of the active drug (Hong et al, 2011).

In general, the basic rule of a reasonable chemotherapy plan is to make sure of the treatment target, the stages of cancer, and be aware of the basic principles of primary indications and common toxicities of drugs. Considering the future of chemotherapy, there will be two tendencies - switching from conventional DNA damaging and apoptosis-promoting agents to more targeted therapeutics like kinase inhibitors, and changing to a strategy more targeted at tumour specific-process like angiogenesis rather than general cytotoxicity cancer treatment strategies (Skeel, 1999).



## *2.2 Pre-clinical Models for Anti-cancer Drug Development*

The introduction of a new drug into the anti-cancer therapeutic area relies on ‘testing models’. Based on the general concept of models, these drug testing models are not necessarily cancer tissue themselves, but can represent basic physiological and pathological features of real cancer. The standards of a good pharmaceutical testing model include – the results obtained from those models should be easy to repeat among different researchers; the whole testing procedure should be clearly controlled and should be relatively easy to be interpreted. By selectively simplifying end details and extracting the essentials of complex real physiological or pathological situations, those models allow researchers to manipulate specific environment parameters and observe the models’ responses. The researchers will then be able to build up the correlation between the controlled variables and the dependent variables.

In the case of testing a potential anti-cancer drug, the variables being controlled can be incubation time, or various concentrations of the tested drug, and the dependent variables are tumour models’ responses to drugs, such as efficacy and toxicity. The results from one model usually needs further confirmation by other models, and the results from pre-clinical models will be used to predict human responses of the drug in the following clinical trials. The results from both pre-clinical models and clinical trials will be presented to certain authorities, like the Food and Drug Administration (FDA). If the drug can be finally approved by those authorities, it will be finally put into the market and still in observation for decades. The development of new drug is a highly risky business, with fairly low success percentage.

### 2.2.1 *In vitro* and *in vivo* testing of anti-cancer drugs

Traditional 2D mono-culture *in vitro* models have been used for many years due to easy evaluation methods, and their relatively easily operated process. However, since those models are mainly monolayers or multiple 2D monolayers of cancer cells isolated from clinical samples, much misleading information may be given due to the lack of intercellular interactions and extracellular matrix (Cukierman et al, 2001). Conventionally, these *in vitro* models must be further confirmed by results from *in vivo* models, which means to test drugs in the living body and is always referred to animal models. In anti-tumour drug testing, the most widely accepted *in vivo* model is xenograft tumour model. In this method, human tumour cells are transplanted into immune-deficient animals, and grown into solid tumour followed by drug treatment (Giacomotto et al, 2010). Many problems are caused by this traditional methodology, including the time and cost consumption, technique requirement and ethical controversy of animal experiments (Fischbach et al, 2007).

High-throughput drug screening (HTS) is based on the modern development of molecular biology, analytical chemistry and information technology. The random chemicals from structure library will be screened by certain models to find out the ones with suitable affinity to certain targets. The microarray technique and the DNA sequencing technique allow the screening of thousands of compounds in a few seconds, and the widely shared information from the internet and databases allows the researchers from different geographical areas to work together to find the suitable structures for certain targets in cancer therapy. Another pathway of finding new drugs is called virtual screening, which is to design molecules from the known target structures (Giacomotto et al, 2010).

However, although the above methods have brought considerable profit to pharmaceutical companies, the limitations of traditional target and model based methodology is being challenged by the lack of known targets, and the failure of recent *in vitro* models to copy some *in vivo* mechanisms. As a result, some drugs showed undesirable results in clinical trials, after they passed the testing of pre-clinical models, and this caused big waste of money and time. To overcome this problem, some researchers are trying to adopt *in vitro* advantages into *in vivo* models, to make the *in vivo* models easier to be manipulated than the traditional *in vivo* models like mice. Those newly developed models include small animals like *Caenorhabditis elegans* (roundworm), *Drosophila melanogaster* (fruit fly) and *Danio rerio* (zebra fish) (Giacomotto et al, 2010). Another effort from a different perspective is to make up the limitations of the recent *in vitro* models, by adding cell-environment and cell-cell interactions into the conventional 2D *in vitro* models. This is implemented by the development of 3D *in vitro* models for cancer drug testing. This resolution has its own advantages compared with the previous method of improving *in vivo* models, because the human cells or tissues can be used *in vitro* testing, allowing the reproduction of a tissue morphology and functions originated from human gene profile, while the *in vivo* models can only use animals, who have genes different from humans.

### *2.2.2 3D models of tumour models*

As mentioned above, to overcome the lacks of extracellular matrix in traditional 2D mono-culture, researchers have been trying to culture cells in a three-dimensional way. Basically, there are two ways to realize this: spontaneous aggregate by cancer cells, or using scaffolds or matrices to support cells (Dietmar et al, 2009; Gurski et al, 2010; Kim et al, 2004), as described below.

#### ***Spheroids***

To create spontaneous aggregates is a straightforward way to create 3D cultures. This method is based on the adherence intrinsic of cancer cells to each other and to other cell types. Holtfreter and Moscona are the pioneers for spheroids culture and observation, who did their observation in 1940-50. From that time on, spheroids were systematically studied for morphology, microenvironment and genetic mechanisms in cell proliferation, differentiation and gene expression regulations by Sutherland in the 1970's, and now become a well accepted representative of 3D cancer cell culture. 1980-1992 was the important period for spheroid technique development. During that period, except responses to radiotherapy, other cancer related process such as the angiogenesis was also studied using 3D culture, and invasion was studied in combination with angiogenesis in the same period. Besides the basic pathological process study, there were also considerable drug-testing feasibility studies. The resistance to drugs was found to be higher in 3D (Desoize et al, 1998), and the drug penetration (Sutherland, et al, 1988), binding and action were also studied in 1980-1992 (Knuechel et al, 1992).

The cell-ECM and cell-cell interactions are believed to be essential for the formation of the spheroids. Integrins are believed to be involved in the process of cell clustering, with a large

number of them can bind to a specific three amino acid sequence (arginine-glycine-aspartate) also known as RGD sequence. The roles of integrins are mainly summarised as the intermediate between extracellular signals and cellular functions such as cell survival and mobility. Apart from cell-ECM interactions, cell-cell adhesions are also crucial in the spheroid formation process. The two groups highlighted in the previous research are cadherins and cellular adhesion molecules (CAMs) (Santini et al, 2000). It was suggested by Kelm et al that their preliminary work indicates a correlation between poor spheroid formation and poor tumourigenicity (Kelm et al, 2003). Measured by the ability of cultured cells to give rise to either benign or malignant progressively growing tumours with viable and mitotically active cells in immunologically nonresponsive animals over a limited observation period, tumorigenicity is considered to be related to cell-adherence molecule such as intercellular adhesion molecule – 1 (ICAM-1). There was report showed that colorectal cancer cell lines expressed higher level of ICAM-1 and CD44 than ovarian cancer cells, which contributes to their higher spheroid formation ability (Santini et al, 2000).

Now the researchers all accept that the advantage of spheroid culture is to rebuild up the link between *in vivo* structure and function in *in vitro* environment. The most popular ways to build up spheroid culture include spinner flask and agar-coating. It is also found that spheroids have differentiated structure mimicking the cell shapes and real tumour microenvironment better than conventional 2D cell culture, thus this model will further give a better simulation of cell gene expression and functions compared with traditional 2D *in vitro* models. The structure of spheroid models featured with the necrosis central area, static internal layer area and active proliferating outer area. A commonly accepted methodology platform of spheroid model analysis has also been built up in pharmaceutical community,

with a set of assays including morphology parameters, such diameter/volume measurement, as well as other function parameters, like cell viability or gene expressions. Considerable research using this combination of morphology and function assay concept already found out many significant differences of growth kinetics, gene expression, drug responses between spheroids and conventional 2D cell cultures.

Recently, the research based on spheroids is diverted into two main directions - to study the basic molecular profile of spheroids, or to compare the drug responses of spheroids and conventional *in vitro* models for the commercial potential of this model. For the basic molecular biology study of spheroids, studies are not only focused on the proliferation, differentiation, and gene expression, but also on the necrosis or the programmed death of cancer cells in spheroids. Some studies demonstrated that the extracellular matrix of spheroids are going reorganizations by the assembly of collagen and fibronectin, integrins, expression of growth factors, and the development of gap junction formation, suggesting the similarity of spheroids with *in vivo* situations (Hsiao et al, 2009; Kelm et al, 2003; Timmins et al, 2004).

The commercial potential of applying spheroids into drug testing for various cancer types has already been investigated by researchers. Prostate cancer cells (Hsiao et al, 2009; Kelm et al, 2003) and breast cancer cells (Timmins et al, 2004) were used to create spheroids. Besides the monoculture of cancer cells for spheroid formation, considering the heterogeneity of the cancer microenvironment, fibroblasts and endothelial cells were also co-cultured with cancer cell formed spheroids (Timmins et al, 2004; Hsiao et al, 2009), and these co-culture spheroid

models are used in investigating cancer progression steps such as angiogenesis and metastasis.

To summarize the past few decades of studies on spheroids, both basic biology and drug responses are considered for this model's big potential in HTS. The 3D sphere structure of spheroids allow the studies of drug penetration, binding and to some level the action mechanisms of the drugs. It is helpful to bear in mind that not all cancer cell lines can form spheroids - some primary cancer cells from biopsy were reported to form spheroids, and some variants of breast cancer cell line MCF-7 were found to have little ability to form spheroids. Even for cell lines which are considered to be able to form spheroids, many parameters include seeding densities, medium ingredients should also be optimized to realize the most reproducible structure. Also, the cancer spheroid model has some limitations, including the small size of aggregates formed, and the uncontrollable shape, thus is usually applied to mimic the early stage of tumours, with no vascularization (Hsiao et al, 2009; Timmins et al, 2004).

**Table 2.1** 3D *in vitro* tumour models

| <b>Methods</b>                |                                 | <b>Advantage</b>                                                                                                                                                                             | <b>Disadvantage</b>                                                                                                                              |
|-------------------------------|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Spontaneous Aggregates</b> |                                 | Relatively easy to operate, without pre-treatment <sup>(Kelm et al, 2003; Hsiao et al, 2009)</sup>                                                                                           | Limited sizes of aggregates, lack of ECM <sup>(Timmins et al, 2004; Hsiao et al, 2009)</sup>                                                     |
| <b>Scaffolds</b>              | <b>Decellularized scaffolds</b> | Maintain the natural complicated structure, straightforward methodology <sup>(Kim et al, 2004; Gurski et al, 2010)</sup>                                                                     | Loss of biological active components, solvent remnant, source variance <sup>(Gurski et al, 2010)</sup>                                           |
|                               | <b>Natural Scaffolds</b>        | High biocompatibility <sup>(Lee et al, 2007; Fischbach et al, 2009)</sup> , physiologically relevant <sup>(Fukuda et al, 2006)</sup> , design flexibility <sup>(Fischbach et al, 2009)</sup> | Undetermined components <sup>(Gurski et al, 2010)</sup> Limited physiochemical properties <sup>(Fischbach et al, 2009; Fukuda et al, 2006)</sup> |
|                               | <b>Synthetic Scaffolds</b>      | Highly controlled and various physiochemical properties <sup>(Gurski et al, 2010; Fischbach et al, 2007; Sahoo et al, 2005)</sup>                                                            | Biocompatibility needs to be tested. Special operation and instruments needed <sup>(Fischbach et al, 2007; Sahoo et al, 2005)</sup>              |



To obtain the supporting matrix for 3D cell culture, the first thought may be the decellularization of an existing organ and seeding it with required cells. This idea has already been tested; heart tissue was reconstructed in the lab (H.C. Ott et al, 2008). However, this method has its limitations including loss of bioactive epitopes and introduction of potentially harmful remnant of solvents during processing. So instead of using natural scaffolds directly, we can design and make new scaffolds using natural or artificial ingredients. Thus various natural originated scaffolds haven been designed and studied, with controllable physical and chemical properties, and some of them even have capabilities to be loaded with growth factors for better cell growth (Dietmar et al, 2009; Gurski et al, 2010; J.B. Kim et al, 2004).

#### ***Natural matrix – Matrigel and alginate***

At the moment, in cancer research Matrigel is one of the most widely used scaffolds originated from natural source. Matrgel is an ECM equivalent extracted from mice tumour (G.Y. Lee et al, 2007). Represented by Mina J. Bissell, cancer researchers around the world did countless research on this material, and the research based on Matrigel eventually led to the wide acceptance of cancer microenvironment theories. Those researchers reported that in Matrigel, cells can restore some features of tissue three-dimensional structure which is usually only observed *in vivo* (Aggeler et al, 1991; Lee et al, 2007; Kanazawa et al, 1992; Streuli et al, 1991; Potter, 2007). For example, in Bissell's lab human breast cancer cells can form tumour-like structures in Matrigel and can be maintained for 4 days (Lee et al, 2007).

Apart from Matrigel, other natural scaffolds have also been used. Alginate is a polysaccharide from the cell walls of algae, and alginate modified by RGD was used for oral

squamous cell carcinoma cells OSCC-3, and can maintain the cell proliferation for 5 days (Fischbach et al, 2009). Chitosan, another type of polysaccharide from the shells of crustaceans, was used to co-culture human hepatoblastoma cells HepG2 and fibroblasts NIH-3T3, and the co-cultures kept proliferating for 9 days (Fukuda et al, 2006). Collagen is one component widely existing in ECM, and rat tail collagen (collagen type I) was used to co-culture breast cancer cells MCF-7, luminal epithelial cells HB4a and fibroblasts hfff2 for 7 days (Holliday et al, 2009).

### ***Synthetic scaffolds***

Compared with naturally originated scaffolds, synthetic scaffolds have advantages like flexibility in engineering design, and certainty and purity of chemical and biological components (Dietmar et al, 2009; Gurski et al, 2010; Kim et al, 2004). Using these pre-fabricated engineered scaffolds, cancer cells can be seeded on artificially modified scaffolds. These scaffolds do not only provide physical support, but also regulate the growth and differentiation of the seeded cells by different cell growth factors being added into the scaffolds.

Because synthetic scaffolds need more evaluations on biocompatibility and toxicity compared with natural scaffold, those most commonly used scaffolds are usually improved by Food and Drug Administration (FDA), including Poly (D,L-lactic-*co*-glycolic acid) (PLG or PLGA) (C. Fischbach et al, 2007; Sahoo et al, 2005) and polylactide (PLLA) (Sahoo et al, 2005). On PLGA, oral squamous cell carcinoma (OSCC-3) cells can continue proliferation for 10 days

(Fischbach et al, 2007). For breast cancer cells MCF-7, using PLLA as scaffold can keep these cells proliferating for 10 days (Sahoo et al, 2005).

Synthetic scaffolds provide a big variety of morphology to be chosen for different culture purposes. Besides these fibre-like or highly porous sponge-like matrix materials, multicellular spheroids can be constructed using commercially available microcarriers, like Cytodex™. Human lung carcinoma cell line A549 was cultured in 3D using Cytodex and treated by Cisplatin for 4 and 24 hours, and it was found the compared with 2D culture, cell sensitivity to Cisplatin was decreased in Cytodex-based 3D culture (Maurer et al, 1999).

In summary, to construct the 3D cancer *in vitro* models, both natural and synthetic scaffolds can be used. Natural scaffolds have intrinsic higher comparability with natural tissue, with a broad spectrum of bioactive ingredients already included in the scaffold to support the growth and differentiation of the seeded cells. Synthetic scaffolds, on the other hand, have stronger physical and mechanical properties and more controllable biochemical features compared with natural scaffolds, but extra engineering techniques are required to make those scaffolds and their biocompatibility should be carefully evaluated. The representative natural and synthetic scaffolds mentioned above are summarised in Table 2.2.

**Table 2.2** Representative scaffolds for 3D cultures

| Name                                                                       | Origin      | 3D scaffold construction                                                                      | Morphology                                                                    |
|----------------------------------------------------------------------------|-------------|-----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------|
| <b>Matrigel</b> (Dietmar et al, 2009; Gurski et al, 2010; Lee et al, 2007) | Mice        | 37°C, 30min-4hrs polymerization (Lee et al, 2007)                                             | Gel (Lee et al, 2007)                                                         |
| <b>Collagen</b> (Dietmar et al, 2009; Kim et al, 2004)                     | Mice        | Alkaline polymerization (Kim et al, 2004)                                                     | Gel (Dietmar et al, 2009; Gurski et al, 2010)                                 |
| <b>Alginate</b> (Gurski et al, 2010; Fischbach et al, 2009)                | Algae       | Ca <sup>2+</sup> (Fischbach et al, 2009)                                                      | Spheroid (Fischbach et al, 2009)                                              |
| <b>Chitosan</b> (Fukuda et al, 2006; Fischbach et al, 2007)                | Crustaceans | Photocrosslinking (Fukuda et al, 2006)                                                        | Sponge (Fukuda et al, 2006)                                                   |
| <b>PLLA</b> (Gurski et al, 2010; Sahoo et al, 2005)                        | Synthetic   | Electrospinning (Dietmar et al, 2009), solvent-evaporation (Sahoo et al, 2005)                | Fibre (Dietmar et al, 2009; Gurski et al, 2010) or sponge (Sahoo et al, 2005) |
| <b>PLGA</b> (Fischbach et al, 2007; Kim et al, 2004; Sahoo et al, 2005)    | Synthetic   | Electrospinning (Kim et al, 2004; Sahoo et al, 2005), solvent-evaporation (Sahoo et al, 2005) | Fibre (Kim et al, 2004; Gurski et al, 2010) or Sponge (Sahoo et al, 2005)     |

### 2.2.3. Co-cultures for 3D tumour models

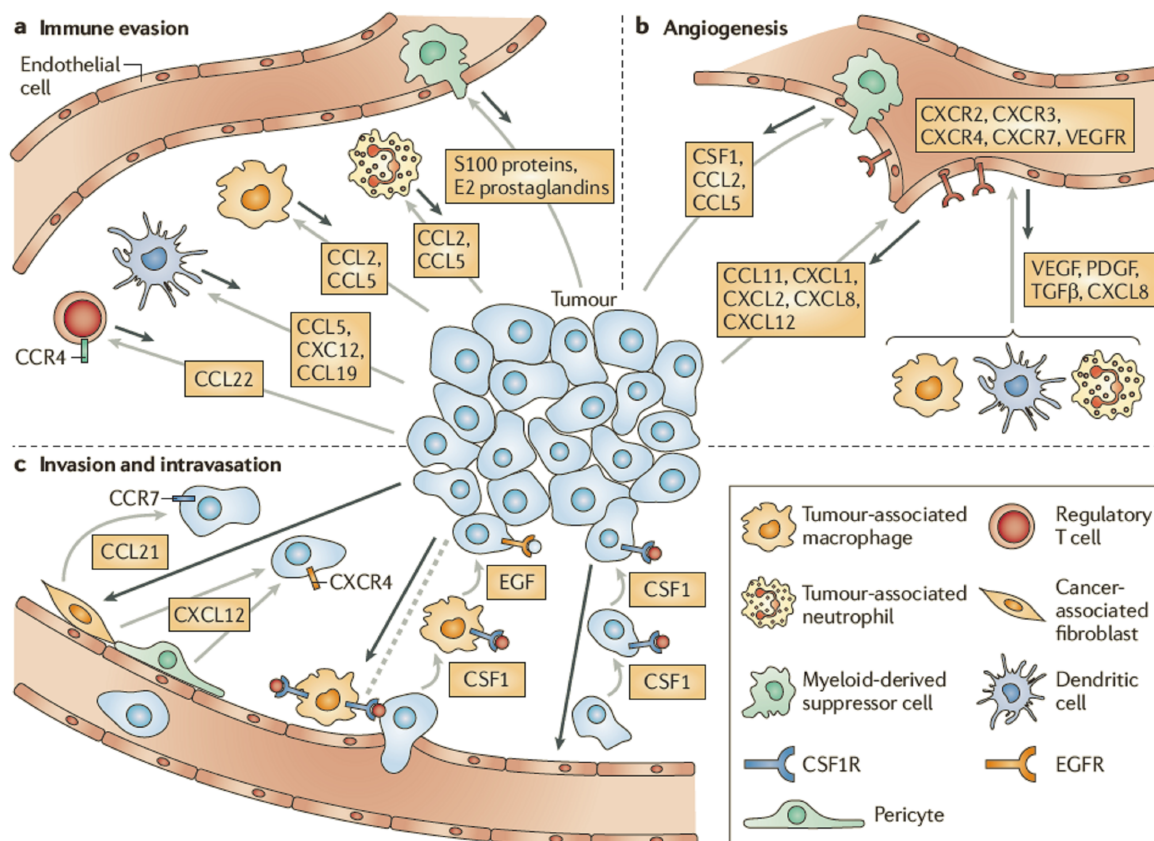
As mentioned in the previous sections, compared with conventional 2D *in vitro* models, cancer cells cultured in 3D give more information on morphology and gene expression alteration in cell malignancy, and reflect the surrounding microenvironment in cancer development (Lee et al, 2007). However, culturing cancer cells alone as *in vitro* models, even in 3D cultures, there is still the lack of interactions between different cell types in *in vivo* environment. Both clinical and molecular studies pointed out other cell types are vital in carcinogenesis and metastasis. Inflammatory cells, blood vessel cells such as endothelial cells, and mesenchymal fibroblasts can be recruited by cancer cells and stimulate cancer cells by soluble or matrix-related factors or by direct cell contact (Albini et al, 2007; Wang et al, 2005).

Thus, co-culturing other cell types with cancer cells in 3D can provide information on interaction between those cells and cancer cells, and can potentially serve as better *in vitro* models for testing therapeutics in more physiologically mimicking conditions (D.L. Holliday et al, 2009). Depending on the specific cancer development stage studied, different cells haven been chosen to be co-cultured with cancer cells, including stromal and supporting cells such as endothelial cells (Timmins et al, 2004) and fibroblasts (Hsiao et al, 2009; Holliday et al, 2009), or normal functional cells like gland cells in breast gland with breast cancer cells (Kelm et al, 2003), and other cells types like immunological cells (Laubli et al, 2009).

Stromal cells such as fibroblast cells provide the pH distribution of tumours, which will then influence the distribution and efficacy of anti-tumour therapeutics, especially those that are only efficacious in specific acidity. As a result of their importance and easiness to be grown

*in vitro*, fibroblasts have been widely studied in co-culture with cancer cells (Hsiao et al, 2009; Holliday et al, 2009). To study the metastasis of specific cancer type, not just fibroblasts, but also other stromal cells can be co-cultured with cancer cells. One example is to study the bone metastasis of prostate cancer, bone-derived stromal cells were co-cultured with prostate cancer cells, and co-evolution of stromal cells and cancer cells was observed by genetic studies (Wang et al, 2005). Besides mesenchymal cells, in microenvironment surrounding cancer cells, inflammatory cells are also crucial in cancer development, by direct effecting on cancer cell aggressiveness, or promoting angiogenesis by chemokines. So co-culture of cancer cells and inflammatory cells like monocytes have been developed for this reason (Laubli et al, 2009).

With the study of inflammatory regulation of cancer progression, clinical and molecular studies both revealed the importance of considering angiogenesis in tumour models (Albini et al, 2007). New capillaries play important roles in tumour growth and metastasis by providing oxygen and nutrients, and cell cytokine factors and growth factors for the growing tumour, and transport tumour cells to other locations (Ferrara et al, 2004; Orr et al, 2000). Considered as the most important supporting cells in cancer angiogenesis, vascular endothelial cells (ECs) have been co-cultured with cancer cells to study tumour angiogenesis. However compared with the research on other supporting cell types such as fibroblasts, co-culture of cancer cells with endothelial cells are still rare in available research reports.



**Figure 2.10 Schematic figure of tumour microenvironment.** Key stages and factors involved in tumour development are included in this figure, including angiogenesis related factors VEGF, PDGF, and the inflammation model of tumour was also elucidated.(Roussos et al, 2011)

Nevertheless, there are many *in vitro* and *in vivo* angiogenesis models available to provide inspiration for the design of 3D co-culture tumour models in the cancer angiogenesis context. These models are traditionally designed to screen angiostatic agents and explore angiogenesis mechanisms. The *in vivo* angiogenesis models can model the multi-step complexity of angiogenesis, and the earliest versions of those models are developed based on the improvement of histology observation techniques and blood flow monitoring techniques like Doppler. In the research labs, the most popular *in vivo* models for angiogenesis are corneal neovascularization assay, chick chorioallantoic membrane (CAM) assay and Matrigel plug

assay. They are reported to have reproducibility and quantification of those methods were discussed in previous research (Auerbach et al, 2003).

Although it is widely accepted that *in vivo* models can mimic the cellular and molecular complexity, in the case of tumour angiogenesis the *in vitro* models have special important positions. This is partially because the recent *in vivo* angiogenesis models such as CAM are still not reproducible and quantitative enough (Norrby, 2006), Another reason for the special attention on angiogenesis *in vitro* models is historically these models played crucial roles in the discovery and studies of most angiogenesis regulating molecules such as VEGF, because in *in vitro* environment the pivotal steps in angiogenesis from EC proliferation to tubule formation can be studied with quantification and reproducibility (Vailhé, et al, 2001).

Nearly all the existing angiogenesis *in vitro* models are featured with ECs cultured alone, and these conventional mono-culture models of endothelial cells cannot mimic the interaction between cancer cells and endothelial cells, thus co-culture models of these two cells have been developed. At first, most of these co-culture of EC-cancer was in 2D but not 3D, leaving the question of whether 3D structural information was missing in the model (N. Khodarev et al, 2002). Thus, efforts have been put into developing further advanced 3D co-culture tumour models to mimic cancer-endothelium interactions, such as co-culture spheroids (Timmins et al, 2004). Meanwhile, there is also argument raised about if there is really a distinct line between those 2D and 3D for some *in vitro* angiogenesis models, suggesting there is potential to improve these conventional 2D models into new 3D models. A good example is the most popular 2D Matrigel-based tube formation analysis. This method was studied systematically, and was highly standardized through considerable amount of research, and has contributed to



the screening of angiostatic agents and characterization of endothelial cell lines (Vailhé, et al, 2001).

So far, for 3D co-culture tumour *in vitro* models, Matrigel has been used for the co-culture of primary tumour endothelial cells with breast cancer cells (Ingthorsson et al, 2010). It is noticeable that endothelial cells cultured on laminin rich ECM represented by Matrigel can be a link between *in vitro* and *in vivo* models, and morphology parameters such as tubule length and tubule areas were evaluated to predict the behaviours of endothelial cells *in vivo* (Staton et al, 2004; Crabtree et al, 2007; Donovan et al, 2001). In these models, tube formation of endothelial cells on Matrigel provide a direct and clear reflection of not just endothelial cell proliferation, but also their migration and tube formation abilities in the angiogenesis process (Crabtree et al, 2007; Donovan et al, 2001).

To summarize, the co-culture of cancer cells is already being considered by researchers who are working on developing 3D tumour models. It is noted that although co-culture of EC and cancer cells were described in some research reports mentioned above, this co-culture research was always an assisting study together with research aims not focusing on angiogenesis, and no further details of EC-cancer interactions were explored (Holliday et al, 2009; Brisson et al, 2002). The difficulties of co-culture should also be considered in the future study of co-culture tumour models, to make sure normal cells such as ECs, who have distinctively lower proliferation activities than cancer cells can grow and maintain functions in co-culture system, without being taken over by cancer cells (Timmins et al, 2004; Hsiao et al, 2009; Holliday et al, 2009).

#### 2.2.4. Perfusion for anti-cancer drug testing

With the development of 3D cell culture, researchers gradually realised that for the thick structures of 3D cultures such as spheroids, there is an increasing demand for improving the chemotransportation, to provide a better homeostasis for sustainable proliferation potential and functions of those 3D structures. Traditional static culture in culture flasks, dishes or well plates cannot satisfy this requirement any more. In conventional 2D culture, simple diffusion is sufficient to maintain the viability of cells in monolayers, while for 3D cultures, the 3D structures makes it difficult for the cells in the core area to get enough oxygen and nutrient supplies from the outside medium, and the waste of these cells also finds difficulty in being transported out (Sailon et al, 2009; Cui et al, 2007). Furthermore, with the development of HTS, perfusion is found out to have better *in vivo* correlation in *in vitro* cancer angiogenesis models. This is because natural existing blood flow perfusion *in vivo* regulates cancer tissue remodelling events including gene expression alteration in metastasis, mechanical features of tissues and cell adhesion. The *in vivo* perfusion in cancer tissue also provides chemokine gradients which will then regulate the polarity behaviours of supporting cells, such as endothelial cells located at basal surface (Song et al, 2009).

Apart from the above technical concerns, introducing perfusion into 3D tumour *in vitro* models also has other advantages compared with conventional static culture. For long-term drug testing or tissue engineering culture which is longer than 72 hours, routine culture medium change every 2-3 days is usually required. This process is labour and time-intensive, with risk accumulation of contamination over time. In perfusion culture, by pushing culture medium continuously and constantly through the cultured tissue by pumps, researchers have less work stress, and contamination risk is lower (Cui et al, 2007). Also, for anti-cancer drug testing, perfusion is better for building up the correlation between tested drug concentration

and tissue response. By supplying testing medium in a continuous manner, perfusion ensures important chemical properties of the culture medium such as pH are maintained at a relatively constant level, avoiding the interrupting to the growth and differentiation of cells, and researchers can focus on the quantitative correlation of the drug concentration and the response of the tested models. On the contrary, in conventional static culture pH and other chemical properties in extracellular environment are fluctuating due to the manual culture medium change, which will then introduces unpredicted influencing factors to quantitatively control the concentrations of tested drugs (Cui et al, 2007; Wu et al, 2006). In a word, perfusion 3D cultures improves the oxygen and nutrient supplies to 3D cell culture, mimics the physiological perfusion of endothelium-cancer tissue interactions, and provides a more stable extracellular environment for anti-cancer drug testing with less research stress and lower contamination risk over long-term culture.

To realize the perfusion culture, there have been various attempts in the past decade, including to culture cells in spinner flasks set upon orbital shakers, or to tumble cultures in rotational bioreactors. However, these methods will only improve the chemotransportation of surface areas of 3D cultures, so a more effective flow-perfusion system needs to be developed. Moreover, most of these developed bioreactors have large-sized complicated structures, and are not transparent at all, so the cells or tissue cultured inside can only be analysed at the end-point, and huge quantities of cells are required for one test (Sailon et al, 2009). Compared with previous perfusion platforms, TissueFlex® is a polydimethylsiloxane (PDMS) based bioreactor developed in our lab. The basic material PDMS was chosen because of its many special advantages, such as non-toxic biological compatibility which has been proven by many studies (Cui et al, 2007; Wu et al, 2006). Compared with previous

perfusion bioreactors, which require large quantity of medium and complex instrument (Sailon et al, 2009), each well of TissueFlex® has the same size as the wells of 24-well plate or 96-well plate, miniaturize tested samples and is relatively easy to handle in the lab, with good comparability to well sizes of conventional well-plates (Cui et al, 2007). Also, the optical transparency of PDMS facilitates real-time observations on tissue morphology, either by inverted microscope or fluorescence microscopy. The medium in the well and the whole bioreactor can be sterilised by standard autoclaving, making it easy to be manipulated (Cui et al, 2007; Wu et al, 2006). Furthermore, studies have shown that PDMS is gas permeable, which allows the sufficient gas exchange for the cultured tissues (Wu et al, 2006).

In conclusion, to solve the problems of recent *in vitro* and *in vivo* models, in the past decade 3D *in vitro* models are developed in the forms of spheroids or scaffold-based 3D models. More recently, to further reflect the intercellular interaction and *in vivo* perfusion environment, advanced culture techniques including co-cultures of different cell types, and perfusion dynamic cultures are being developed for 3D *in vitro* tumour models. These 3D *in vitro* culture methods keep the advantages of *in vitro* cultures such as easy manipulation and clear quantification, and at the same time introduce a culture environment mimicking *in vivo* physiological environment. The concept of 3D *in vitro* tumour models will fill the gap between *in vitro* and *in vivo* studies, providing more reliable pre-clinical data.

## ***2.3 Challenges for 3D Tumour Models***

### ***2.3.1 Requirement of high-throughput screening and in vivo correlation***

To satisfy the screening requirement for an increasing pool of anti-cancer candidates, highly standardized culture plates like 96-well, 384-, and even 1536-well plates and commercially available microplate readers encouraged the development of high-throughput screening (HTS) in pharmaceutical research and industry. Now there are countless commercial testing kits and other commercial research products available in the market, with standard manufacturer manuals. Also, the positive interactions between industry and research catalyst inspired by HTS experience further encourage the requirement for a more user-friendly working environment for the pharmaceutical researchers - a clean and tidy working bench, an add-mix-measure protocol, and highly reproducible, reliable results which are relatively easy to process and interpret. However, for a lot of the 3D tumour models, these aspects have been missing – forming scaffolds takes longer time than just seeding cells, the mixture of cells and scaffolds introduces risks of heterogeneity of tested media, and the thickness of the samples, as well as the components of scaffolds increased the difficulty of end-point analysis. To give a further analysis of the comparison between 2D and 3D in HTS potential, we will take a closer look at each step of HTS standard procedure.

HTS is first developed based on biochemical reactions like enzyme linked immunology assay (ELISA). However, when people realized the cells in which drugs will take actions are far more complicated than the artificial buffer for biochemical assays, cell-based HTS was then developed. Instead of using 'testing tubes', living cancer cells were seeded on the pre-treated surface of culture plates usually made from tissue culture plastic, and then cultured in the presence or absence of tested drugs for a particular period. At the end point of treatment, some reaction reagents will be added to each well, and there will usually be some colour or

fluorescent change of the reaction reagent caused by the metabolism of cells. Then the whole plate will be incubated at a certain temperature for a certain period, to ensure there is a stable relation between the microplate readout and the cell function which researchers are interested in. At the end the microplate reader will give the researchers the data read out from each well, and researchers will transform these data into quantified original cell function measurements. However, traditional biochemical testing does not have a testing environment mimicking the very complicated physiological environment of real cancer cells. In 1960's, the development of human tissue culture gave a new dimension to the HTS technique. By culturing human cells in sterilised containers, researchers can test anti-cancer drugs in a well-controlled environment, and use the microplate reader to produce results quickly. This standardized procedure can be transferable to 3D *in vitro* tumour models, but extra optimisation must be carried out for each step for the developed 3D models – 1. Consider the manufacturing process of the scaffold and the mixture of cells and scaffolds, and compare whether to form the 3D cultures in the plate, or to form the 3D cultures first in a large batch and then distribute them into the plate later. The former has higher risks of heterogeneity of tested wells, but the latter has higher risk of introducing contamination into the culture; 2. The thickness of the samples, as well as the components of scaffolds increased the difficulty of end-point analysis, thus the testing protocol from 2D cultures must be pre-tested and modified to accommodate the 3D cultures.

Moreover, to design successful *in vitro* HTS assays of 3D tumour models, many other factors need to be considered. The first consideration is if the cultured populations of cells in culture are homogeneous. The passages of cells, the seeding densities, cell cycle phases of cells all should be controlled for the same experiments (Riss et al, 2004). To interpret the results, it is

important to bear in mind the readout of one well in a microplate is the average of the whole cell population in the well, so this might be a reason why researchers usually put the results of HTS microplate testing together with the results from individual cell images taken under microscopes, to give both details of single cells together with the representative data from the whole tested populations (Riss et al, 2006; Catalano, 2006). Secondly, to get large-scale of cell number amplification to satisfy the cell number requirement for 3D cultures, some additional factors need to be considered. For example, oxygen and nutrient supply to the cells in 3D structures, and cell density which can influence morphology and other functions of 3D cultures. Based on previous research, the general seeding density fall into the range of  $5 \times 10^4$  cells/mL to  $2 \times 10^5$  cells/mL ( $5 \times 10^3/\text{cm}^2$ - $2 \times 10^4/\text{cm}^2$ ), but for each experiment the seeding density of each cell type needs to be optimised (Tovey, 1980). Thirdly, for the following drug testing, the general requirements for drug solution preparation need to be followed especially carefully for 3D culture testing. For example, the dry compounds can be stored at  $-20^\circ\text{C}$  to  $-70^\circ\text{C}$  over desiccant, while the aliquots of liquid stock drug solutions must be stored at  $-70^\circ\text{C}$ . The drug loss in sterilization process should be minimized by operation details, such as to use the highest concentrations of drug solutions for the first few millilitres of filtrate, to saturate the cellulose nitrate or acetate filtrate membrane (In the case of nylon, there is no worry about the absorption). Other tips include to decrease the work load and risk of inactivation of drug solution in the process of filtration, to use sterile tool to weigh pure dry powder into a sterile container is enough, even for a relatively long testing time.

As the gap filling for traditional *in vitro* and *in vivo* models, it is important to consider the *in vitro-in vivo* correlation of 3D tumour *in vitro* models. The first factor to be considered for this concern is the choice of cells for each protocol. In general, the earliest generation of cells

used *in vitro* testing, which is primary culture of cancer cells isolated from autopsy has intrinsic better correlation to *in vivo*, compared with cell lines. The cell lines are genetically modified to make up the quantity limitation of primary cells – they can proliferate theoretically infinitely in lab to create large numbers of cells to satisfy the statistical requirement of drug testing. These commercially available cell lines enhance the dominance of monolayer, mono-type cell models which have been used until now. However, recent research also pointed out that for some cell types, cell lines lose many essential properties of the primary cells, so primary cells have to be used. For instance, in this thesis the endothelial cells used are primary cultured human umbilical endothelial cells (HUVECs).

The second consideration for a better *in vivo* correlation is the choice for an end-point analysis profile, in general there are three types of cell function measurements - cell viability, cell integrity, and cell apoptosis. Cell viability is usually the first parameter tested in *in vitro* cytotoxicity or other function evaluation. There are several ways to do this kind of testing, including to measure the total protein content, to trace radioisotope incorporation into synthesized DNA or glucose, or to use tetrazolium dye reduction as a measurement of cell metabolic rates. As the tetrazolium methods are more sensitive, easy to use by colourimetric microplate reader, and compared with radioactive reagents used in the isotope incorporation this method is safer for researchers, many reagents have been developed for this method. These include MTT, XTT, WST-1 and AlamarBlue (Wilson, 2000). For cell integrity, the most popular idea to test the integrity of cell membrane, and lactate dehydrogenase (LDH) release is usually used as a marker. Other measurements in addition to cell viability and cell integrity include cell apoptosis, which is a natural cell process triggered by intrinsic programme in cells. The most common marker for apoptosis is Caspase-3, which is an



enzyme involved in cell apoptosis cascades (Riss et al, 2004). To better reflect the complexity of *in vivo* process, usually more than one parameters are combined together to give a whole picture, which can be different stages of a physiological process, or different aspects of a pathological development.

Last but not least consideration for a better *in vivo* correlation in 3D *in vitro* testing is the presentation of HTS results. Originated from 2D *in vitro* cultures, the responses of 3D models can be demonstrated to a series of dosage, either linear or exponential, or to a range of exposure time. The classic example is the programme developed by National Cancer Institute (NCI) for finding new anti-cancer candidates, called NCI-DP60, which is to use the most commonly used 60 cell lines to screen candidates from databases (Paul, K.D. et al, 1989). As a marker of drug toxicity or efficiency, IC<sub>50</sub> is the concentration of the drug causing 50% of cell death of tested cancer cells. This value can be read out from the log transformation of the response function to the dosage. It is already reported that 3D cultures can have significant IC<sub>50</sub> compared with 2D models, with a better *in vivo* correlation. It is also suggested that even for the same passage of cells seeded at the identical density with mostly the same cell cycle phase, the response pattern to drug dosage and exposure time rely on the mechanisms of drug actions. Thus, using maths tools it is possible to figure out the *in vivo* mechanisms of drugs based on the response patterns of *in vitro* responses (Paul, et al, 1989; 1993). This can be a new tendency to build up the *in vitro-in vivo* correlation for *in vitro* HTS testing in the future, as a good alternative to the conventional molecular method.

In a word, with the development of HTS and application of other disciplines in 3D tumour models such as computer modelling and statistical analysis, there are considerable evidences

showing 3D tumour models have a better correlation with *in vivo* experiments, compared with conventional 2D models. This confirms the potential of 3D models bridging *in vitro* and *in vivo* gaps (Dietmar et al, 2009; Kim et al, 2003; Gurski et al, 2010).

### *2.3.2 Morphology – opportunities and challenges by 3D co-culture model*

Morphology, the shape and structures formed by cells cultured *in vitro* has special significance for data analysis in three-dimensional (3D) cultures – the 3D environment can restore some of the original 3D structures of cancer tissue, such as tumour spheroids, and the parameters related to those 3D structures, such as diameters, can provide markers to evaluate the drugs. One good example is for angiogenesis assay, 3D *in vitro* models of tubule formation in Matrigel can be used to measure pro-angiogenesis/anti-angiogenesis abilities of tested medicine, while the conventional 2D *in vitro* culture of blood vessel cells cannot provide such information (Staton et al, 2004).

Morphology assays for drug testing is developed on the basis of the advancement of microscopy. The most common microscopes used for drug testing are inverted light microscope, confocal microscope and multi-photon microscope. The digital images collected by digital camera connected to the microscope can be composed of pixels or vectors. Pixel images are more commonly used in cell imaging, and one pixel is a square of uniform gray values, and the grey values range from 0 (pure white) to 4095 (pure black) in 12-bit digital camera system. The digital images can also divided into 2D and 3D. A threshold is usually set and then the images can be recorded and processed by commercially available or public open image processing software. Measurements used by image-based assays can be intensity

values obtained by grey values, for example, fluorescent intensity, or morphology parameters such as lengths or areas. Pixels with grey value lower than the threshold value will be recognized as the background, and the ones with higher grey values will be recognized as the objects of interest. In most image processing software, a histogram of grey value is provided to the researchers, to allow them set the sensible threshold based on their professional experience. In other words, the ultimate rule of image recognition is still the visual system of trained and experienced researchers (Catalano, 2006).

Although compared with 2D culture, 3D tumour models opened new opportunities in image processing, there are intrinsic challenges for 3D culture image taking and processing – the samples are usually thicker than 100 micrometres, and there is sometimes signal interference from scaffold auto fluorescence. A potential resolution is the multiphoton microscope (MPM). Multiphoton microscopes can collect a series of images automatically from different depths, at a relatively mild testing environment, and then imaging software can be used to reconstruct the original 3D structures of cells, which makes it a good choice for thick tissue imaging, especially in 3D *in vitro* testing system. The successful application of this technique will be shown in results of this thesis.

In particular for co-culture 3D imaging, there is another challenge – to track each cultured cell type separately in image analysis. The first way to solve this problem is to tell the cells apart afterwards, using immunochemistry techniques to label specific marker proteins in specific cell types. For the first method, depending on purposes certain pre-treatment before loading on an instrument might be required before the imaging. For example, enzyme digestion, or calcium-chelating cell detach by EDTA will be used to obtain cell suspension

from the original 3D culture (Lee et al, 2007). It should be noted that some membrane proteins might be influenced in this pre-treatment, and the morphology structures used for analysis might be interrupted in the enzyme digestion or other efforts to separate the cells. For the in situ fixing and imaging, which means to fix the cells directly at the culture surface for later imaging, though the 3D structure information will be presumably better protected, there are also other technical challenges, including the increased risk to ruin the structure sample with traditional fixing solution recipes in immunofluorescence staining. Luckily, researchers also give specially designed solutions to fix specific 3D culture samples, to reduce the interruption to 3D structure in pre-treatment (Lee et al, 2007).

Another solution for cell tracking in co-culture is to label each cell type before mixing them together, using living cell tracing probes which are commercially available now. This technique allows the real-time tracing of cells' behaviour in co-culture. There are many colours available commercially, including red, green, orange, purple etc. However, living cell probes are relatively new techniques, and some researchers pointed out that for certain cell types, the strong fluorescence can only maintain for up to 72 hours. For longer culture time these dyes might be pumped out of the cells and fluorescence will gradually fade away (Korff et al, 2007). Another more stable way of pre-culture labelling is to use molecular techniques introducing fluorescence protein expression genes into living cells. Examples include green fluorescence protein (GFP) and red fluorescence protein (RFP) (Hwa et al, 2007). However, the molecular modification requires months of lab work and expertise in molecular biology, and even in the most successful cases, there are still cells negative in fluorescence expression, which needs to be filtered out by culture medium containing selective antibiotics, and still, these fluorescent cells might lose fluorescence after certain passages anyway.

In summary, 3D *in vitro* models give researchers more information on tissue morphology for end-point analysis, but there are also new challenges facing the cancer research community, including cell tracking and image collection from a thicker, more complicated system. Last but not least, it is important to keep the results from morphology analysis as objective and reproducible as possible. This can be done by considering the advantages and limitations of certain instrument for image analysis. For example, normal inverted microscope has lower resolution but is quicker to use for morphology in HTS, while multiphoton microscope (MPM) can provide much more detailed original structural information, but usually contain much fewer cells in one investigated area. So it is vital to choose areas randomly to ensure the objectiveness of the results. In research reality, information from other quantitative HTS measurements such as cell viability can be combined with morphology analysis, to give a full profile of the drug being tested (Tsunoda et al, 2010).

### *2.3.3 Other challenges of 3D co-culture - Gene and Metabolism profile, pH & redox environment*

As mentioned in previous sections, by culturing cancer cells with related other cell types in 3D microenvironment, 3D co-culture *in vitro* tumour models provide a better simulation of intercellular and cell-ECM interactions in cancer development. The evidences were found not only in cell viability testing and tissue morphology, but also by advanced molecular biology studies on gene expression and metabolism. These evidences include markers which relate to cell proliferation and migration, like human epidermal growth factor (HER2). It was found that HER2 and MARK signalling were only activated in 3D models (Pickl et al, 2009). Further drug testing showed that HER2 – targeting agents, like Trasuzumab, responded in a

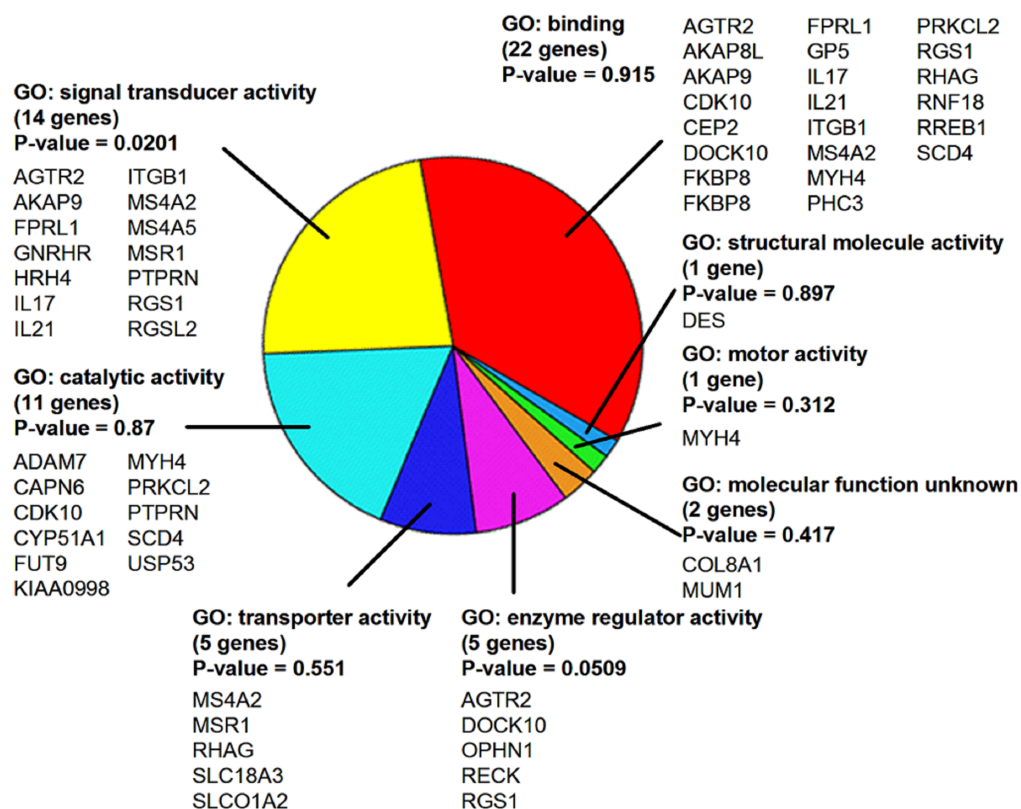
manner highly dependent on the existence of 3D environment (Weigelt et al, 2010). One factor which needs to be taken into consideration is that for some proteins involved in proliferation, the phosphorylation level can be a better reflection of proliferation pathway activation. One example is HER2 (Taniyama et al, 2008).

One recent research report summarized the different gene expression profiles of 2D and 3D culture, based on the function categories of expressed proteins. Interestingly, the structural and binding proteins are more similar between those two models with P-value as 0.897 and 0.915 respectively, while more difference was observed for enzyme regulating proteins, with P-value as 0.0509. The proteins regulating signal pathway turned out to be the most different between 2D and 3D models, with the P-value as 0.0201. Apart from proteins involved in cancer cell proliferation, other examples showing the difference between 3D and 2D culture include tissue polarity markers Laminin V, which is marker for basement membrane, and the F-actin, which is the marker for apical membrane. The distribution of those two markers were examined in the 3D models to validate the models, then through this research it was found out that the expression of some DNA repair-related genes like TP53 were suppressed by activated KRAS, but this relation was not observed in 2D. This possibly contributes to the higher drug resistance in 3D *in vitro* tumour models (Tsunoda et al, 2010).

Besides gene and protein expressions, to consider the microenvironment of cancer cell growth other chemical and physical properties of ECM, such as pH distribution, and reduction-oxidation environment should also be considered in cancer testing models. For decades, pH in tumour ECM has been considered to be more acidic than normal tissue, but only recently it is found that not only the acidity will influence cancer cells' functions, but

will also alter properties of some anti-cancer drugs activities, especially those drugs with weak acid or base properties (Starzec et al, 2003). Another chemical property in the cancer microenvironment to be considered for 3D tumour models is the reduction-oxidation (redox) situations around cancer cells, because it is shown that certain anti-cancer drug, like tirapazamine (TPZ), can only act in 3D tumour models but not 2D model (Tung et al, 2011). For 3D *in vitro* co-culture tumour models, another reason that redox properties is so important is other cell types apart from cancer cells are also sensitive to redox environment. Take co-culture models of endothelial cells and cancer cells, as an example, it has been long recognized that angiogenesis process in cancer development is highly regulated by oxidative signal in the environment, and pathways such as VEGF/VEGF-R2 is very sensitive to redox properties in extracellular environment (Miller et al, 2009).

To summarise, many researchers have demonstrated the significant difference of 2D and 3D *in vitro* models in terms of cell viability, drug responses, morphology measurements, gene expressions and reliance on microenvironment properties such as pH and redox, but the observations specifically on 3D co-culture models are still limited and there are more challenges such as cell tracking. But to solve these problems or to improve the situations can be significant for the development of a further advanced 3D models, with a better mimicking of intercellular interaction in 3D cancer microenvironment.



**Figure 2.11** *Gene ontology analysis of genes distinguishing cells grown in 2D and 3D culture conditions.* Gene Ontology analysis of the 41 of the 96 genes shown in Figure 5 for which gene ontology annotations were available. Genes encoding proteins involved in signal transduction are significantly overrepresented in this set ( $P = 0.0201$ ), while genes encoding proteins involved in the regulation of enzyme activity almost achieved statistical significance ( $P = 0.0509$ ) (Tsunoda et al, 2010).



## ***2.4 Aims and Scope of Thesis***

The aim of this study is to develop advanced in vitro three-dimensional (3D) cancer models, to address the challenges mentioned in the literature review above. The important cell-cell and cell-ECM interactions are missing in 2D in vitro models and often show large differences compared with clinical trials, while animal testing have intrinsic financial, ethical and the genetic relevance issues. Using engineering methods, human-origin cells can be used to build up cancer tissue in vitro, to mimic the intercellular and cell-ECM interactions in vivo, to test drugs in a well-controlled testing environment, with lower cost and less stress experienced by researchers, and more clinically relevant data.

To approach this aim, the project consists of different complexity. The first step is to characterise the perfusion effect on simple 3D cancer models, to further explore the possibility to mimic physiological perfusion environment in cancer angiogenesis. The next step is to build up a co-culture cancer cells to re-establish the interactions between cancer cells and their surrounding other cell types, such as endothelial cells who have crucial roles in cancer angiogenesis. To accompany this new model, new methodology will be developed. In the third step, different 3D culture scaffolds as well as different culture methods will be compared, to give more clues for how the choice of scaffold materials and processing will influence system's viability and drug response. Finally, perfusion and co-culture will be combined, to reveal the possibility of a new advanced 3D cancer in vitro model for long-term drug testing, and how perfusion will influence the culture homeostasis will be briefly investigated.

Based on the above aim and objectives, 3D tumour models were developed and investigated in four chapters via different perspectives – Chapter 3 introduced perfusion based on the

TissueFlex<sup>®</sup> system, to test out the feasibility of using this system to improve medium supply to 3D tumour models. 3D co-culture models of cancer cells and endothelial cells were developed in Chapter 4, and mono-cultures of the above two cells types were compared with the corresponding co-culture. In Chapter 5 Matrigel was compared with a relatively inert scaffold alginate; the Matrigel sandwich showed the more morphological information compared with homogeneous Matrigel. The final Chapter 6 tests the integration of co-culture and perfusion techniques, for an advanced model mimicking cancer-endothelium interactions and perfusion in tumour niche. Throughout these four chapters, representative anti-cancer drugs with various acting mechanisms were tested to validate the developed models, including 5-fluorouracil (5-FU), Cisplatin, Paclitaxel, and Tarapazamine (TPZ). A variety of quantitative and morphology parameters were applied in this process, and angiogenesis-related measurements were compared particularly with other parameters.

## Chapter 3. Spheroid Colorectal Tumour Model and Effects of Perfusion

### 3.1 Introduction

The development of three-dimensional (3D) drug screening models is an emerging but promising branch of anti-cancer therapeutics discovery and evaluation technologies. On one hand, conventional *in vivo* tumour model, which is based on transplanting human cancer cells into immunodeficient mice, has shown its limitations with intrinsic interspecies differences, long experiment time, and high cost (Nyga et al, 2011). On the other hand, studies pointed out those results obtained from conventional two-dimensional (2D) *in vitro* models failed to predict the clinical responses of anti-cancer drugs (Friedrich et al, 2007). This is due to the fact that the 2D monolayer cell cultures used in those models failed to mimic 3D spatial relationships of cells in tumour (Cukierman et al 2001). Compared with 2D conventional *in vitro* models, 3D *in vitro* models better mimic the intercellular interactions and the connections between cells and extracellular matrix (ECM), providing a better microenvironment for the proliferation, differentiation, and gene expression of cancer cells (Bottaro et al, 2002). Thus, three-dimensional cancer model is an important bridge between conventional 2D *in vitro* model and conventional *in vivo* animal experiments, combining the intracellular interactions in 3D tissue environment with the financial and ethical advantages of *in vitro* models.

Multicellular tumour spheroids (MCTS) are self-aggregates formed by homogeneous or heterogeneous cancer cells. Research has found that there might be multiple layers with different functions and features in MCTS. In a model presented by previous researchers, the surface layer of the spheroid consists of cells in active proliferation, while the core is mainly formed by necrotic and dead cells due to the inadequate nutrient and oxygen supplies. Between the surface and core areas there is also a static cell group (Brown et al, 1998). In

MCTS model, the cell-cell and cell-ECM interactions can be reconstructed in a 3D environment, mimicking the initial stage of solid tumours *in vivo* (Kelmet et al, 2003). Compared with conventional 2D *in vitro* models, MCTS models provide a more predictable chemotherapy response profile for clinical practice (Elliott et al, 2011).

To create MCTS, the most commonly used methods include suspension, liquid overlay (for example, forming spheroids on top of agar or agarose coating), and rotation culture for large quantity spheroid production. The diameter of MCTS is one crucial parameter for the culture and drug testing. Previous research has shown that MCTS with 200  $\mu\text{m}$  average diameters could be used to study the communications between cells and their extracellular environment (Lambert et al., 2004). However, this small diameter range is not suitable for studies concerning drug responses in pathological environment as the MCTS lack the feature of proliferation-static-necrosis gradient caused by oxygen transfer deficiency (Sutherland, 1988). On the other hand, research also found that further necrosis would be caused by spheroid diameters larger than 600  $\mu\text{m}$ , and impact on the accuracy of the following drug testing (Friedrich et al, 2009). So, in this research we used MCTS with the average diameter around 400  $\mu\text{m}$ .

Although proven to be a promising new model for future drug testing, one limitation of MCTS models is that the nutrient and oxygen supplies to the cells in the spheroid center rely mainly on passive diffusion, in the lack of vascular structure. With the growth of spheroid diameters, the percentage of dead cells in the core will increase due to longer diffusion distance, while in physiological microenvironment this oxygen and nutrient supply deficiency will be solved by blood perfusion. This lack of physiological flow in static culture caused

difficulty of correlating the final drug testing data to the drug response *in vivo* (Inamdar et al, 2011). Furthermore, the repeated medium change in long-term culture will cause the fluctuation of pH for cell culture medium, making it difficult to quantify the final drug responses of the cells, because pH and other environment changes will influence the cell metabolisms and other functions (Tostões et al., 2012; Wu et al, 2011). Perfusion culture provides a solution to this problem by passing the medium continuously through the culture wells, thus providing a relatively stable culture environment, and a more efficient nutrient supply system, which mimicking the *in vivo* environment in a better way than static cultures (Wu et al, 2008).

Here we developed an *in vitro* 3D perfused cancer spheroid model for anti-cancer drug study by using TissueFlex® microbioreactor (Cui et al., 2007). This perfusion spheroid model was designed to improve Perfused spheroids model cell viability with increased diameter of spheroid increase over a relatively long culture period (17 days). To elucidate the influence of perfusion on anti-cancer drug responses in cancer spheroids, three anti-cancer drugs with different cytotoxic mechanisms were tested. Overall, perfusion culture provided a better homeostasis for cancer cell growth, in a more controllable working platform for long-term drug testing. For traditional anti-cancer drugs 5-fluorouracil (5-FU) and Paclitaxel, higher sensitivity was observed based cell viability inhibition and spheroid integrity interruption, respectively. On the other hand, for hypoxic-dependent drug tirapazamine (TPZ) there was no significant difference observed between static and perfusion culture.

## **3.2 Materials and Methods**

### **3.2.1 Cell line culture and maintenance**

Colorectal cancer cell line DLD-1 cells (ATCC, UK) and human breast cancer cell line NCI/ADR were kind gifts from Dr. Richard Callaghan (Nuffield Department of Clinical Laboratory Sciences, University of Oxford). The cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM, Lonza, UK) supplemented with 10% (v/v) fetal bovine serum (FBS, Life Technologies, US) and 1% (v/v) penicillin (100U/mL)-streptomycin (100µg/mL) (PAA, US). All the cells were cultured in a humidified incubator (37°C in an atmosphere of 5% CO<sub>2</sub>). Cells were passaged when they reached 80% confluence. Before cell seeding for spheroid formation, single-cell suspension was made by mild enzymatic dissociation using a trypsin/EDTA solution (PAA, US). Stock cultures were transferred every 3-4 days by seeding appropriate numbers of cells of  $(0.5-1) \times 10^6$  (depending on cell type and doubling time) into T75 culture flasks. Avoid repeated dissociation of monolayers within less than two days, to ensure the spheroids would be formed with the cell membranes being interrupted not too frequently. Cells could be frozen in a 30% fetal bovine serum and 10% DMSO supplemented DMEM and stored in liquid nitrogen, but after being defrosted the cells had to be passaged for at least once before being seeded to ensure the optimal spheroid formation abilities, and this recovery period is usually 8-12 days.

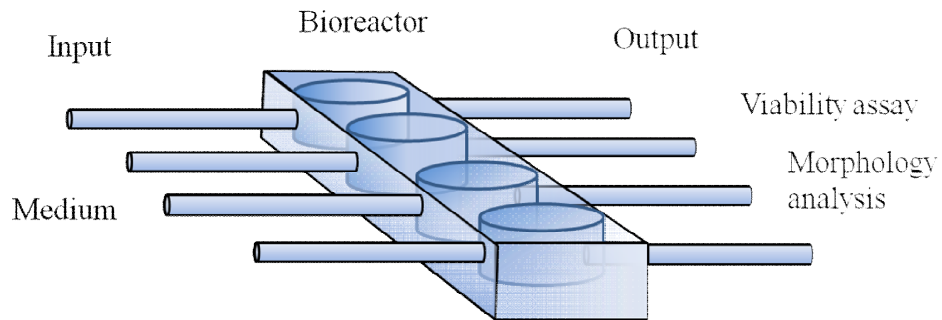
### **3.2.2 TissueFlex<sup>®</sup> microbioreactor production and surface modification**

TissueFlex<sup>®</sup> bioreactors were made by the crosslinking of poly (dimethylsiloxane) (PDMS) polymers (SYLGARD 184 kit by Dow Corning, UK). The polymers and crosslinking reagent is mixed at the ratio of 10:1, then the mixture was poured into an individually designed module. The module was then put into vacuum conditions to remove the bubbles in the

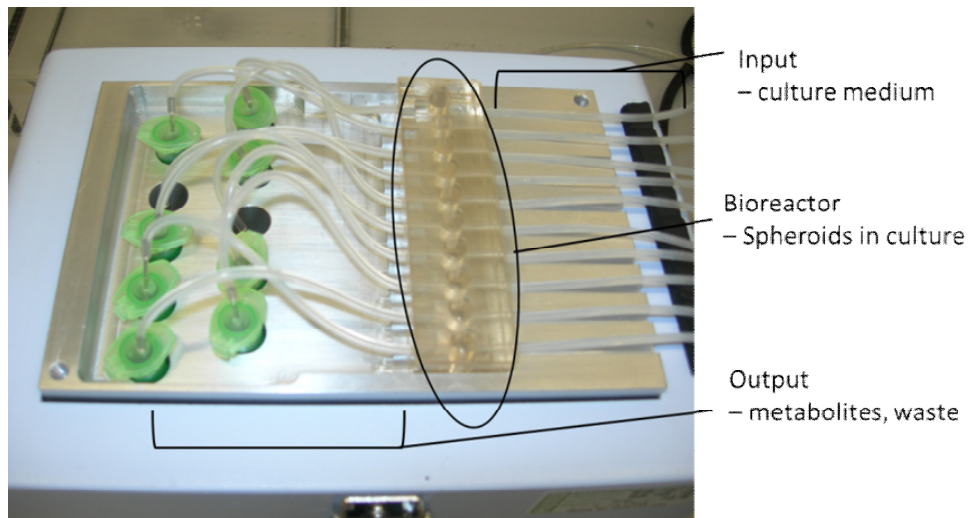
mixture. The input and output of the bioreactor are made of Tygon tubing (Cole-Parmer, UK), with the inside diameter as 0.75946mm (0.0299 inches), and the outside diameter as 2.4892mm (0.098inches). The bubble remove step is important for keeping the homogeneity of the inside environment of the bioreactor. After all the visible bubbles were removed, to solidify the bioreactor, the module with the mixture was then baked at the temperature of 60 °C overnight. Alternatively, the crosslinking process can be finished in two hours at the temperature of 70 °C. Since PDMS is hydrophobic, so the surface of PDMS needs to be modified to allow the following agarose coating to form a concave surface. Briefly, prior to agarose coating, 100 µl of 0.1M sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) solution was added in each well of bioreactors, and the bioreactors were incubated overnight at room temperature. Next day H<sub>2</sub>SO<sub>4</sub> solution was completely removed, and each well was washed thoroughly with dH<sub>2</sub>O. After washing, 100µl dH<sub>2</sub>O was added to each well, and bioreactors with lids were autoclaved following the standard procedure. Wells should always be kept wet in order to maintain modified hydrophilic PDMS surface. Distilled H<sub>2</sub>O was completely removed from each well of bioreactors just before agarose solution was ready to use for coating.

TissueFlex is a single direction, multiple parallel perfused micro-bioreactor system. As shown in schematic diagram Figure 3.1 (A), where the culture media or testing compounds are individually perfused through inlet tubing by a syringe pump (Harvard Apparatus, Standard Infuse/Withdraw PHD 2000) to the bioreactors where 3D micro-tissues are housed in the chambers and the spent medium can be collected separately through the outlet tubing for further analysis. The perfusion rate is 1.38µL/hr, which equals to 100µL/72hrs, to make it comparable to the static culture, in which 100µL culture medium was changed every 72 hours. The special optical properties of PDMS material allows 3D micro-tissues in the bioreactors to

**A**



**B**



**Figure 3.1.** (A) Schematic diagram of the bioreactor. The output is collected in eppendorf tubes and the input is controlled by mini syringe pumps, while the well size is comparable to normal 96-well plate. The anti-cancer drugs can then be tested on spheroids, by diameter measurement, end point viability assays or further investigation under multi-photon microscope (MPM). (B) PDMS-based perfusion platform. The special optical properties of PDMS material allows the TissueFlex bioreactor to be transparent and it is convenient to view and monitor the inside culture content.



be viewed and monitored during culture period. The size of the wells is comparable to normal 96-well plates. In the study, the spheroids were formed in the bioreactors prior to testing anti-cancer drugs through diameter measurement, viability assays and morphological analysis using multi-photon microscope (MPM).

### *3.2.3 Spheroid formation*

0.15 g of agarose (A9539-100G, Sigma-Aldrich) was added to 10 ml of serum-free medium (1.5% w/v) in appropriate beaker, and the beaker was sealed with aluminum foil or lid and autoclaved for 20 min at 120 °C, 2 bar. The beaker was then taken out of autoclave at about 90 °C and transferred to a sterile biosafety hood, kept in a water bath at 60 °C. 60 µl agarose solution was added to each well of bioreactors or 96-well plates (flat bottomed) under sterile condition. Bioreactors and plates with agarose were left in the hood to cool down to room temperature at least for 20 min to allow the agarose to solidify. Bioreactors and plates are recommended to be used immediately. Alternatively, they can be stored in sterile condition at room temperature for up to 10 days after preparation.

For spheroid formation, 100 µl cell suspensions with cell densities ranging from  $5 \times 10^3$  to  $3 \times 10^4$  cells/ml was added to each well. Cell spheroid growth was monitored by measuring diameter of spheroid formed and cell viability (AlamarBlue assay) over 14 day's culture. The optimal initial cell seeding densities to form spheroids of diameters of 200 µm, 400 µm and 600 µm in 4 days culture were investigated.

#### *3.2.4 Diameter measurement*

The images of spheroids were taken at Day2, Day4, Day6/7, Day8/9, Day10/11, and Day14 using an inverted microscope (Nikon). For DLD-1 cell line, in each well there was only one spheroid formed in the centre of the well. Ten wells were seeded by cells in parallel for each group. The diameters were measured by image processing software ImageJ (NIH, US), and the diameters of ten spheroids were measured and averaged for each group.

#### *3.2.5 Viability assay*

Cell metabolic activity was assessed by AlamarBlue assay (DAL1025, Invitrogen) at each time point by adding 10 µl AlamarBlue solution in each well and incubating for 2 hours. After incubation, 50 µl of medium from each well was carefully taken and the fluorescence intensity at 560 nm excitation and 590 nm emission was measured using a fluorescent microplate reader (WALLAC VICTOR2 1420 multilabel counter model, Perkin Elmer, UK). The indicative cell growth rate at different time point was normalised with reading obtained from Day2. 100 µl of cell-free culture medium with 10 µl AlamarBlue solution is set as a blank.

#### *3.2.6 Live/dead imaging using multiphoton microscope*

##### *Cell labelling*

Cell viability and morphology were monitored using live/dead viability/cytotoxicity kit (L3224, Invitrogen). The dead cells were stained by ethidium homodimer-1 (EthD-1) with red fluorescence, which is not permeable to living cells. To stain the living cells, the acetomethoxy derivative of calcein (calcein AM) was used. This compound has no fluorescence, but will be transported through cell membranes of living cells, and degraded into calcein, which has green fluorescence. Prior to staining, spent culture medium was briefly removed and the spheroids were rinsed with phosphorous buffer saline (PBS) twice.

Spheroids were incubated with PBS containing 4  $\mu$ M calcein AM and EthD-1 in dark at 37°C for 10 min. Then samples were washed with PBS prior to mount on slides with mounting medium (Vector labs, UK) and visualized using a multi-photon microscope.

#### Image acquisition

3D microscopic images were obtained from a modified BioRad Radiance 2100 MP Multiphoton Microscope (MPM, Zeiss; Jena, Germany). Near infrared (NIR) laser beams ( $\lambda = 800$  nm) were obtained from a tunable 76 MHz femtosecond pulsed Ti: sapphire laser (Mira 900-F, Coherent, Ely, UK) pumped by a 10 W multiline argon ion laser (Verdi; Coherent). A Nikon S Fluor 10  $\times$  objective with a numerical aperture of 0.30 and working distance of 2.0 mm was used for all images. The emission filters selected were: 525 nm for green and 595 nm for red. All serial optical sections from individual datasets were loaded into Imaris 7.6.1 (Bitplane Ag, Zurich, Switzerland) software for processing and analysis.

#### 3.2.7 Drug treatment

DLD-1 spheroids of diameters of 400  $\mu$ m were used to study the effect of drugs 5-fluorouracil (5-FU) (Sigma, UK), paclitaxel (Sigma, UK) and tirapazamine (TPZ) (Toronto Research Chemicals Inc.). Stock drug solutions of 10 mM were prepared in dimethyl sulfoxide (DMSO), and stored at -20°C. The working drug solution (one thousand times dilution in culture medium) was prepared before each experiment. 10  $\mu$ M was chosen as the tested concentration for both 5-FU, TPZ and paclitaxel, which is also a concentration close to clinical drug dosage (Ando et al., 2008; Hong et al., 2011).

Spheroids were initiated in 4 days static culture in TissueFlex® bioreactors and in 96-well plates prior to exposure to drug. None drug treatment group (medium containing 0.1% DMSO) was used as controls. For perfusion culture, desired volume of medium containing drug or control (0.1% DMSO) was filled in 2 ml syringes at perfusion rate of 10µl/h, and perfusion experiment was set up in the bio-safety cabinet following a standard procedure. For both static 2D and 3D spheroid culture set in 96-well plates, 100 µl of medium containing 2 folds final concentration drug or control (0.2% DMSO) was added in each sample well where initiated cell spheroids with 100 µl medium were situated (total volume of culture medium per well was 200 µl containing 1 fold final concentration of drug). Conventional 2D culture was set as control, with everything else being the same as the 3D spheroid experiments. Spheroids/cells were exposed to drugs or control DMSO in perfused bioreactors and/or static 96-well plates for 72 hours.

Diameters of spheroids were monitored at 24, 48, and 72 hour of drug incubation, and cell viability was monitored at 24, 48 and 72 hour by sampling 50 µl of spent medium from each well. For all samples cultured in static 96-well plates, top up fresh 50 µl of drug solution in each corresponding well after 50 µl of sample being taken. The average percentage cell viability for each drug concentration was calculated by normalizing to the untreated spheroid control.

### *3.2.8 Data analysis*

Data were presented as the mean  $\pm$  the standard error of the mean (SEM) of three independent experiments (n = 3), each performed in triplicate. A two-tailed Student's t-test was used with \*p < 0.05; \*\*p < 0.01 being regarded as statistically significant difference.

### 3.3 Results and Discussions

#### 3.3.1 Optimisation of spheroid formation

To optimise the spheroid formation, different parameters which may affect spheroid formation and growth, including agarose type, cancer cell type, seeding density and culture time, were investigated. Both morphology and spheroid viability were used to evaluate these culture parameters. As shown in Figure 3.2, breast cancer cell line NCI/ADR could only form small aggregates with uncontrollable sizes (Figure 3.2 (A) a), while colorectal cancer cell line DLD-1 was found to form one wholesome spheroid in the centre of each well, with reproducible average diameters and growth kinetics. Besides, gel strength of agarose will also influence the morphologies of spheroids, as shown in Figure 3.2 (A) b. the gel formed by one and half percent of agarose 2576 (w/v) (Sigma-Aldrich) could not support cells to form a integral spheroid structure in the centre. By contrast, on gel formed by 1.5% agarose 9539 (w/v) (Sigma-Aldrich) cells formed nice and firm spheroids (Figure 3.2 (A) a).

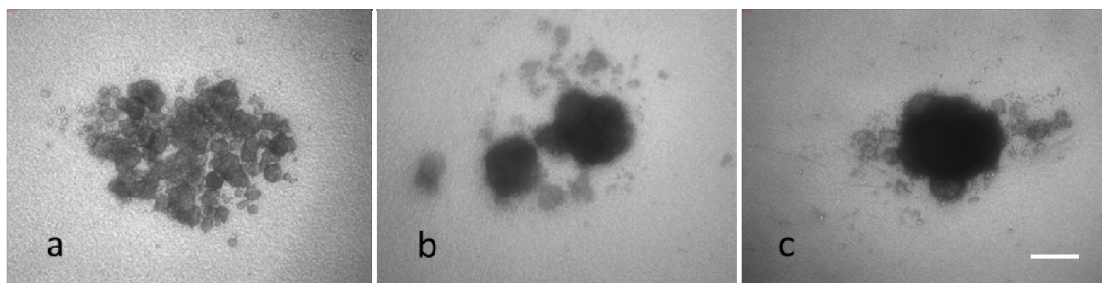
Based on the information provided by the supplier Sigma-Aldrich, agarose 2576 only has the strength as 400g/cm<sup>2</sup> at 1.5% while agarose 9539 has the strength as 1200g/cm<sup>2</sup>. In addition, the gel point of agarose 9539 is 36±1.5°C, which means it will form gel at room temperature quickly, compared with agarose 2576, with gel point as 17°C, which makes it much harder to form a gel after the coating. Therefore 1.5% agarose 9539 and colorectal cancer cell line DLD-1 was used for the following spheroid growth kinetics and drug sensitivity testing.

To find out the optimal initial seeding density for spheroid formation, which is determinant for the growth behaviour and drug responses of spheroids, the growth rate of DLD-1 at cell densities ranging from 5×10<sup>3</sup> to 3×10<sup>4</sup>cells/ml in 11 days culture was studied. We found that after four days of culture, DLD-1 could form spheroids with average diameters ranging from

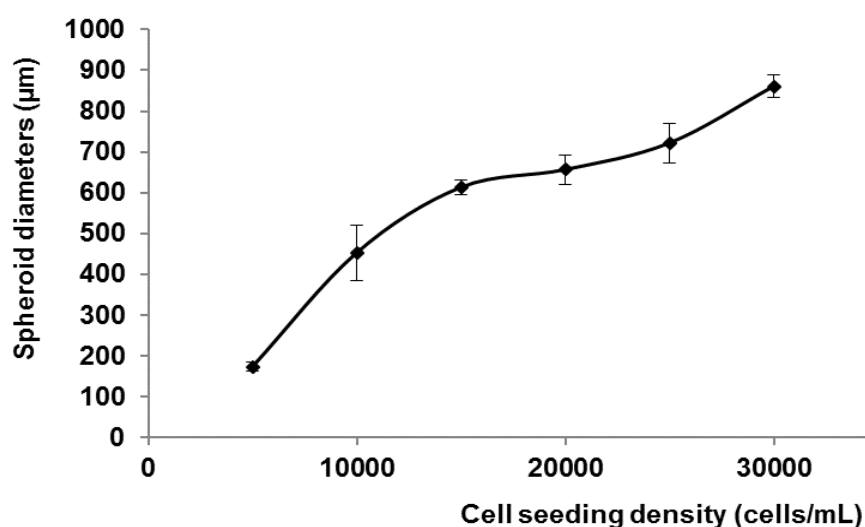
174 to 861  $\mu\text{m}$ , as shown in Figure 3.2 (B). With the optimal seeding density of  $1\text{--}1.5\times 10^3$  cells in each well, the average diameter of the spheroids was 453  $\mu\text{m}$  after four days of culture. Based on previous research, spheroids with average diameter around 400  $\mu\text{m}$  were considered optimal for efficient anti-cancer drug testing (Friedrich et al., 2009).

Seeding density has a profound impact on cell growth and functions. Therefore, the growth curves of spheroids with seeding densities 500, 1000 and 2000 cells/well were further studied for extended culture period of 11 days. It was observed that either low or high seeding density would have a negative impact on spheroid size growth, based on spheroid diameter growth curve (Figure 3.3 (A)). For example, from Figure 3.3 (A) we can see that at day 11, the diameters of the spheroid originated from seeding density 500 cells/well and 2000 cells/well increased only by 32.7% and 7.8% respectively, compared with day 2. By contrast, with the seeding density of 1000 cells/well, the diameters of spheroids increased one fold in 11 days culture (from 316 $\mu\text{m}$  at day 2 to 656 $\mu\text{m}$  at day 11). This result was consistent with Figure 3.2 (B). Interestingly, the tendency of cell metabolic activity over 11 days was not totally consistent with spheroid diameter growth curve obtained from the same sample. For all the three seeding densities of 500, 1000, 2000 cells/well, the cell metabolic activities kept increasing over 11 days (Figure 3.3 (B)). It implies that for all these spheroids with various diameters, the cells inside the spheroid remained metabolically active, with no necessary linear correlation with the morphology sizes of the spheroids containing those cells. In another word, there should not be many dead cells in the cores of these spheroids due to potential oxygen deficiency. This suggests that combination of morphology and metabolism studies are important for the following culture evaluation and drug testing, which might provide information emphasizing different aspects of spheroid features.

**A**



**B**



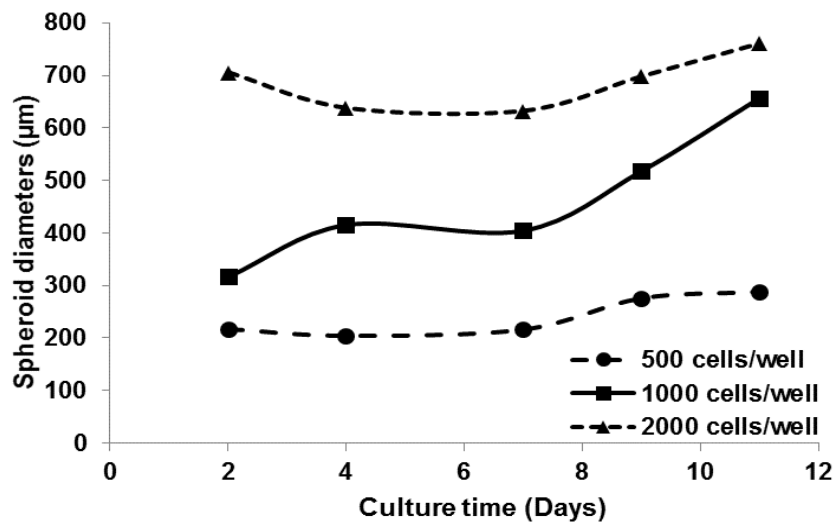
**Figure 3.2.** (A) The optimisation of agarose type and cell type for spheroid formation. From left to right: a. NCI/ADR on agarose 9539, b. DLD-1 on agarose 2576, c. DLD-1 on agarose 9539. All the cells were cultured on agarose coating for 4 days. On unsuitable agarose type coating, or with certain type of cell line, only scattered cell clusters with varied diameters was observed. *Scale bar: 200μm* (B) Initial cell seeding density optimisation on day 4. Seeding density of  $1 \times 10^5/\text{mL}$  was chosen eventually for the following experiments, because the optimal initial diameter (400 μm) was formed at this initial seeding density. Note that results in (A) were selected as representative images from three independent experiments, and data in (B) were summary of three independent experiments.

There were some points in culturing at which spheroid sizes decreased, suggesting the formation of dense compact spheroids. For example, at initial seeding density of 2000 cells/well, the spheroid diameter at day 4 was smaller than day 2. (This phenomenon was also observed in kinetics study such as in Figure 3.3 (A). After day 15, the diameter of spheroids in both static and perfusion culture actually decreased). The growth plateau possibly resulted from growth inhibition as the over saturation of available growth space limited cell proliferation and unbalance of physical and chemical microenvironment down regulated biological activities. Thus the higher cell density has, the larger the size is, the quicker growth plateau appears, which explains why in Figure 3.3 (A), the diameter growth curve of spheroids with initial seeding density 2000 cells/well has a more notable 'reverse growth' at day 2-7, while the initial seeding density 1000 cells/well went through a plateau at day 4-7, and 500 cells/well kept growing until day 9.

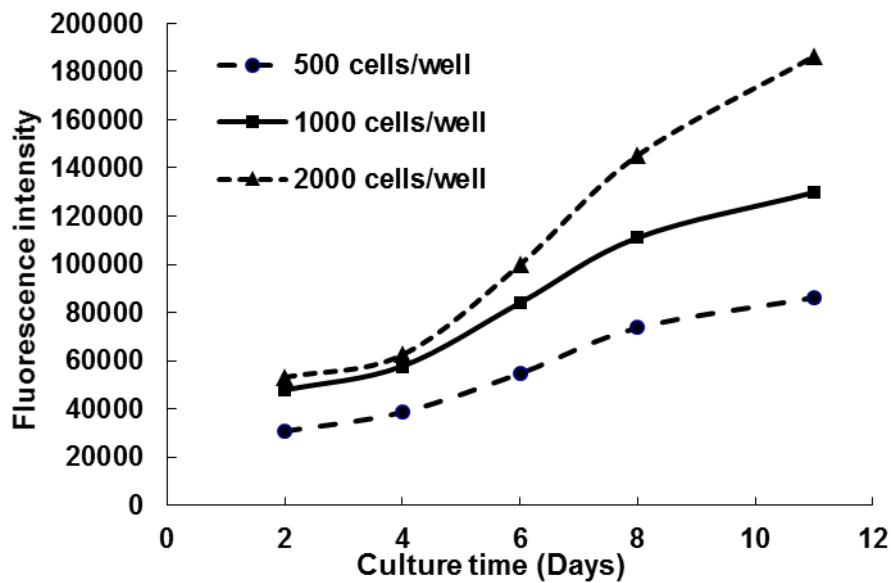
The plateau or decrease of spheroid size growth is possibly due to the enhancing interactions between cells and extracellular matrix, which causes more compressed organisation of cells in the spheroids. Nevertheless, another interesting point shown in this study is that the sizes of the spheroids do not necessarily reflect the cell metabolic activities inside those spheroids. As shown in Figure 3.3 (B), for all the three tested initial seeding densities 500, 1000, and 2000 cells/well, the overall cell metabolic activities kept growing over 11 days. An explanation for the difference between size and metabolic activity is that the size does not necessarily reflect the cell numbers or activity, and the microenvironment provided by relatively smaller but more compact spheroids can still support the survival for cells residing in those spheroids.



A



B



**Figure 3.3** (A) The spheroid growth curves originated from different seeding density, based on spheroid diameters. (B) The spheroid growth curves originated from different seeding density, from the same sample as (A), based on AlamarBlue viability test. The fluorescence intensity is correlated to cell metabolic activities. (A) and (B) were from the same sample group, but were analyzed in two different assays, showing that different assays emphasize different features of spheroids.

### 3.3.2 Kinetics of spheroids growth in static and perfusion cultures

Based on the previous study, the first four-day spheroid initiation period under static culture condition is vital to form spheroid with intact spherical structure. Therefore for all the following testing DLD-1 cells were seeded at the initial density of 1000 cells/well in agarose-coated TissueFlex® 10-well bioreactors and cultured for four days prior to perfusion for another 13 days. Spheroid in static culture was used as control. Figure 3.4 (A) showed that spheroids in both static and perfusion cultures kept growing until day 15. After day 15, the sizes of the spheroids in both culture conditions decreased. This was consistent with previous finding shown in Figure 3.4 (A), for spheroids seeded with the initial seeding density as 2000 cells/well, the spheroid stopped growing when their diameters reached 650µm. This is consistent that in Figure 3.4 (A) in static culture at day 15 when the spheroids stopped growing, they had average diameter as 649.15µm. It is noticeable this growing limitation caused by sizes was extended by perfusion significantly – even at day 12 the perfused spheroids already reached the average diameter of 650µm, but in contrast to the growth stop happening to statically culture spheroids, the perfused spheroids kept growing until their average diameter was 741.54µm.

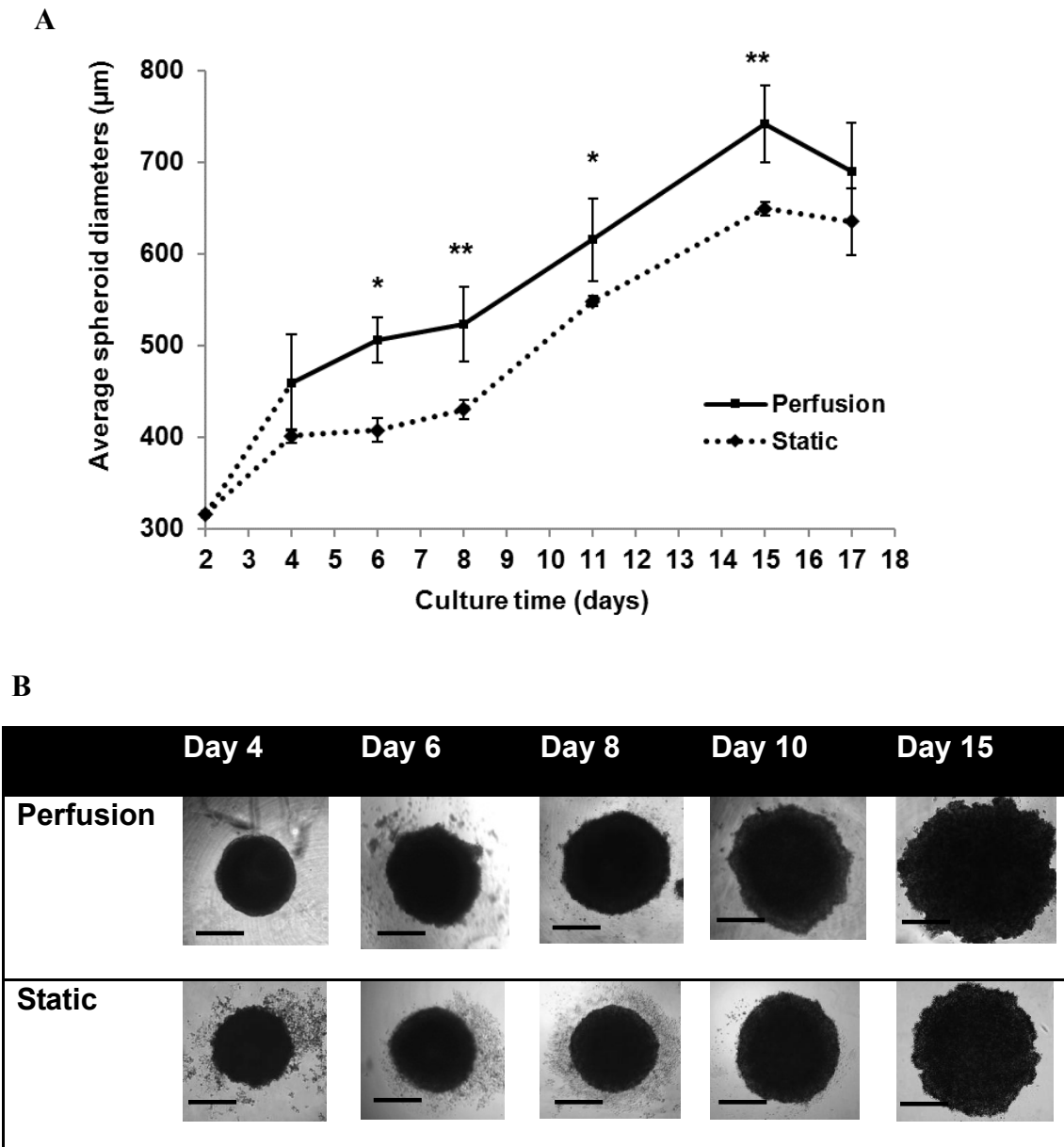
As shown in Figure 3.4 (A) the diameter of spheroids formed in bioreactors under static culture condition was larger than those statically cultured in 96-well plate from day 4, however no significant difference was observed. The average diameter of perfused spheroids was significantly larger than statically cultured spheroids from day 6 ( $p < 0.05$  by Student's t-test). From day 6, the kinetic growth curve of spheroid diameters suggests that perfusion culture supported spheroid size growth. Based on Student's t-test analysis, at day 6 and 10,  $*p < 0.05$ ; at day 8 and 15,  $**p < 0.01$ . Representative images were demonstrated in Figure 3.4 (B). One possible explanation for this enhancing effect on spheroid growth by perfusion is

that the improved cell culture chemostatic the efficient medium supply and continuous metabolic waste removal by perfusion improved the chemostasis of cell culture environment. Previous research from other labs also has shown the improved survival of the culture environment at a more stable level than static culture, and suggested that the perfusion improved the fluctuation of local microenvironment around cells by continuously pumping fresh medium in and removal of waste out of system, rather than abrupt change after a certain period of time (Wu et al, 2006).

In Figure 3.5, multiphoton microscope (MPM) was used to image the live/dead cell distribution comparing static and perfusion cultures. MPM is particularly suitable to image three-dimension of cell morphologies and micro-tissue constructs. While in con-focal microscopy the observable sample depth is about 100 $\mu$ m in general, MPM can investigate samples with the thickness of up to 1mm (Graf et al, 2010). In this study, spheroid structures, and cell viability was determined using MPM. The inside of dense structure of spheroids can be easily visualized and captured by laser power assisted MPM. Figure 3.5 showed detailed live-dead cell distribution in 3D spheroids where dead cells were mainly distributed in the central areas of spheroids in static culture whereas small areas of dead cells observed scattered within the spheroids in perfusion culture. The observation was in agreement with cell metabolic activity data. These data demonstrated that perfused culture of 3D cancer spheroids in TissueFlex® effectively improved spheroid growth by maintaining cell microenvironment steady throughout long culture period.

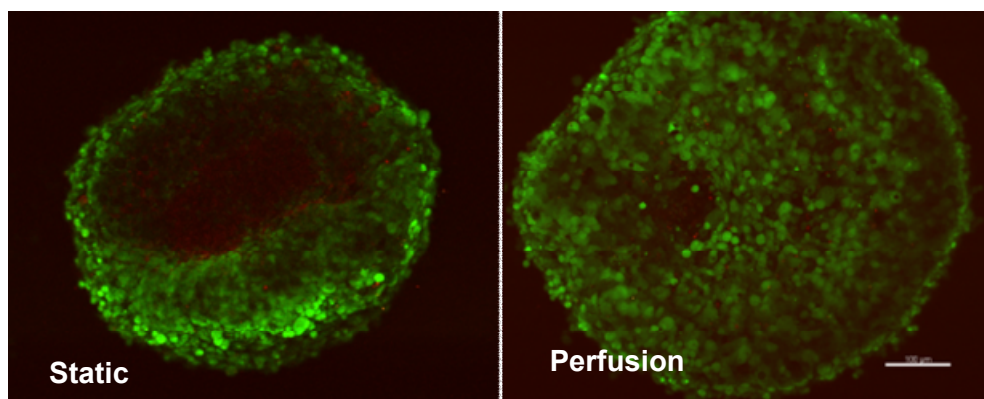
In summary, results in Figure 3.4 and Figure 3.5 demonstrate that cancer spheroids perfused in bioreactor TissueFlex® enhanced size growth at day 6-17 compared with static cultured

spheroids. The MPM 3D imaging further confirmed that there were less dead cells in the core area of perfused spheroids than in the statically cultured ones. Based on previous research, the explanation is that perfusion improved the diffusion capabilities of culture medium through the 3D structure of cultured tissue, and maintained the hemostasis of the culture environment at a more stable level than static culture. The perfusion eases the fluctuation changes of local microenvironment situated around cells by continuously pumping fresh medium in and removal of waste out of system (Wu et al, 2006). In addition, the introduction of perfusion to the culture environment mimic the physiological perfusion in tumours, thus perfused 3D models may provide more accurate prediction of drug toxicity and efficacy (Elliott et al, 2011). In static culture, limited diffusion of nutrients and waste products across the matrix *in vitro* may potentially cause dysfunction of organelles and degradation of cellular integrity for some highly metabolic active cell types such as hepatocytes and islets (LeCluyse et al, 2012). On the other hand, cumulated toxic by-products of metabolism in spent medium in static culture may cause oxidative stress and lead to the rapid loss of cellular function and viability over short culture period *in vitro* (Richert et al., 2006). Besides, the intermittent medium change process in conventional static culture would result in large fluctuations in the culture microenvironments (Sittinger et al, 1997; Wu et al., 2006). In addition to the above considerations, for long-term drug testing perfusion culture also has technical advantages such as to prevent potential interruption of cells from manually medium change, to reduce the potential contamination generation during frequency of medium change and thus to save the time and labor for researchers (Wu et al., 2008).

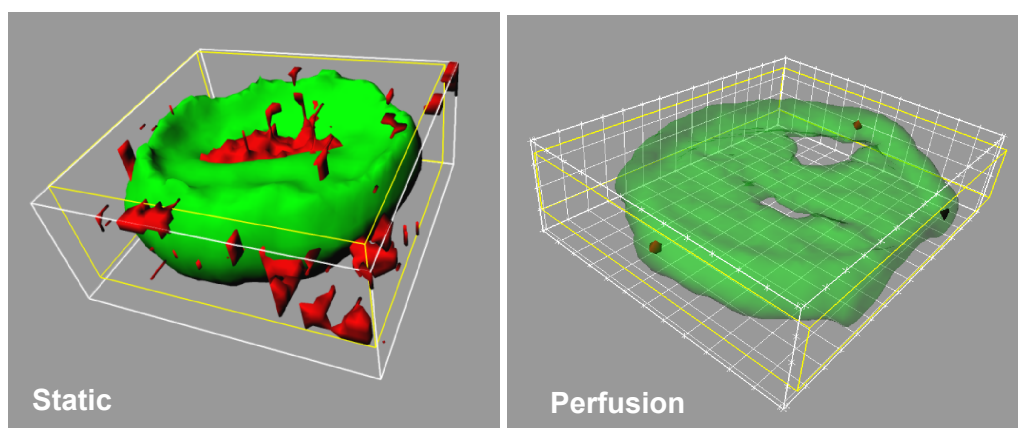


**Figure 3.4.** Growth kinetics of the spheroids under perfusion and static cultures. Between Day 3 and Day 12, significantly different diameters were observed between static and perfusion spheroids. (A) Spheroid growth curve; \*  $p < 0.05$ , \*\*  $p < 0.01$ . (B) Representative images of spheroids from Day 1 to Day 11. The experiments were repeated independently for 2 times, and for each time point 5 spheroids were measured and averaged together. Student's t-test was applied to compare the significant difference between the perfusion and static groups.

**A**



**B**



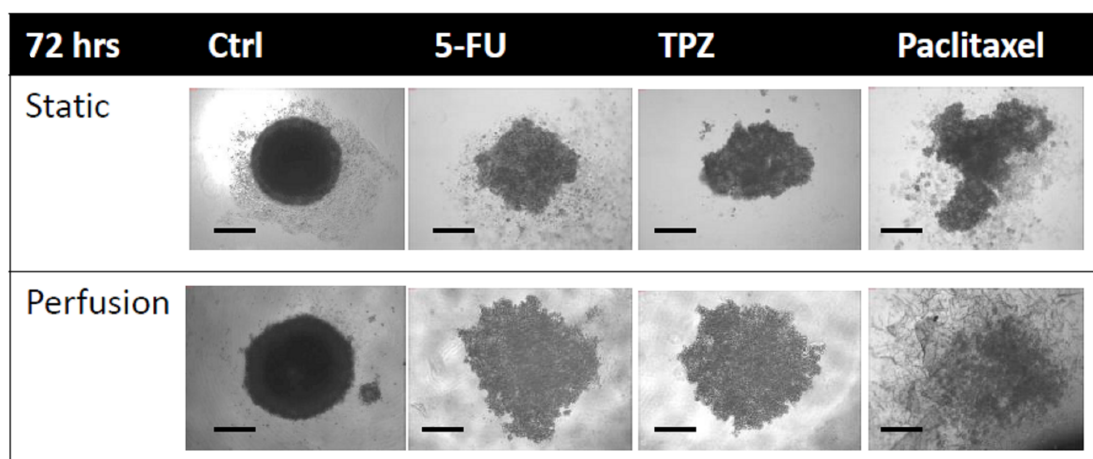
**Figure 3.5** Multiphoton microscope imaging for live/dead staining comparing spheroids in static and perfusion cultures. (A) Live/dead cell imaging of spheroids in static and perfusion cultures. Middle section of a representative MPM image was chosen; (B) 3D reconstruction of corresponding samples in (A). *Scale bar: 100 $\mu$ m.*

### 3.3.3 Anti-cancer drug testing

Three anti-cancer drugs, TPZ, 5-FU and paclitaxel, were used to evaluate the drug responses of Perfused spheroids model and standard static spheroid culture. 5-fluorouracil is a conventional anticancer drug that inhibits cellular proliferation. TPZ is a hypoxia-triggered cytotoxin that causes DNA damage. Paclitaxel binds to  $\beta$ -subunit of tubulin and thereby interferes with microtubule growth and as a result cells are arrested in their growth and consequently go to apoptosis. Based on optimised protocol, the spheroids were statically cultured in both agarose-coated bioreactors and normal 96-well plates for 4 days to initiate spheroid formation prior to introducing drug. The diameters of spheroids formed and cell metabolic activities were determined on day 5 prior to drug testing. Traditional cytotoxic anti-cancer drug 5-FU was compared with TPZ, whose effect will only be obvious under hypoxia condition (Hong et al., 2011), and with paclitaxel, which mainly influences microtubules in proliferating cells (Zhou et al, 2005).

Routine microscope imaging was applied to all drug testing, and the representative images from spheroids treated by the above three drugs were summarised in Figure 3.6. As shown in Figure 3.6, light field phase microscopy shows that thickness decrease and structure integrity interruption were observed in all the drug-treated samples, compared with the control group. In perfusion group, the structure interruption is more obvious compared with statically culture group, especially in the case of Paclitaxel – in static group there was still one single three-dimensional structured spheroid remained in the centre of the culture well, while in perfusion group the whole structure was nearly lost, with cells aggregates scattered randomly over the whole well. It was also noticeable that for 5-FU and TPZ, the structures of the perfused spheroids seemed looser, compared with statically treated spheroids. Thus, the

results from light field inverted microscopy suggest perfusion can enhance the structure change caused by drug treatment for all the drugs being tested.



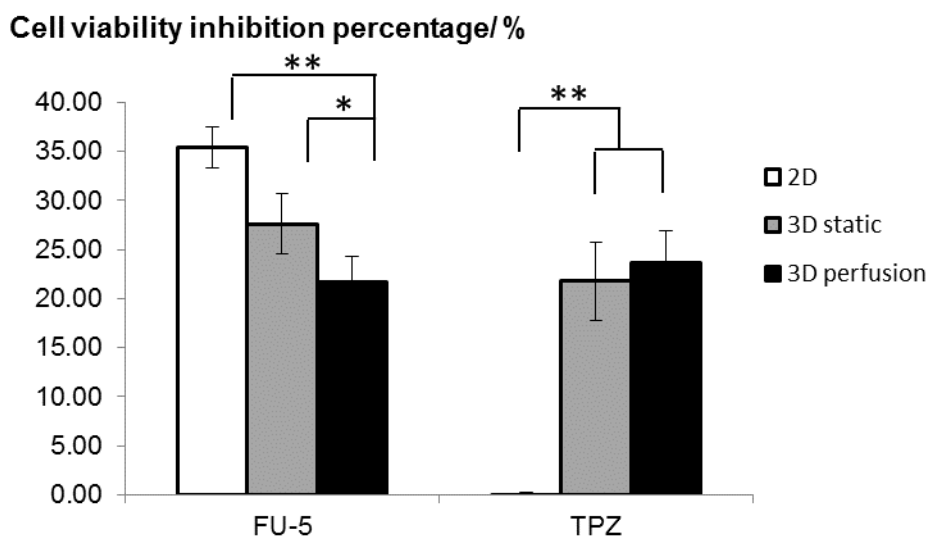
**Figure 3.6** Representative images of spheroids after treatment with different drugs. Different morphologies were observed, probably due to different acting mechanisms of the tested drugs.

The viability tests comparison between 5-FU and TPZ is shown in Figure 3.7. After 72 hours of drug treatment, completely different drug response profiles were observed for 2D, 3D static and 3D perfusion spheroids. For 5-FU treated groups, Student's t-test revealed statistically significant difference between the average inhibition percentages of 3D static model (27.6%) and perfusion spheroids (21.7 %). The highest inhibition effect was observed in 2D monolayer model (35%), while 3D perfusion model showed the lowest drug response (21.7%). 5-FU was mainly acting as a proliferation inhibitor for cancer cells, but the 3D spheroid structures were shown to increase the drug resistance, possibly due to the reinforced intercellular interactions between cancer cells, which may set more barriers to the drug diffusion into inner cells in the spheroids. Interestingly, though there was no significant



difference between the inhibition percentages of 2D and 3D cultures ( $p=0.0854$  by Student's t-test), significant difference was observed between 2D and 3D perfusion cultures ( $p = 0.00706$  by Student's t-test). The enhancement of cell growing and function environment due to 3D structures was possibly further improved by the perfusion condition, in which a lower drug response was observed. This is consistent with our hypothesis that perfusion can improve the local microenvironment for cancer cells.

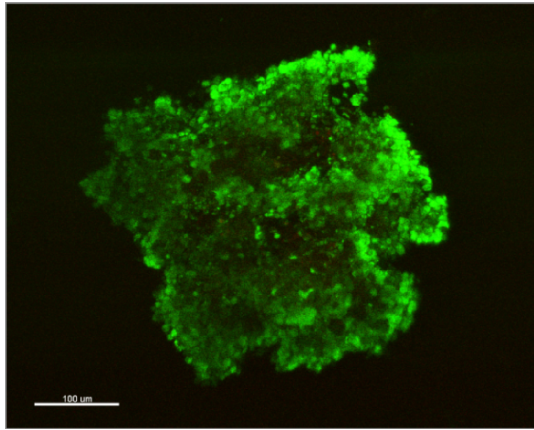
Considering TPZ treated group, significant drug inhibition was observed in 3D models, with 21.8% in static culture and 23.7% in perfusion (Figure 3.7). However, different from 5-FU, no significant difference was detected between the two 3D models. Different from the cytotoxicity mechanism of 5-FU, TPZ relies on the hypoxic situation, thus a significant difference was observed between results from 2D and 3D models, which is consistent with other groups' research (Tung et al., 2011). In 2D monolayer model, all the cancer cells were exposed to the same sufficient oxygen supply, so no significant drug response was recorded. This situation was totally changed in 3D spheroid models, in which the cancer cell aggregates created an oxygen gradient from the surface with full exposure the oxygen, to the centre where is hypoxic. Our results imply that although the perfusion can improve oxygen and nutrient supply to the surface of the spheroids, the oxygen ingredient through the whole spheroid structure will probably not be changed significantly.



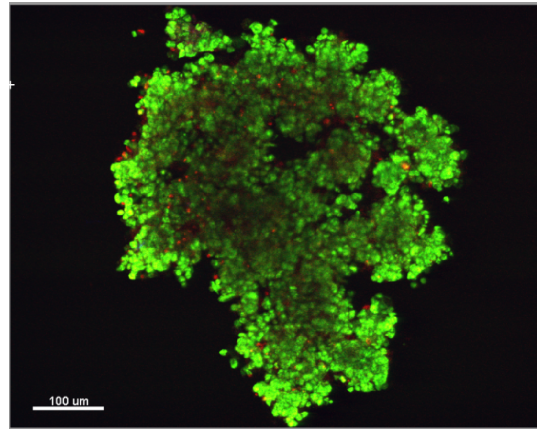
**Figure 3.7** Cell viability testing comparing perfusion and static spheroids. Both drugs were applied at the concentration of 10  $\mu$ M.

Different from the cytotoxic drug 5-FU, who mainly acts by killing cells, paclitaxel mainly acting on proliferating cells by interfering with microtubule assembling (Frankel et al, 1997; Zhou et al, 2005), and we expected the morphology studies would reveal more details in the comparison between static and perfusion culture. As we expected, the morphology studies on paclitaxel showed obvious difference between 3D static and perfusion cultures, as in Figure 3.6 and 3.8. The spheroids statistically treated by Paclitaxel still had some 3D structures, while the spheroids perfused with Paclitaxel totally lost the spheroids structures after 72 hours, with only single cells or small aggregates scattered in the culture well, as shown in Figure 6 and 8. There was significant morphology change in 3D static culture, while spheroids treated by paclitaxel nearly lost all the structure integrity. Furthermore, MPM imaging showed large quantities of dead cells (stained as red in Figure 3.8) in this integrity loss process. However, why perfusion can enhance the structure interference effect of paclitaxel to spheroids needs to be further studied, but we assume that the improved

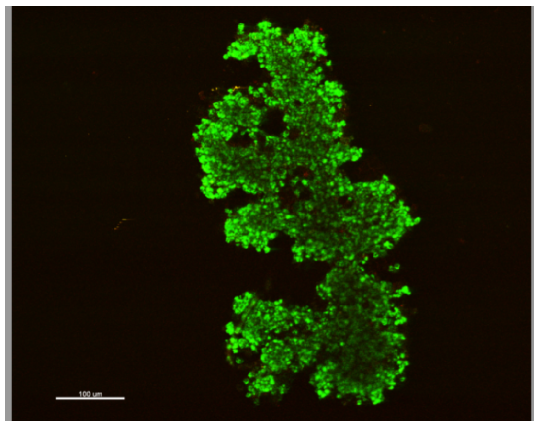
intercellular 3D structures due to perfusion contributed to the amplified effect of paclitaxel on spheroids, as demonstrated in results from previous sections (Figure 3.3) and previous research (Cui et al., 2007; Wu et al., 2008).



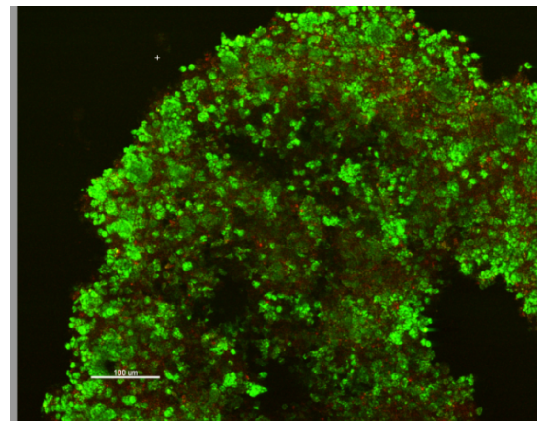
(A)



(B)



(C)



(D)

**Figure 3.8** Representative MPM images of spheroids after treatment with different drugs. Different distribution patterns of live-dead cells can be observed using MPM under the treatment of drugs with different acting mechanisms. (A) 5-FU treatment in static culture; (B) 5-FU treatment in perfusion culture; (C) Paclitaxel treatment in static culture; (D) Paclitaxel treatment in perfusion. *Scale bar: 100 μm*

In short, the introduction of perfusion has different influences on drugs with different acting mechanisms. Cell viability showed a lower drug response in perfusion culture for traditional cytotoxic drug 5-FU, while no significant difference was observed for hypoxic-dependent cytotoxic drug TPZ. For microtubule targeting paclitaxel, a more significant spheroid integrity interruption and large quantities of dead cells were observed using advanced imaging technique MPM, highlighting the importance of imaging analysis in anti-cancer drug evaluation. Future research will use different cancer cells to test the feasibility of this model. Other supporting cells such as endothelial cells in co-culture with cancer cells will also be considered, to improve the physiological relevance of the model.

### 3.4 Summary

In this study, we established an *in vitro* 3D perfused spheroid model for anti-cancer drug testing. We demonstrated that TissueFlex® is better mimicking physiological flow in real tumour than static culture. The perfusion can maintain the local micro-environment around the culture cells and spheroids for long-term culture, enabling the directly link between extracellular conditions to cell/tissue function. TissueFlex® also enables precisely control the chemostatic environment in a mid-throughput and parallel manner during the drug dosing period by constantly supplying fresh media, which may contain drugs at preset doses, and removing waste products through pumping system that mimics the circulatory system in the body. Therefore this perfused colorectal cancer model could be a more predictive *in vitro* model for long-term anti-cancer drug efficacy testing and cytotoxic mechanism study.

It is noted that the perfusion rate of this study is quite slow (less than 10 $\mu$ L/hr). Under this slow perfusion rate, it is usually considered that diffusion is the main pathway for mass transfer in the culture medium, especially at the directions perpendicular to the perfusion direction (Heiskanen et al, 2009). One direct factor influencing drug diffusion rate is the drug concentration gradient across cell membranes. It is possible that perfusion facilitate the diffusion process, as in perfusion culture the molecule reservoir around the spheroid is continuously refreshed by the continuous supply of the drug, while in the static culture the drug concentration will go down after a fraction of applied drug molecules penetrated into the cell. It is interesting that previous reports pointed out the penetration percentage of 5-FU is nearly 100% over 24 hours while only 20% Paclitaxel penetrate over a 62.5 $\mu$ m of multilayer cell culture (Choi et al, 2011). Based on the above factors, it is likely that as for 5-FU the full penetration happened very quickly even in the static culture, but in perfusion culture the drug

concentration gradient was improved in the perfusion culture compared with the static culture, thus the difference between the static and perfusion treated groups was more significant.

It is also relevant to consider the different albumin binding affinities of the tested drugs in this chapter, as 10% foetal bovine serum (FBS) was added in the culture medium before and during the drug treatment, and about 60% of the serum protein was albumin so the potential binding between albumin and tested drugs will influence the drug efficiency (Trynda-Lemiesz et al, 2004). There was report suggesting there was binding existing between bovine serum albumin (BSA) and 5-FU (Abdi et al, 2012), and in the case of Paclitaxel though the main research was focused on human serum albumin the percentage of bound Paclitaxel can be as high as 90% (Trynda-Lemiesz et al, 2004). This drug-albumin binding can reduce the free drug concentration in the medium and influence the abilities of the drugs to penetrate through the spheroids. It was observed in this chapter that perfusion enhanced the effects of Paclitaxel, as shown in Figure 3.8. It is possible that contrary to the static culture in which most Paclitaxel molecules were bound to albumin and the effects were potentially underestimated, in the perfusion culture the available free drug in the medium reservoir was consistently refreshed and facilitate the penetration process of the drug.

In a word, from the results of this chapter show that how the perfusion factor will influence the drug testing results vary depending on different drugs, and multiple factors, including the mechanisms of the drugs to enter the spheroids, and formation of drug-albumin complex should be considered together when interpreting the perfusion data and compare them with the static culture.

## Chapter 4. Sandwich Co-Culture of Breast Cancer Cells with Endothelial Cells

### 4.1 Introduction

Angiogenesis, the formation of new blood vessels, is crucial in cancer progression. Endothelial cells are the main participants in this process, and the new capillary networks formed by them will then provide nutrients and oxygen to cancer tissue by perfusion (Albini, et al. 2007; Kerbel, et al. 2002). Moreover, the interactions between cancer cells and endothelial cells influence the dynamics of cancer tissue growth by paracrine pathways, and cancer cell response to anti-cancer therapeutics will also depend on cancer-endothelium interactions (Buess, et al. 2009; Ingthorsson, et al. 2010). Compared with traditional cytotoxicity strategy, anti-angiogenesis therapy is emerging to become a promising new strategy for treating cancer because of improved targeting to cancer tissue with lower toxicity to normal tissue. This is because endothelial cells are relatively stable genetically compared with cancer cells, thus there is less possibility for accumulated mutation to cause drug resistance in the therapy and the drug can be applied at lower concentration with higher efficiency but much lower toxicity (Kerbel, et al. 2002).

To investigate the effects of anti-cancer drugs on angiogenesis regulation, *in vitro* and *in vivo* models have been developed. *In vivo* testing is the testing of drugs in a whole physiological environment, i.e. an animal model, but they have ethical and scientific controversies (Auerbach et al. 2003; Staton et al. 2004). *In vitro* models, on the other hand, use cultured cells isolated from the human body to test the efficiency and toxicity of anti-cancer drug candidates. This method is relatively clearly quantified with less ethical issues, but lacks the intercellular and cell-extracellular matrix interactions of the *in vivo* environment.

To mimic *in vivo* intercellular interactions between cancer cells and endothelial cells, *in vitro* tumour models co-cultured with endothelial cells have been built up and studied (Khodarev et al. 2002; Kozien et al. 2000; Reid et al. 2009). The Transwell system is a well-established co-culture method, with one cell type loaded on the inserts and the other cells seeded on the wells. Using Transwell, it is found that being co-cultured with cancer cells could stimulate endothelial cell growth and induce the angiogenic molecules such as Tie-2 (Khodarev et al. 2002). Another research in which cancer cells were co-cultured in direct contact with endothelial cells in culture flasks found that cancer cells could stimulate osteoprotegerin (OPG) produced by endothelial cells, which was then related to endothelial cells' angiogenesis abilities (Reid, et al. 2009). Although the above *in vitro* co-culture models revealed some aspects of cancer angiogenesis, they are mainly 2D models and lack cell-extracellular (ECM) interaction in 3D microenvironment of *in vivo* models. Also, due to the limitations of 2D monolayer cell culture, these studies were carried out for relatively short time, up to 4 days of co-culturing time (Brisson et al, 2002; Kozien et al, 2000).

In order to overcome the above limitations of 2D *in vitro* models, three dimensional (3D) *in vitro* cancer models have been developed. These models are considered to bridge the gap between traditional *in vitro* and *in vivo* models (Gurski et al. 2010). 3D models can be built up by hydrophobic surface to form cancer spheroids or by seeding cells in scaffold. One study co-culturing cancer cells and endothelial cells in 3D environments demonstrated the 3D environment factors influenced the growth rate of cancer stem cells, which could not be recapitulated by 2D models (Hsiao et al. 2009). Another report based on co-culture of cancer cells and endothelial cells in Matrigel illustrated the stimulating effects of co-cultured endothelial cells on cancer cell growth (Ingthorsson et al. 2010). These studies are evidence



that 3D *in vitro* models can better mimic the cancer angiogenesis microenvironment compared with 2D models.

Due to the limitation of their models, the tubule formation ability of endothelial cells was lost in the above discussed co-culture models, and as a result no morphology information was provided by these 3D co-culture models for further analysis. In addition, these studies only characterised the developed co-culture models, but no further drug testing was reported for these models. Based on the limitations of previous *in vitro* tumour model co-cultured with endothelial cells, the aim of this study was to develop a 3D *in vitro* co-culture model which features vascular network and tumour structures for use in cancer angiogenesis study and testing for drugs with anti-angiogenesis potential.

## **4.2 Materials and Methods**

### **4.2.1 Cell Preparation**

For the culture process of human breast cancer cell line NCI/ADR, please see Section 3.2.1 in Chapter 3; Human umbilical vein endothelial cells (HUVECs, Lonza, UK) were cultured in endothelial growth medium (EGM-2, Lonza, UK) and passage 2-6 were used for the study. HUVECs older than P6 were discarded as they lost tube formation ability.

### **4.2.2 Sandwich co-culture of NCI/ADR with HUVECs and co-culture ratio optimisation**

A 3D culture protocol based on the Matrigel sandwich structure was adapted from previous research (Lee et al. 2007) and illustrated in Figure 4.1. Briefly, to form the bottom layer of Matrigel, 120 $\mu$ L pure Matrigel was added into each well of 24-well plate (NUNC) which had been pre-chilled on ice, and then the plates were incubated at 37°C for 30min, allowing the Matrigel to polymerise. Then HUVECs were seeded onto the polymerised gel layer at the density of  $1.4 \times 10^5$  cells/mL, in 250 $\mu$ L endothelial basic medium (EBM-2, Lonza, UK) supplemented with 2% FBS (Life Technologies, US) and 1% penicillin-streptomycin (PAA). HUVECs were allowed to attach to the Matrigel and form tubules for 4 hours before NCI/ADR cells were added.

After 4 hours of HUVECs seeding, NCI/ADR cells in 10% Matrigel (v/v) in 250 $\mu$ L pre-chilled EBM-2 supplemented with 2% FBS and 1% penicillin-streptomycin were added onto the polymerised Matrigel at the density of  $5 \times 10^4$  cells/mL. The plate was then left at 37°C to allow the top layer of Matrigel to polymerise. The co-culture system was maintained in EBM-2 supplemented with 2% FBS and 1% penicillin-streptomycin at 37°C, 5% CO<sub>2</sub> for 24 hours before further drug testing. For the co-culture ratio optimisation experiment, the

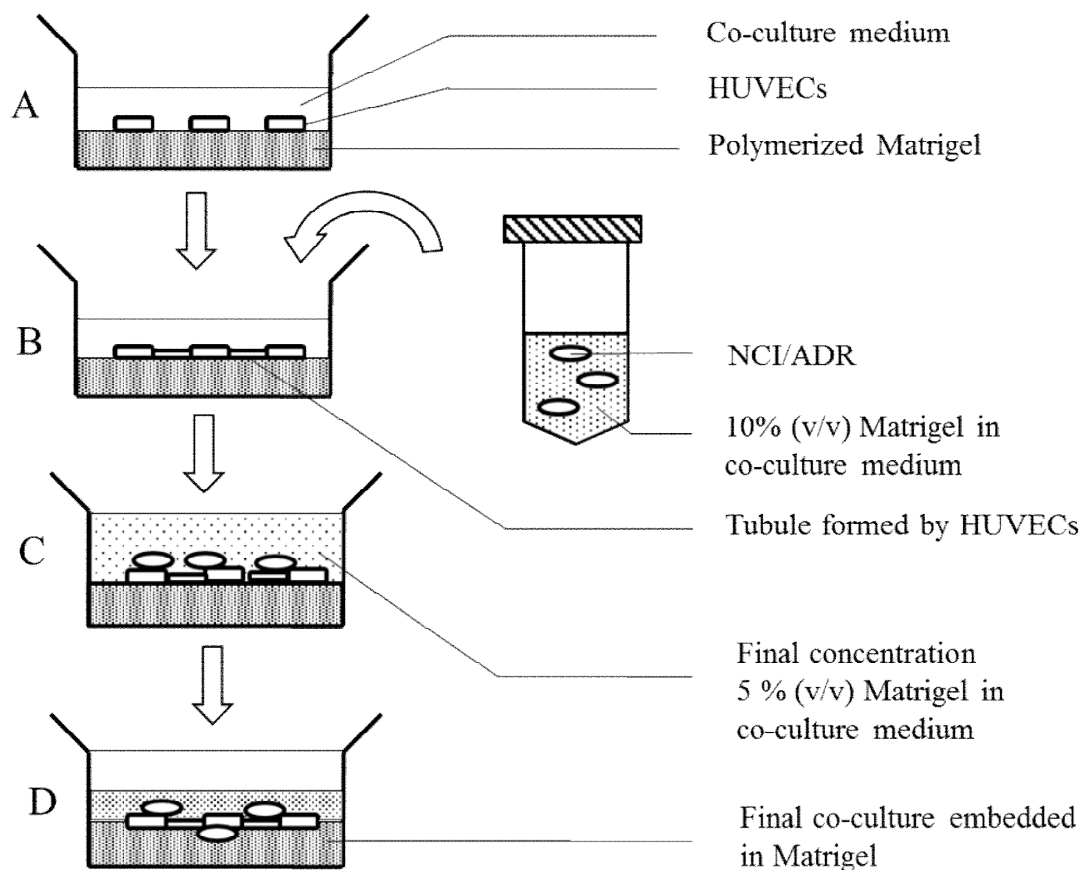
seeding density of the HUVECs remained  $1.4 \times 10^5$  cells/mL, while the density of NCI/ADR as  $1.4 \times 10^4$ ,  $5 \times 10^4$ ,  $1.4 \times 10^5$ ,  $4.2 \times 10^5$ . The ratios of HUVECs: NCI/ADR as 10:1, 3:1, 1:1, 1:3 were tested.

#### *4.2.3 Sandwich mono-culture of NCI/ADR*

The process of mono-culture of NCI/ADR in 3D was the same as the procedure described in co-culture. The only difference was that no HUVECs were added, so NCI/ADR were cultured alone between 120  $\mu$ L bottom layer of and 25  $\mu$ L top layer of Matrigel.

#### *4.2.4 Sandwich mono-culture of HUVECs*

The process of mono-culture of HUVECs in 3D was the same as the procedure described in co-culture. The only difference was no NCI/ADR cells were added following the seeding of HUVECs, so HUVECs were cultured alone between 120  $\mu$ L bottom layer of and 25  $\mu$ L top layer of Matrigel.



**Figure 4.1** Co-culture procedure. (A) HUVECs cultured on Matrigel; (B) Tubule network structure were formed by HUVECs on Matrigel after 4 hours, and then NCI/ADR cells in medium containing 10% Matrigel were added; (C) Tubule network structure covered by unpolymerised Matrigel containing NCI/ADR cells; (D) 24 hours later, co-culture structure formed.

#### 4.2.5 Monolayer culture of NCI/ADR

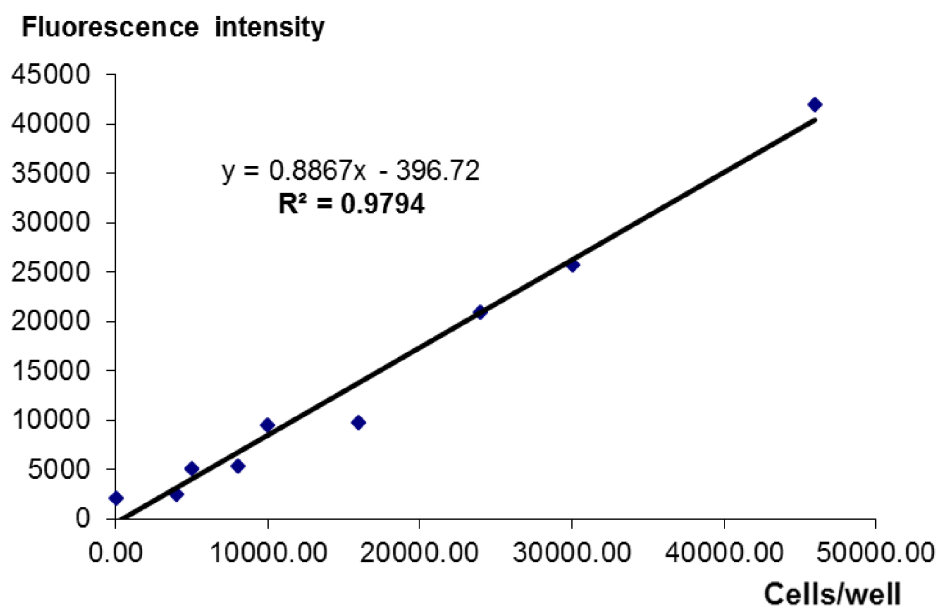
In the process of mono-culture of NCI/ADR in 2D, cells were seeded at the density of  $1 \times 10^4/\text{mL}$ ,  $500 \mu\text{L}/\text{well}$  in 24-well plates (NUNC, Denmark). The protocol was following based on the standard *in vitro* procedure of National Cancer Institute (Shoemaker et al, 2006).

#### *4.2.6 Preparation of drug solutions for testing*

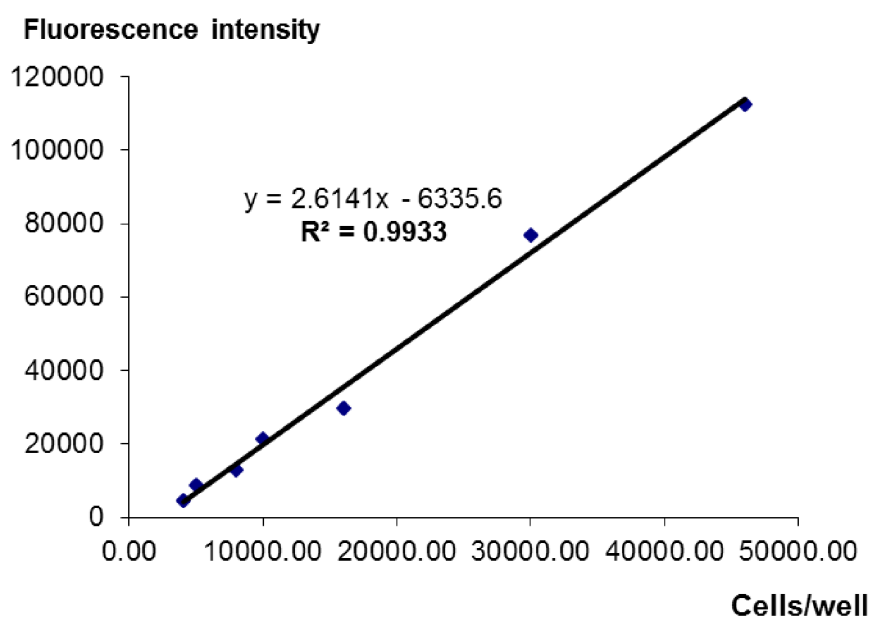
Cisplatin or Paclitaxel (Sigma-Aldrich, US) was dissolved first in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, US) at concentration of 10mM as stock solutions and stored at -80°C. The stock solutions were then diluted into the desired concentration with EBM-2 supplemented with 2% FBS and 1% penicillin-streptomycin just before use.

#### *4.2.7 AlamarBlue assay for cell viability testing*

AlamarBlue (Life Technologies, US) cell viability testing was conducted based on the instructions from the manufacturer. The amount of fluorescence is proportional to the number of living cells and corresponds to the cell metabolic activity. Cells were incubated with EBM-2 containing 10% AlamarBlue for 2 hours at 37°C. The fluorescence intensity was measured at 560 nm excitation and 590 nm emission using a fluorescent micro-plate reader (WALLAC VICTOR2 1420 multilabel counter model, Perkin Elmer, UK). The inhibition percentage of anti-cancer drug on the viability of cells was calculated as below: Percentage of Inhibition =  $\frac{[\text{cell viability of sample treated by anti-cancer drug} - \text{cell viability of untreated sample}]}{\text{cell viability of untreated sample}} \times 100\%$ . AlamarBlue standard curves for NCI/ADR and HUVECs were plotted based on a gradient of known cell seeding densities of NCI/ADR and HUVECs and the absorptions were measured and the linear correlation was processed by Excel, shown as the figure below.



A



B

**Figure 4.2.** Linear correlation of AlamarBlue fluorescence intensity to cell numbers study. (A). HUVECs; (B). NCI/ADR. Certain numbers of cells were seeded into each well of 96-well plates, and the seeding densities were shown in the figure as cells/well.

#### *4.2.8 Colony sizes and angiogenesis parameters measurements*

Inverted fluorescence microscope (Nikon, Eclipse TE 200) was used to collect images for colony diameter measurement and angiogenesis parameters evaluation. The collected images were analysed using ImageJ (NIH, US). For colonies formed in both mono-culture of NCI/ADR and co-culture of NCI/ADR with HUVECs, the diameters were measured as  $d = (a \times b)^{1/2}$ , in which  $a$  is the longest Ferret diameter of the colony and  $b$  is the shortest one (Friedrich et al. 2009; Hsiao et al. 2009; Kelm et al. 2003). The reduction percentages of colony sizes were calculated as  $R(A) = [(average\ colony\ diameter\ of\ model\ (A)\ treated\ by\ anti-cancer\ drug - average\ colony\ diameter\ of\ untreated\ model\ (A)] / colony\ average\ diameter\ of\ untreated\ model\ (A) \times 100\%$ .

The angiogenesis ability of HUVECs was measured using three parameters adapted from previous research (Donovan, et al. 2001): Distribution of tubule length, tubule area and number of branches. For tubule length measurement, a line was drawn along the structure, and the length of which was derived from number of pixels by initial calibration of the image. The tubule area was also measured using ImageJ (NIH, US), and the number of branches was counted. Five views were randomly chosen for each sample, with at least one from the centre of the well. It was ensured that same size field is analysed for each image processed.

#### *4.2.9 Cell labelling and immune staining*

NCI/ADR cells were genetically modified to show green fluorescence. Enhanced green fluorescence protein (EGFP) under the control of human elongation factor alpha Promoter (EF1  $\alpha$ ) was inserted in the 5' LTR region of replication incompetent, vesicular stomatitis virus (VSV)-g pseudotyped self-inactivating lentivirus (SIN) particles. A deletion in the

promoter/enhancer region of the U3 portion of the 3'LTR ensures self-inactivation of the lentivirus construct after transduction and integration in the host cells.

In a 24 well plate, 60 % confluent monolayers of NCI/ADR cells were transduced with EF1 $\alpha$ -GFP lentivirus at a multiplicity of infection (MOI) of 10. Cells were typically cultured for 48 hours to ensure lenti virus integration, after which cells were trypsinised and EGFP positive colonies were picked up under fluorescent inverted microscope (Zeiss, Axiovert25), by a sterile Gilson pipette. Cells were further passaged in DMEM media containing 10% FCS (Life technologies, US), 1% penicillin/streptomycin (PAA, US) and the selection marker puromycin (Sigma) at a concentration of 5  $\mu$ g/ml until stable expression of EGFP was obtained in all cell population.

HUVECs in 3D culture were stained for von Willebrand Factor (vWF) using a protocol modified for sandwich Matrigel (Lee, et al. 2007). The samples were first rinsed by PBS-glycine (100mM glycine in PBS) three times, then blocking buffer (10% goat serum (Sigma), 1% goat F(ab')<sub>2</sub> anti-mouse immunoglobulin G (Caltag, UK) in staining buffer (PBS supplemented with 0.2% TritonX-100, 0.1% BSA, and 0.05% Tween 20) was used to block the unspecific binding site of samples. The samples were then incubated with the primary antibody (mouse IgG2B anti-human vWF A2 domain (R&D, US)) at 4°C overnight, rinsed twice with PBS, and followed by the incubation in the second antibody rabbit anti-mouse IgG labelled with TRITC (Sigma-Aldrich, US) at 4°C for 1 hour. Then the samples were washed once with staining solution and once with PBS. Finally diaminophenylindole (DAPI) hard-set mounting medium (Vector Labs, UK) was used to mount the samples and the sample will be ready for imaging.



Microscopic images of 3D constructs were obtained from a modified BioRad Radiance 2100 MP Multiphoton Microscope (MPM, Zeiss; Jena, Germany). Near infrared (NIR) laser beams of 800 nm from a tunable 76 MHz femtosecond pulsed Ti: sapphire laser (Mira 900-F, Coherent, Ely, UK) pumped by a 10 W multiline argon ion laser (Verdi; Coherent) were used to excite the samples. A Nikon S Fluor 10 × objective with a numerical aperture of 0.30 and working distance as 2.0mm was used for all images. The emission filters selected were: 495 nm for blue, 525 nm for green and 595 nm for red. Finally, all serial optical sections from individual datasets were loaded into Imaris 7.6.1 (Bitplane Ag, Zurich, Switzerland) software for processing and analysis.

#### *4.2.10 Data analysis and statistics*

All experiments were triplicated and repeated three times if not stated otherwise. Student's *t*-test was used to compare parameters between different groups.  $P < 0.05$  was considered to be significantly different.

### *4.3 Results and Discussions*

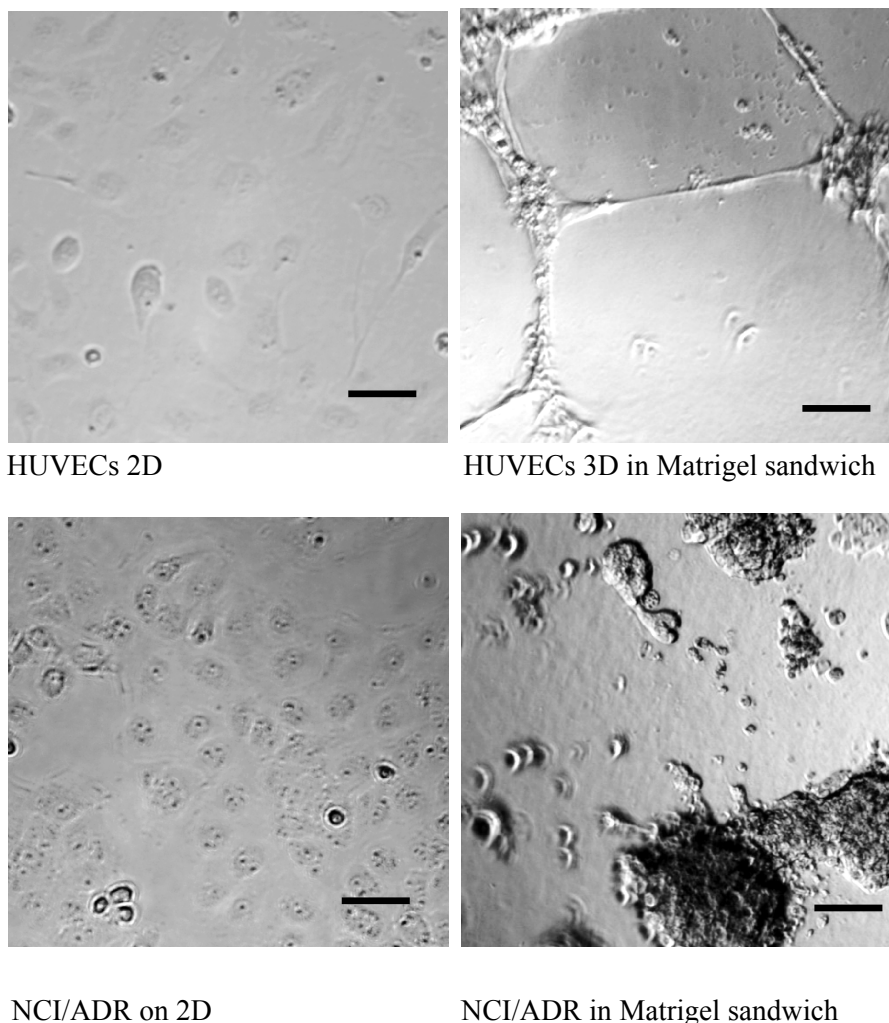
#### *4.3.1 Morphology change of cancer cells and endothelial cells in Matrigel sandwich culture*

Human umbilical cord vein endothelial cells have different morphology compared with cancer cells NCI/ADR cultured on their own. HUVECs can only form a cell monolayer, while cancer cells can grow in an overlapping way, with multiple layers. The cell nuclei of HUVECs also look different from the ones in NCI/ADR – cell nuclei in cancer cells are more condense, with thick and irregular chromatin. Compared with HUVECs, the nucleus of NCI/ADR is larger, even the diameter of the whole cell is slightly smaller than the average diameter of HUVECs. The chromatin in the nucleus is distributed unevenly. Also, cancer cells show denser nucleolus, and usually more than one nucleolus existing in one nucleus. Grown on scaffolds, these two types of cells showed different morphologies: HUVECs form tubules on top or in the sandwich culture of Matrigel, while NCI/ADR tend to form clusters either on the surface or inside of scaffolds. HUVECs lose tubule forming abilities inside Matrigel or alginate. This morphology characteristic work is shown in Figure 4.3.

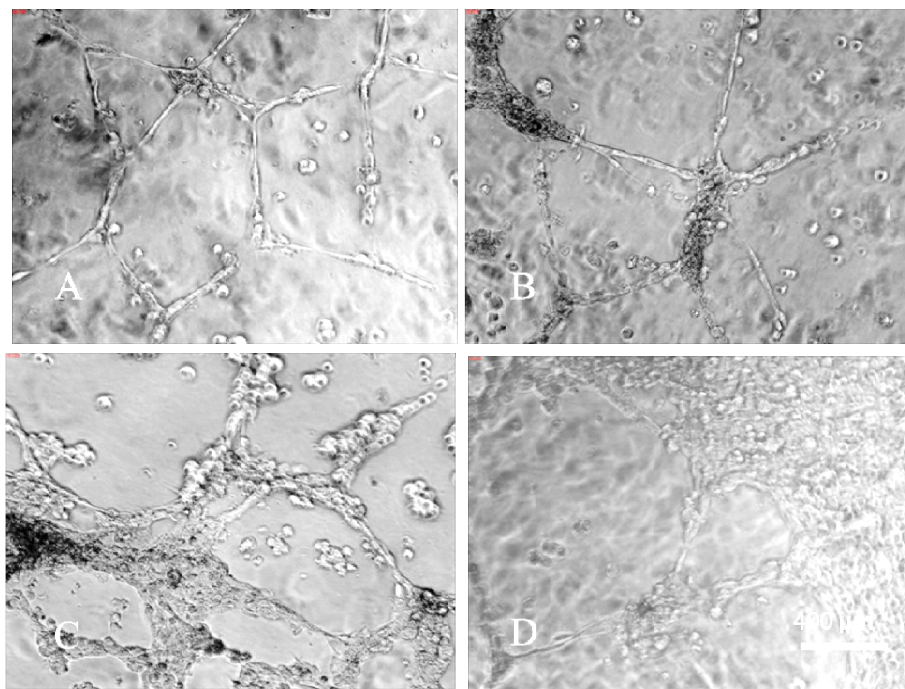
#### *4.3.2 Co-culture ratio optimisation*

The ratio of endothelial cells HUVECs to cancer cells NCI/ADR in the co-culture system was first optimized, considering that NCI/ADR has higher proliferation activity than HUVECs, and the optimised co-culture seeding density ratio is essential to ensure HUVECs can proliferate in co-culture without being taken over by NCI/ADR in long-term culture. As shown in Figure 4.5 (A), after 24 hours of co-culture, with HUVECs: NCI/ADR ratio as 10: 1, the cancer cells could not proliferate because of low seeding density. In Figure 2(B), when this ratio decreased to 3: 1, it was observed that cancer cell colonies were formed. Under the ratio 1: 1 in Figure 4.5(C), cancer cells revealed high proliferation activity, covering the surfaces of tubule structures formed by HUVECs, and the overtaking effect of NCI/ADR was even more significant at higher ratio of the cancer cells, as in 2(D). Thus, HUVECs: NCI = 3:

1 was chosen as the ratio to initiate the co-culture. This requirement for optimised ratio reflects the complex relations between cancer cells and endothelial cells – higher density of cancer cells will definitely compete with endothelial cells for nutrients in culture medium, but cancer cells can secrete growth factors like vascular endothelial growth factor (VEGF), which can stimulate the growth of endothelial cells, thus appropriate density of cancer cells can help maintain the co-culture structure in long term (Albini et al. 2007).



**Figure 4.3** Morphology comparisons between 2D and 3D cultures. (Left) cancer cell line NCI/ADR; (right) primary cultured human umbilical vein endothelial cells HUVECs. *Scale bar: 50 $\mu$ m.*

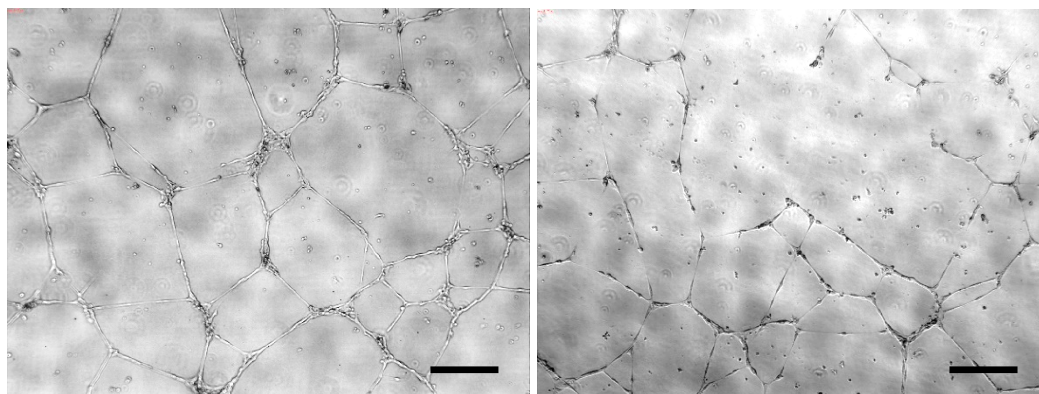


**Figure 4.4** Co-culture ratio optimisation. (A) HUVECs : NCI/ADR = 10 : 1, where cancer cells did not form colonies; (B) HUVECs : NCI/ADR = 3 : 1, where HUVECs showed tubule structure and cancer cells formed colonies; (C) HUVECs : NCI/ADR = 1 : 1, where HUVECs are overtaken by cancer cells (D) HUVECs : NCI/ADR = 1 : 3, where cancer cells covered almost whole surface area of culture surface. The pictures were taken 24 hours after the initial cell seeding.

#### 4.3.3 The morphology comparison of co-culture and single cell type cultures

It was firstly noted that for HUVECs cultured alone in Matrigel sandwich, at day 2 the tubule structures already showed the sign of degradation, as shown in Figure 4.6. Thus, for culture time more than 48 hours the growth of co-culture and NCI/ADR cultured alone in Matrigel were compared for up to 10 days, as shown in Figure 4.7. Overall, tubules and clusters were formed in co-culture, as shown in Figure 4.7(A), and these structures were maintained for up

to 10 days. In Figure 4.7(C), 2 days after co-culture, the primary colony formed, and some colonies attached to the tubule structure of HUVECs. This co-culture structure featured with colonies and tubules kept growing as shown in (E) - (I). Take (I) as an example, 6 days after co-culture, tubule structures kept on growing, and the colonies attaching to the tubules have larger diameters compared with the colonies grown on the non-tubule area; (K) 8 days after co-culture, significant growth of colony-like structures and tube-like structures can be observed, with more colonies over 200 $\mu$ m diameters attaching to tubule structure; (M) 10 days after co-culture, the structures were still stable, with more colonies of cancer cells with considerable sizes attaching to the tubule structures. Compared with co-culture, the morphology of NCI/ADR cultured alone in Matrigel sandwich only showed colonies formed, though these colonies also kept growing for up to 10 days. It is noted that at day 1 there were also some tubule-like structures observed, as shown in Figure 4.7(B). However, the self-adherence abilities of cancer cells seemed to take over this structure and finally mainly colony-like structures were observed later from day 2 to day 10.

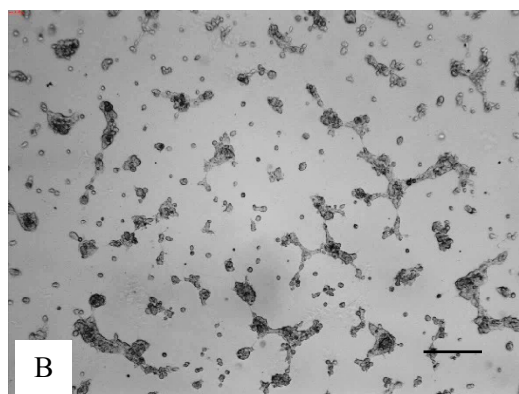
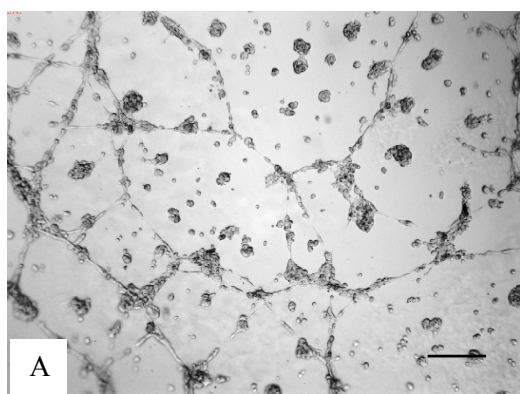


24 hours

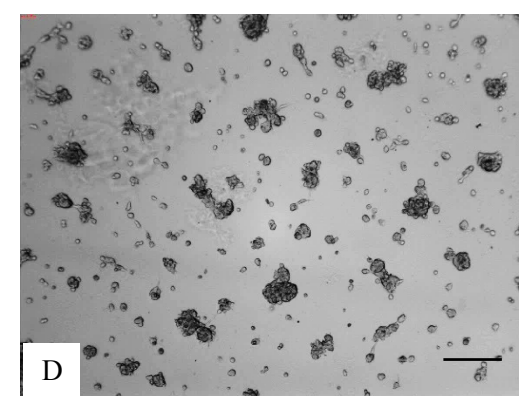
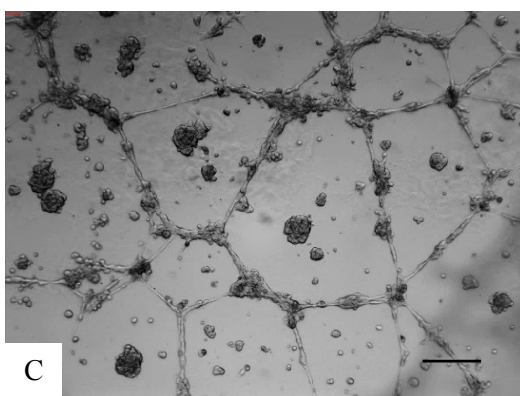
48 hours

**Figure 4.5** HUVECs tubule structures degraded over 48 hours, in comparison of co-culture shown in **Figure 4.6** as following. In HUVECs mono-culture the tubule structure degraded significantly on day 2 (48 hours). *Scale bar: 400 $\mu$ m*

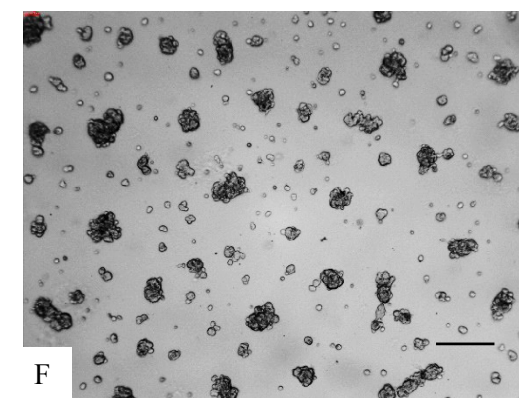
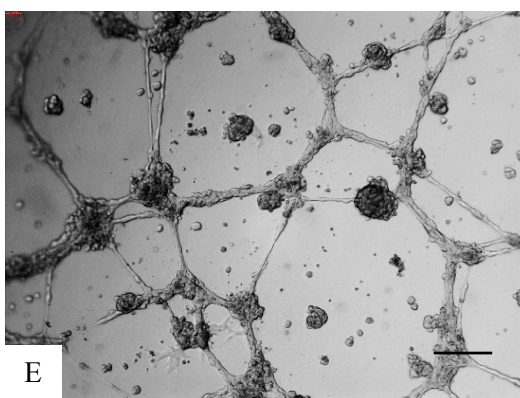




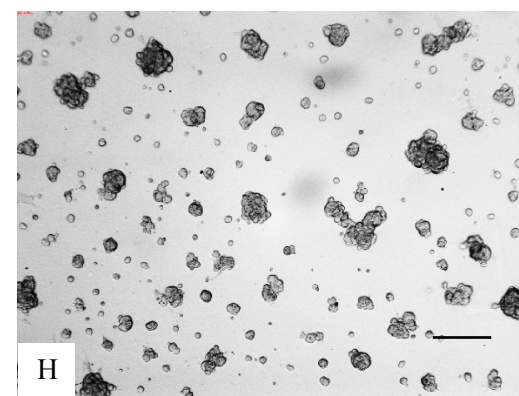
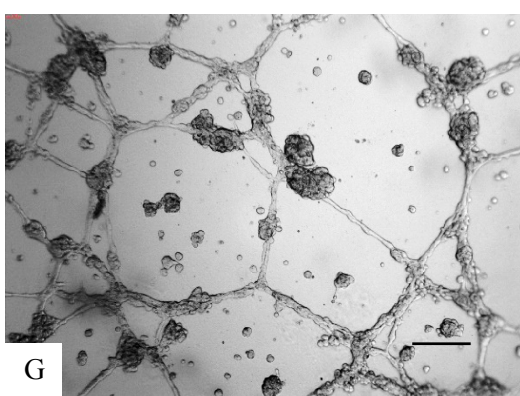
**Day 1**



**Day 2**

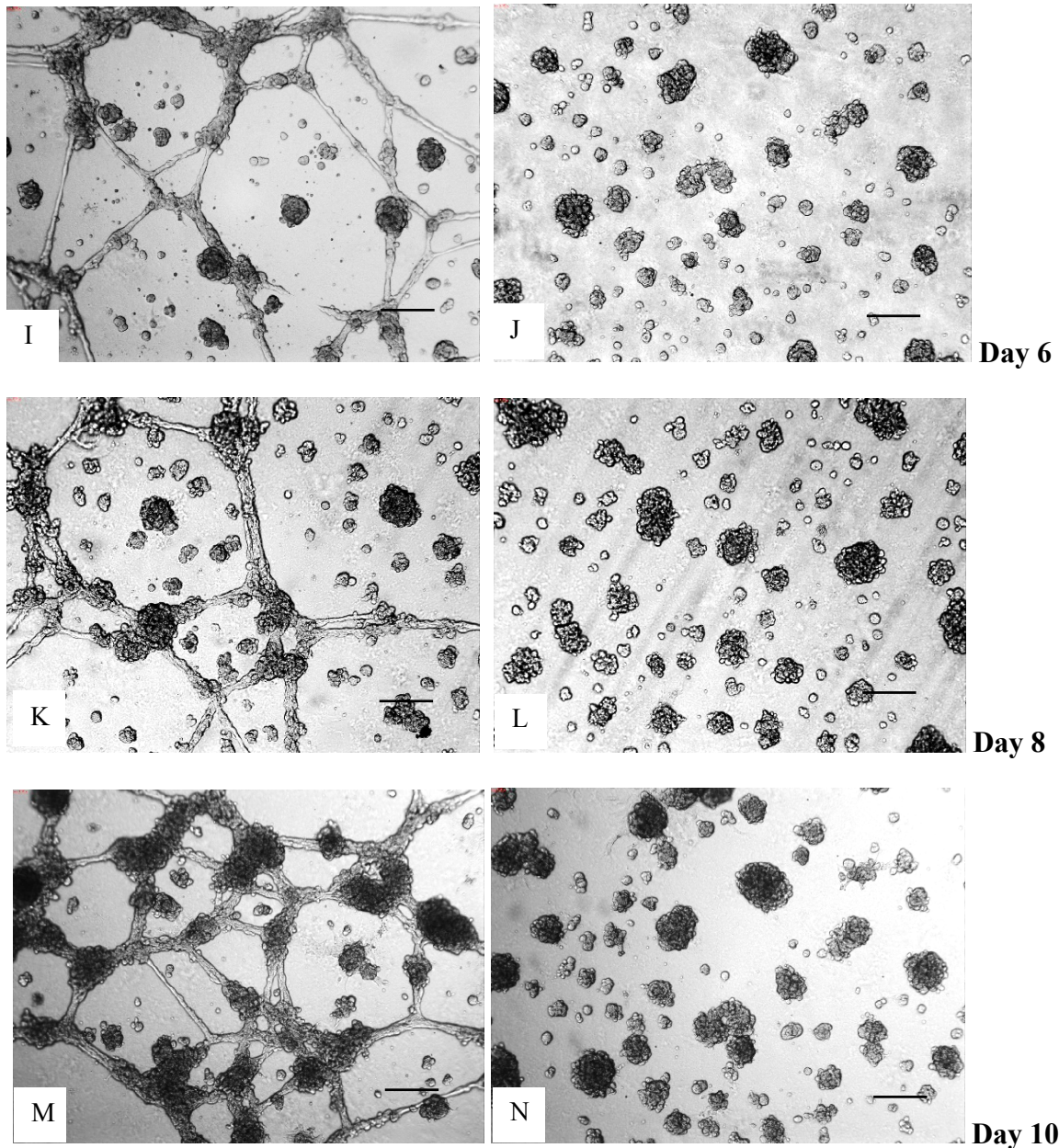


**Day 3**



**Day 4**

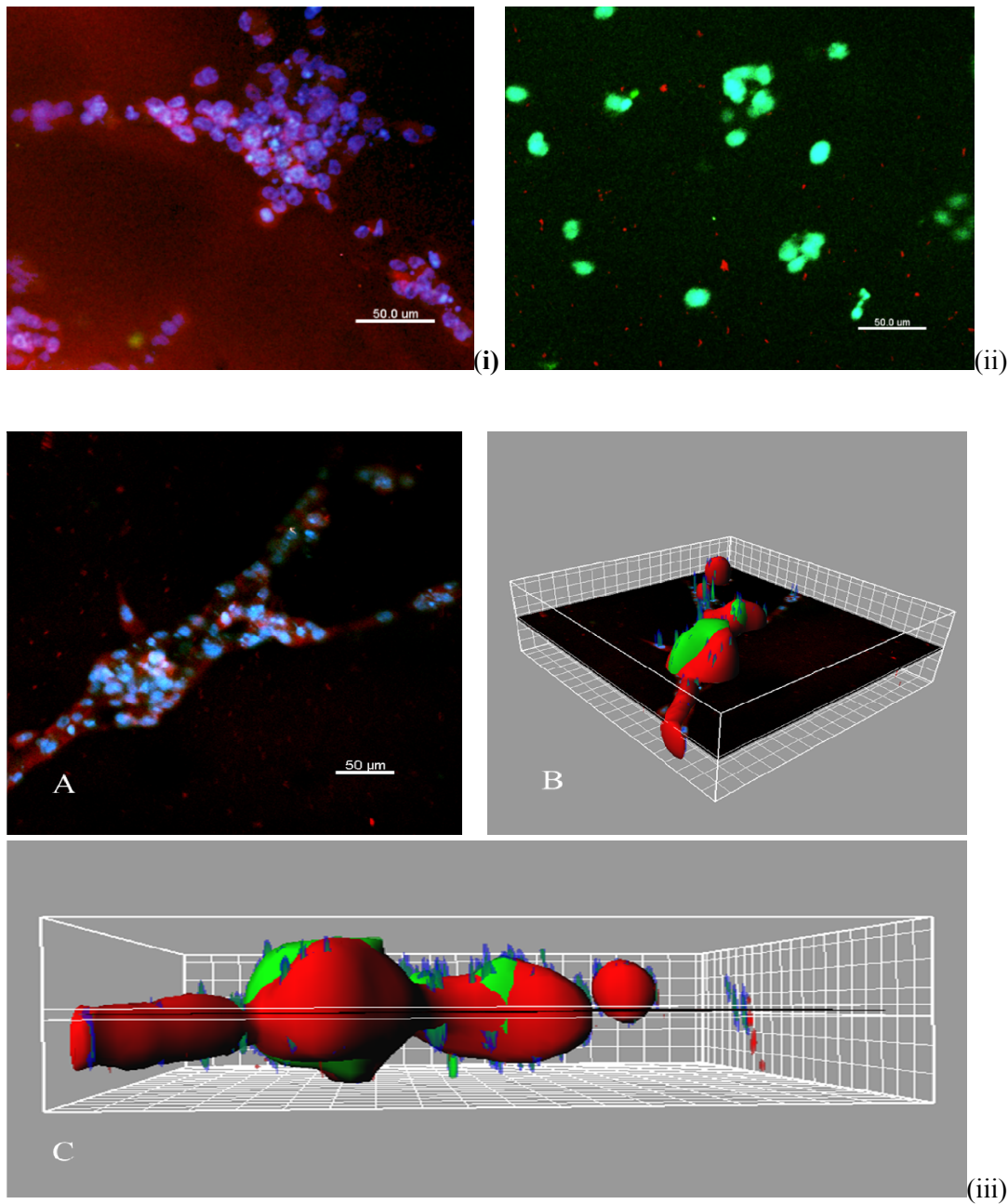
**(Figure to be continued in the next page)**



**Figure 4.6** Co-culture in 3D can keep proliferation for up to 10 days. **Left panel: Co-culture; right panel: NCI/ADR cultured alone.** (A) day 1 co-culture, (B) day 1 NCI/ADR; (C) 2 days co-culture, (D) 2 days NCI/ADR, primary colony formed; (E) 3 days co-culture, (F) 3 days NCI/ADR; (G) 4 days co-culture, (H) 4 days NCI/ADR; (I) 6 days NCI/ADR, (J) 6 days NCI/ADR, colony-like structures kept on growing; (K) 8 days co-culture, (L) 8 days NCI/ADR, significant growth of colony-like structures and tube-like structures, with the colonies on tubules larger than isolated colonies; (M) 10 days co-culture, (N) 10 days NCI/ADR, the structures were still stable. Scale bar: 200µm.

To obtain more structure details of the co-culture structure, multiphoton microscopy (MPM) was used to get more details of cell types in colonies and tubule structures at day 5 of co-culture. Endothelial cells were marked using the endothelial cell-specific molecule vWF labelled by red fluorescent TRITC, and the cancer cells NCI/ADR expressed green fluorescence protein (GFP). From the MPM image in Figure 4(A) and the following 3D processed result in Figure 4(B), it was confirmed that after 5 days of co-culture, HUVECs were still not overtaken by cancer cells NCI/ADR, and NCI/ADR were adhering to the tubule structure formed by HUVECs, in the form of 3D colonies. This also confirmed that the optimised co-culture ratio of HUVECs to NCI/ADR is 3:1 is feasible for a relative long-term co-culture. Moreover, the reconstructed 3D image showed that the co-culture system has an average thickness of around 200  $\mu\text{m}$ , which is calculated with the help of the scale bar and grid frame in Figure 4(C), implying that the two types of cells have formed 3D structure in Matrigel based on the fact that diameters of a single cell of HUVECs and NCI/ADR are both around 20-60  $\mu\text{m}$ .





**Figure 4.7** 3D structure analysis by multiphoton microscope (MPM). Green: GFP-expressing NCI/ADR; Red: vWF staining by TRITC. (i) HUVECs control, (ii) NCI/ADR control; (iii) Co-culture of HUVECs and NCI/ADR. (A) Slice image by MPM; (B) 3D reconstruction. Cancer cells expressed green fluorescence protein (GFP), and endothelial cells were marked red by endothelial cell specific molecule vWF. Cell nuclei of both cell types were marked blue by DAPI. (C) Section of 3D reconstruction. All grid measurement is 20μm.

Our results suggest the maintaining effect of cancer cells on the tubule structures formed by endothelial cells, and our model is more suitable for long-term testing (longer than 72 hours) compared with traditional angiogenesis assay on Matrigel. Although HUVECs were reported to be able to form tubule structures, these tubules only lasted for up to 72 hours (Ingthorsson et al, 2010), which was confirmed in our own results as shown in Figure 2 (E) as in the control group – only after 48 hours, the tubule structures were mostly degraded. In contrast, in our co-culture model, the tube-like structures were maintained for at least 10 days. The maintenance effect of cancer co-culture on endothelial cell formed tubule structures can be explained by confirmed stimulating effects of cancer cells on proliferation, migration and tubule formation of endothelial cells. These morphological alterations of endothelial cells were also found to be coordinated with gene profile changes preferable for angiogenesis, and growth factors like VEGF and integrins were found to be up-regulated, with the former stimulating the growth of endothelial cells, and the latter contributing to endothelial cell differentiation in tubule formation (Khodarev et al, 2003).

Previous research on the 3D mono-culture of cancer cells in Matrigel has pointed out the similarities of those cell aggregates to cancerous tissue. These structures were thought to be able form due to the three-dimensional (3D) structure signals restored by Matrigel, the laminin-rich extracellular matrix. Signal pathways like  $\beta$ 1-integrin and epidermal growth factor receptor (EGFR) function in a parallel way in traditional 2D culture, which requires to block both pathways at the same time in order to reduce the cancer cell growth below 20% of control, with no down-regulation of expressions of either  $\beta$ 1-integrin or EGFR, while in 3D they can be integrated in a reciprocal way in which inhibiting either of those two ways will be enough to induce growth-arrest and down-regulation of both  $\beta$ 1-integrin or EGFR.(Lee, et al.

2007; Wang et al, 1998). Compared with mono-culture of NCI/ADR cells on Matrigel, this co-culture system can be applied to studies on cancer angiogenesis, potentially providing the information on both cell types in extracellular matrix at the same time, combining the morphology of cancer cells and endothelial cells in 3D structures. For 3D cell culture, Matrigel can be used as embedded matrix, sandwich layers, and simple coating (Staton, et al. 2004). When embedded in Matrigel, endothelial cells were found to always lose the ability to form tubule-like structures (Ingthorsson, et al. 2010). However, our results show that in 3D sandwich culture, endothelial cells can form tubule structure of around 400µm in length which can then be maintained for up to 10 days. Possible explanation is that the initial 4 hours allows HUVECs to attach to Matrigel prior to cancer cell seeding, or that the Matrigel bottom surface was essential to initiate the tubule structure formation by HUVECs.

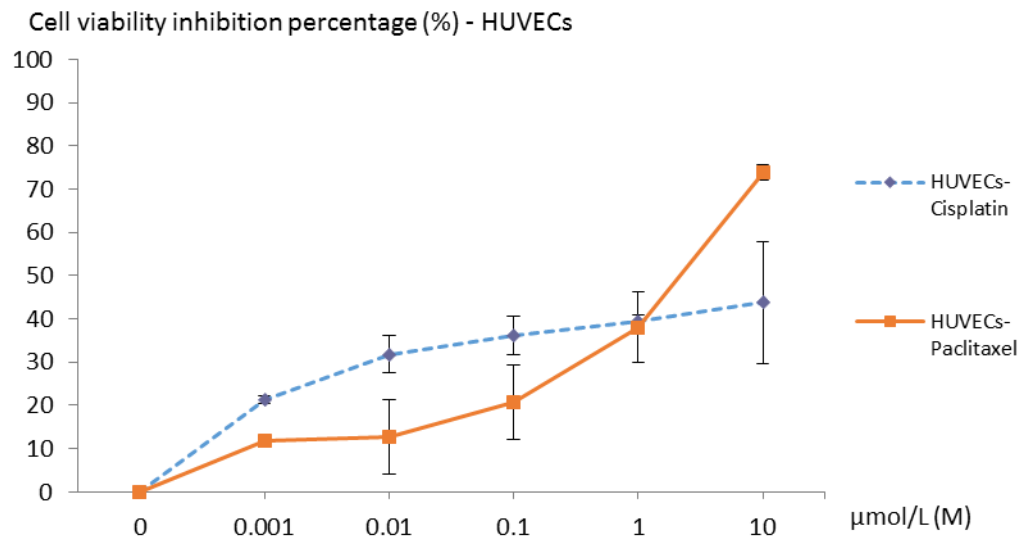
#### *4.3.4 Viability and morphology change of HUVECs and NCI/ADR mono-cultures in response to Cisplatin and Paclitaxel*

Although dose –dependent responses of Cisplatin and Paclitaxel have been studied in 2D models in other researchers' work, the anti-cancer drug testing on 3D cancer models or endothelial cells is still limited (L'Azou et al, 2005; Shoemaker et al, 2006; Vacca et al, 2002). Thus it is worth doing the drug testing on mono-cultures of cancer cells line NCI/ADR and endothelial cells HUVECs before testing the drugs on co-culture system, which can also provide controls for the later co-culture drug testing.

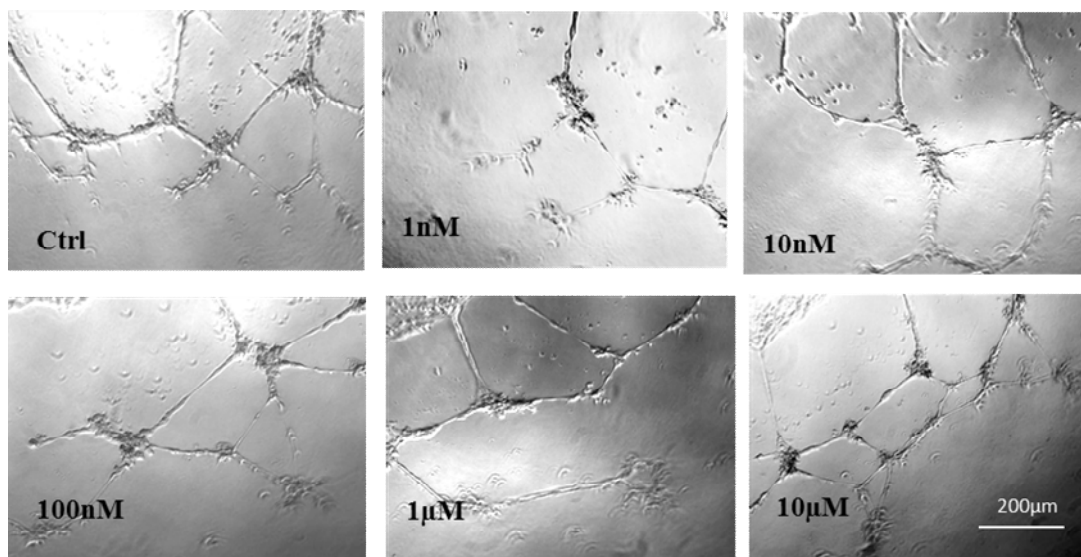
For cell viability inhibition assay shown in Figure 4.8(A), the toxicity of both drugs increased with positive correlation to the drug concentration. However, Cisplatin and Paclitaxel show different dose-dependence patterns on endothelial cells HUVECs cultured in Matrigel sandwich. Cisplatin showed significant toxicity from concentration as low as 1nM, while for Paclitaxel this inhibiting effect is lower than Cisplatin at the same concentration. However with an increase of concentration, the toxicity of Paclitaxel on HUVECs increased at a higher rate than the Cisplatin, until at concentration 1 $\mu$ M the inhibition effect of Paclitaxel started to exceed Cisplatin. Between 1 $\mu$ M to 10 $\mu$ M, the inhibition percentage of Paclitaxel increased from 38.10% to 74.01%, while the inhibition percentage of Cisplatin only increased to 43.76% at 10 $\mu$ M.

The effect difference of Cisplatin and Paclitaxel is also reflected in the morphology of HUVECs in Matrigel sandwich. As shown in Figure 4.8(B), there was barely tubule structure change with the increase of Cisplatin dose, while in Figure 4.8(C) there was dose-dependence

tubule structure interruption by Paclitaxel. It is noted that since the tubule structures degraded after 48 hours, thus the drug testing on HUVECs tubules should be less than 48 hours.



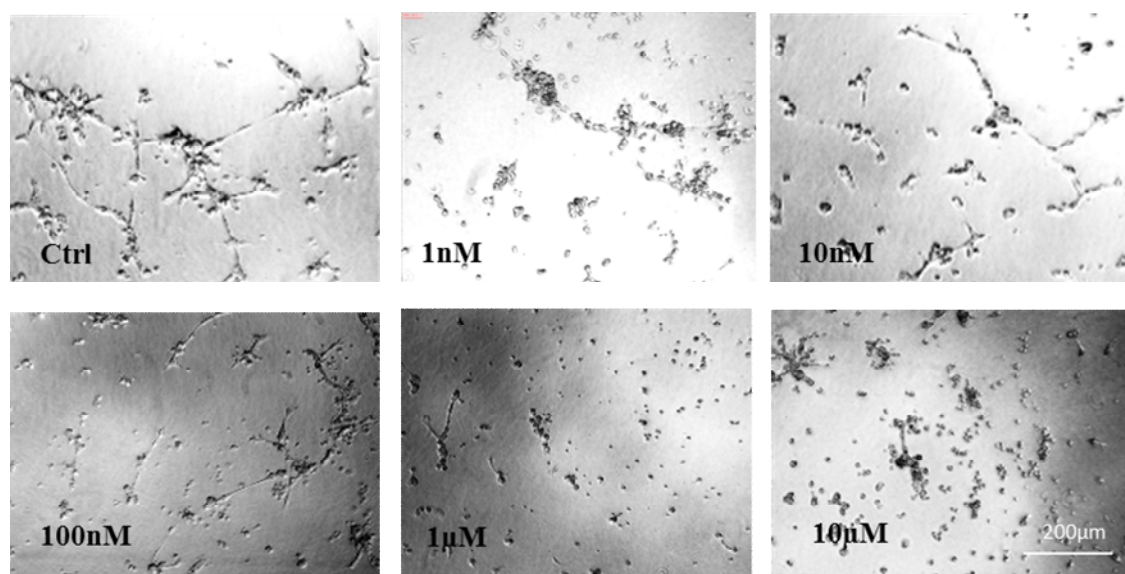
(A)



(B)

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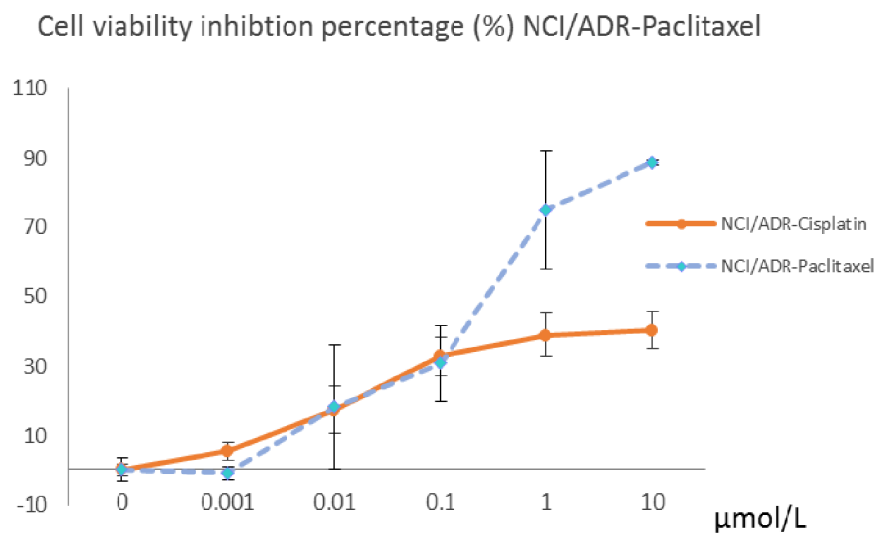


(C)

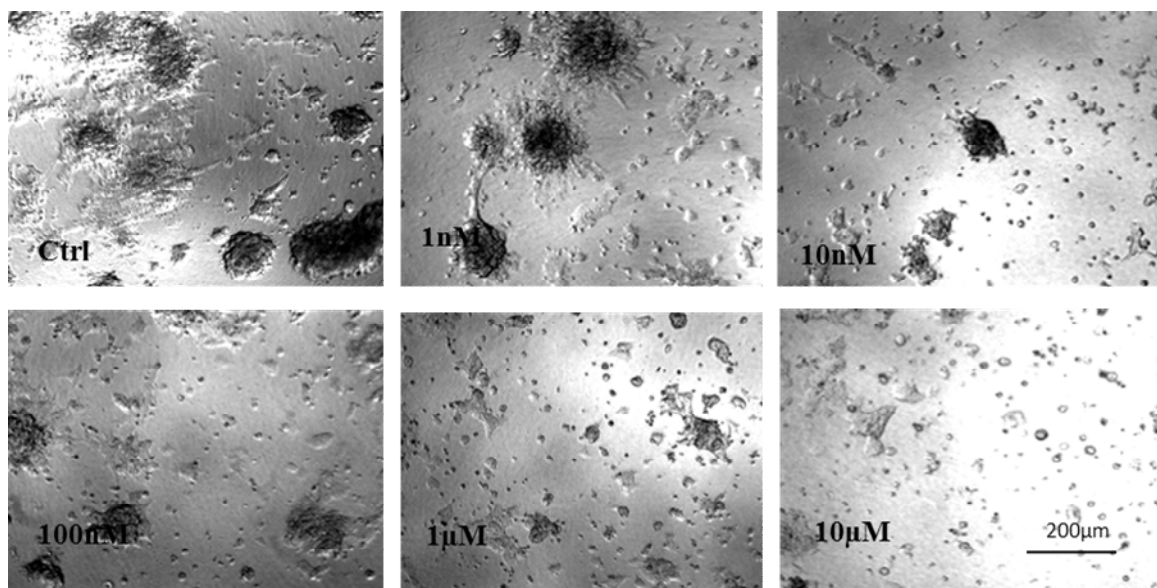
**Figure 4.8** Dose-dependent responses of HUVECs mono-cultured in Matrigel sandwich treated with Cisplatin or Paclitaxel. (A) Cell viability inhibition percentage for Cisplatin and Paclitaxel; (B) Representative images by Cisplatin of different concentrations; (C) Representative images by Paclitaxel of different concentrations.

Figure 4.9(A) demonstrates that for NCI/ADR cultured alone in Matrigel sandwich, the inhibition percentages of Cisplatin and Paclitaxel overlap from 10nM to 100nM, with the inhibition changing from about 18% at 10nM to 30% at 100nM. However, Paclitaxel shows significantly higher inhibition on NCI/ADR at concentration 1μM, with the percentage as 74.97% while the inhibition percentage of Cisplatin at the same concentration is only 39.01%. This difference is even bigger at 10μM, with the inhibition percentage of Paclitaxel as high as 88.86% whereas Cisplatin only causes 40.38% viability inhibition of NCI/ADR in Matrigel

sandwich. It is noted that the morphology change correlates well with the viability change – Cisplatin treated NCI/ADR colonies in Matrigel sandwich shows gradual size decrease over the increase of Cisplatin concentrations in Figure 4.9(B), and a sharp decrease of colony size was observed for Paclitaxel at 1 $\mu$ M compared with 100nM, as shown in Figure 4.9(C), corresponding to the cell viability inhibition exceeding of Paclitaxel than Cisplatin at 1 $\mu$ M.



(A)

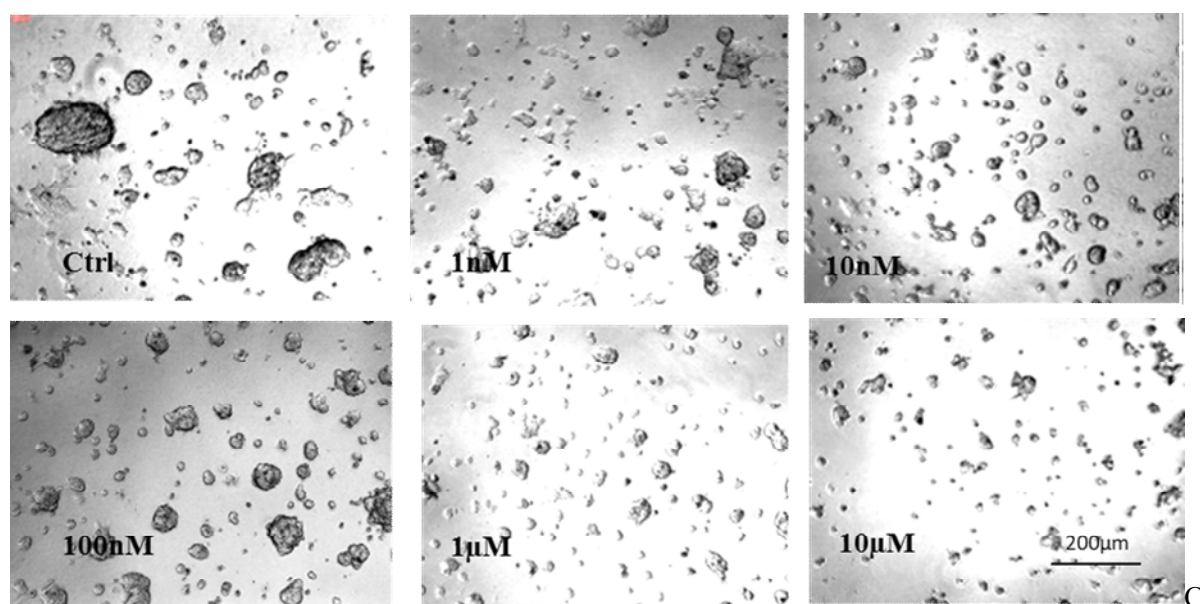


(B)

(figure to be continued in the next page)



(figure continued from last page)



(C)

**Figure 4.9** Dose-dependent responses of NCI/ADR mono-cultured in Matrigel sandwich treated with Cisplatin or Paclitaxel. (A) Cell viability inhibition percentage for Cisplatin and Paclitaxel; (B) Representative images by Cisplatin of different concentrations; (C) Representative images by Paclitaxel of different concentrations.

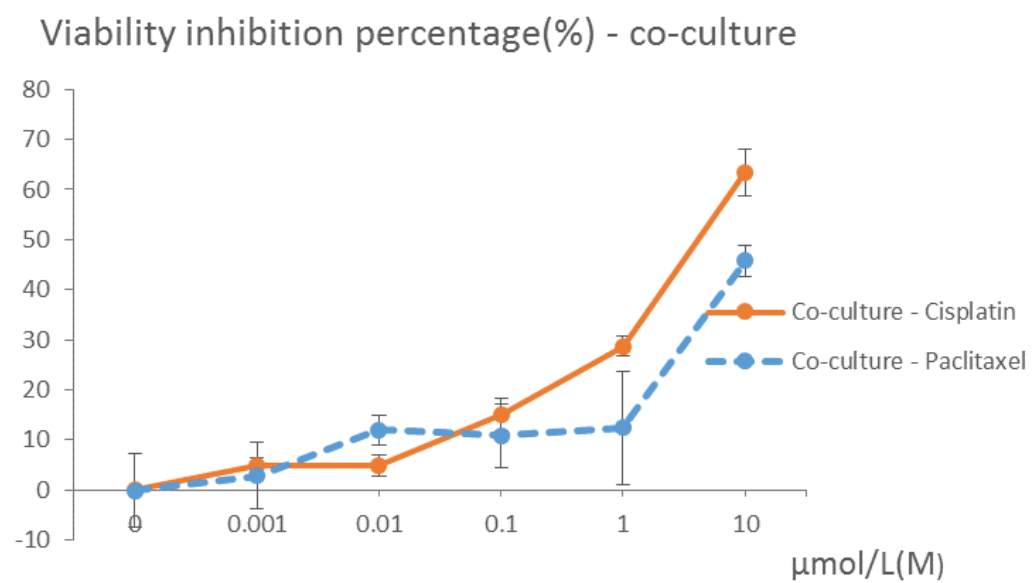
In the above results, it is noticeable that morphology change can be well consistent with the viability inhibition of the drug, such as in terms of HUVECs treated with Paclitaxel, or NCI/ADR treated by Paclitaxel. But this is not always the case. For example, in the HUVECs treated by Cisplatin, though there is a dose-dependence viability inhibition, the tubule structure change is hardly visible even at the highest concentration 10μM. The response of morphology change can also be more sensitive than viability change, as a significant colony size decrease was observed NCI/ADR treated by Cisplatin from 100nM to 1μM, which is not reflected well in the viability assay.



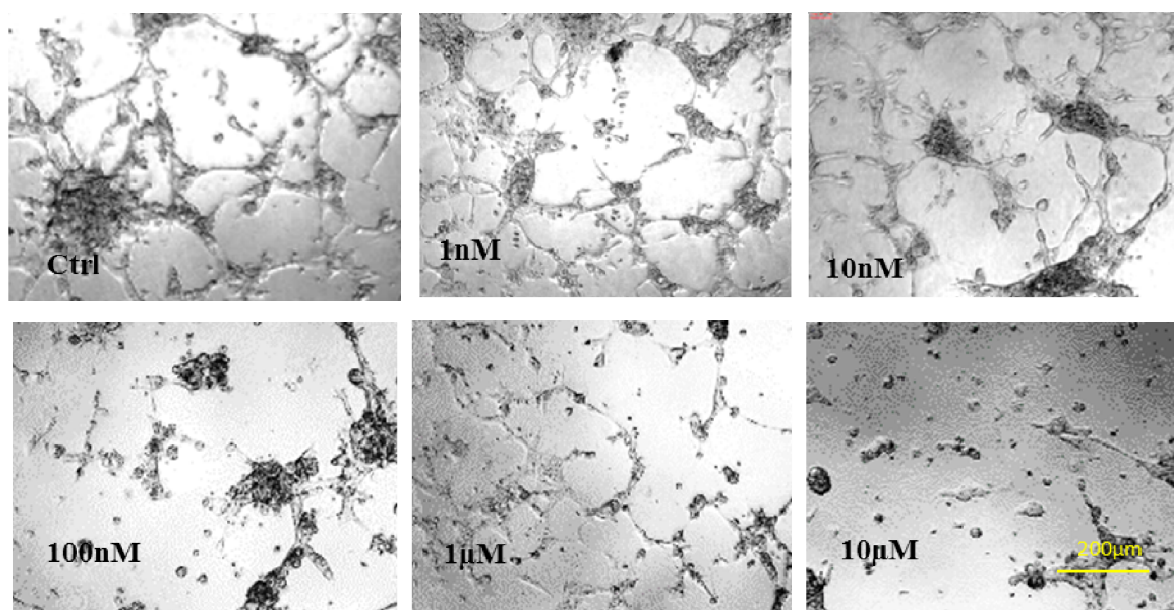
#### *4.3.5 Viability and morphology comparisons of co-culture and mono-cultures in drug testing*

It is important to compare the simple value sum of mono-culture of NCI-ADR and HUVECs to the viability value of the co-culture of these two cells, to clarify if the interactions between those two cell types play roles in the drug responses of the models. It is noted that HUVECs showed consistent low viability under drug concentration from 0.01 $\mu$ M to 100 $\mu$ M. While the co-culture has highest viability compared with the mono-cultures of either NCI-ADR or HUVECs. Report has pointed out that the maximum testing time of HUVECs formed tubules is 24 hours, due to the low viability of HUVECs under tubule formation medium, which has low nutrient. Our results are consistent with the previous report of other researchers. In conclusion, we can exclude the effect of HUVECs from the final drug testing, and this assumption forms a basis for the following chapters, which the comparison between mono-culture of NCI-ADR and co-culture of NCI-ADR and HUVECs were focused on.

Although the contribution of HUVECs to the total viability value of co-culture model is limited, the drug response of co-culture is still lower compared with mono-culture of NCI-ADR only under the same concentration of drug, either for Cisplatin or Paclitaxel, from concentration 0.01 $\mu$ M to 100 $\mu$ M. This suggests that even HUVECs alone have limited viability after 24 hours of culture in the co-culture medium, when cultured with NCI-ADR the effect of HUVECs is still significant. The possible explanations are the mutual stimulating effects of endothelial cells and cancer cells, on proliferation, migration, metastasis and extracellular matrix degradation abilities, which have been reported by other researchers before.



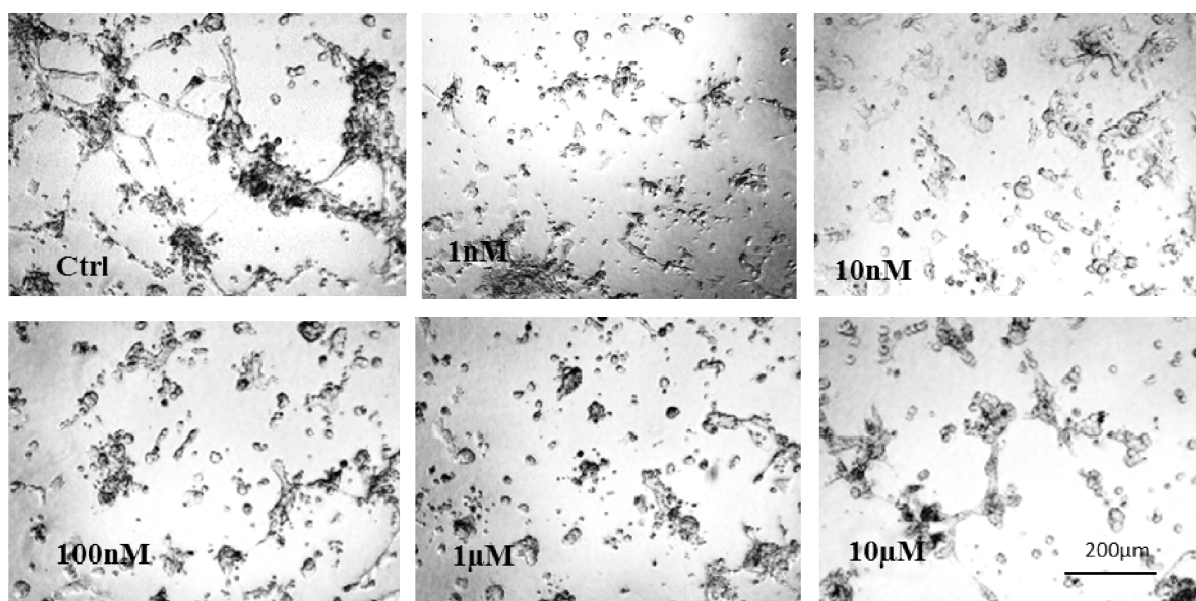
(A)



(B)

(figure to be continued in the next page)

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(C)

**Figure 4.10** Dose-dependent responses of co-culture of HUVECs and NCI/ADR in Matrigel sandwich to Cisplatin and Paclitaxel. (A) Cell viability assay; (B) Representative images by Cisplatin of different concentrations; (C) Representative images by Paclitaxel of different concentrations.

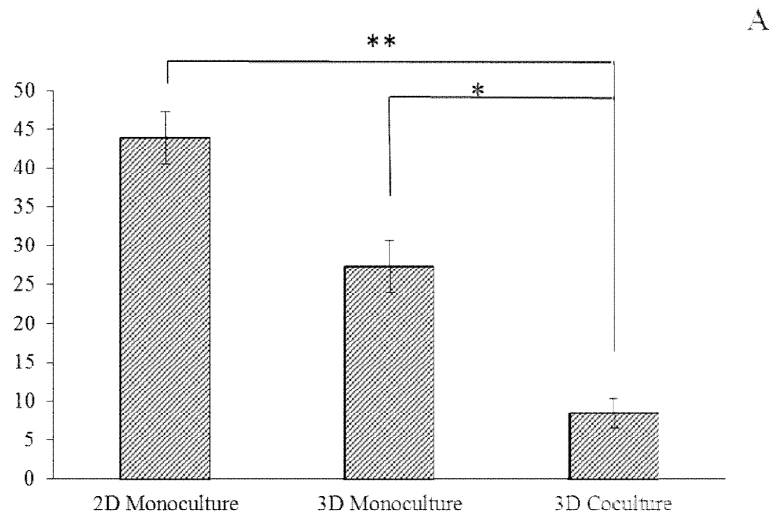
To investigate the effect of endothelial cells on drug responses of the co-culture model, cell viability tested by AlamarBlue assay and average colony sizes analysed by microscope images of co-culture and mono-culture models were compared. All the groups were treated by either 0.5μM Cisplatin, or 1μM Paclitaxel for 48 hours. For AlamarBlue cell viability assay, data from traditional 2D culture was used as a control for both 3D mono-culture and 3D co-culture. Cell viability assay results in Figure 6(A) shows that conventional 2D monoculture had the highest drug sensitivity among three culture methods (43.9%), and the inhibition percentage of 3D co-culture (8.5%) is lower than mono-culture (27.4%). The difference in drug response between 2D and 3D *in vitro* models were also observed by other

researchers, and the explanations for this response difference include drug penetration, oxygen deficiency and contact-dependent drug resistance caused by 3D multicellular structures (Friedrich et al. 2009).

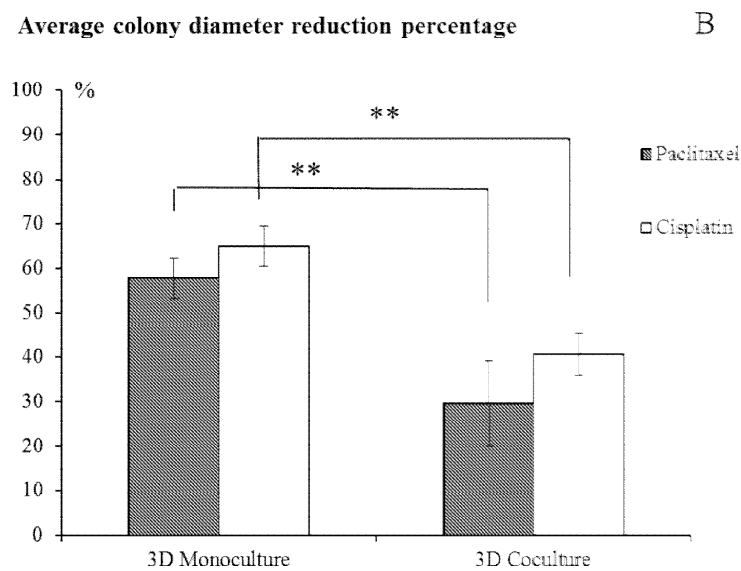
In 2D monolayer cultures, Cisplatin and Paclitaxel showed different acting patterns on cancer cells and endothelial cells – for IC50 assay, Cisplatin treated cancer cells showed higher drug response than endothelial cells, and this is supported by previous report of systemic studies on many cancer cell lines by NIH in US. However, Paclitaxel showed significant inhibiting effects on both cell types at much lower concentration (L’Azou et al, 2005). Our results also show the higher drug resistance in co-culture model compared with mono-culture of cancer cells. For both Cisplatin and Paclitaxel, there were significant differences in colony size reduction between sandwich mono-culture and sandwich co-culture ( $p < 0.01$ ). Compared with cancer cells cultured alone, cancer cells in co-culture system formed colonies with bigger diameters including improved cell survival (Brisson et al, 2002), increased colony sizes and improved colony formation capabilities of cancer cells co-cultured with endothelial cells compared with cancer cells culture alone were reported by other researchers (Ingthorsson et al, 2010). Furthermore, this mutual interaction between cancer cells and endothelial cells was even amplified in 3D culture, compared with 2D culture. For instance, interleukin (IL-8) expression was increased by 17 fold in 3D culture compared with 2D culture, and IL-8 is an important chemokine for angiogenesis (Fischbach et al, 2009). The angiogenesis promoting effect of 3D culture will in return induce the growth and malignancy of cancer cells, and thus increase the drug resistance of cancer cells.

The presented sandwich co-culture cancer model has shown some potential in providing more information and close to physiological condition *in vivo* in drug testing. However, there are still some limitations of this co-culture model which should be taken into consideration when using this model. The drugs, Cisplatin and Paclitaxel, used in this study would normally be injected intravenously as part of the chemotherapy for cancer treatment, which means that they have to be transported and distributed via circulation system, penetrate through blood vessel walls, and then finally reach and act on cancer tissue. This complicated absorption and distribution process was not able to be represented in the *in vitro* model by its nature. Secondly, the scaffolding material, Matrigel, was suggested to influence the cell growth and behaviours because it contains many growth factors, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), transforming growth factor (TGF) etc. so extra caution should be taken when interpreting the data based on Matrigel system (Hughes et al., 2010). On the other hand, 3D cancer models based on well-defined scaffolding materials which can provide similar biological cues for cells would be more desirable to reduce the unnecessary complexity in the model.

**Inhibition Percentage/%**



**Average colony diameter reduction percentage**



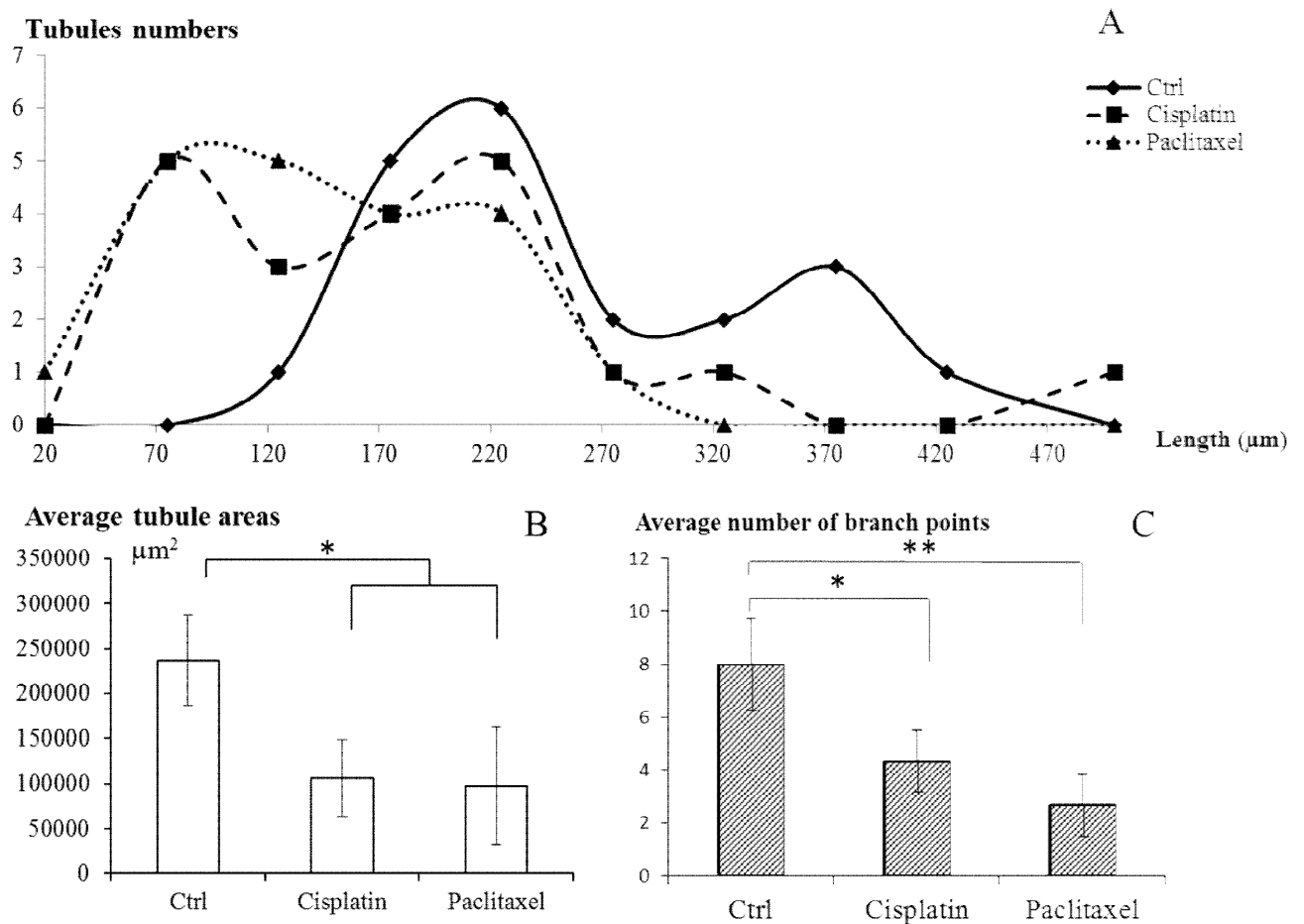
**Figure 4.11** Higher drug resistance of co-culture than mono-culture (A) Total viability of different cultures after being treated by Cisplatin 0.5 $\mu$ M for 48 hours \*\*  $p < 0.01$  compared to 2D mono-culture. \*  $p < 0.05$  compared to 3D mono-culture. (B) Reduction of colony sizes of mono-culture and co-culture. \*\*  $p < 0.01$  compared to mono-culture treated by the same drug. The differences are compared by Student's t-test Experiments were repeated 3 times independently.

#### *4.3.6 Co-culture model provided information on angiogenesis*

To further investigate the potential to use the co-culture system for information on both cancer cell proliferation and angiogenic abilities of endothelial cells, two widely accepted anti-cancer drugs with different acting mechanisms were tested on the system and the drug responses were compared. Paclitaxel interferes with the dynamics of microtubules, which participate spindle formation in cell mitosis, thus block cell proliferation effects (Zhou et al, 2005). Cisplatin, on the other hand, impairs DNA by interacting with purine bases to promote formation of DNA crosslinks (Siddik et al, 2003). To evaluate angiogenesis regulating effects quantitatively, three measurements were used in this study - distributions of tubule length, tubule areas and numbers of tubule branches. This angiogenesis abilities evaluation profile was adapted from previous research (Donovan et al. 2001).

Our results show that both Cisplatin and Paclitaxel show inhibiting effect on all three angiogenesis-related parameters. In Figure 5(A), in the control group, the majority of tubules have the average diameter of around 220 $\mu$ m, while after treatments of either Cisplatin or Paclitaxel considerable amount of tubules decreased their lengths to average length of 70 $\mu$ m. The difference between Cisplatin and Paclitaxel treated groups is that the effect of Paclitaxel is more significant, with most of tubules have diameters around 70 $\mu$ m, while for Cisplatin group there were still equal amount of tubules had the average length of 220 $\mu$ m. In Figure 5(B), Cisplatin and Paclitaxel caused the tubule areas to decrease significantly from 237461  $\mu$ m<sup>2</sup> to 106437  $\mu$ m<sup>2</sup> and 97374  $\mu$ m<sup>2</sup>, respectively. However, there was no statistical significance between the tubule area reduction percentages between these two drugs. In contrast, Figure 5(C) shows that Paclitaxel has significantly stronger inhibiting effect on the branching growth of tubules formed by endothelial cells, which is 75% decrease, when compared with the 50% decrease of branch points when treated with Cisplatin. Compared

with Cisplatin, Paclitaxel caused not only a further shift of distribution of tubule length to the shorter side, but also more reduction in the number of tubules.



**Figure 4.12** Tubule parameters analysis. (A) Tubule structure length distribution in response of drugs; (B) Tubule area reduction; (C) Reduction of branch points. Cisplatin  $0.5\mu\text{M}$ , Paclitaxel  $1\mu\text{M}$ ; Cultures are treated for 48 hours; 5 random areas are selected from 3 parallel groups of each treatment. \* $p<0.05$  \*\* $p<0.01$  compared with control using Student's t-test.



Overall, Paclitaxel has more significant inhibiting effects on the angiogenesis abilities (tubule length distribution and tubule branch point number) of the co-culture model, and supporting evidence for this result were found from previous research, including the official database of National Cancer Institute (NCI). Based on NCI database, Cisplatin and Paclitaxel have similar IC<sub>50</sub> on NCI/ADR cell line (Cisplatin 2.5  $\mu$ M, Paclitaxel 1.0  $\mu$ M) (Scherf, et al. 2000), but the IC<sub>50</sub> difference of these two drugs on HUVECs is about 2500 times: after 24 hours of treatment, IC<sub>50</sub> of Cisplatin on HUVECs is around 50 $\mu$ M (L'Azou, et al. 2005), while the IC<sub>50</sub> of Paclitaxel in HUVECs is 2nM (Vacca, et al. 2002). In other words, responses of HUVECs to those two drugs are very different, though they have similar cytotoxicity on NCI/ADR. This also suggests that compared with mono-culture model of NCI/ADR, co-culture with HUVECs can not only give information on cancer cell response, but also provides extra information on the anti-angiogenic effects of the tested drugs.

One potential explanation for this difference in anti-angiogenesis effects of Cisplatin and Paclitaxel is their different acting mechanisms. Cisplatin has a square planar structure which can bind to DNA to interrupt DNA synthesis, and inhibit cell proliferation (L'Azou et al. 2005). Paclitaxel has a different mechanism: it mainly interacts with cytoskeletal microtubule to block the formation of mitotic spindle, and thus induces cell necrosis. Previous studies have pointed out the particular dependence of endothelial cell functions on cytoskeleton dynamics, including proliferation, migration and other steps in angiogenesis process (Vacca et al. 2002; Zhou et al. 2005), which may explain the stronger inhibition effects of Paclitaxel on HUVECs shown in the current study.

#### 4.4 Summary

In this chapter, our study developed a 3D cancer model co-cultured with endothelial cells for anti-cancer drug testing. Apart from general use in anti-cancer drug testing as a 3D *in vitro* cancer model, this co-culture model has special significance in anti-angiogenesis drug testing. In this study, we used angiogenesis parameters like tubule length, branch points and tubule area combining with other 3D model parameters like colony sizes and viabilities, which gives potentially the information on both cancer cell growth and angiogenesis at the same time. This data analysing method is more efficient and the model is potentially closer to *in vivo* cancer microenvironment, compared with culturing cancer cell mono-culture model. However, since there are still more cell types besides endothelial cells in cancer microenvironment, and Matrigel is a complex extracellular matrix replacement, in our future study, more molecular studies comparing the features of co-culture and mono-culture 3D cancer cell models in Matrigel will be conducted, to give more details of endothelial cells and cancer cells interactions, and to further validate the drug testing results. Co-culture with more cell types will also be investigated in our future research.

It is important to consider the possibilities that Cisplatin and Paclitaxel bind to albumin in the culture medium as FBS was used in the culture medium, and albumin is the main protein in serum. This binding between drugs and albumin can potentially decrease the free drug concentration, to eventually affect the efficiency of the applied drug (Trynda-Lemiesz et al, 2004). A quite detailed study on the binding of Cisplatin and bovine serum albumin (BSA) pointed out that when the concentration of Cisplatin is at 5 $\mu$ M, after 45.5 hours there was 23.6% Cisplatin binding to the albumin (Cole et al, 1980). On the other side, it was suggested by both *in vitro* and *in vivo* data that the binding percentage of Paclitaxel to albumin was higher compared with Cisplatin, in the case of human albumin the binding of Paclitaxel can be

90% (Trynda-Lemiesz et al, 2004). As a result, at the same concentration, the free drug concentration of Cisplatin can be higher than Paclitaxel, which is a factor must be taken into consideration when comparing the results from the treatment of these drugs, such as the data shown in Figure 4.11 and Figure 4.12. On the other hand, Albumin is an important carrier of endogenous and exogenous hydrophobic molecules in the human circulation, as its abundant existence in the plasma (~ 60% of total plasma protein). Paclitaxel is a hydrophobic molecule with low aqueous solubility, and with special processing, it can binds to albumin with high affinity. The binding of Paclitaxel to albumin can potentially exploit endogenous albumin-mediated pathways to increase drug delivery to the tumour. In fact, a trend of recent research on advanced anti-cancer drug delivery is to design the albumin-Paclitaxel conjugate to increase the efficiency of the drug (Gradishar et al, 2006). It is possible that endothelium in the co-culture system intake the albumin together with the Paclitaxel bound to the protein, which facilitate the intake of the drug as an end result and compensate the effects of free drug concentration reduction caused by BSA binding.

## Chapter 5. Tissue Engineering Approaches to Develop Three-Dimensional Breast Tumour models

### 5.1 Introduction

To mimic *in vivo* intercellular interactions between cancer cells and endothelial cells, 3D *in vitro* tumour models co-cultured with endothelial cells have been developed, which are considered to bridge the gap between traditional *in vitro* and *in vivo* models (Hsiao et al, 2009; Gurski, et al. 2010). These models include scaffold-free 3D structures such as cancer spheroids ((Hsiao et al, 2009; Timmins et al, 2004), and 3D cancer tissue with cancer cells seeded cells in either natural (Lee et al, 2007; Fischbach et al, 2009) or synthetic scaffolds (Fischbach et al, 2007; Sahoo et al, 2005). Apart from mimicking the 3D microenvironment of cancer tissue by culturing cancer cells themselves in 3D tissue engineered structure, to reconstruct the interactions between cancer cells and other cell types in the microenvironment, co-culture techniques have been applied. The examples are endothelial cells (Timmins et al, 2004) or fibroblasts (Hsiao et al. 2009) co-cultured with cancer cells in spheroids, co-culture of endothelial cells with breast cancer cells in homogenous Matrigel (Ingthorsson, et al. 2010), normal functional breast gland cells with breast cancer cells in spheroids (Kelm et al, 2003) etc. These studies showed evidence that 3D *in vitro* models can better mimic the cancer development dynamics compared with 2D models. In Chapter 4 a 3D *in vitro* co-culture model was developed, which maintained the tubule formation abilities of endothelial cells to feature vascular network and tumour structures, and the feasibility of this co-culture model in cancer angiogenesis study was tested with Cisplatin and Paclitaxel.

Matrigel was used in Chapter 4 as the scaffold. Previous research has pointed out that different scaffold materials can influence the final drug response of the 3D *in vitro* models. Matrigel is a complex mixture of extracellular (ECM) components and bioactive factors such as vascular endothelial growth factors (VEGF), thus it is useful to clarify if there is any influence on drug testing from this biochemical complexity of Matrigel. As a control, another scaffold material, alginate which is widely accepted for 3D *in vitro* culture, is used and compared with Matrigel. Alginate is a linear copolymer formed by linked blocks or sequences of homopolymeric blocks of (1, 4)-linked  $\beta$ -D-mannuronate (M) and its C-5 epimer  $\alpha$ -L-guluronate (G). The specific structures vary depending on the origins of the alginate. Alginate gel can form in the presence of divalent ions like  $\text{Ca}^{2+}$ , at room temperature. This crosslinking ability at physiological temperature provides alginate a special advantage compared with other polysaccharide gels, which protects the functions of cells or tissues in the process of gelling and encapsulation. Alginate has been used on anti-cancer drug testing (Chen, Gupta, & Cheung, 2010), and endothelial cells-based angiogenesis study (Perets et al., 2003). In this chapter, alginate was used as scaffold for 3D co-culture of cancer cells and endothelial cells, and the comparisons of alginate encapsulated co-culture and co-culture embedded in Matrigel were made and discussed.

Moreover, previous researchers have pointed out that to make a 3D environment for *in vitro* models, different 3D matrix formation techniques are available based on different situations and requirements (Lee, et al. 2007; Ingthorsson, et al. 2010). For Matrigel, cells can be embedded in homogeneous Matrigel (Lee, et al. 2007; Ingthorsson, et al. 2010), or they can be cultured in a sandwich-like structure (Lee, et al. 2007). However, the previous work of other researchers has not compared the characterisations and drug responses of these different

3D matrix formation methods. In this chapter, cancer cell were co-cultured with endothelial cells in both ways – embedded and in a sandwich structure, and both viability and multiple morphology features, as well as drug tests using a few representative anti-cancer drugs, were investigated and compared.

Previously in Chapter 3 TissueFlex® demonstrated the potential effects of perfusion on 3D cancer models. It was suggested that by better mimicking physiological flow in real tumour, and maintaining the micro-environment homeostasis and relatively stable drug concentration around the 3D cell culture, perfusion enables a better correlation between the tested anti-cancer drugs and the cultured tissue's function. Therefore this perfused colorectal cancer model could be a more predictive *in vitro* model for long-term anti-cancer drug efficacy testing and cytotoxic mechanism study. Thus, in this Chapter all the testing was carried out in perfusion culture. Overall, the aim of this chapter is to apply different scaffolds and 3D culture methods to the co-culture of breast cancer cells and endothelial cells, to elucidate the effects of scaffold type and culture method on the drug responses of these co-culture models.

## ***5.2 Materials and Methods***

### ***5.2.1 Cell Preparation***

Human breast cancer cell line NCI/ADR is cultured in high-glucose DMEM (Lonza) supplemented by fetal bovine serum (FBS, Invitrogen) 10% (v/v); Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial growth medium (EGM-2, Lonza). HUVECs must be passage 2-6. Passages older than P6 will lose tube formation abilities.

### ***5.2.2 Sandwich co-culture of NCI/ADR with HUVECs***

See Section 4.2.2.

### ***5.2.3 Embedded co-culture of NCI/ADR with HUVECs***

To create a 3D embedded co-culture of HUVECs and NCI/ADR, at first 5  $\mu$ L Matrigel was coated on the surface of each well, then  $2.5 \times 10^4$  NCI/ADR cells with  $7 \times 10^4$  HUVECs were suspended in 75  $\mu$ L Matrigel together for each well of the 10-well bioreactor. After 30 minutes of polymerization, then 60  $\mu$ L EBM-2 (Lonza) supplemented with 2% FBS (Invitrogen) and penicillin 100U/mL-streptomycin 100 $\mu$ g/mL (PAA) was added to each well (Mina Bissell et al, 2007). The final cell seeding density of both embedded and sandwich cultures are the same for both HUVECs and NCI/ADR, to allow the comparison between two culture methods. This procedure is summarized in Figure 5.1(A).

#### *5.2.4 Alginate beads production*

Alginate was dissolved into 0.9% NaCl solution at the concentration of 1.2%, and then autoclaved to sterilize the mixed solution. After the alginate solution was prepared, HUVECs and NCI/ADR cells were trypsinized and suspended in alginate solution. The cell seeding density used for cell-alginate beads has been studied by previous research (Hauselmann et al, 1992), and the seeding density used in this study was  $1 \times 10^5$  cells/mL. After the suspension of cells into alginate solution, 22G needles were used (Wang et al, 2001), and the alginate-cell mixture was dropped into 102 mM  $\text{CaCl}_2$  to form alginate beads. After at least 10 min cross-linking reaction between calcium and alginate, the beads were washed with endothelial cell basic medium (EBM, Lonza, UK) 3 times. For 24-well plates, 10 drops were used for each well, cultured in 1mL co-culture medium, and cultured at standard cell culture environment (37°C, CO<sub>2</sub> 5%). The alginate bead production is shown in Figure 5.1(B).

#### *5.2.5 Preparation of drug solutions for testing*

Cisplatin and Paclitaxel were purchased from Sigma-Aldrich, and were both dissolved first in DMSO (Sigma) as stock solutions into concentration of 10mM. When used for testing, the stock solutions were diluted into the desired concentration with EBM-2 supplemented with 2% FBS.

#### *5.2.6 TissueFlex® microbio reactor perfusion culture*

See Section 3.2.2.

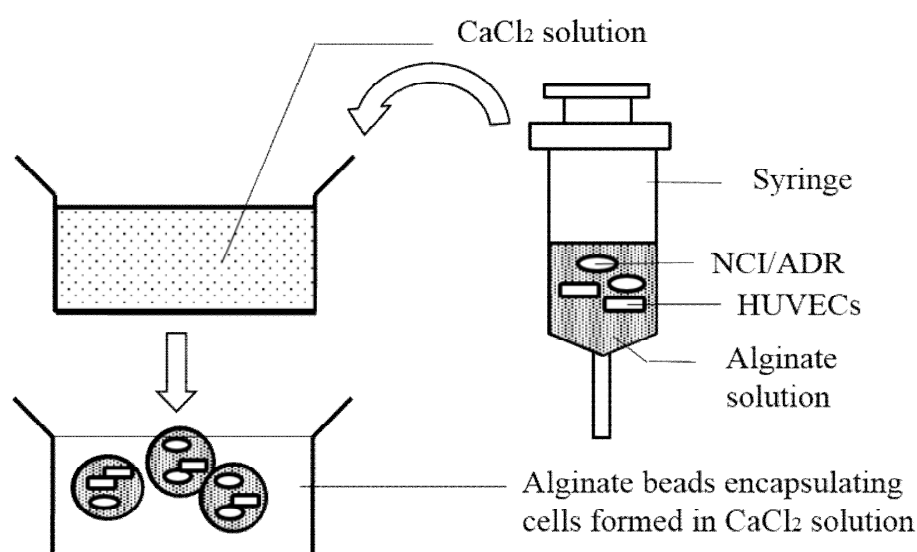
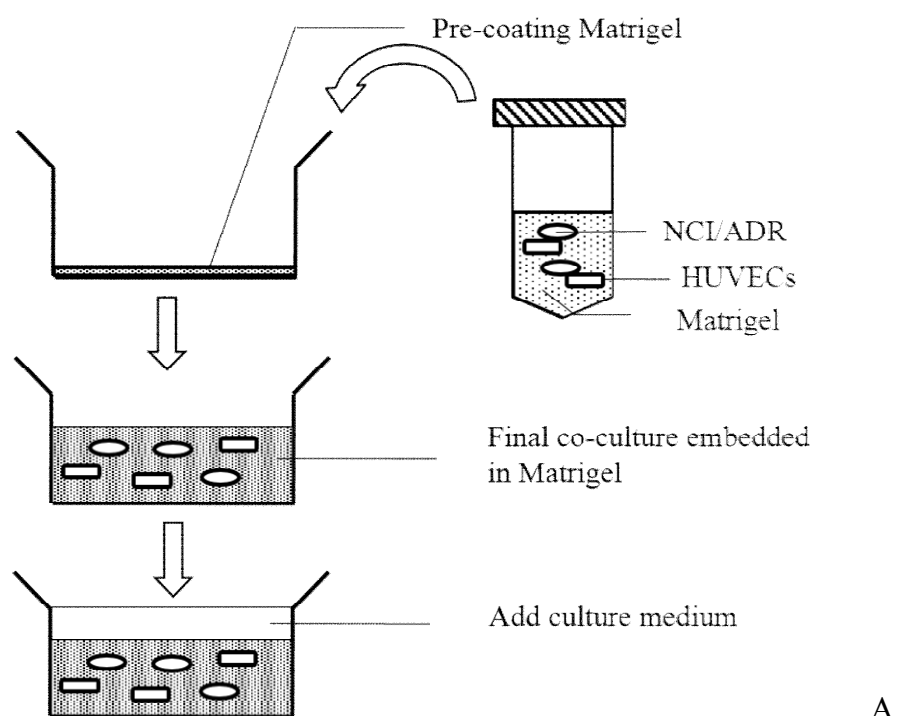


### *5.2.7 Drug treatment*

For static culture in standard 96-well plates, 100 µl of medium containing 2× final concentration drug or control (0.1% DMSO) was added in each well of a 96-well plate (total volume of culture medium per well is 200 µl now). For perfusion culture, the desired volume of medium containing drug or control (0.1% DMSO) was filled in 2 ml syringes, and the perfusion experiment was set up in the bio-safety cabinet following a standard procedure. The perfusion rate is 10µl/hr. Without special mentioning, the AlamarBlue assay and colony diameter analysis were carried out after 72 hours of drug treatment, either in static or perfusion culture.

### *5.2.8 AlamarBlue assay for cell viability testing*

AlamarBlue (Life Sciences) cell viability testing was conducted based on the instructions from manufacturer. Cells were incubated with EBM-2 containing 10% AlamarBlue for 2 hours at 37°C. After incubation, carefully take 50 µl of medium from each well and the fluorescence intensity at 525 nm excitation and 590 nm emission length was measured using a fluorescent micro-plate reader. The incubation time was optimized by measuring the linearity of fluorescence intensity with known cell numbers. When the incubation time is 2 hours, cell numbers of both HUVECs and NCI/ADR have a linearity  $R^2 > 0.99$  to fluorescence intensity (Data not shown). The cell growth rate with time was normalised with reading obtained from Day2. 100 µl of culture medium with 10 µl AlamarBlue reagent is set as a cancer drug – cell viability of untreated model (A)]/ cell viability of untreated model (A) × 100%.



**Figure 5.1** Matrigel and alginate based culture methods. (A) Matrigel embedded co-culture procedure. The surface of each well was first coated by 5  $\mu$ L Matrigel, then 75  $\mu$ L Matrigel containing NCI/ADR cells and HUVECs was added; (B) Alginate encapsulated co-culture procedure. 1.2% alginate in 0.9% NaCl solution mixed with cells were added drop by drop through a 22G needle into 102 mM CaCl<sub>2</sub> to form alginate beads.

#### *5.2.9 Live/dead imaging using multiphoton microscope*

Cell viability and morphology were monitored using live/dead viability/cytotoxicity kit (L3224, Invitrogen). The dead cells were stained by ethidium homodimer-1 (EthD-1) with red fluorescence, which is not permeable to living cells. To stain the living cells, the acetomethoxy derivative of calcein (calcein AM) was used. This compound has no fluorescence, but will be transported through cell membranes of living cells, and degraded into calcein, which has green fluorescence. Prior to staining, spent culture medium was briefly removed and spheroids were rinsed with PBS twice. Spheroids were incubated with PBS containing 4  $\mu$ M calcein AM and EthD-1 in darkness at 37°C for 10 min. Then samples were washed with PBS prior to mount on slides with mounting medium and visualized using a multi-photon microscope. 3D microscopic images were obtained from a modified BioRad Radiance 2100 MP Multiphoton Microscope (MPM, Zeiss; Jena, Germany). Near infrared (NIR) laser beams ( $\lambda = 800$  nm) were obtained from a tunable 76 MHz femtosecond pulsed Ti: sapphire laser (Mira 900-F, Coherent, Ely, UK) pumped by a 10 W multiline argon ion laser (Verdi; Coherent). A Nikon S Fluor 10  $\times$  objective with a numerical aperture of 0.30 and working distance as 2.0mm was used for all images. The emission filters selected were: 495 nm for blue, 525 nm for green and 595 nm for red. All serial optical sections from individual datasets were loaded into Imaris 7.6.1 (Bitplane Ag, Zurich, Switzerland) software for processing and analysis.

#### *5.2.10 Statistics*

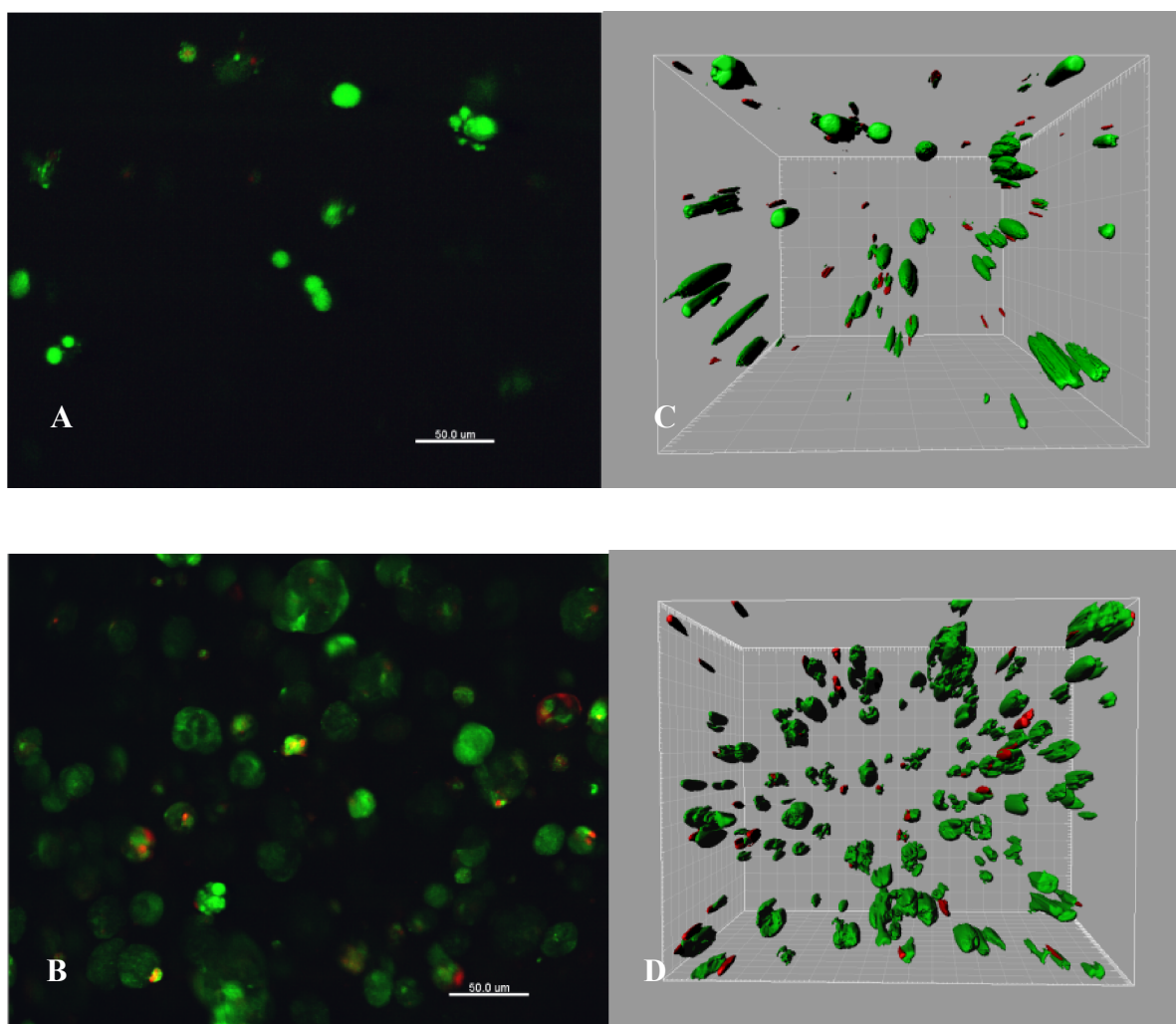
Besides specific instructions, all experiments were repeated three times independently. Student's t-test was used to compare the significant difference between different culture methods.  $P < 0.05$  was considered to be significantly different.

## 5.3 Results and Discussions

### 5.3.1 Comparisons between Matrigel and alginate co-cultures

Matrigel is a widely accepted replacement of extracellular matrix (ECM), as a solubilised basement membrane preparation. However, because it contains a wide range of growth factors and other bioactive components such as integrins, it is useful to clarify the influence of Matrigel itself on the co-culture response to the anti-cancer drugs. Thus, an inert scaffold, alginate is used as a comparison in anti-cancer drug testing represented by Cisplatin and Paclitaxel.

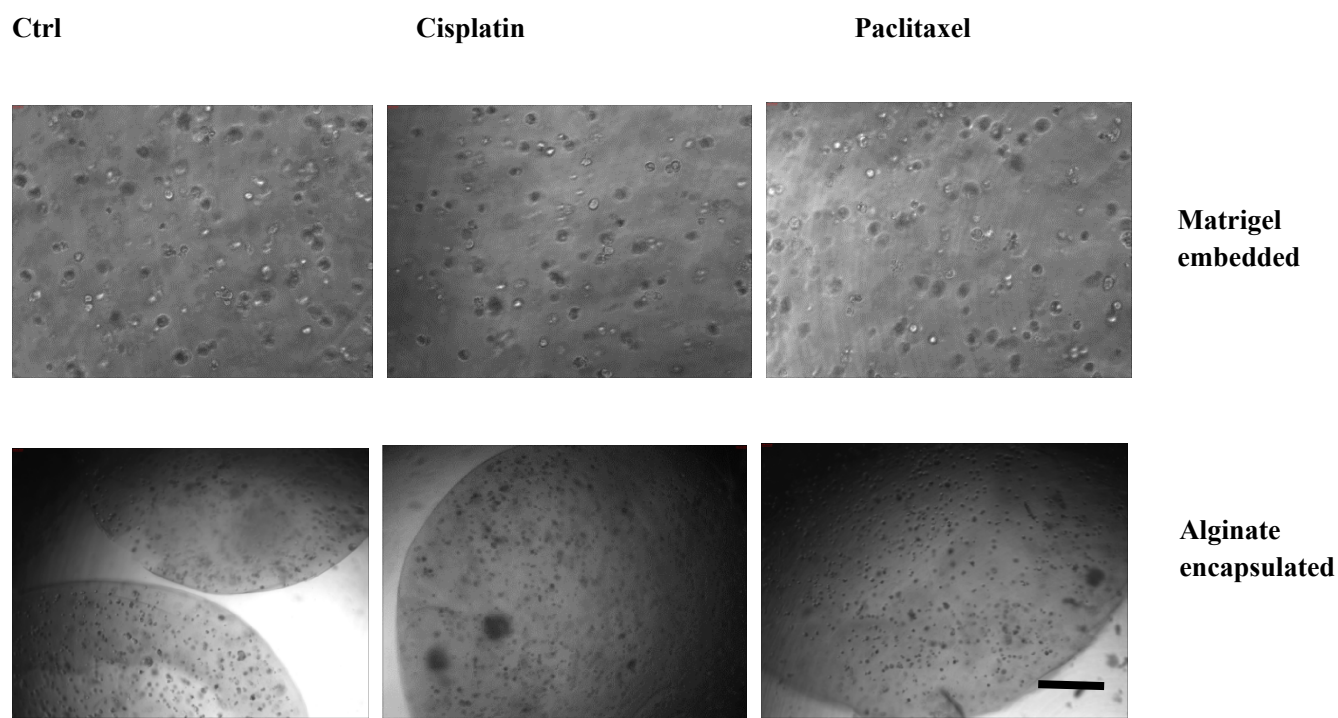
Interestingly, based on the morphology shown in Figure 5.2, although both alginate encapsulated co-culture and Matrigel embedded co-culture did not form tubules, in alginate encapsulation there were more spheroids formed in 72 hours. One potential explanation of this advantage of alginate facilitated spheroid formation is the hydrophobic properties of alginate leaves no binding possibilities for the cells, thus the self-adherence of cancer cells were the main intercellular interactions and caused more cells to form spheroids in the culture. On the contrast, Matrigel has a high hydrophobicity thus the cells preferred to attach to the matrix rather than gathering together. The larger and more cell spheroid formation do not necessarily relate to higher cell viability. Another noticeable point is the higher number of spheroids in alginate does not necessarily lead to high overall cell viability. This is supported by the fact that Matrigel is a mixture of bioactive components such as growth factors and integrins, which helps to increase the cell growth in the Matrigel, while alginate is much more inert and non-active, thus cancer cells and endothelial cells need to rely on their own autocrine system for growth factors etc.



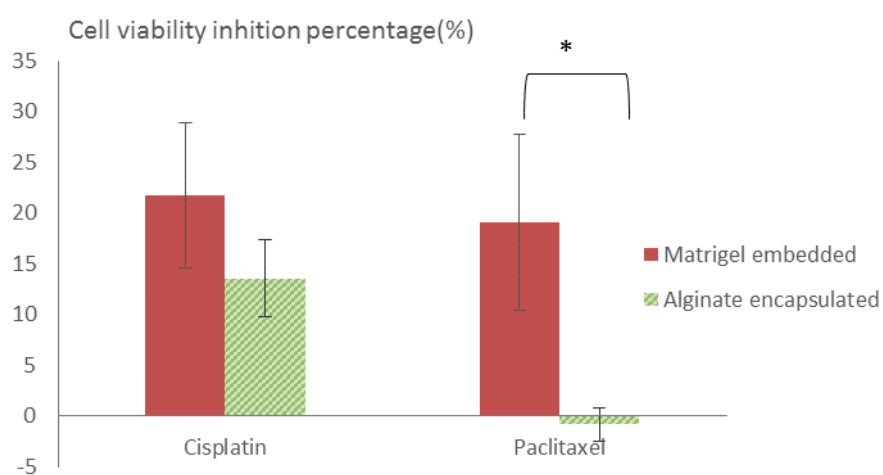
**Figure 5.2** (A) Matrigel embedded culture; (B) Alginate encapsulated culture; (C) 3D image reconstruction by Imaris for Matrigel embedded culture; (D) 3D image reconstruction for alginate encapsulated culture. *All grid measurement is 20 $\mu$ m.*

For the drug testing, as shown in Figure 5.3(A) it seems that both cultures showed no significantly morphology changes, such as observable reduction of spheroid numbers or diameters. However, the cell viability comparisons shown in Figure 5.3(B) still reveal different levels of inhibition on cell metabolism activity in both co-culture systems. For the responses to Cisplatin 10 $\mu$ M, the viability inhibition percentages of Matrigel and alginate co-cultures do not have significant statistical difference, though the average inhibition

percentage of Matrigel embedded co-culture ( $21.74 \pm 2.66\%$ ) is higher than alginate encapsulated co-culture ( $13.56 \pm 3.78\%$ ). The responses to Paclitaxel, on the other hand, has a more dramatic difference between Matrigel and alginate co-cultures, with the inhibition percentage of Matrigel as  $19.07 \pm 2.95\%$  whereas no significant inhibition effect was observed in alginate encapsulated culture. The results in Figure 5.3 suggest— 1. The cell viability change is not necessarily accompanied by observable morphology change in 3D cultures. Thus to make conclusion just based on morphology is not reliable for drug testing. 2. Matrigel can have potential influence on co-culture's response to anti-cancer drugs, but for some drug, such as cytotoxic drug Cisplatin, the influence of bioactive Matrigel on 3D co-culture response is not significant. 3. The bioactive factors in Matrigel can be necessary to predict the clinical effects of anti-cancer drugs. In the case of this study, while the inhibition effect of cytotoxic drug Cisplatin was not significantly different in Matrigel compared with alginate, cytostatic drug Paclitaxel showed an average of 19.07% viability inhibition only in Matrigel system with no significant inhibition was detected in alginate co-culture at all. Since Paclitaxel is commonly used in clinical practice already it can be assumed to be an effective anti-cancer drug, thus the negative results obtained from alginate system could be misleading and indicates a shortcoming of the model.



A



B

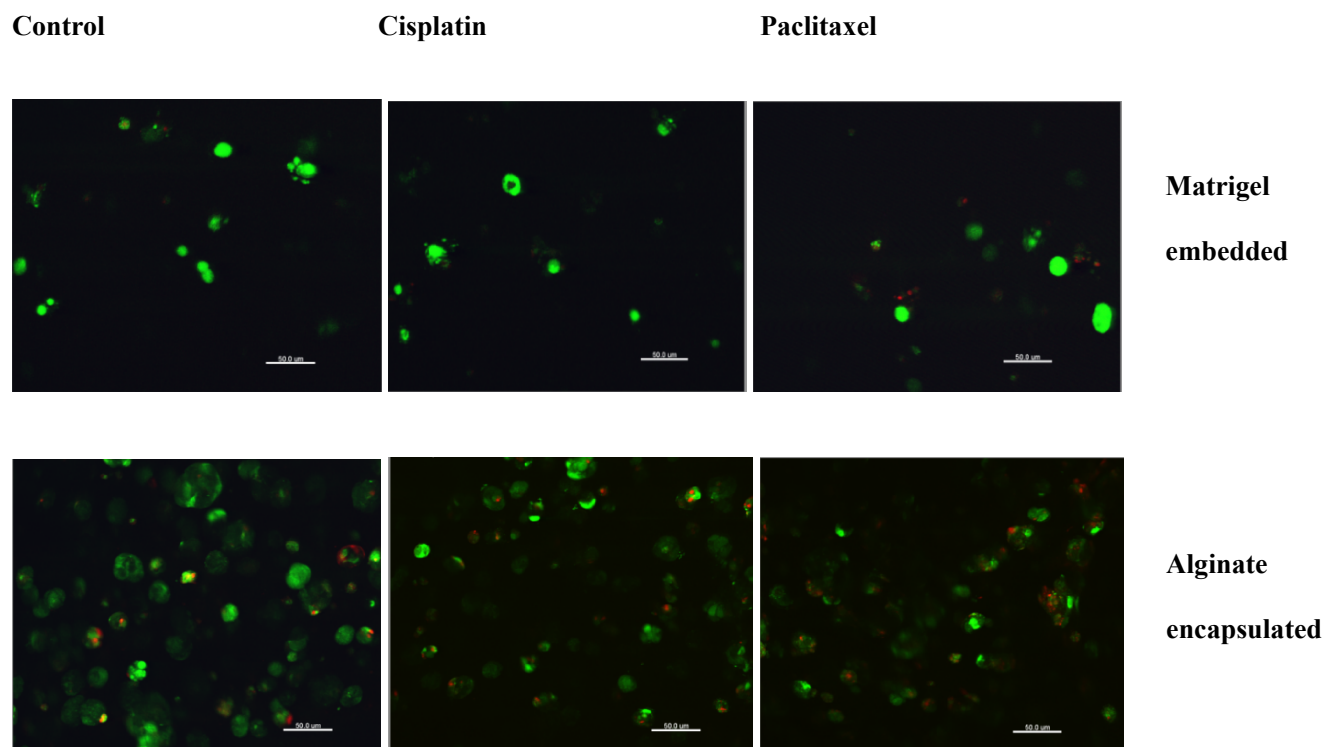
**Figure 5.3** The viabilities and morphologies of Matrigel sandwich, embedded and alginate encapsulated cultures and drug testing. (A) Morphology comparison of three cultures and drug testing. *Scale bar: 50 $\mu$ m*; (B) AlamarBlue viability test. \*  $p < 0.05$  by Student's t-test.

To give more information on how many cells were actually killed by the tested drugs in Matrigel and alginate co-cultures, multiphoton microscope (MPM) was used for the live/dead imaging, as shown in the Figure 5.4 below. For both Cisplatin and Paclitaxel, alginate showed a higher dead cell percentage (for Cisplatin,  $p = 0.0026$ , for Paclitaxel,  $p = 1.33217\text{E}-05$  by Student's t-test). Under Cisplatin treatment, in Matrigel co-culture there was only around 10% dead cells, while in alginate co-culture this dead cell percentage was about 30%. In terms of Paclitaxel, the difference is even bigger – Matrigel co-culture had about 15% dead cells while the dead cell ratio in alginate co-culture was as high as about 70%. The high dead cell percentage in alginate is not too hard to understand, taking into consideration that alginate can barely provide such a bioactive supporting, biological compatible extracellular environment compared with Matrigel. This lack of growth enhancing microenvironment is strengthened by even larger diameters of spheroids formed in alginate, initiated by the high cell aggregation due to the hydrophobic nature of alginate – the cells in the core areas of those larger spheroids could not get enough nutrients for sustainable growth and eventually died.

One complicated situation is the live/dead percentage results in Figure 5.4 (B) do not seem to be consistent with cell viability results shown Figure 5.3(B) – Cisplatin caused 30% cell death in alginate and 10% in Matrigel, and Paclitaxel did not change the overall cell viability of alginate co-culture even there were 70% cells dead in the whole group. This can be explained by a subtle feedback loop of the whole cell population - the death of those sufficient cells allow the surviving cells to finally get access to enough nutrients and other resources, to reveal a better metabolic activity. The end result of this complicated and dynamic interaction between co-culture spheroids in alginate with Paclitaxel is considerable



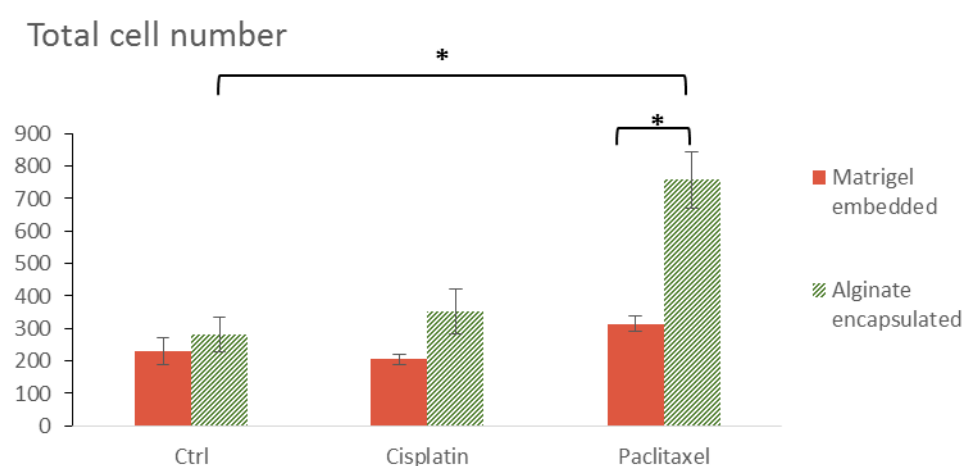
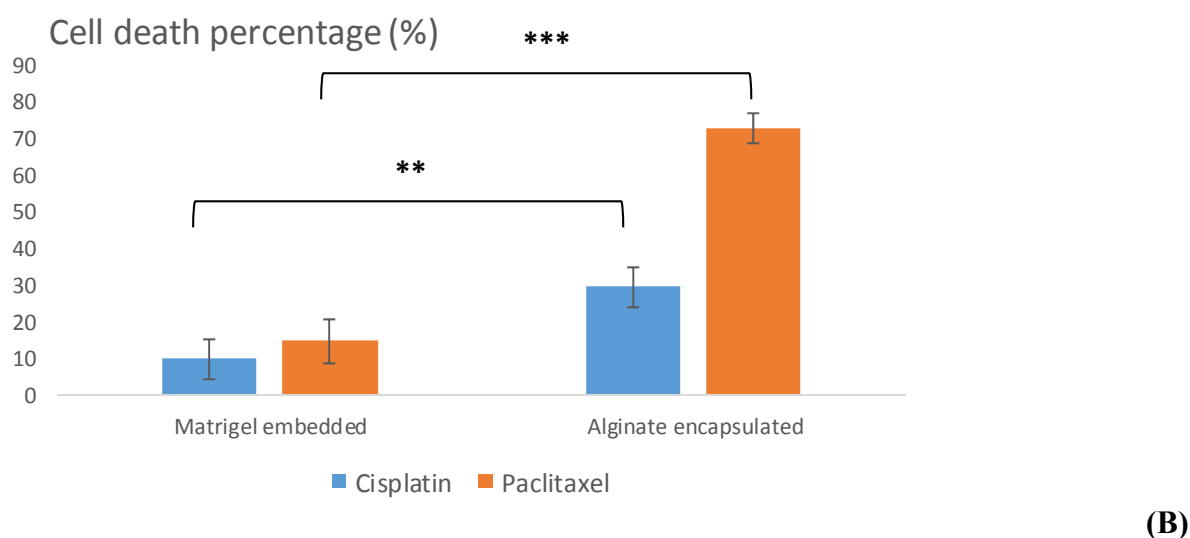
cell death after 72 hours, but the overall cell viability remained nearly the same. This hypothesis finds support in Figure 5.4(C), which shows a significant total cell number increase in the Paclitaxel treated group. This result shows an actual consistency between the results of Figure 5.3(B) and 5.4(B) – the increased total cell number combined with the 70% cell death percentage, leads to the net result of no significant change of overall cell viability. However, why Paclitaxel leads to such significant cell number increase still needs further research.



(A)

(figure to be continued in the next page)

(figure continued from last page)



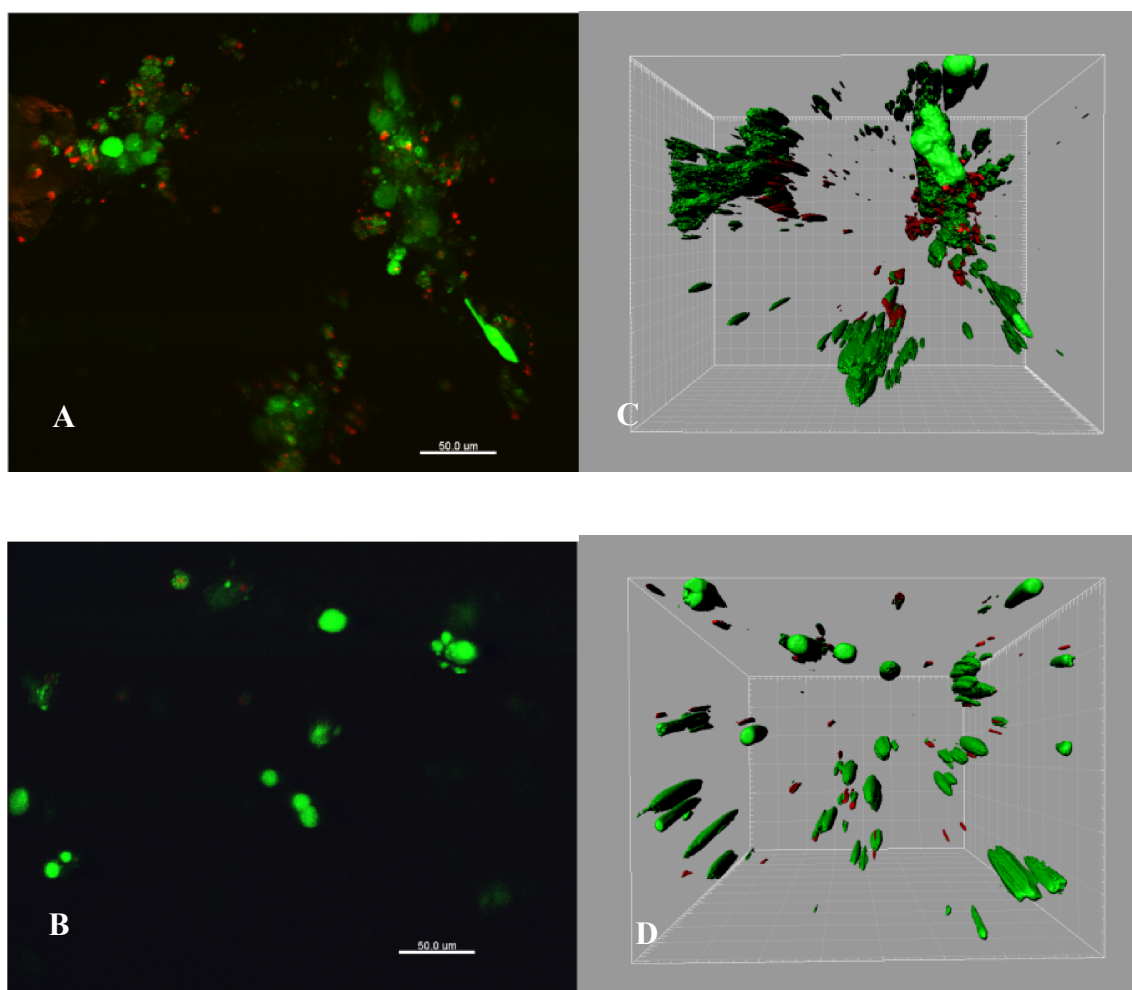
**Figure 5.4** Live/dead cell counting comparison. (A) Representative MPM imaging of Matrigel embedded co-culture with alginate encapsulated co-culture treated by Cisplatin or Paclitaxel. Green – live cells, red – dead cells. Scale bar: 50  $\mu$ m; (B) Percentages of live/dead cell comparing Matrigel embedded and alginate encapsulated co-cultures by Cisplatin or Paclitaxel treatment; (C) Total cell numbers of each group \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  by Student's t-test.

In summary, cell viability inhibition is the overall behaviour of a cell population group, and the result of cell viability should be interpreted together with other details such as live/dead percentage to give clearer information of what happened under certain drug treatment. It is found the drug testing comparisons that compared with Matrigel embedded co-culture, the alginate encapsulated co-culture has more dead cells in general, though the spheroids formed in alginate have larger diameters and there are more spheroids per unit area alginate beads than homogeneous Matrigel. The live/dead staining results suggest a possible explanation for the deviation of cell viability results from the morphology analysis – even there were more cells, because of the existence of large numbers of dead cells, the co-cultures in alginate bead do not have correspondingly high metabolic activities.

### 5.3.2 Comparisons between Matrigel sandwich and embedded culture methods

To compare the features and drug responses of the co-culture models in different methods based on Matrigel, an investigation of those two models in parallel culture conditions were carried out and similar to previous section 5.3.1, two representative anti-cancer drugs – Cisplatin and Paclitaxel were tested on the model to compare the drug responses of those two Matrigel co-culture methods. As shown in Figure 5.5, MPM 3D imaging shows that there were only tubules formed in Matrigel sandwich culture, but no tubule structure was observed in Matrigel embedded culture, even with the same cell seeding densities (NCI/ADR  $2.5 \times 10^4/\text{mL}$ , HUVECs  $7 \times 10^4/\text{mL}$ ) and the culture time (72 hours). It is noticeable that this result is consistent with previous report from other researchers, in which Matrigel embedded co-culture of breast cancer cells and endothelial cells did not show tubule formation (Ingthorsson et al, 2010). Summarizing the results from this study and the above study by Ingthorsson et al (2010), the initial surface for endothelial cells to form *in vitro* network could be necessary. Sandwich Matrigel can provide this surface to initialize the tubule formation, while 3D homogenous Matrigel cannot provide this surface. It is noted in previous research, the endothelial cells can remain alive and functional, but relatively static.

It is noted in MPM imaging, there are more red dead cells shown in co-culture sandwich compared with embedded co-culture. This is possibly due to the dense and complicated 3D structure in sandwich co-culture expanded from this initial seeding surface of endothelial cells, in which the thickness of sandwich co-culture is around  $200\mu\text{m}$ , while the single cell is only  $20\text{-}60\mu\text{m}$ . As a result, the competition for nutrients in culture medium between cells are more intensive in sandwich culture, thus cause more cell death; whereas in homogeneous environment of embedding co-culture there was larger growing surface for each cell.

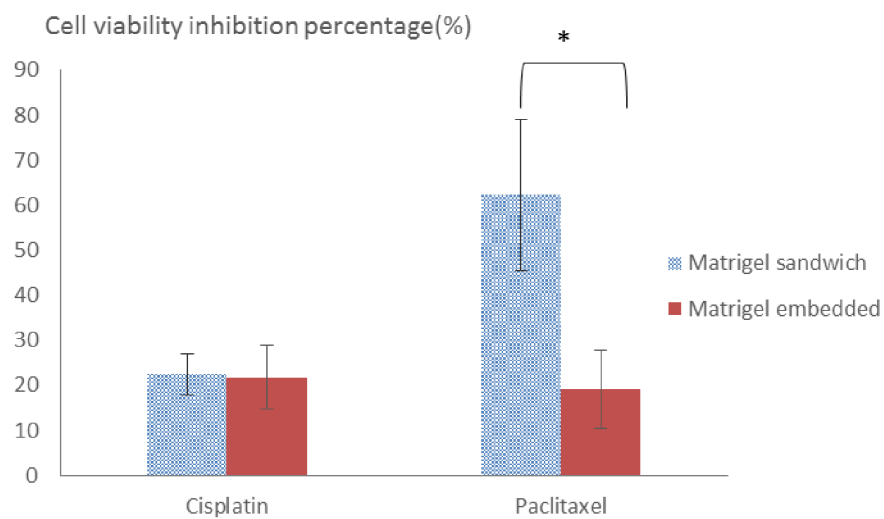
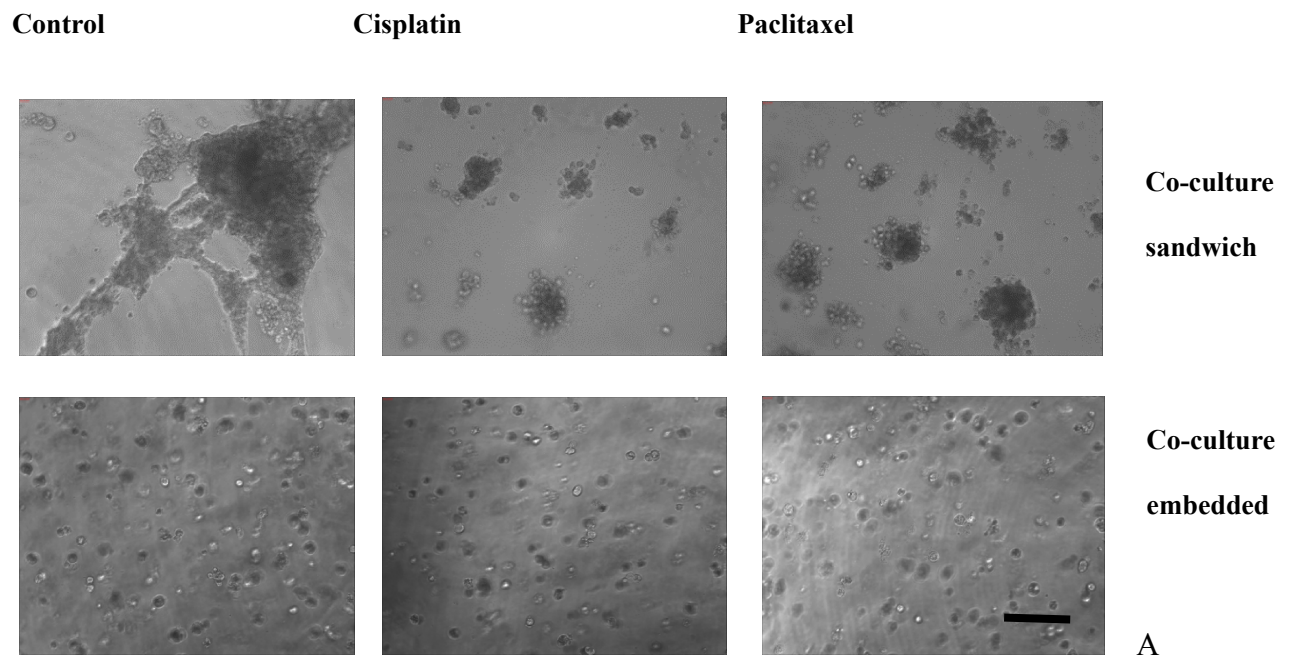


**Figure 5.5** (A) Matrigel sandwich culture; (B) Matrigel embedded culture; (C) 3D image reconstruction by Imaris for Matrigel sandwich culture; (D) 3D image reconstruction by Imaris for Matrigel embedded. *All grid measurement is 20 $\mu$ m.*

Cytotoxic Cisplatin and cytostatic Paclitaxel were used to compare the responses of two co-culture methods. As shown in Figure 5.6(A), the morphology of Matrigel sandwich co-culture responded to both anti-cancer drugs by losing tubule structures. Compared with this tubule-loss morphology change, the morphology change in Matrigel embedded co-culture is not that obvious. For the viability inhibition percentage results shown in Figure 5.6(B), it is noticeable that similar to Figure 5.3(B), Cisplatin was not sensitive to the change of co-culture methods, showing no significant difference between Matrigel sandwich and

embedded co-cultures with inhibition percentage as about 20% for both co-culture methods. Interestingly, as in Figure 5.3(B), the results in Figure 5.6(B) here also showed a significant difference of co-culture methods on viability inhibition caused by Paclitaxel ( $p = 0.029$  by Student's t-test). Matrigel sandwich co-culture the viability was decreased by  $62.24 \pm 4.10\%$ , while in Matrigel embedded co-culture there was only  $19.07 \pm 2.95\%$  decrease at the same concentration.

From the above comparisons between Matrigel sandwich and embedded co-cultures, firstly it is noticeable that morphology variety can potentially provide a quick way to predict a drug's effect in early screening, such as the tubule structure in Matrigel sandwich can suggest the inhibiting effects of those two drugs at the very first glance, while in Matrigel embedded co-culture further testing must be carried out. Secondly, cytotoxic drug Cisplatin did not show significant different inhibition on two Matrigel co-culture methods. This result together with the data in section 5.3.1 Figure 5.3(B) suggest Cisplatin is not sensitive to different culture methods, possibly due to its cytotoxic mechanism, by killing cancer cells via DNA-structure interruption whatever the extracellular environment is. Also because Cisplatin is a small molecule, so it will penetrate the 3D structures easily. Thirdly, Paclitaxel, on the other hand, is very sensitive to different culture scaffolds and methods, possibly because it relies on the cell cycle status of cells, and its larger molecule will also make it hard to penetrate certain 3D structures. The viability of Matrigel sandwich has a higher sensitivity to the treatment of Paclitaxel, suggesting this organization method for co-culture of cancer cells and endothelial cells might provide more cells in active proliferation status in the whole co-culture cell population.

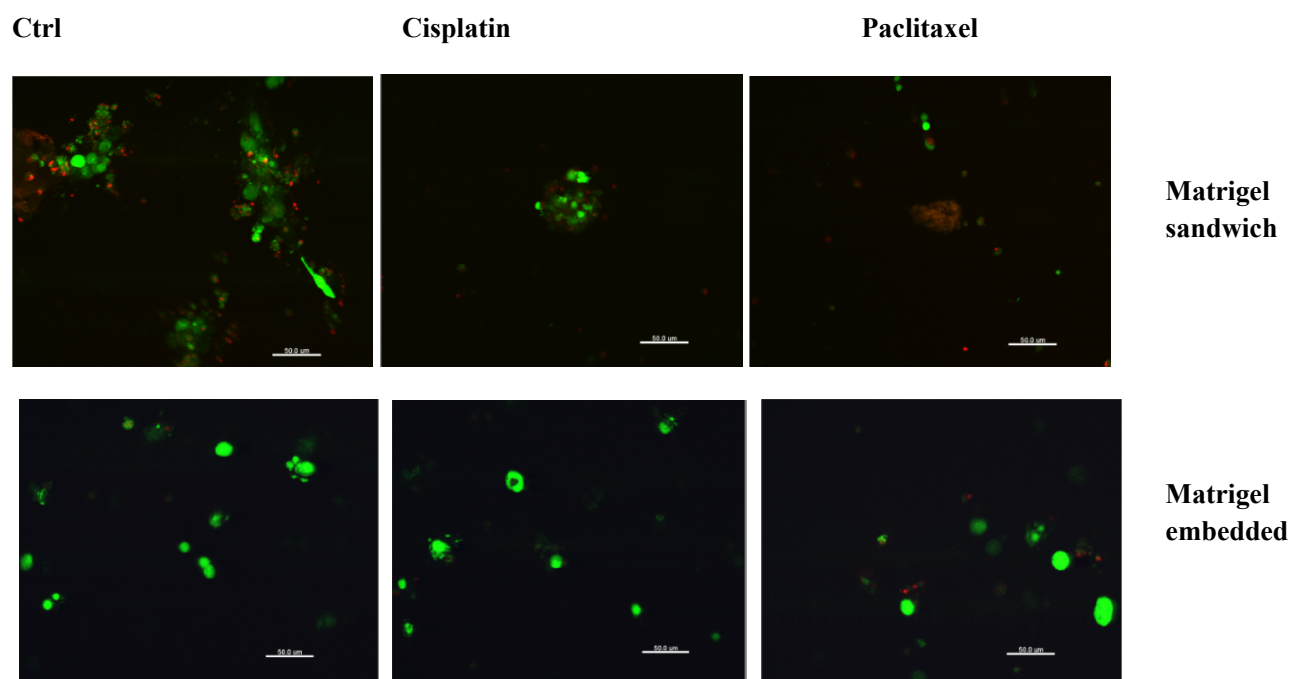


**Figure 5.6** Drug testing results comparison between sandwich co-culture and embedded co-culture. (A) Representative inverted microscope imaging of different groups; (B) Inhibition percentage comparison. Scale bar: 50  $\mu\text{m}$ . \*  $p < 0.05$  by Student's t-test.

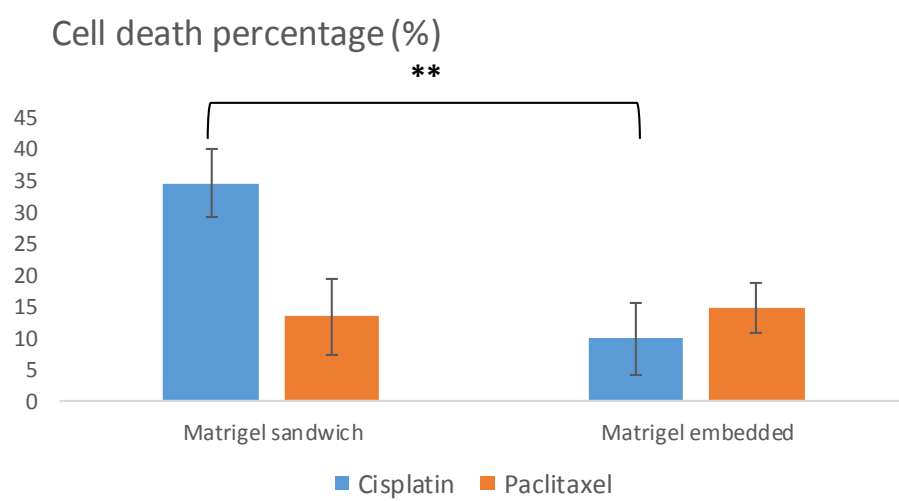
Comparison of live/dead percentages for Cisplatin or Paclitaxel treated groups of Matrigel sandwich co-culture and Matrigel embedded co-culture are shown as in Figure 5.7 below. For Cisplatin,  $34.55 \pm 8.02\%$  cells were dead in Matrigel sandwich culture while there was only  $9.86 \pm 5.43\%$  dead cells counted in Matrigel embedded co-culture. This leads to a significant difference by Student's t-test for the live/dead cell ratios of those two methods, with  $p = 0.0032$ . Interestingly, the live/dead ratios for both Matrigel culture methods treated by Paclitaxel showed high similarity, with dead cell percentage  $13.31 \pm 4.15\%$  in sandwich co-culture and  $14.69 \pm 6.14\%$  in embedded co-culture.

This results again shows the viability and live/dead ratio can be inconsistent with each other, as in previous section 5.3.1. As mentioned in Figure 5.6, Cisplatin treated groups did not show significant difference between different Matrigel culture methods, but in Figure 5.7 (B) there is a significant difference between the live/dead ratio of the two co-culture methods; On the other hand, Paclitaxel had significantly different inhibition effects on the viability of Matrigel sandwich and embedded co-cultures, while the live/dead ratio of both co-culture methods remained about 85:15. Total cell number counting shown in Figure 5.7(C) demonstrates that compared with Matrigel embedded model, Matrigel sandwich model showed significant difference in all the three groups: control group, Cisplatin and Paclitaxel treated groups, with  $P < 0.05$  by Student's t-test comparing the control group with both drug treated groups.





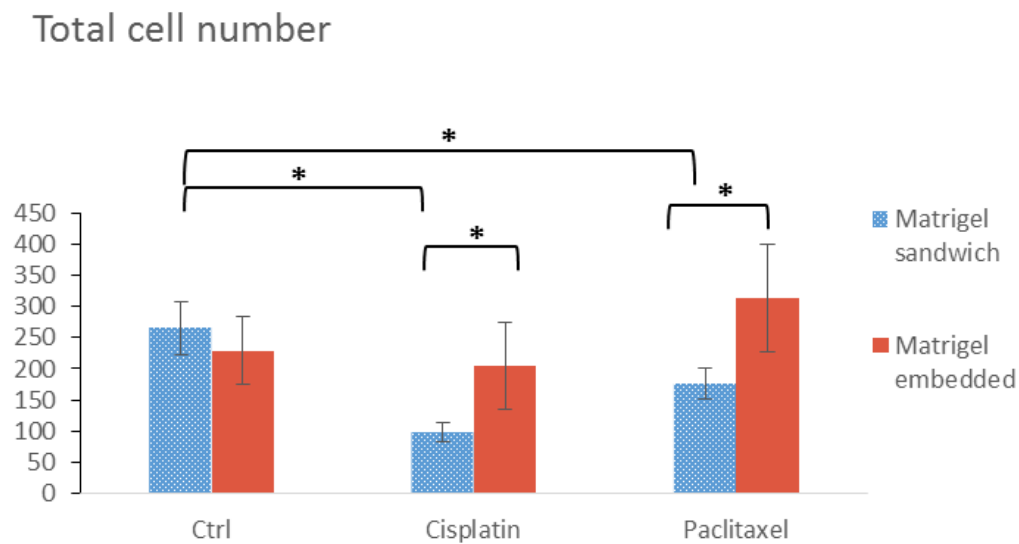
**(A)**



**(B)**

(figure to be continued in the next page)

(figure continued from last page)



(C)

**Figure 5.7** Live/dead cell counting comparison. (A) Representative MPM imaging of Matrigel sandwich co-culture with Matrigel embedded co-culture treated by Cisplatin or Paclitaxel. Green – live cells, red – dead cells. Scale bar: 50  $\mu$ m; (B) Percentages of live/dead cell comparing Matrigel sandwich and Matrigel embedded co-cultures by Cisplatin or Paclitaxel treatment; (C) Total cell numbers of each group \* $p < 0.05$ , \*\*  $p < 0.01$  by Student's t-test.

#### *5.4 Summary*

Matrigel is a highly flexible and one of the most widely used naturally originated scaffold for 3D culture. However, the complexity of its biochemical properties and high flexibility of different application methods also leave researchers with a lot of open questions. In this chapter, to better understand the influence of Matrigel bioactive features and specifically sandwich culture method on co-culture's response to anti-cancer drugs, two controls were used – alginate, as an inert scaffold control, to elucidate the influence of Matrigel bioactive components influence; and Matrigel embedded co-culture, serving as a control for the sandwich co-culture method, to see if there is any difference between just mixing cancer cells and endothelial cells directly, and to co-culture them in an organized sandwich. In preliminary test, collagen (type I extracted from rat tails, BD UK) was also briefly tested together with alginate and Matrigel, but no tubule by endothelial cells were observed, so the results were not shown here. In addition to the fact that collage is also a mice-originated scaffold with some bioactive components for cell growth, alginate would be a better inert control to compare with Matrigel. Thus for the formal experiments it was Matrigel and alginate were studied in detail.

Alginate is a biomaterial used in pharmaceutical and food industry, with potential as a biodegradable and biocompatible scaffold for drug delivery and tissue engineering (Yamamoto et al., 2010). Alginate beads will degrade over long-term culture, just like Matrigel, however, compared with Matrigel, alginate still shows advantage of higher stability over long-term culture. Another consideration is that although considered as a relatively inert material compared with Matrigel, research still suggests that alginate oligosaccharides can activate endothelial cells via VEGF-mediated mechanism (Kawada et al, 1999). This means that alginate is not totally inert, which has two sides for drug testing on alginate co-culture

beads – the endothelial cells in those beads have better growth potential for long-term culture, but the interpretation of the following data needs consideration of the angiogenesis-active feature of alginate derived molecules in culture medium.

The penetration of drugs through scaffolds is a common concern in designing tissue engineered models for drug testing. In this study, both Cisplatin and Paclitaxel are small molecules – Cisplatin molecule is less than 1nm (Boulikas, 2007), and the longest diameter of Paclitaxel is about 1.4nm (Mastropaolo et al, 1995). Meanwhile, the pore sizes of both Matrigel and alginate are quite big compared with those two drug molecules: the average size of Matrigel pores is about 2 $\mu$ m (Zaman et al, 2006), and alginate beads gel has a pore size range of 6.8-16.6nm (Klein et al, 1983). Based on the above facts, we can assume the two drugs Cisplatin and Paclitaxel should be able to penetrate the scaffolds via diffusion.

In summary, the technical comparisons between Matrigel embedded co-culture and alginate encapsulated co-culture are shown in the table below. Overall, Matrigel embedding technique is a more time-effective way for co-culture, without extra gel formation step, and can be used immediately after the mixing of cancer cells and endothelial cells (Lee et al, 2007). Alginate encapsulation method, on the other hand, requires more time for the polymerisation of alginate, which includes adding alginate-cell suspension into calcium solution, and pre-culture of alginate co-culture beads is necessary for the spheroid to settle properly in the scaffolds for further drug treatment (Kawada, et al, 1999). However, since both methods do not show the capabilities of tubule formation featured by endothelial cells, the morphology analysis potential is limited for both methods.

Table 5.1 Comparisons of Matrigel embedded co-culture and alginate encapsulated co-culture

|                                        | Matrigel embedded      | Alginate encapsulated      |
|----------------------------------------|------------------------|----------------------------|
| <b>Time</b>                            | <4 hours               | >4 hours                   |
| <b>Cost</b>                            | More expensive         | Less                       |
| <b>Pre-treatment</b>                   | Pre-coating of surface | No                         |
| <b>Gelling procedure</b>               | Temperature-dependent  | Calcium solution is needed |
| <b>Viability</b>                       | Low                    | Low                        |
| <b>Morphology</b>                      | No tubule              | No tubule                  |
| <b>Pre-culture before drug testing</b> | No                     | 4 days                     |
| <b>Drug response</b>                   | Not very sensitive     | Not very sensitive         |

Red: disadvantages; Green advantages.

As a widely used biological origin scaffold, the methods of using Matrigel have widely been studied. Overall, there are two most popular methods – mix the cells with Matrigel, or use Matrigel for coating. Both methods have been studied by other researchers in developing their 3D *in vitro* tumour models (Lee et al, 2007; Ingthorsson et al, 2010), and some researcher even compared the two methods already (Lee et al, 2007). But so far in the research on co-culture of endothelial cells with cancer cells, drug testing has rarely been reported, and nearly no drug response comparing those two methods was reported. There was a much higher viability for sandwich model compared with embedded model. This is possibly

due to the limited movement abilities of cells in embedded culture, and much lower endothelial cell density in the same unit area in a more three-dimensional space, compared with sandwich culture, though the later one also showed certain level of 3D structure, and is claimed to be three-dimensional based on previous report of other researchers (Lee et al, 2007).

The two Matrigel co-culture methods are compared in the table below, focusing on the practical aspects of embedded co-culture and sandwich co-culture techniques. Embedding cells in Matrigel is obviously quicker, with a 3D structure formed immediately. However, as shown clearly in the table, embedded co-culture uses more Matrigel, which can be significantly more expensive than sandwich co-culture, especially for high-screening application which requires hundreds of parallel tested samples. Moreover, the following results in Matrigel sandwich have more information concerning the tubule formation abilities of the co-culture system, with higher viability, which can provide information in the drug testing.

Perfusion culture platform developed in Chapter 3 was used in this comparison study. The reason for this is to provide a more comparable culture environment for different scaffolds, and different culture methods. The continuous supplies of nutrients and clearance of metabolic waste of cells help to improve the homeostasis of the culture system, as well as to maintain other physiochemical properties such as pH at a more stable level (Sailon et al, 2009; Cui et al, 2007). Moreover, because the culture was carried out in a dynamic environment, the communications of cancer cells and endothelial cells were more effective than in static

culture, and the comparison results can better reflect the real *in vivo* situation (Song et al, 2009).

Table 5.2. Comparisons of Matrigel sandwich co-culture and Matrigel embedded co-culture

|                      | Matrigel sandwich                                                     | Matrigel embedded                                                                      |
|----------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------------------------|
| <b>Time</b>          | >4 hours                                                              | <4 hours                                                                               |
| <b>Cost</b>          | Less (e.g. 120uL Matrigel is needed for each well of a 48-well plate) | More Matrigel needed (e.g. 500 uL Matrigel is needed for each well of a 48-well plate) |
| <b>Pre-treatment</b> | No                                                                    | Pre-coating of surface                                                                 |
| <b>Viability</b>     | Significantly higher                                                  | Lower                                                                                  |
| <b>Morphology</b>    | Tubule formation potential                                            | No tubule                                                                              |
| <b>Drug response</b> | More sensitive                                                        | Less sensitive                                                                         |

Red: disadvantages; Green advantages.

In summary, different scaffolds can influence both viability and morphology behaviours of the co-culture, as shown in the comparison between Matrigel and alginate co-culture. Even for same scaffold, different co-culture methods will also influence the following drug testing results, as demonstrated in the comparison between Matrigel sandwich and embedded co-cultures. Overall, Matrigel sandwich co-culture is the only scaffold-method combination showing tubule structures featured by endothelial cells, thus, to get more morphology-related for potential tumour angiogenesis assays, Matrigel sandwich is a suitable choice.

## Chapter 6. Effects of Perfusion on 3D Co-culture Breast Tumour model with Endothelial Cells

### 6.1 Introduction

Three dimensional (3D) co-culture tissue engineered *in vitro* cancer models have shown their potential to bridge the gap between traditional *in vitro* and *in vivo* models. However, one limitation still challenges the development of advanced *in vitro* cancer models is that for the drug which aims at therapy for a long period, results obtained from *in vitro* results failed to predict the drug responses in clinical trials (Matsubara et al, 2006; Altan et al, 1998). This failure of prediction is largely due to the traditional way of long-term *in vitro* testing - the repeated medium change every few days in long-term culture will cause the fluctuation of pH and other culture environment for cells. Many researchers have pointed out that subtle change of environment parameters like pH will influence cell metabolisms and other behaviours (Wu et al, 2011; Tostões et al, 2012).

The pH of the surrounding microenvironment influences behaviours of the cultured cells or tissues including biochemical drug resistance, which is indicated by overexpression of drug-efflux proteins like P-gp and Breast Cancer Resistant Protein (BCRP). The drug resistance can also be influenced by other special physical and chemical microenvironment factors such as hypoxia (Brent et al, 2003). Since evidences show resistance to anti-cancer drugs is one main cause of failure of chemotherapy and using pre-clinical data to predict drug response in clinical trials (Matsubara et al, 2006; Altan et al, 1998), it is possible that the rapid extracellular environment change in static culture influence the drug resistance of the cultured tissue, and then the results obtained from these tissue models will fail to predict the real resistance of *in vivo* tissues, which has a far more stable extracellular environment in living bodies.



For angiogenesis assays for anti-cancer drug testing, the maintenance of consistent drug-testing environment is particularly crucial, as found out by *in vivo* studies, angiogenesis inhibitors are more effective when they are kept at constant concentrations in circulation (R. Kerbel et al, 2002). Thus, we can foresee that the limitation on the ability to maintain the extracellular environment in traditional static culture will cause an interruption to the metabolism and drug resistance of cultured tissues, which will then cause misleading interpretation of their response to the drugs. Furthermore, consistently changing the culture medium environment by medium change makes it difficult to quantify the correlation of drug dose with the responses of the models, causing the difficulty of interpreting the data (Wu et al, 2011). Thus, the maintenance of a stable homeostasis of extracellular environment is important to keep the consistent responses of cultured tissue, and necessary for a better correlation building for drug testing.

While the fluctuated pH in traditional static cell culture became a barrier for the wide application of 3D model in anti-cancer drug testing, as its dependence on manual change of culture medium in anti-cancer drug assay makes it impossible to keep constant concentration of tested anti-cancer drugs, on the other hand, perfusion can provide a better oxygen and nutrient supply to the cells cultured in 3D environment, with a better homeostasis for the cells, lower risk of contamination and less time and efforts of researchers caused by frequent medium change in this long-term culture, and with a better resolution to the quantification problem (Cui et al, 2007; Wu et al, 2011). There are also other advantages of perfusion compared with static culture – the automatic medium supply system saves efforts and time of researchers changing the medium by their hands, with lower risk of introducing contamination during long period culture. Moreover, the consistent and continuous medium

supply with low perfusion rate (usually  $<10\mu\text{L/hr}$ ) minimizes the interruption to the 3D tissue integrity caused by shear stress of medium flow change, while in static culture the specific morphology of 3D cultures were under the risk of being interrupted by manual medium change.

The objective of this chapter is to examine the effects of perfusion and the underline mechanisms of those effects. Considering the sensitivity of cancer cells to environment pH and the fact that perfusion will influence the pH values of the culture medium by consistent medium supply, the mechanism study of this chapter will focus on the pH measurement of the culture medium. The previous two chapters provide basis for this chapter - in chapter 3, perfusion platform was tested on well-build spheroid model, providing the possibility of long-term drug testing; then in chapter 4, a 3D co-culture method of endothelial cells and cancer cells has been developed. In this chapter, the co-culture model was integrated into the tested perfusion platform, and long-term drug testing was applied using Paclitaxel, the anti-cancer drug which shows anti-angiogenesis efficacy as well as inhibition effect on cancer cells. The morphology and drug response were compared between perfusion and static cultures.

## **6.2 Materials and Methods**

### **6.2.1 Cell Preparation**

Breast cancer cell line NCI/ADR was used for this study, and their preparation is shown in Section 5.2.1.

### **6.2.2 Live cell staining for co-culture**

Cancer cells NCI/ADR was stained by 5-chloromethylfluorescein diacetate (CellTracker Green CMFDA, Molecular Probes, UK). The stock solution was prepared in DMSO with concentration of Green CMFDA as 10mM. Then the stock solution was diluted into working solution with the final concentration as 10 $\mu$ M, by EBM-2 without any other supplement. The cells in T75 culture flask were incubated with 2mL working solution for 60 minutes, at 37°C, 5% CO<sub>2</sub>, which is the normal static cell culture environment..

Endothelial cells HUVECs was stained orange by 5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (CellTracker Orange CMTMR, Molecular Probes, UK). The stock solution was prepared in DMSO with concentration of Green CMFDA as 10mM. Then the stock solution was diluted by EBM-2 into final concentration as 5 $\mu$ M. 1 mL working solution was incubated with HUVECs seeded in T25 culture flask, for 45 minutes at 37°C, 5% CO<sub>2</sub>.

The images were taken either by a fluorescence microscope (Nikon, UK) for analysis. Green channel was used for cancer cell colony formation analysis, and red channel was used for angiogenesis related parameter analysis. For further 3D imaging, multiphoton microscope (MPM) can be applied.

### *6.2.3 Sandwich co-culture of NCI/ADR with HUVECs*

A modified 3D culture protocol was developed to form the Matrigel sandwich structure for the co-culture system. For the detailed process see Section 4.2.2.

### *6.2.4 Preparation of drug solutions for testing*

Paclitaxel were purchased from Sigma-Aldrich, and were both dissolved first in DMSO (Sigma) as stock solutions into concentration of 10mM. When used for testing, the stock solutions were diluted into the desired concentration with EBM-2 supplemented with 2% FBS.

### *6.2.5 TissueFlex<sup>®</sup> microbioreactor production*

TissueFlex<sup>®</sup> bioreactors were made by the crosslinking of polymers. The polymers and crosslinking reagent is mixed at the weight ratio of 10:1, then the mixture was poured into an individually designed module. The module was then put into vacuum conditions to remove the bubbles in the mixture. The bubble remove step is important for keeping the homogeneity of the inside environment of the bioreactor. After all the visible bubbles were removed, to solidify the bioreactor, the module with the mixture was then baked at the temperature of 60 °C overnight. Alternatively, the crosslinking process can be finished in two hours at the temperature of 70 °C. However, the lower temperature is encouraged because some invisible small bubbles in the mixture might take longer time to reach the surface of the bioreactor to eventually get out of the mixture, so we assume lower temperature with longer and milder solidifying conditions will improve the remove of those small bubbles.

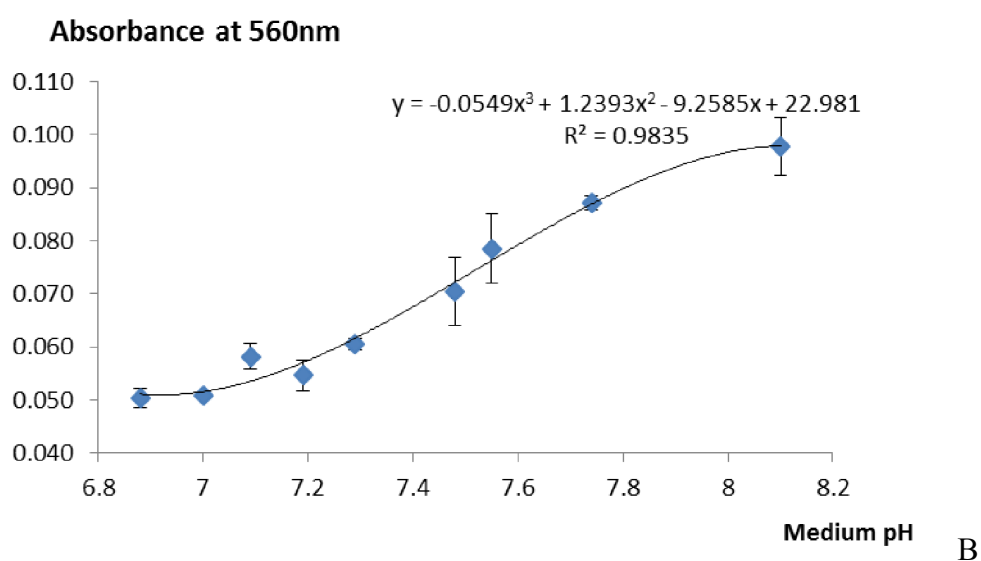
#### *6.2.6 pH testing and maintenance*

The pH of culture medium was determined by a PH 213 microprocessor pH meter (HANNA Instruments, US). Briefly, the pH meter was calibrated first using the buffers which are suitable for the tested pH range, then the probe was placed in the culture sample and the data was recorded. To obtain culture medium with different pHs, 1 mol/L hydrochloric acid (HCl) or 1 mol/L sodium hydroxide (NaOH) was added to the culture medium, and when the desired pH was reached, the medium was sterilised by a syringe filter with the pore diameter as 22µm (Millipore, UK), and then used for cell culture.

Because the quantity of the medium is < 2mL, the pH meter could not satisfy the precision and technical requirement to test the pH of medium. Thus, as an indication of pH, the absorption of the medium at 560 nm was measured for each sample, on the microplate reader (WALLAC VICTOR2 1420 multilabel counter model, Perkin Elmer, UK). This is based on the fact that phenol red will change colour in relation to the medium pH (Truppo et al, 2010). Both linear and polynomial correlation were built and compared.



A



**Figure 6.1** pH measurement and calibration based on absorption at 560nm. (A) Culture medium shows different colours under different pH values. From left to right, pH = 6.8, 7.3, 8.1. The medium shows yellow, red and pink colours respectively. (B) polynomial correlation building of pH and absorption (n=3).

#### *6.2.7 Drug treatment*

For static culture in standard 96-well plates, 100 µl of medium containing 2× final concentration drug or control (0.1% DMSO) was added in each well of 96-well plate (total volume of culture medium per well is 200 µl now). For perfusion culture, desired volume of medium containing drug or control (0.1% DMSO) was filled in 2 ml syringes, and perfusion experiment was set up in the bio-safety cabinet following a standard procedure. The perfusion rate is 10µl/hr. Morphology parameters of co-culture and cell viabilities were monitored at 3, 7 days and 14 days of drug incubation.

#### *6.2.8 AlamarBlue assay for cell viability testing*

AlamarBlue (Life Sciences) cell viability testing was conducted as shown in Section 4.2.7.

#### *6.2.9 Colony sizes and angiogenesis parameters measurements*

Inverted fluorescence microscope (Nikon, Eclipse TE 200) was used to collect images for colony diameter measurement and angiogenesis parameters evaluation. Then the collected images were analysed using imaging analysis software ImageJ from NIH. For the diameters of 3D cell colonies formed in both mono-culture of NCI/ADR and co-culture of NCI/ADR with HUVECs, the diameters were measured as  $d = (a \times b)^{1/2}$ , in which a is the longest Feret diameter of the colony and b is the shortest one (Friedrich, et al. 2009; Hsiao, et al. 2009; Kelm, et al. 2003). The reduction percentages of colony sizes were calculated as  $R(A) = ((\text{average colony diameter of model (A) treated by anti-cancer drug} - \text{average colony diameter of untreated model (A)}) / \text{colony average diameter of untreated model (A)}) \times 100\%$ .

The angiogenesis ability of HUVECs was measured using three parameters: Distribution of

tubule length, tubule area and number of branches. For tubule length measurement, a line was drawn along the structure, and the length was transferred from number of pixels into  $\mu\text{m}$  by calibration of ImageJ (See Materials and Methods in Chapter 3). The tubule area was also measured using ImageJ, and the numbers of branches were counted manually (Donovan, et al. 2001).

#### *6.2.10 Statistics*

Besides specific instructions, all experiments were repeated three times independently. Student's t-test was used to compare the significant difference between different culture methods.  $P < 0.05$  was considered to be significantly different.



## 6.3 Results and Discussions

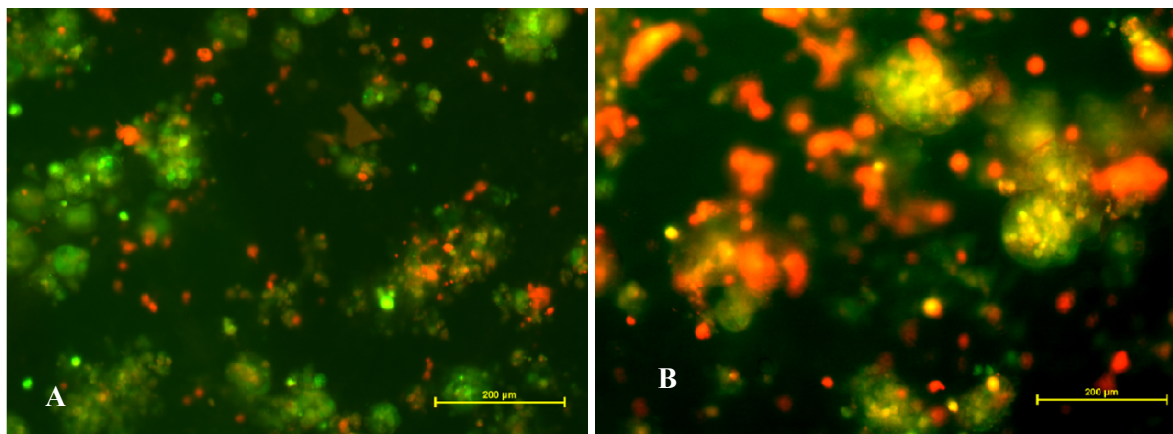
### 6.3.1. Long-term test comparing static and perfusion cultures

In Chapter 3, the results show that co-culture sandwich model can be maintained for 10 days. The hypothesis was perfusion would reveal further advantage for long-term culture. The long-term investigation was facilitated by an improved cell tracking strategy, by applying the live cell probes CellTracker Green CMFDA and CellTracker Orange CMTMR (Molecular Probes, UK). The feasibility pre-test confirms that the cells labelled by those probes can be passaged, frozen and defrosted with maintained fluorescence, and the fluorescence can be maintained for 14 days through the perfusion experiment. By using the living cell probe, the two cell types can be relatively easily investigated under fluorescence microscope, without extra fixation steps in immunochemistry, which could contribute to cell loss and lead to compromised data precision.

As shown in Figure 6.2, the perfusion group shows larger green fluorescent colonies formed by cancer cells compared with the ones in static group. Although the tubule structures formed by HUVECs started degradation over long term culture (14 days), the orange fluorescence confirms that the endothelial cells are still alive after such a long culture period. In the static group, the fluorescent areas for both cancer cells (green) and endothelial cells (orange) were significantly smaller than the perfusion group. This result shows that perfusion culture can increase the quantities of both NCI/ADR cancer cells (green) and HUVECs (orange). The possible explanation for this enhanced viability is that the homeostasis was maintained better in the perfusion group, compared with static culture, and this improved homeostasis allows the co-culture cells to grow in the environment with pH closer to optimal pH compared with traditional static culture. Another noticeable point in the Figure 6.2 is that the total cell viability in co-culture is larger than the sum of viabilities of NCI/ADR and HUVECs cultured

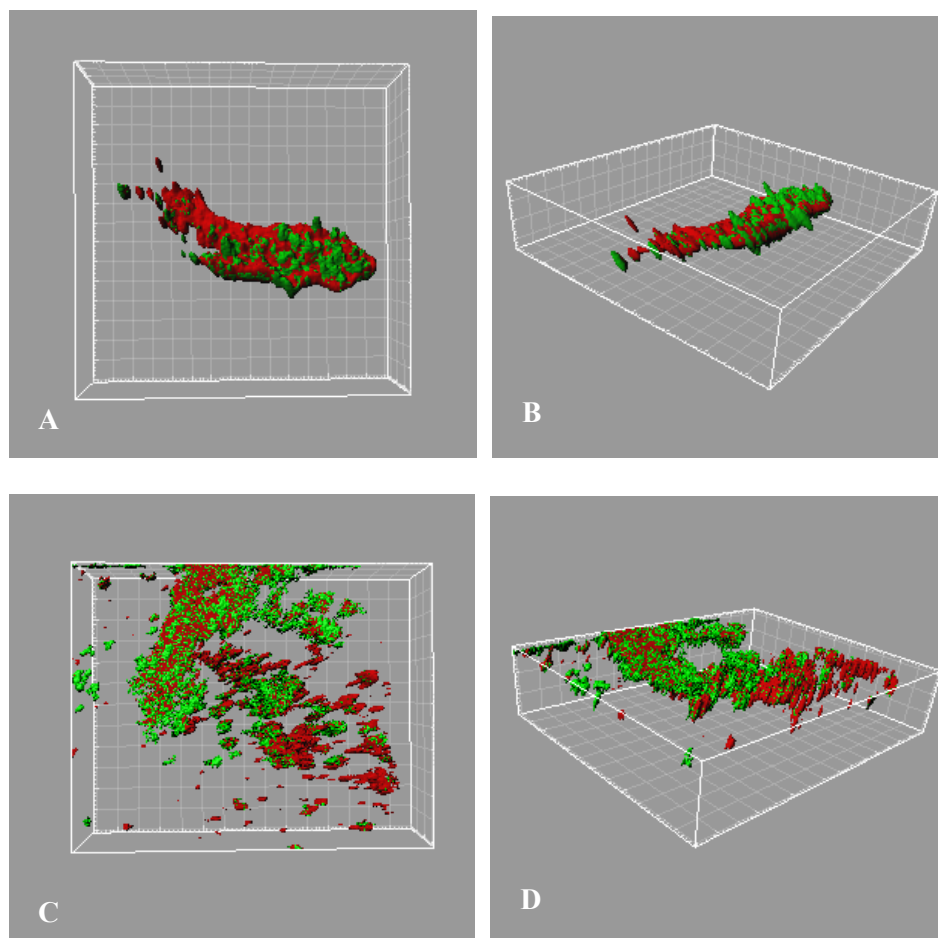
alone. This suggests that co-culturing those two cell types together must have enhancing effects on cell viability.

From Figure 6.2 we can see the integrity of tubule structures formed by endothelial cells is not sufficient for further image analysis, which is a limitation of this co-culture model over long-term culture. Nevertheless, compared with static group after 14 days of culture, the perfusion group still has more orange fluorescence labelled area – This still supports that perfusion itself is a way to enhance cell proliferation, especially for long term culture.



**Figure 6.2** Viability comparison of perfused and static co-culture. (A) static co-culture; (B) perfusion co-culture. Both groups were co-cultured for 14 days. Cancer cells NCI/ADR were stained green by CellTracker Green CMFDA, and endothelial cells HUVECs were stained orange by CellTracker Orange CMTMR (Molecular Probes, UK). Scale bar: 200µm.

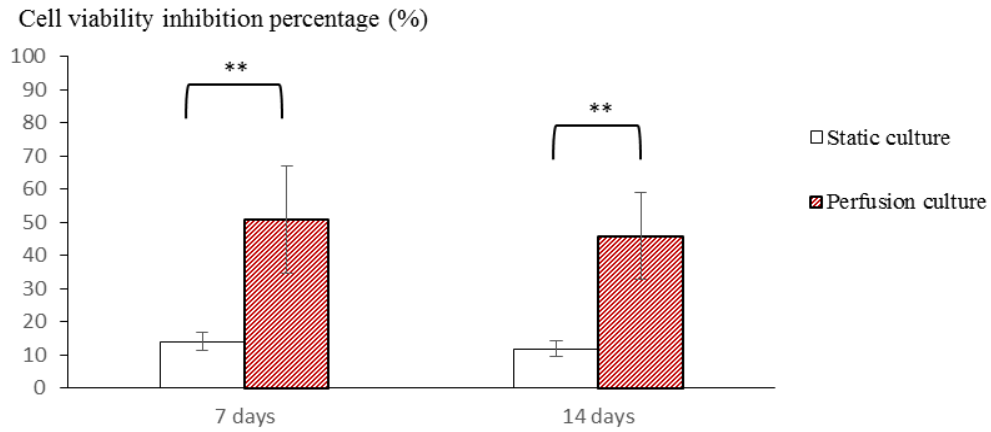
Multiphoton microscope imaging was also used to compare the perfusion and static co-culture. Compared with normal fluorescence microscope imaging, from MPM 3D reconstruction of co-culture structures it is clearer that though in perfusion co-culture after 14 days, the branches of tubule network were largely degraded, the tubule frames of co-culture formed by endothelial cells (red) and cancer cells (green) still exist with stable morphology features. On the contrast, in static co-culture, the morphology of co-culture tubule network was degraded and the co-culture cell mixture was scattered in homogeneous Matrigel.



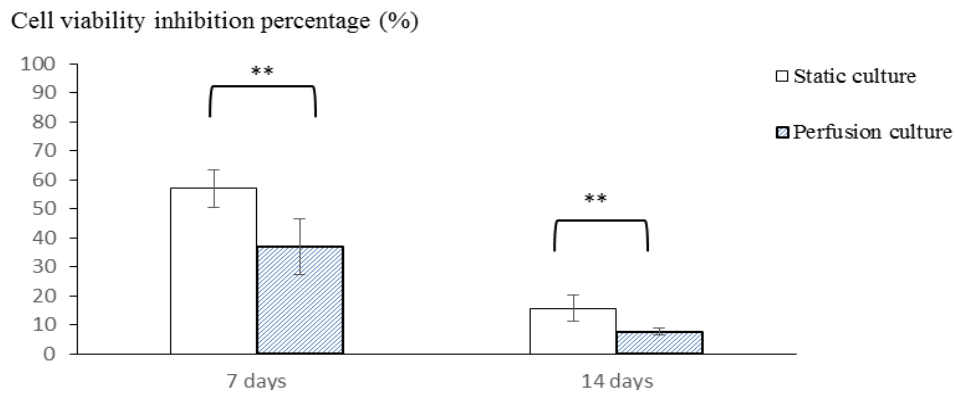
**Figure 6.3** Multiphoton imaging of representative samples comparing static and perfused co-cultures over 14 days of culture. (A)-(B), perfusion co-culture; (C)-(D), static co-culture. Green – cancer cells NCI/ADR, Red – endothelial cells HUVECs. Grid unit: 20 $\mu$ m. Perfusion enhanced tubule formation while increased the ratio of endothelial cells in co-culture.

### *6.3.2 Effects of perfusion on cell viability and 3D colony formation*

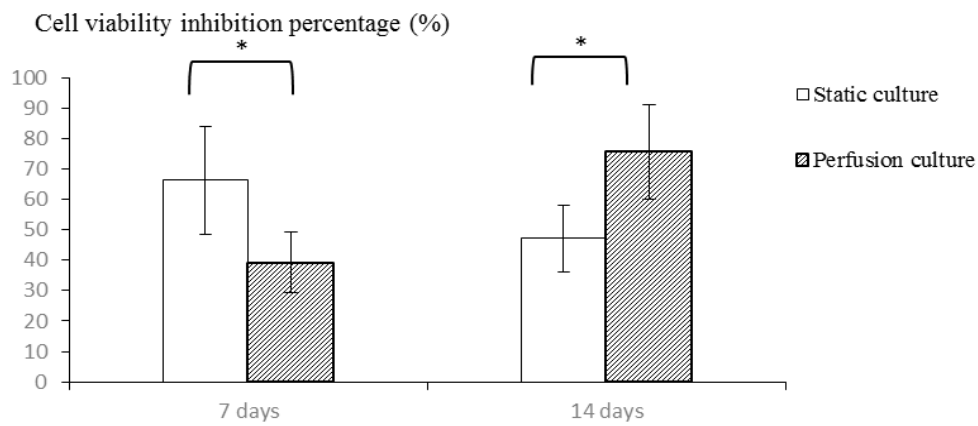
Both cell viabilities by AlamarBlue and colony sizes were used in the comparison of perfusion culture and static culture, and mono-cultures of HUVECs and NCI/ADR were treated under the same conditions as the co-culture of those two cell types (same seeding density for the same cell type). The mono-cultures of HUVECs and NCI/ADR show some interesting different acting patterns of Paclitaxel on those two different cell types. As shown in Figure 6.4 (A), in perfusion Paclitaxel had nearly as much as five times higher inhibiting effects on the viability of cultured HUVECs at both end-points ( $p < 0.01$  by Student's t-test). On the contrast, for NCI/ADR cultured alone in sandwich Matrigel, perfused Paclitaxel had lower inhibition effects on the cancer cells with significant difference ( $p < 0.01$  by Student's t-test). In a word, static and perfused cultures of HUVECs and NCI/ADR caused different responses to Paclitaxel. For endothelial cells, perfusion enhanced the viability inhibition effects on HUVECs, while in cancer cell line NCI/ADR the perfusion actually caused lower drug response, or higher drug resistance. Another difference in the responses of HUVECs and NCI/ADR to Paclitaxel is in general, drug resistance in NCI/ADR increased from day 7 to day 14, while for HUVECs the inhibition percentage of Paclitaxel did not change obviously. This is because endothelial cells have higher genetic stability compared with cancer cells, thus lower drug resistance can be induced over long-term application of medicine (Albini & Sporn, 2007), which again proves the potential of angiogenesis-related cancer therapy. The different responses of HUVECs and NCI/ADR can potentially explain the reverse of perfusion effects on Paclitaxel's inhibition of co-culture viability. As demonstrated in Figure 6.4(C), the response of perfusion group was lower than the response in static group at day 7, with significant difference by Student's t-test ( $p < 0.05$ ). However this trend was changed at day 14, in which the Paclitaxel in perfusion showed significantly higher inhibition effects compared with static co-culture ( $p < 0.05$  by Student's t-test).



**A**



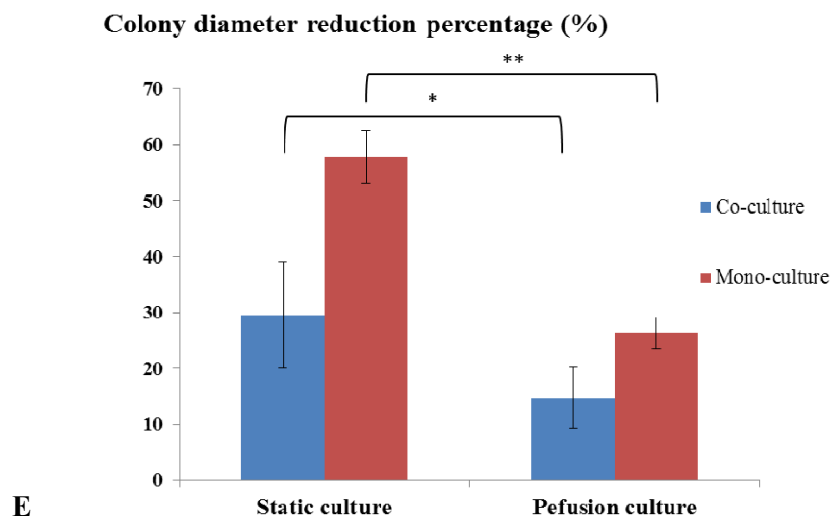
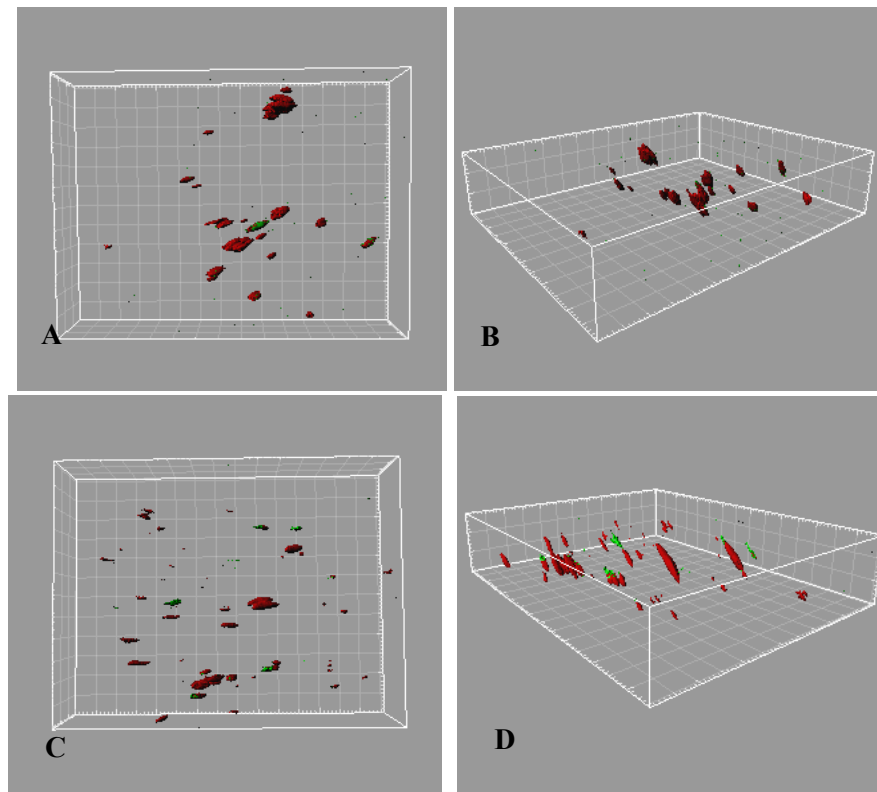
**B**



**C**

**Figure 6.4 Inhibition percentage of Paclitaxel on static and perfusion cultures.** (A) HUVECs cultured alone; (B) NCI/ADR cultured alone; (C) Co-culture viability testing. End-point assays by AlamarBlue was applied at day 7 and day 14. \*  $p < 0.05$  compared with static co-culture, \*\* $p < 0.01$  compared with static mono-culture.

Apart from cell viability comparison, colony size reduction in Paclitaxel (1 $\mu$ M) treated groups were also compared between static and perfusion systems to better investigate the effects of perfusion in 3D model testing. This is necessary because Paclitaxel treatment also showed an inhibition effects on colony formation in both static and perfusion systems. The results in Figure 6.5 (B) show that as for the colony sizes, the perfusion co-culture had lower drug response compared with co-culture in static culture. Moreover, as shown in Figure 6.5(B), the mono-culture of NCI/ADR was also considered in colony size measurement and comparison, and the co-cultures still had higher drug resistance than mono-cultures in general. Interestingly, this consistent higher drug resistance, or lower drug response observed in perfusion culture compared with static culture, is consistent with results from static culture testing demonstrated in Chapter 4. This implies that the positive crosstalk between cancer cells and endothelial cells in static culture could still exist in the perfusion system, and the perfusion system keeps the potential of using a co-culture model to investigate interactions between those two cell types, which mimic the real cancer progression microenvironment, such as cancer angiogenesis.



**Figure 6.5** Multiphoton imaging of representative samples treated by Paclitaxel for 14 days. (A)-(B), co-culture statically treated by Paclitaxel 1μM for 14 days, and the medium was changed every 3 days (72 hours); (C)-(D), co-culture perfused by Paclitaxel 1μM for 14 days, at perfusion rate of 6-10μL/hr. (E) Reduction of colony size. The experimental group was treated by Paclitaxel 1μM. \*  $p < 0.05$  compared with static co-culture, \*\* $p < 0.01$  compared with static mono-culture.

### *6.3.3 Effects of perfusion on angiogenesis parameters*

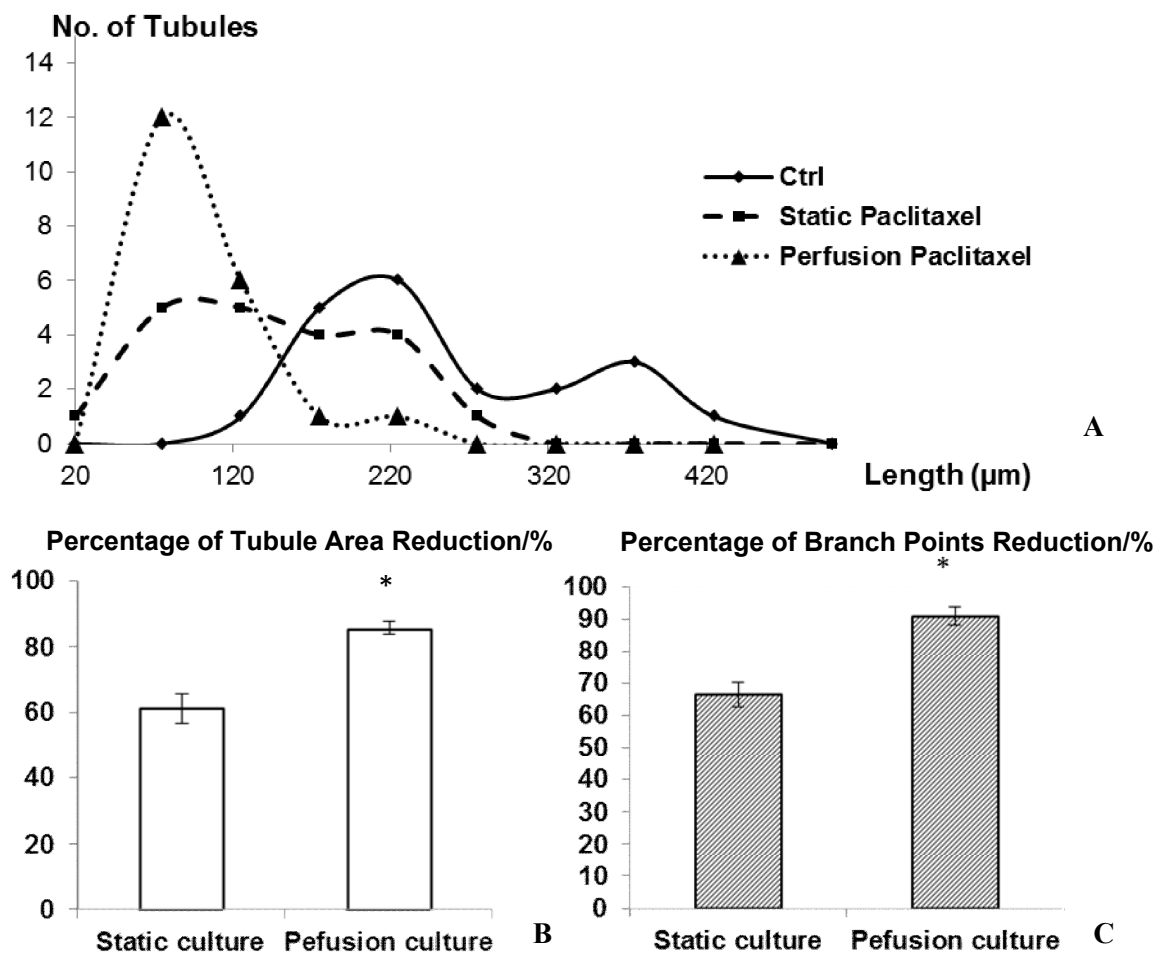
Higher sensitivity of the perfusion system to the anti-angiogenesis effect of Paclitaxel was observed. As shown in Figure 6.6, compared with static culture, in perfusion culture Paclitaxel caused more significant decrease of tubule length and the reduction of tubule area was 25% more in the perfusion system compared with the static system. Moreover, there was dramatic reduction of branching in the group perfused by Paclitaxel, compared with the group incubated with Paclitaxel in static condition.

For the tubule length distribution, the length of the majority (92.3%) of tubules shifted from 220 $\mu$ m to particularly 70 $\mu$ m in perfusion group (68.18% decrease), compared with static group 52.6% tubules had the length ranged from 70 $\mu$ m – 80 $\mu$ m, 42.10% tubules still had the length around 220 $\mu$ m, which is the length value for majority tubules in control group. This shows a more sensitive response in perfusion group.

Concerning the tubule area, the perfusion group had about 80% reduction in Paclitaxel (1 $\mu$ M) treated perfusion group compared with untreated perfused group. This inhibition effect has a significant difference (Student's t-test,  $p < 0.05$ ) from static system, which had an inhibition percentage around 60% in Paclitaxel (1 $\mu$ M) treated group compared with static untreated group.

Regarding the branch points, the Paclitaxel treated group in perfusion system witnessed a 90% decrease compared with 70% reduction of branch points in Paclitaxel treated group in static culture. This difference also shows a statistical significance of  $p < 0.05$  by Student's t-test.

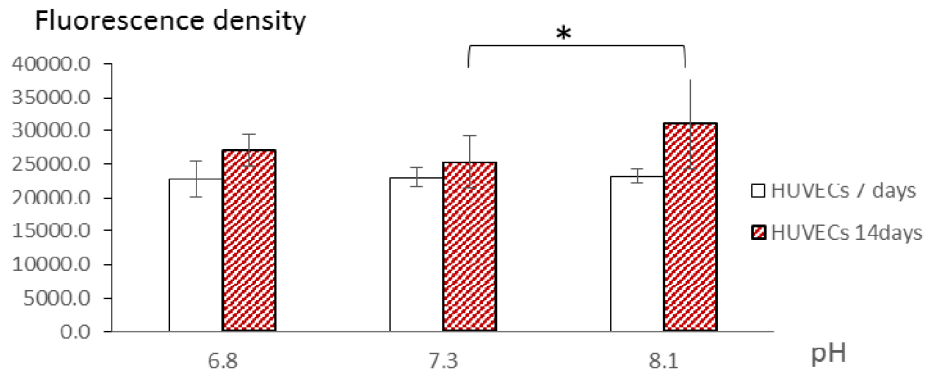




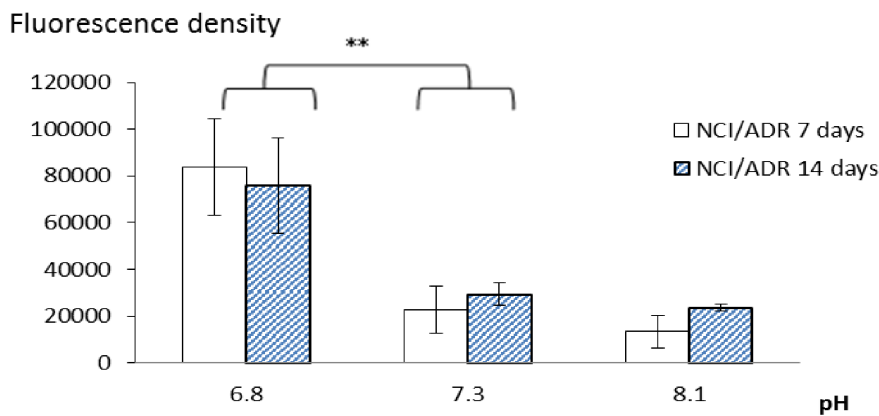
**Figure 6.6** Angiogenic parameters measurements in static and perfusion cultures. (A) Tubule length distribution; (B) Tubule area reduction; (C) Branch point numbers reduction. \* $p < 0.05$  compared with static culture group by Student's T-test. 5 random areas are selected from 3 parallel groups.

#### *6.3.4. pH of culture medium influences the growth of co-culture system*

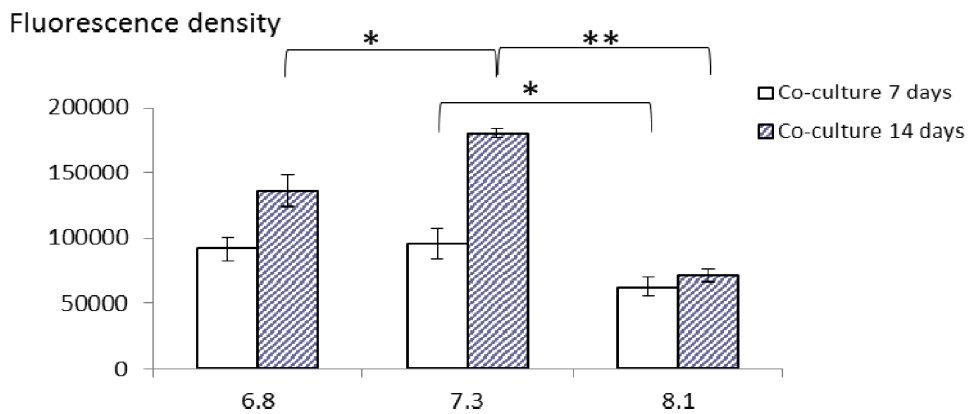
pH 6.8, 7.2 and 8.1 were chosen as the initial starting pHs for co-culture, based on previous research as representative and relevant pHs for cell and tissue culture (Ceccarini et al, 1971). As shown in Figure 5.2, different cell types require various optimal medium pH values. Based on Figure 5.2 (A), though at day 7 there was no significant difference among the selected pHs, at day 14 the best growth pH for HUVECs is relatively alkali pH 8.1 ( $p < 0.05$ , Student's t-test compared with pH = 7.3). As for cancer cells NCI/ADR cultured alone in Matrigel sandwich, even at day 7, there was a significant difference shown in the group cultured at pH 6.8, with  $p < 0.01$  compared with the pH 8.1 group and  $p < 0.05$  compared with the pH 7.3 group (Student's t-test). Another point demonstrated by Figure 5.2 is that the optimal pH for the co-culture of both cell types can be different from either of those cell types, as shown in Figure 6.7 (C), the highest cell viability of the co-culture was reached at pH of 7.3, with  $p < 0.01$  compared with the pH 8.1 group and  $p < 0.05$  compared with the pH 6.8 group (Student's t-test). Though at day 7 there was a similar trend observed among different pH groups (cell viability pH 7.3 > pH 6.8 > pH 8.1), a statistically significant difference was only observed between the pH 7.3 group and the pH 8.1 group ( $p < 0.05$  by Student's t-test), with no significant difference observed between pH 7.3 and pH 6.8. Based on previous research, cancer cells prefer a lower pH microenvironment, which is supported by Figure 6.7 (B). For HUVECs, interestingly, 7.3 is the pH of endothelial cell culture medium (EGM-2 and EBM-2) provided by Lonza, implying endothelial cells survive well at this pH. The preference of HUVECs for pH 8.1 shows a slightly different preference of HUVECs over a long-term culture of 14 days. Overall, the results in Figure 6.7 pointed out that the performance of the co-culture could be very different from the responses of a single cell type, and this unpredictability of co-culture characterisation should be taken into careful consideration in data interpretation.



A



B



C

**Figure 6.7** Cell viabilities were influenced by initial culture medium pH. (A) HUVECs only, in Matrigel sandwich culture. pH=8.1 was the optimal pH for HUVECs over 14 days of culture; (B) NCI/ADR only, in Matrigel sandwich culture. pH=6.8 was preferred, at both day 7 and day 14 end-point of assay; (C) Co-culture of HUVEC and NCI/ADR, in Matrigel sandwich. At day 14, pH=7.3 was the most suitable pH for the co-culture, which could be the overall behaviour of HUVECs and NCI/ADR. \*  $p < 0.05$ ; \*\*  $p < 0.01$  by Student's t-test.

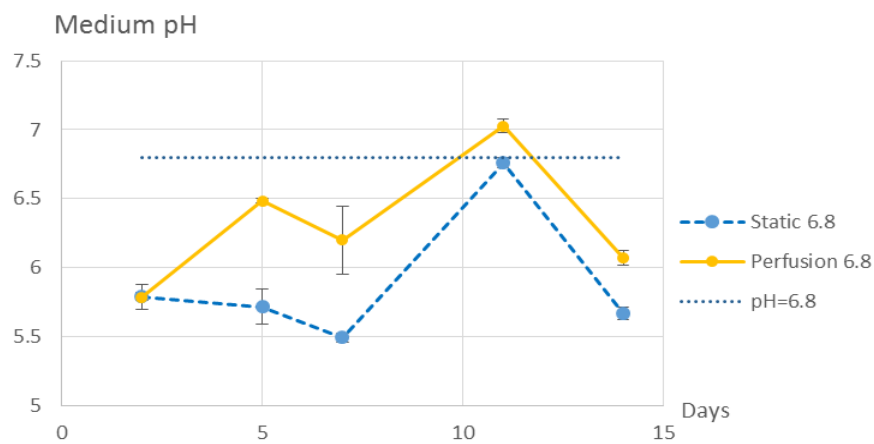
To confirm that culture pH can be better maintained in the perfusion platform compared with static culture, pH values of the culture medium were compared. For starting pH = 6.8, starting pH = 7.3, starting pH=8.1, the comparisons are shown as in Figure 6.8 (A), (B), (C) respectively. Overall, comparing the pH values of the medium of static and perfusion groups at the same time point, it is found that for perfusion cultures, the pH was maintained at a closer level to the original pH value of the culture medium, though oscillation was still observed. On the other hand, in static cultures, the medium pH went significantly lower than the medium pH in perfusion cultures. At day 2, there was no significant difference between static and perfusion cultures, and this is probably because that for a short term culture (<72 hours), perfusion does not show significant advantage. The medium pH of both static and perfusion cultures gradually increased over time, possibly because the cells in co-culture gradually reach the growing limit and cell metabolism and proliferation were not that active anymore.

The pH values of the culture medium at day 7 and day 14 were taken out as representatives to compare static and perfusion cultures, in correspondence to the pH-dependent viability test presented in previous Figure 6.7. As shown in Figure 6.9, starting from initial culture pH 6.8, at day 7 the pH of static culture was as low as 5.49, while in the perfusion medium the pH value was 6.19 ( $p < 0.01$  by Student's t-test); similarly, from initial pH = 7.3, the pH of static and perfusion co-cultures were 6.82, 7.19 respectively; and from initial pH = 8.1, the pH of static and perfusion co-cultures were 6.86 and 7.94 respectively. In other words, in static culture, at day 7 the pH value of the culture medium co-culture system was actually 6.82, while the perfusion system the culture medium had pH value of 7.19. From Figure 6.7(C), it is clear that the co-culture viability at pH=7.3 was higher than the viability at pH = 6.8. Thus,

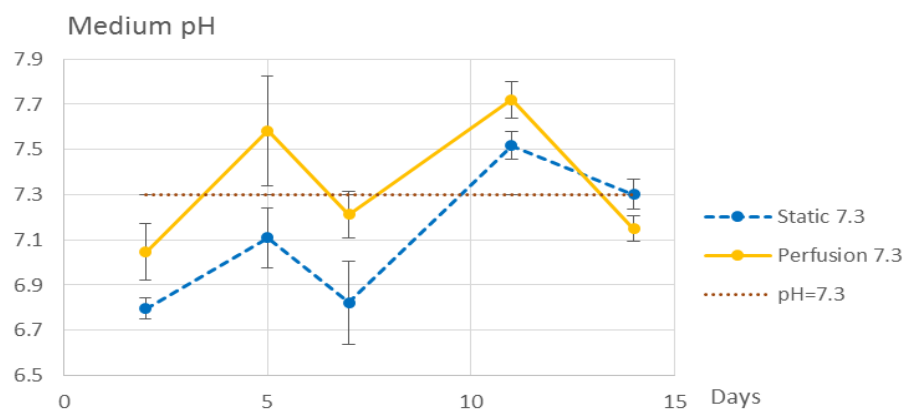
combining the results in Figure 6.7(C) and Figure 6.9(A) we can expect that the co-culture viability in perfusion with medium pH around 7.19 would be higher than the co-culture viability in static culture in which the medium pH was around 6.82.

Based on Figure 6.8, for a culture medium with the starting pH 7.3, which is the case in the cell viability comparison of static and perfusion cultures in previous section, the static group had the pH varied from  $6.79 \pm 0.04$  to  $7.52 \pm 0.06$ , and the medium pH was below 7.3 for most of the time especially before day 10. On the contrary, the pH of perfusion group starting from the same initial pH varied from  $7.01 \pm 0.12$  to  $7.72 \pm 0.08$ , with a more even fluctuation around the base line pH = 7.3. Thus, combining this result with the fact shown in Figure 6.7(C) that the viability is higher at culture pH=7.3 than pH=6.8, the explanation for improved cell viability in perfusion compared with static culture could be that perfusion maintained the culture medium pH at a level more suitable for the growth of co-culture.

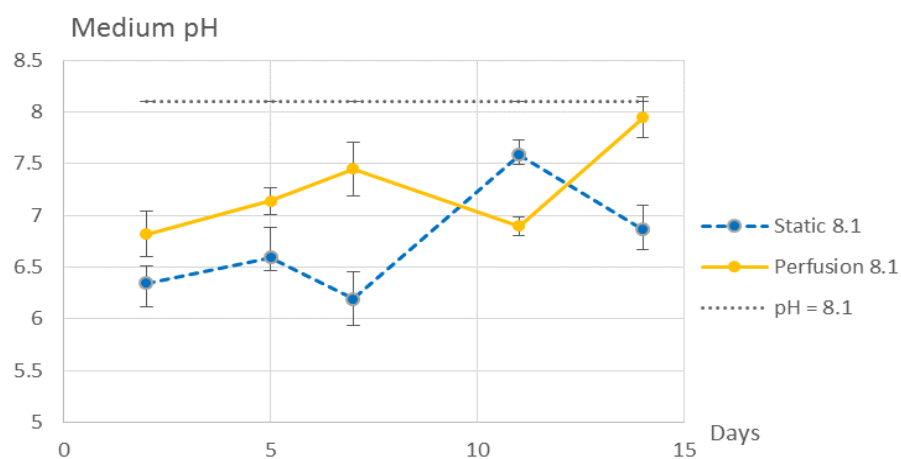
Overall, from Figure 6.7 it is proved that cancer cells are sensitive to culture medium pH, and endothelial cells also showed some response to the change of pH. The co-culture showed the highest viability at the relatively neutral pH (7.3). In Figure 6.8 and 6.9 the perfusion demonstrated its advantage of maintaining the culture medium pH around the preferred pH value, thus providing some mechanism explanation for the enhanced viability of the co-culture in perfusion compared with the co-culture static culture.



**A**



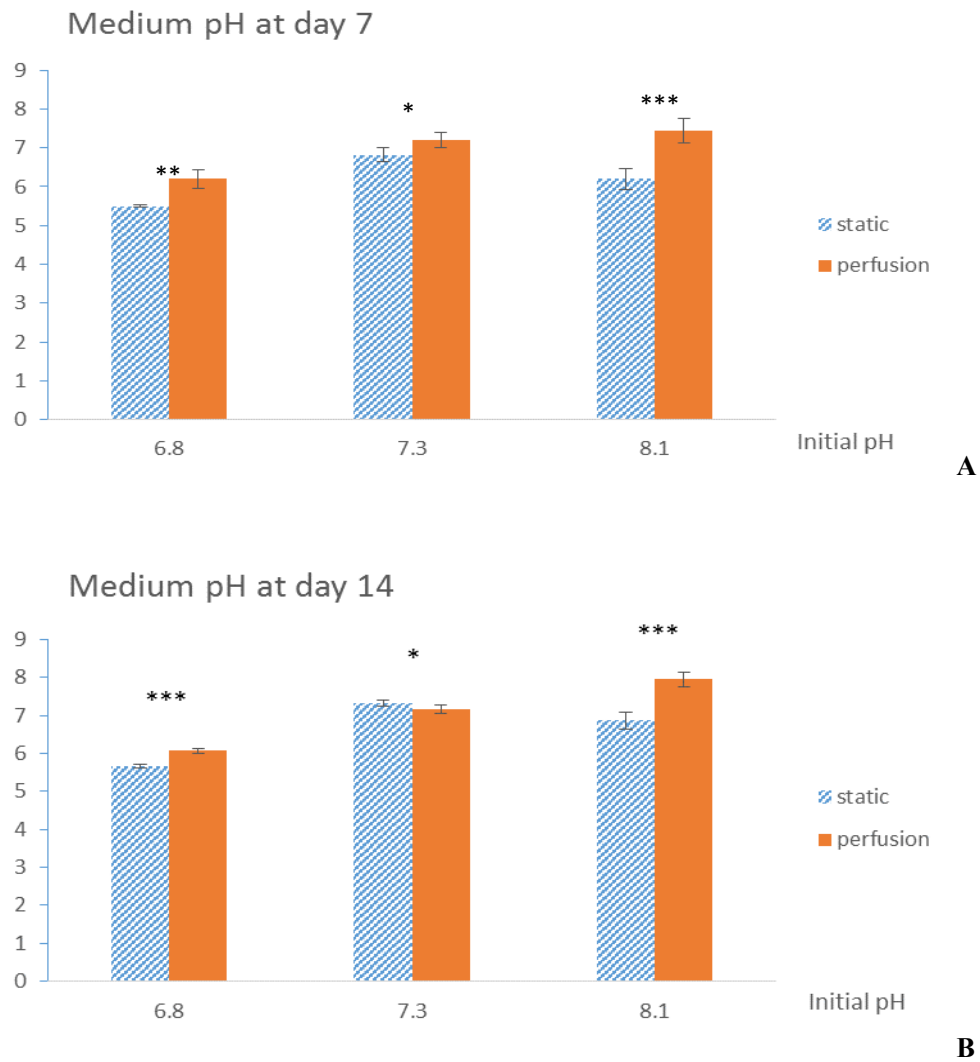
**B**



**C**

**Figure 6.8** pH monitoring for co-culture perfusion co-culture and static co-culture. (A)

Starting pH 6.8; (B) Starting pH 7.3; (C) Starting pH 8.1.



**Figure 6.9** The pH values of culture medium values for static and perfusion co-culture at end-point day 7 and day 14. Significant difference was observed between static and perfusion cultures initiating at three pH values. Starting with pH 8.1, the perfusion and static groups show most significant difference between them, while in the group with initial pH = 7.3 the difference was not so significant. \*  $p < 0.5$ , \*\*  $p < 0.001$ , \*\*\*  $p < 0.001$  by Student's t-test.

#### *6.4 Summary*

In Chapter 3 a PDMS- based perfusion platform has tested on widely accepted 3D tumour model spheroids, and the effects of perfusion on 3D tumour models were briefly tested. Then the co-culture of cancer cells NCI/ADR and endothelial cells HUVECs in Matrigel sandwich was built and characterised in Chapter 4, however the cell showed morphology of apoptosis and the co-culture structure degraded after 10 days. To extend the co-culture time in order to investigate anti-cancer drug effects over long-term, in this chapter, long-term testing (7-14 days) using perfusion system was carried out, and the perfusion system showed potential for long-term drug testing with Paclitaxel used as an model drug.

Apart from longer time of co-culture morphology and viability investigation, compared with previous chapters, another progress in this Chapter is live cell staining probes were used. This method can resolve the difficulty in differentiating the effects on different cell types in co-culture drug testing as described in Chapter 4. As shown in the multiphoton images shown in Figure 6.2 and 6.3, we can see there were still considerable cells surviving at day 14, which is in strong contrast to static culture. The results in section 6.2 show that perfusion, especially in long-term culture, can enhance the total cell viability. In following section 6.3 both cell viability and colony sizes were compared between static and perfusion cultures, revealing that the perfusion group had lower size reduction response to the tested drug, but higher viability inhibition percentage of the same drug was observed at 14 days of end point viability test. The angiogenesis parameter analysis in section 5.4 based on the tubule morphology formed by endothelial cells showed higher response to Paclitaxel in the perfusion group, compared with the static group. This effect of perfusion was confirmed by all the three parameters, including tubule length distribution, tubule areas, and branch point number counting.



The mitosis inhibitor Paclitaxel was chosen as the tested drug in this chapter, because other research has shown that the potential of Paclitaxel as a model for long-term anti-cancer drug testing experiments (Y. Dong et al, 2013), and showed different behaviours over 2D and 3D models (F. Perche et al, 2012). It is interesting that the results show that at day 7, static culture has a higher drug response compared with the perfusion group ( $p < 0.01$  by Student's t-test), but this trend was turned to be opposite at day 14, while the perfusion group showed higher inhibition percentage than the static cultured group ( $p < 0.01$  by Student's t-test). The effect switch by perfusion might be attributed to multiple factors, considering the diffusion of drug is influenced by both concentration driving force and resistance of the tissue barrier, and the complexity of the perfusion effects. It is necessary to consider the fact that Paclitaxel is highly hydrophobic, it is widely accepted that it will diffuse through cell membranes and interstitial space easily (Choi et al, 2011). There is also evidence showing that perfusion will increase the diffusion rate of hydrophobic small molecules (van Doorn et al, 1974), and increase of perfusion will facilitate in vitro penetration (Crutcher et al, 1969), suggesting perfusion will facilitate the diffusion process. It is possible that at day 7, though the equilibrium of drug concentration was built quicker in the perfusion group (Carrier et al, 2002) to facilitate the drug penetration, at the same time the enhanced extracellular matrix synthesis by perfusion culture (Davisson et al, 2002) increased the difficulty of drug penetrating the tissue. Overall the effect was a less significant drug response in the perfusion culture group. . Nevertheless, at day 14, because the perfusion provides a better drug penetration environment due to the consistent drug concentration gradient over the cell membrane compared with the fluctuating drug concentration in the static culture, as well as a weakened interstitial barrier for the drug to penetrate through the 3D cell culture, and the drug penetration will increase over time with the interstitial space widened up by the

apoptotic skilling abilities of Paclitaxel (Choi et al, 2011) the drug response of the perfusion culture showed higher sensitivity to Paclitaxel over this long-term perfusion treatment.

Considering the importance of pH for cell viability and other behaviours, to test the hypothesis that perfusion can improve the co-culture viability by maintaining the culture medium pH at a more stable level, in this Chapter a detailed pH-dependence experiment was carried out. The viabilities not only for the co-culture of cancer cells and endothelial cells, but also mono-culture of those two cell types were compared to elucidate the contributions of those two cells in the behaviours of the co-culture. The results of the pH-dependence test show the high dependence of cell viabilities on the pH of the culture medium, and different cell types showed different preference for culture medium pH. For specific cell type, the higher cell viability may be achieved under certain pH even with the other culture parameters remaining the same (e.g., initial seeding density), for example, the cancer cells NCI/ADR preferred more acidic pH 6.8 while HUVECs had higher viability at pH=8.1 (though not significant until day 14). It is noticeable that apart from the influence of perfusion on culture medium pH, the sheer stress would also be a potential influence factor. Though the sheer stress was not large in low rate perfusion, previous research on cardiac tissue showed that even low rate perfusion can improve the function and survival of cell culture (Carrier 2002). In addition, a preliminary study by the author (not shown here) suggested that cell morphology can be potentially influenced by the mechanic stimuli caused by the perfusion culture, in combination with other people work showing the relation between cell morphology and function of vascular endothelial cells (Santini et al, 2000), it is possible that the response of endothelial cells play some important roles of the drug responses of the whole co-culture system.

Overall, the results of this chapter show the potential and advantages of perfusion culture in the application of co-culture model testing, with higher sensitivity to the tested representative drug Paclitaxel. However, because of the high complexity and biological activity of Matrigel (Kleinman et al, 2005; Hughes et al, 2010), further comparisons between Matrigel and relatively inert scaffolds should be further compared, to further reveal the influence of Matrigel in co-culture system's performance in drug testing.

## Chapter 7. Conclusions and Future Work

### 7.1 Conclusions

In this thesis, advanced three-dimensional tumour models for anti-cancer drug testing have been developed. The techniques of co-culture and perfusion were successfully applied in these models. The co-culture of endothelial cells and cancer cells demonstrated that the interplay between cancer cells and other supporting cell types, such as vascular endothelial cells can be mimicked to certain level via 3D advanced tissue engineered models, and the drug responses on these models can be different from the data obtained from models based only on cancer cells. The perfusion platform showed the potential for long-term drug testing, with improved cell viability and morphology integrity. Furthermore, the co-culture and perfusion techniques can be combined together, to mimic the microenvironment of cancer development reflecting the cancer tissue growth for a longer period with consideration of interactions with endothelial cells. The effects of perfusion on the co-culture system should be interpreted in the context of multiple factors, including chemical environment such as pH of the culture medium, different cell types and their responses to perfusion, the acting mechanisms of the tested drugs, as well as the culture time and end-point testing time.

The perfusion system was first tested on a spheroid model - a well understood 3D model of cancer. This model was chosen because it is the most widely accepted model, with considerable previous report supporting its feasibility in anti-cancer drug testing using 3D *in vitro* model. To simplify the results at this stage, no artificial extracellular matrix was introduced, and the parameters were straightforward, only concerning the dimensions of the spheroid. In order to test the feasibility of using tissue engineering to mimic the intercellular interactions in cancer microenvironment, a breast cancer model co-cultured with endothelial

cells was developed. Drug testing using co-culture model in Matrigel was applied, and results were compared. It was found that co-culture has the potential to influence anti-cancer drug testing results, and the pattern of influence depends on the acting mechanisms of the drugs. Two types of material have been used to create 3D co-culture models – Matrigel, and alginate. They have been chosen because of their representative features – Matrigel is a widely used material to represent extracellular matrix, but its components are very complicated, leading to difficulty in interpreting data; alginate has well-studied chemical features, but it is very inert and has no biological activity. It is found that alginate embedded co-culture did not show any tubule formation, and the tubule structure was only observed in the Matrigel sandwich model, but not in co-culture in homogenous Matrigel. With the basis of above perfusion and co-culture testing, drugs were eventually tested on a co-culture model in perfusion. From the drug testing results, it is found that perfusion enhanced the viability and angiogenic abilities of the co-culture model. The effects of perfusion on different parameters vary, for example cancer cell colonies sizes and anti-angiogenesis parameters show opposite trends comparing perfusion and static culture systems.

To interpret the results in this thesis, because multiple factors were tested, it is important to view the results across the chapters to have a clear picture of the potential effects of a single factor on the developed models and their drug responses. For instance, to have a whole picture of the effects of co-culture with endothelial cells on the cancer models, we can analyse the results from both static culture (Chapter 4) and perfusion culture (Chapter 6). Taking the results from those two chapters together, we can see the co-culture of endothelial cells with the cancer cells can improve the survival of those two types of cells, compared with endothelial cells or cancer cell cultured alone in the same method. For the responses to

anti-cancer drugs, the introduction of endothelial cells will influence the drug response in some cases, as shown in the testing with Cisplatin and Paclitaxel.

Similarly, to consider effects of the perfusion in general, it is helpful to take results from both Chapter 3 and Chapter 6 into consideration: on both the mono-culture of cancer cells in spheroid form and the co-culture of cancer cells and endothelial cells in Matrigel sandwich culture, we can see perfusion enhanced the viability and improved the nutrient supply to the cultured 3D structures, especially in long-term culture (17 days for colorectal cancer cell spheroids in Chapter 3, and 14 days for co-culture of endothelial cells and cancer cells in Matrigel sandwich co-culture). One interesting result is the drug testing results from both perfusion co-culture and mono-culture system showed higher sensitivity to Paclitaxel, a drug mainly acting on microtubules, though results for other drugs have more complicated responses to the introduction of perfusion. This could imply that a close link of perfusion conditions to the functions of microtubules. However, further studies need to be conducted to confirm this and other factors such as how the perfusion might influence the diffusion process of Paclitaxel, and the binding of Paclitaxel to albumin in the culture medium should also be taken into consideration.

In conclusion, the developed models in this study demonstrate the importance of the conversations between blood vessel cells and cancer cells, which are crucial in the development of cancer tissue and resistance of cancer cells to the therapeutics. In other words, cancer cells working together with blood vessels are much stronger than cancer cells alone. This is proved by the fact that co-culture with endothelial cells enhanced the viability of cultured tissue, with a lower drug responses to the tested anti-cancer drugs. Another novelty in this thesis is the application of engineering tools in developing tumour models. By

integrating a well-built perfusion platform into those tissue engineered models, the improved perfusion 3D cancer testing system has a better nutrient supply and more a stable culture environment. This improvement makes the methods set out in this thesis more suitable for long-term drug testing, which suggests a resolution to a major limitation for anti-cancer drug testing.

Overall, the successful application of co-culture and perfusion suggest that tissue engineering can provide a novel dimension to solve the problems in traditional *in vitro* cell-based testing and *in vivo* animal-based experiments. Using engineering methods, human-origin cells can be used to build up cancer tissue *in vitro*, to mimic the intercellular and cell-ECM interactions *in vivo*, and to test drugs in a well-controlled testing environment, with lower cost and less stress experienced by researchers compared with animal testing. These advanced three-dimensional tumour models have the potential to reduce animal testing, not only in future cancer research, but also in other areas of biotechnology and the pharmaceutical industry.

## 7.2 Future work

In this study, a co-culture of cancer cells and endothelial cells was built up for anti-cancer drug testing, especially for Paclitaxel, the anti-mitosis anti-cancer reagent with inhibiting effects on both cancer cells and endothelial cells. Both the viability and morphology parameters demonstrated by this co-culture model were investigated and compared, and the drug responses of both Matrigel sandwich and embedded cultures, as well as Matrigel and alginate were compared in drug testing. Although Matrigel sandwich co-culture showed potential for an efficient drug testing platform with tubule structures formed to mimic the angiogenesis process in endothelium-tumour interactions, the tubules can only be maintained for 10 days and even with perfusion the tubule structure will degrade, though endothelial cells were still observed to be alive. In other co-culture method such as Matrigel embedded

co-culture or an alternative scaffold, alginate, the tubule structures were not observed. In future study, in order to maintain the tubule structures for longer, the scaffold for the co-culture needs to be improved.

Though Matrigel is widely applied in pharmaceutical research and has approved positive effects on cell proliferation, its physical and mechanical properties are limited, and it is rich in complex and yet not fully characterised bioactive components. Thus, Matrigel is not an ideal scaffold yet, and other scaffold materials should be considered for future study, such as alginate with some biological modification. Another potential direction for future study is the improvement of perfusion liquid. At the moment it is the optimised perfusion medium based on standard culture medium (EBM-2, Lonza) was used for the perfusion. If a medium mimicking real human blood or body fluid was applied, the behaviours of the co-culture might be closer to the *in vivo* situations. However, ethical issues must be taken into consideration for this possibility of using human originated perfusion medium.

Another limitation is that for angiogenesis, even though endothelial cells are the main participants, there are other cell types like fibroblasts involved. Thus in future study, more cell types should be considered for co-culture. This could lead to a more complex co-culture system with higher difficulty of data interpretation, and challenges of cell labelling of each individual cell type. However, the building up of co-culture system with more cell types will help the 3D co-culture cancer *in vitro* models to mimic the *in vivo* behaviours of cancer issue at a better level. In the final stage of the author's DPhil study, the co-culture of cancer cell line NCI/ADR, primary cultured human umbilical vein endothelial cells (HUVECs) and human breast gland fibroblasts was explored briefly. Some interesting results were revealed



including the morphology change and viability variation compared with the two cell co-culture of cancer cells and endothelial cells. This preliminary study needs further results to support a few hypotheses, but it definitely demonstrated the significance of this study direction in the future. In the future study, new techniques, such as 3D printing, can also be tested to see if the spatial relations between those co-cultured cells can be further optimised, and if new models in addition to sandwich model can be developed. In this study, mainly breast cancer models were developed. In future research, models for more cancer types should be developed, such as colorectal cancer etc., to further test the feasibility of the developed models.

Future studies can also extend the developed new models into testing of more anti-cancer drugs, with the possibility of correlating the viability and morphology measurements to the acting mechanisms of these drugs. It is noted in this thesis the hypoxic specific anti-cancer drug TPZ showed some interesting results in the comparison study, and in the future study the new generation of anti-cancer drugs highly targeting at cancer tissue such as TPZ can be tested on the developed 3D models. Another type of anti-cancer drug that can be considered to be tested is tumour angiogenesis regulating drugs, such as specific angiogenesis inhibitors. For example, angiostatin and endostatin, the two most widely accepted endogenous anti-angiogenesis molecules, as well as the combination of those two drugs can be tested. Commercially available angiogenesis regulators such as Avastin can also be applied on the new models, to see if the new tissue engineered *in vitro* models can reflect the real clinical responses of this drug, which is already proved to be quite different from its pre-clinical data.

Finally, in future research, ideally the newly developed 3D *in vitro* tumour models should be validated in comparison with *in vivo* studies, or even better, in comparison with the clinical results of the same tested drugs. This validation study will answer the question of whether tissue engineering really has the potential to mimic the *in vivo* behaviours of the tested drugs in physiological situations. In summary, the results in this thesis show both advantages and limitations of the Matrigel sandwich breast cancer *in vitro* model co-cultured with endothelial cells, and the results from these results will give clues to future study which can apply more cancer cell and supporting cell types, new scaffold materials, improved perfusion platform into the testing of a wider range of anti-cancer drugs.

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