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Isolate and identified cancer stem cells by using MCF7 human breast cancer cell line, using mammospheres formation in two different methods.

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Abstract

Cancer represents one of the most significant challenges facing biological and clinical research. A higher understanding of the theory and mechanism of cancer will allow scientists to develop an active target therapy of cancer diseases. It has been suggested in several studies that a tumour that has been derived from many cells that have the ability to rapidly reproduce many more new cells and increase the tumour. Cancer stem cells are one of these types of cells which have been isolated in different parts of the body such as in the breast, prostate and colon. Breast cancer stem cells (BCSCs) have been shown in several studies to comprise of small parts of cancer cells which lead to an increase the prognosis of breast cancer. This type of tumour cells able to develop tumour generation. The focus of this research will be in breast cancer (BC) which still the main issue of public concerns. As a result of many studies which show that BC remains the popular cancers among women in the world. In China, for example, more than million cases each year. In spite of all the concentration to discover a new, fast diagnosis method and treatment, BC still forms the second causes of death among women in the world. The majority of cancers incidence are belonged to uncontrolled on the rapid growing of the cells.

In this project, MCF7 human breast cancer cell line used to isolate cancer stem cells by using mammospheres formation in two different methods.

The main target of this work was to isolate cancer stem cells from human breast cancer cell lines and compare with the parental cells, single and cancer stem cells which we have identified by using spheres formation assay.

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Introduction

Cancer is defined as a gathering of diseases which is portrayed by the uncontrolled development of cells. If this rapid growth of cells is left to continue, the result is death (American Cancer Society, 2015; Hue et al., 2013). This malignancy represents one of the most significant challenges in clinical, biological and medical research. Normally the body includes trillions of living cells which can divide and grow quickly in younger people to allow their body to grow while it will grow more slowly in elderly people. In the case of cancer, the cells begin to grow and reproduce abnormally, without control. (American Cancer Society, 2014, 2015; Xu et al., 2015) These cancer cells are different in their generation due to continuous growth of cells that form more and more cancerous cells. These cells are called cancer cells because of the lack of control in the growth and invasion of other parts of the body. This invasion is called metastasis. Cancer cells are caused by damage to their DNA, which, unlike normal cells, do not repair or have apoptosis occur. DNA damaging may occur due to genetic causes, environment (for example, ultraviolet radiation from the sun) or without any clear reason at all. Because the cancer cells do not renew or perish, they reproduce rapidly, creating cells that the body is not in need of (American Cancer Society, 2008, 2010; Van-De Vijver et al., 2002).

There is a variety of different cancers that inhabit various parts of the body. Therefore, it requires a good diagnostic method to discover the particular area being affected by cancer to have an effective treatment. Cancer is classified into two types, benign and malignant. Benign cancer is a tumorous growth that grows slowly and does not spread to the rest of the body. This means is not as dangerous as malignant cancer because when it is removed there is no recurrence. (American Cancer Society, 2014; King and Robins,2006). Malignant cancer is a tumerous growth that *can* spread to other areas of the body and cause different cancers (Pecorino, 2005).

There is evidence to suggest that the proliferation happens in the cells because of the changes in the genome and epigenome (Wang *et al*., 2012; WHO, 2014;Wie et al.,2011). Cancer forms several diseases that

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spread in different parts of the body which are known as malignant tumours or neoplasms. Studies show that cancer can be controlled if there is an early diagnosis and if some of the risk factors are avoided. Some of these risk factors are smoking, drinking alcohol, lack of physical activity and obesity. All of these are external factors while the genetic mutation, changes in hormones, immunity and race are examples of internal factors (King and Robins, 2006; Sagar *et al*., 2007). Data show that cancer is very popular in the UK. Approximately 331,500 cases of cancer were diagnosed in 2011 (Wang *et al*., 2012). Diagnosis is difficult because there are more than 200 types that all need different diagnosis methods (Cancer Research UK, 2012; Sagar *et al*., 2007). Treatment of cancer is either by surgery, chemotherapy or by radiotherapy (American Cancer Society, 2014; Weinberg, 2007). The biology of cancer includes several stages that lead to a formation of a tumour by carcinogenesis, in which the tumour is induced and formed. This process includes three steps, initiation, promotion and progression (King and Robins, 2006; Weigelt, Kreik and Reis-Filho, 2009).

CANCER STEM CELLS

For a decade, several studies suggested the theory that tumours are arranged in a hierarchy of heterogeneous cells which includes cancer stem cells (CSC) (Chilet *et al*., 2014;Hermann et al.,2010). CSCs provide an exceptional understanding of cancer, growth, resistance to drugs and cancer recurrence (Velazquez *et al*., 2009; Baker, 2012; Sehawat et al.,2012). Cancer stem cells can be defined as a small subpopulation of cancer cells that produce cancer-initiating cells (Liang *et al*., 2013; Collins et al.,2005). Studies assume that to clarify the origin of CSCs two factors need to be illustrated; the number of mutations and stem cells. It is believed that CSCs originate from self-renewal stem cells and progenitor cells (Sagar *et al*., 2007; Shackleton *et al*., 2009; Hardt and Dittmer,2001). These cells are able to regenerate rapidly and differentiate to form a tumour. Another feature of CSCs is that these cells are resistant to several chemotherapy and radiotherapy treatments (Wang *et al*., 2014; Saadin and White, 2013;Whica,2009). Cancer stem cells are known as cancer-initiating cells or tumour

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initiating cells. These cells had been isolated for the first time in 1994 by Charles *et al*. AL-Hajj *et al*. conducted a study that indicated that CSCs found in solid tumours and leukaemia can contain breast carcinoma (Lorico and Rappa, 2010). Cancer stem cells can be found in a different type of tumour such as breast, brain, colon, ovary, prostate and pancreatic (Wang *et al*., 2012). These cancer stem cells are found in a small percentage of tumours. It was believed that one single cancer stem cell might proliferate and produce a greater expansion of heterogeneity of tumour cells. More research needs to be done to find the ideal treatment for these cells due to their high resistance to drug therapy and radiotherapy. Studies have shown that CSCs form important evidence related to the theory of heterogeneity among cancer cells. These cells are characterised by a hierarchy of subpopulations of tumour and non-tumour in cancer stem cells (Heitzer *et al*., 2013; Ponti *et al*., 2006). Different studies illustrate the theory of cancer stem cells, which show they are tumorigenic, they can differentiate and they can self-renew to produce daughter cells (Charaf-Jauffret *et al*., 2008). Cancer stem cells are generated from cellular or tissue level while the tumour proceeds from a cellular system that is exhibited by Stemness (Max *et al*., 2006). Cancer can be connected with CSCs due to several cancers depending on cancer stem cells (Max *et al*., 2006). CSCs can be an ideal pathway of prognostic factors with malignancy tumours (such as breast, colon and prostate) which is assessed in the diagnostic procedures (AL-Hajj *et al*., 2003; Wang *et al*., 2014).

STEM CELLS

Stem cells can be defined as the cells that are derived from the haematopoietic system. These cells originated from bone marrow or peripheral blood (Sagar *et al*.,2007; Reya *et al*.,2001), and have the capacity of self-renewal, are distinguishable and drug resistant (Velazquez *et al*.,2009;Heitzer *et al*.,2013;Liang *et al*., 2013, Llipoulous *et al*.,2011; Tuck and Miranker, 2010;Dick *et al*.,2008). It has been suggested that stem cells need to be eliminated to stop the growing of the cancer stem cells to another part in the body. The majority of cells in the body stay for a short period. Therefore, the body renews them continuously (Baker,

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2012). Stem cells can be found in every tissue in the body. The other main properties of these cells are their ability to make exact copies of themselves in a process called self-renewal. Also, they can differentiate into other cells types that make the organs of the body (Reya *et al*., 2001; Cyranoski,2012). Stem cells have been discovered for the first time in 2003 in a solid tumour by using U-M centre. We knew that stem cells have been involved in cancer for more than 20 years (Saadin and White, 2013). These cells form ideal targets in biomedical research by targeting molecular pathways because the function of these cells is: self-renewal and differentiation (Tuck and Miranker, 2010). In addition to that, stem cells share the same features with cancer stem cells, for that reason the issue will be more complicated and need a high focus on these cells (Badve and Nakshatri, 2012).

BREAST CANCER STEM CELLS

It has been identified in several studies that breast cancer stem cells are heterogeneous in their morphology and function (Senbanjo, Miller and Hawkins, 1986), which occurs because of the self-renewal features and initiation tumour. 40% of breast cancer recurrence cases have been reported as initiated from CSCs (Shaw *et al*., 2012; Morel *et al*., 2008; Graham and Graeber, 2014). Data show there are more than 1,000,000 BCSC cases and 410,000 deaths each year (Pham *et al*., 2012). In recent studies, the origin of BCSCs has been debated. There is evidence to suggest two concepts that try to clarify the origin [figure1.3] (Velazquez *et al*., 2012). One of them refers to CSCs as being sustained from deregulated normal cells' self-renewal pathway. This would be occurred because of the similarities between normal cells and cancer stem cells. Another theory suggests that BCSCs might result from (EMT) epithelial-mesenchymal transition (Velazquez *et al*., 2011; Fillmore and Kuperwasser, 2008; Velazquez *et al*., 2012). Studies show that there are different methods to investigate cancer stem cells because it is hard to identify CSCs from only using the microscope due to their high capacity to form tumours and metastatic (Conley *et al*.,2012).Therefore, several biochemical techniques need to be developed to detect BCSCs (Van De Vijver *et al*.,1999).

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EVIDENCE OF PERDENSE CANCER STEMS CELLS

There is evidence provided by Lic *et al*., suggesting that pancreatic cancer cells with a high frequency of CD44 markers have a greater tumourgenicity. Another important piece of proof of the existence of BCSCs was proposed by AL-Hajj *et al*, where they found that tumour-initiating cells can identify from BC (CD44+/CD24- low) cells (Lic *et al*., 2007).

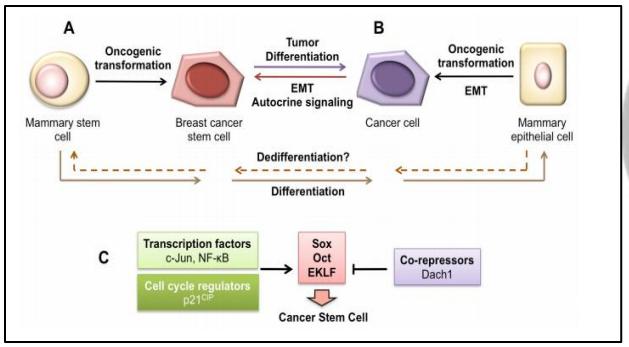

Figure 1.1 the formation of cancer stem cells

Figure1.2 shows the formation of cancer stem cells. There are two models of how to generate CSCs. In the first theory, the tumour cells originate from stem cells that are called the linear hierarchy. In the second model, CSCs initiate from evolved EMT to produce cancer cells or as a response to the tumour microenvironment. (Velazquez *et al*., 2012).

The Function of breast cancer stem cells:

SELF-RENEWAL AND DIFFRENTATION

The major important function of stem cells is their capacity of self-renewal. Studies show a variety of pathways that can applied in this feature such as Notch, Hedgehog and Wnt. Another important function of BCSCs is the differentiation of non-stem breast cancer cells. It has been estimated that retinoic acid and ALDH+ form a crucial pathway to manage on the differentiation of BCSCs. The inducing in retinoid signalling trigger to reduce mammospheres formation. Then will lead to induce gene expression in the distinguishable breast cancer cells and down-regulated those genes (Velazquez *et al*., 2012; McDermott and Wicha, 2010).

DRUGS RESISTANCE

It has been clarified that in several papers that BCSCs resist to chemotherapy and radiotherapy (Germain *et al*., 2012) Using assistance chemotherapy will increase part of CD44+ tumour cells. It is estimated that drugs resistance in BCSCs is related to self-renewal cells pathways, which involve Notch, Wnt, Hedgehog and HER-2. For

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example, Notch-1 overexpression is related mainly to the drug resistance and radioresistance of breast cancer stem cells. Notch pathway has the capacity to raise the anti-apoptotic gene and D1. The latter will improve the Notch-1 activity by inhibition the expression of cell regulation. Different studies proof that D1 is imperative as a target to expand stem cells (Velazquez *et al*., 2011).

METASTASIS

The breast cancer initial tumour is responsible for the metastasis. This process refers to the invasion of cancer from one part to each part in the body. This procedure remains till now under debates. There is evidence proposed that BCSCs form an essential roles in metastasis because it will increase the cell movements, infestation and gene expression. All of these roles need to the end to metastasis (Strati *et al*., 2011).

THE MOLECULAR SIGNALLING PATHWAYS OF CANCER STEM CELLS

BCSCs are growing very slowly, therefore, it is required a signalling patterns for the reproduction and self-renewal (Nguyen *et al*., 2010; Wicha, 2009). Research suggest that it is worthy to determine the main signalling pathways in CSCs that consider as the main cellular target for treat cancer stem cells. This includes the identification of the cell surface markers which related to cancer stem cells (Foltz *et al*., 2009). In the experiments, research shows that CSCs markers such CD44, CD133 represent the fundamental process of the isolation breast cancer stem cells (Kim *et al*., 2005). A study by AL-Hajj *et al*. was the first step to isolate solid tumour indicating that reverse and forward cancer rely on the microenvironment of BCSCs (AL-Hajj *et al*., 2003). There are three important signalling pathways which is involved in the reproduction of stem cells and cancer stem cells such as Shh-Sonic hedgehog Wnt/Catenin, Notch, Hox gene and Bmi-1 which help in the transformation of CSCs from stem cells and normal stem cells organization (Nguyen *et al*.,2010; Bjerkvig *et al*.,2005; Sagar *et al*.,2007). These different pathways have been formed overexpressed in spheres from breast cancer cell lines such as; MAPK, Notch and Wnt which are related to BCSCs markers aldehyde dehydrogenase (Lorico and Rappa, 2010; McDermott and Wicha, 2010).). It is

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estimated that Wnt signalling important pathway to maintaining cancer stem cells and it is originated from Drosophila gene wingless. Also, it was suggested that the dysregulation of this pathway leads to produce cancer (Nguyen *et al*.,2010). More recently, studies show the high connection between Wnt signalling, FGF, Hedgehog and TGF-β, which lead to stopping the regulation of EMT, which involves snail and TWIST. Another pathway was Hedgehog, which increase spheres formation (Saadin and White, 2013). In addition, this pathway includes different ligands which allow for this signalling such as; sonic Hedgehog (Shh), Desert Hedgehog (Dhh) and Indian Hedghog (Ihh). All of these ligands can bind to the receptor and the latter will form essential pathway in stem cells reproduction. Moreover, the inhibition of these pathways signalling will lead to induce the reduction of the generation of BCSCs (Nguyen *et al*., 2010). For all these function, Notch pathways as a several papers indicate its involvement in normal cells and cancer stem cells self-renewal (Wicha,2009; Morel *et al*.,2008).

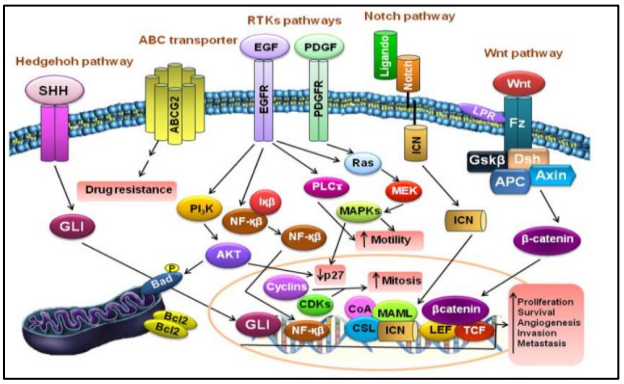

Figure 1.2 the main signalling patterns that indicate the Wnt, Hh and Nothch pathways that are related to breast cancer stem cells (Vera-Ramirez et al., 2010)

HETEROGENITY OF CANCER STEM CELLS

The evidence of CSCs is very argumentative by researchers. Cancer stem cells form a group of tumour cells which produce and initiate tumour (Li *et al*.,2009; AL-Hajj *et al*., 2003; McDonald et al.,2012).Cancer stem cell has the capacity for self-renewal and differentiation to heterogeneous subpopulation (Velazquez *et al*.,2009; Swanton,2012; Gerlinger et al.,2012). Several experiments have been approved that tumour emerges of a heterogenic of cells. Particularly, CSCs, which involve a specific population of heterogenic cells. A variety of evidence assumed that phenotypic population have been isolated from breast cancer stem cells and characterised by CD133+ and CD44+. Several markers

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expressed and used in isolating CSCs (Li-Heidt *et al*., 2007). This isolated cells can generate phenotypic heterogeneity from the parental cells (Reya *et al*., 2001). Intratumour heterogeneity is frequently observed in across multiple tumour types. There is evidence suggest that Increase in the heterogeneity result to resistance to many targeted therapies (Heitzer *et al*., 2013; Dick *et al*., 2003). The theory of heterogeneity in breast cancer remains unclear till now. It is believed that cancer stem cells have contributed to tumour heterogeneity (Badve and Nackshatri, 2012). Heterogeneity clearly refers to a tumour which involve in different types of cells. The majority of studies supported the idea of heterogeneity of breast cancer stem cells because they found that different group BCSCs express a variety of cell surface markers such as CD133 which is important markers in breast cancer stem cells (Lassus *et al*.,2010;Wei *et al*.,2011). In study carried out by Murohashi et al., they used different cell lines to detect the expression of genes. The co workers, found that these cell lines had a variety of CD44 markers to identify breast cancer stem cells (Murohashi et al.,2010; Ababneh et al.,2013).

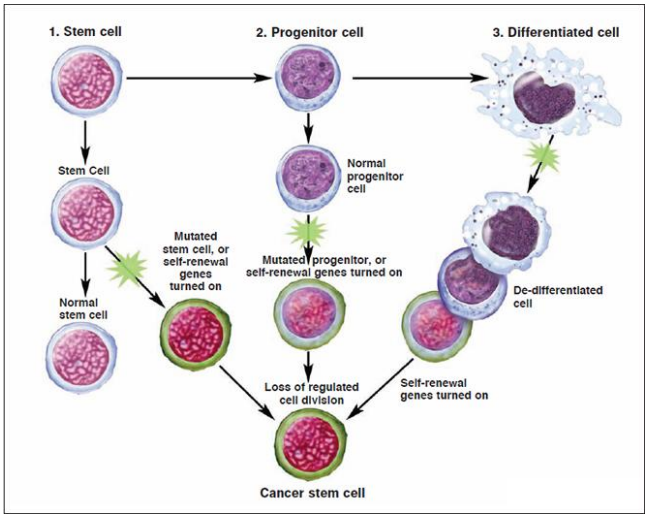

Figure 1.3 models of cells which are initiated cancer stem cells. (Velazquez et al.,2009)

ISOLATION OF CANCER STEMS CELLS

CSCs as a small population of tumour cells, therefore, identify and isolate these bulk of cells consider as a significant challenge for recent studies (Saadin and White, 2013). Enriching of cancer stem cells allows to discover an ideal solution for diagnosis and prognosis. By wiping outgrowth foundational microorganisms particularly, which is the hotspot for malignancy development, it can minimise the requirement for surgery and chemotherapy. Cancer stem cells are available to a little extent of the tumour, consequently it is trusted that traditional chemotherapies could just execute separating cells, which form the main part of the tumour, however, are not

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able to produce new cells (Razmkayshi et al.,2012). The number of inhabitants in CSCs could remain untouched. Studies have demonstrated that undeveloped tumour cells had been impervious to illumination treatment and chemotherapy medications, for example, It is an anthracycline drug that acts by intercalating DNA strands, bringing about complex development which hinders DNA and RNA combination. In this way, ideal strategies must be looked into to target undifferentiated disease organisms particularly, by going for their multiplication and development pathways. Hindering these pathways might thus stop their quick rate of tumour proliferation (Phue *et al*., 2011). Isolation and characterization of CSCs considered as the main challenges for clinical and biological researchers (Jaggupilli and Elkord, 2012).There are three techniques have been used widely to identify BCSCs. Firstly, fluorescence-activated cell sorting(FACS), which rely on CD44markers, CD133 and other markers. Secondly, identification of side population (SP) that one effluxes Hoechst 33342. Finally, mammosheres assay (Pham *et al*., 2012; Saadin and White, 2013; Yan et al.,2013;Brugnoli et al.,2013). CSCs can be isolated by using putative surface markers. Using these markers genes that establish more isolated cells such as CD44, CD133 can found in a different type of tumour; breast, colon and lung. It is estimated that these markers work very effectively when they emerge with each other (Jaggupilli and Elkord,2012; Bc-Sun et al.,2013;Sheridan et al.,2006). Ponti *et al*., were the first to detect a new method to isolate mammospheres from human breast cancer depends on the mammospheres forming assay in serum-free medium on the non-adherent flask. This technique could be used in the culture of normal epithelial cells. Therefore, this is important because using molecule to adhere the cells which are attached strongly to each other, while when we added trypsin the cells will separate from each other and float making clusters of cells in different shapes. This means that adding trypsin will change the form and movement of cells. Ponti and co-workers found that CD44+ phenotype is the same to malignant mammospheres that discovered by AL-Hajj *et al*. and co-workers (Lorico and Rappa, 2010; Sahlberg et al.,2014). Theoretically, breast cancer stem

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cells have been approved by several studies in vivo and in vitro.it is assumed that CSCs cause imitation, metastasis of cancer. In order to study cancer stem cells in details, it is required to identify the biomarkers of CSCs that form an essential target for treatment. Using molecular methods allow research to investigate different types of protein which used as markers specific for cancer stem cells. It is noteworthy to understand that BCSCs have been enhanced by characterising breast cancer cells for CD44 by culturing of cells in non-adherent conditions to produce mammospheres (Wang *et al*., 2014). Using serum free media provides an ideal method to enhance cancer stem cells. Several studies have been used low attachment surface and serum-free media which lead to producing clusters of stem cells. One of these studies was used neural cells that generate neurospheres after using the conditions above. Dontu *et al*., was the first were used non-adherent culture and serum free media. The result were BCSCs can be used to enrich by using this technique. The concept behind that is the cells will die in these conditions and the other cells that survive called mammospheres. In these study, they demonstrate that by using this simple method in vitro and culture, mammospheres method allow to generate tumorigenic CSCs from the other population of tumour cells. Therefore, spheres formation assay can be an ideal target to study cancer and solve the issue that validate with cancer stem cells theory (Saadin and White, 2013;). This project will shed light in vitro toward the determination and generation of proposed breast cancer stem cells in human BC cell line (MCF7) by using direct spheres assay to confirm the presence of CSCs in the tumour.

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- Methodology

Materials and chemicals

Material for tissue culture

This equipment has been used in the tissue culture method, as presented in a table (3.1).

Laboratory materials for tissue culture

The laboratory material	Suppliers
Class-II safety cabinet	Microflow Biological Safety, UK
Centrifuge (refrigerated)	Eppendorf, UK
Humid 37°C CO_2 incubator	Forma Scientific, UK
Micropipets P10, P20,p100 and p1000	Gilson, UK
Water distillation unit	Millipore, USA
Inverted microscope & imaging system	Nikon, Japan
Inverted microscope	Olympus, Japan
Tubes size $25cm^3$	SARSTEDT, Germany(reference number: 62.547.254)
$T75cm^3$ and tissue culture flasks(red)	SARSTEDT 83.3911.002 Germany
Water bath	Grant, UK (Fisherbrand)
$75cm^3$ ultra low adherent tissue culture flasks(ORANGE)	Corning, USA LOT 3814
5mL, 10mL and 25mL disposable pipettes	Sarstedt, Germany
10ul, 200ul and 1000ul Micropipettes tips	Sarstedt, Germany
0.2um and 0.45um sterile filters	Sarstedt, Germany

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Haemocytometer	Minisart Sartorius, Germany

Table 3.1 laboratory material for tissue culture.

Chemical and reagents for tissue culture

Chemical	Suppliers	References
Dulbecco's Modified Eagle Medium (DMEM)	Lonza, UK	
Epidermal Growth Factor	Sigma-Aldrich, UK	(Kreso and O'Brien 2008)
Foetal Calf Serum (FCS)	HyClone, UK	
Trypan blue	Sigma-Aldrich, UK	
Trypsin-EDTA	Lonza, UK	
DMSO (Dimethyl Sulphoxide, 100ml	Sigma-Aldrich, UK	RNBB7017
bFGF (Fibroblast Growth Factor Basic	Sigma-Aldrich, UK	

Table 3.2 chemicals and reagents for tissue culture

METHODS

BREAST CANCER CELL LINE

The human breast cancer cell lines MCF-7 that were provided kindly by Dr Selman Ali from Nottingham Trent University were used in this project. These cell lines were cultured to isolate prospective cancer stem cells.

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CELLS REVIVAL AND MAINTENCE

Initially, the cells were harvested after being in the freezing media which contains Nitrogen. They were removed and then centrifuged at 1500rpm for 5 minutes. The cells were planted in Dulbecco's Modified Eagle Medium (DMEM), which contains insulin 5mg/ml and epidermal growth factor (EGF) long/ml, in adherent tissue culture flasks size T75. The new culture was incubated at 37◦c with 5% CO2. The cells were regularly checked every two days to see the percentage of the growth if cell splitting is possible. Normally, the cells take two days to reach 70 to 80% confluence. After the growth of cells had been checked under the microscope, the cells were collected, halved and replanted in new flasks and fresh media.

CELLS CULTIVATION TO PRODUCE SPHERE

Culturing of cells in non-adherent, non-differentiate growth condition

When the cells have a greater confluence than 80%, they need to be reseeded. The media was removed from the flask gently and the cells were washed twice with PBS. Trypsin was then added (1-2 ml depending on the flask size) to the cells and incubated at 37°C for 5 minutes. The reason for this is to help the cells float in the flask and stop attachment to the flasks surface. By adding serum- containing a medium, the effect of trypsin was attenuated. The cells were suspended and centrifuged and a white cells pellet was seen at the bottom of the new tube. The cells were planted as pellet and the supernatant was discharged. The fresh media that was added contains (DMEM) and 10% of (FCS). Lastly, the cells were incubated at 37'C in 2 flasks each time, one as normal culturing and the other for producing spheres. This method was repeated for several (5-10) times to confirm the results.

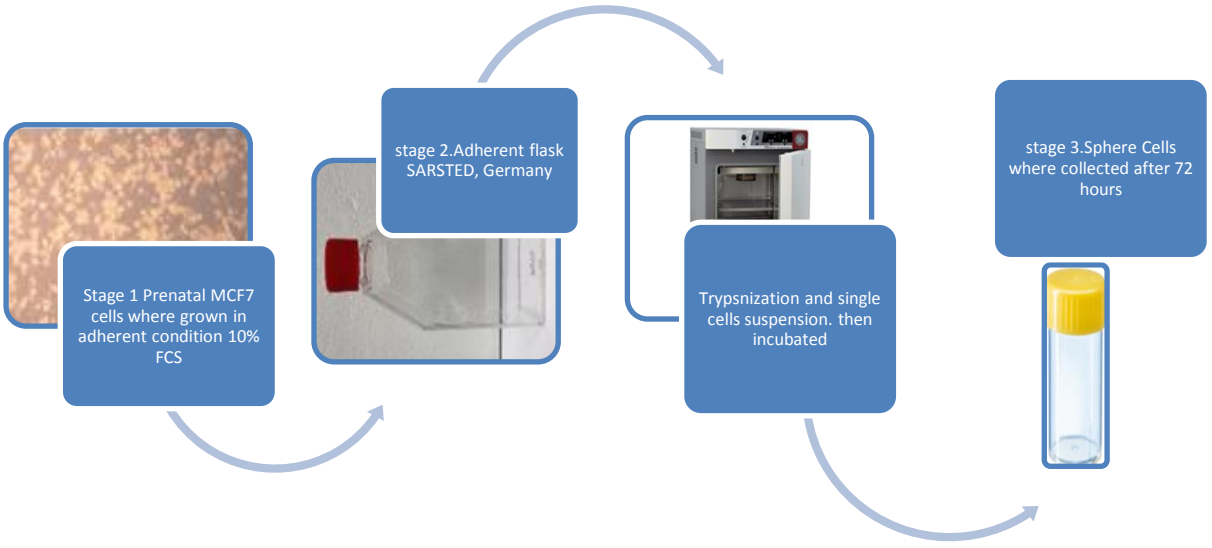

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Figure 3.1 Direct Sphere Formation Assay Using Adherent Conditions

3.2.3.2 Culturing cells in Ultra-low Attachment surface polystyrene e (U shape with Vent cap)

The cells were cultured using a low attachment surface (Corning Flask). The cells were split into two and washed by PBS and trypsin. The cells were grown in a Corning Flask with new media by adding 15% serum free media which includes DMEM/F-12 1µl, BFGF 10ng, EGF 20ng, Insulin 5µg and the percentage of foetal calf serum FCS was increased.

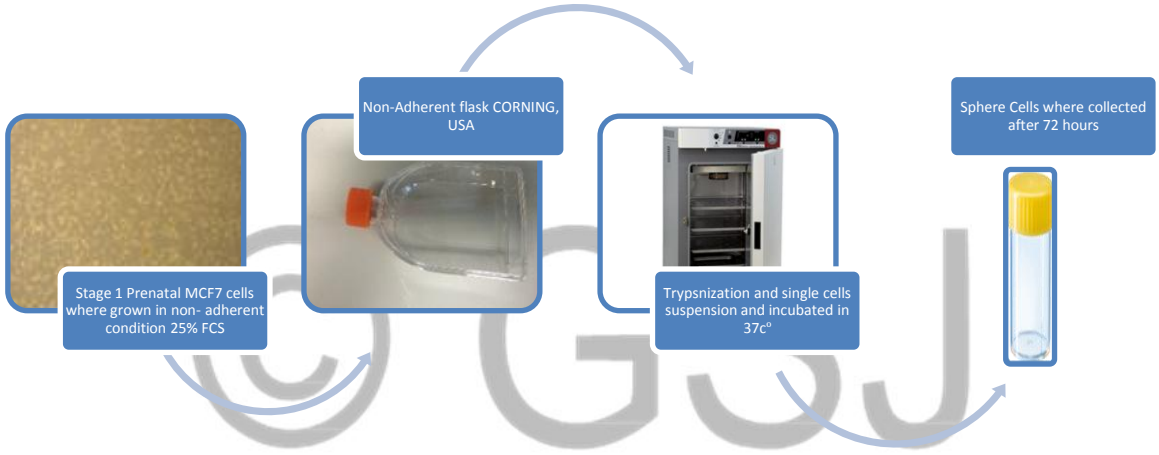

Figure 3.2 Direct Sphere Formation Assay Using Non-Adherent Conditions

COUNTING THE CELLS

This step was applied in order to know the number of the cells by using a haemocytometer. The pellet was taken from the cultured cells and suspended in 1ml of media then 100ml of the suspension was diluted with 900ml of trypan blue in a 1 to 2 dilution. The last dilution was then loaded to the haemocytometer. The cells were counted under the microscope with an x10 magnification (when the cells were not painted with trypan blue). The number was timed by 10^5 to calculate the total of cells per 1ml of suspension. The total number of cells in a large flask size T75 was around $1.2 * 10^6$ cells/ml.

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FREEZING THE CELLS

The cells were prepared for freezing, to be kept for a long time. The cells were collected and suspended in 1million cells for each 1ml of freezing cell media. The latter was prepared by adding 10% Dimethyl disulfoxide (DMSO) to keep the cells alive and DMEM media with 10% of FCS. Then the cells were filtered and collected in small freezing sterile tubes and were maintained at -80 ^{o}C for a long period of time.

RESULTS

TISSUE CULTURE RESULTS

Producing mammosheres of MCF7 cells by using standard conditions (normal flask and normal media)

Figure 4.1spheres formation assay of MCF7 cells by using normal culturing condition (media with serum and adherent flasks).

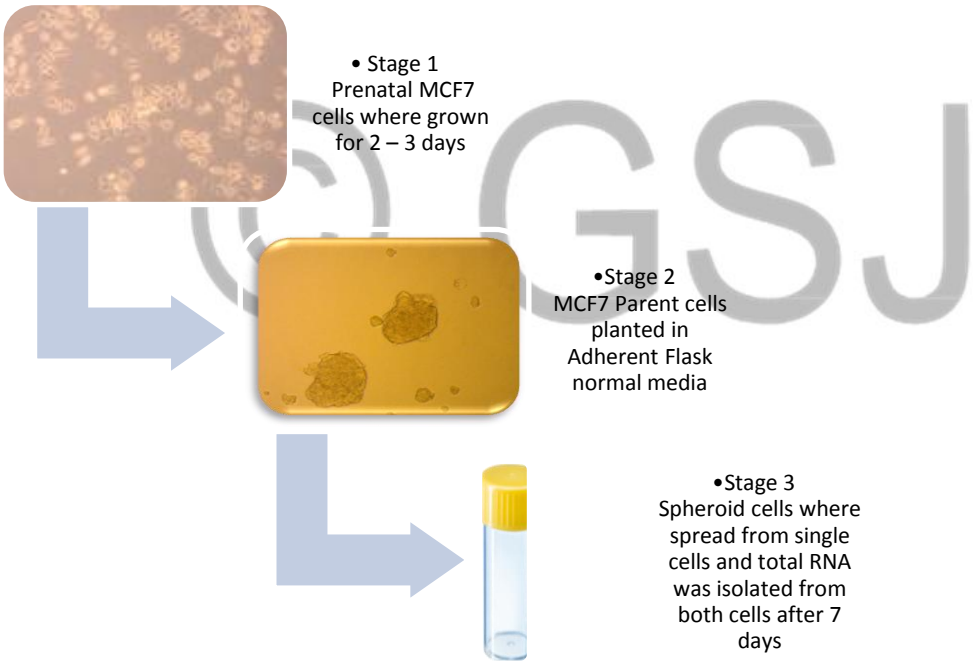

Figure 4.1 Phenotypic morphology of MCF7 cells by using standard conditions.

This experiment includes several stages. The first phase, the parental MCF7 cells were grown under standard conditions for five days (normal conditions such as normal flask and normal media). In stage 2, the parental MCF7 were split into two groups. The first population were normal cells and the second one was spheres in the normal flask. In stage 3, the spheroid was separated from the single cells. The RNA was isolated from parental, single and spheres cells respectively.

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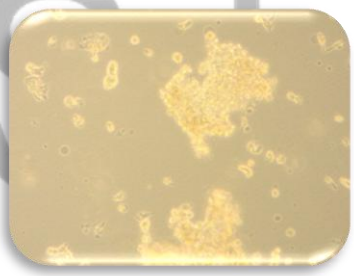
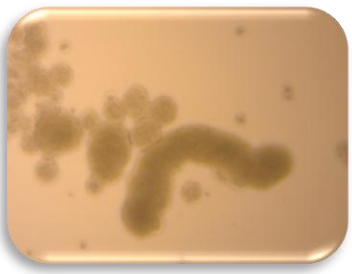
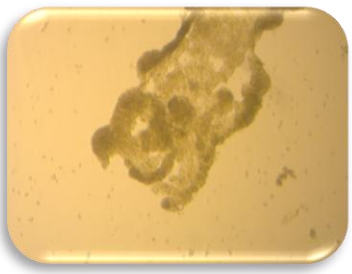
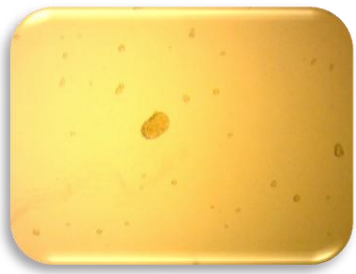
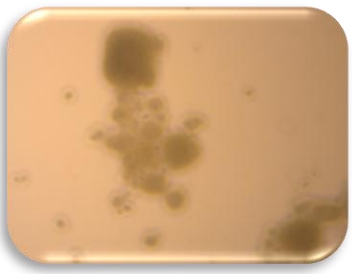
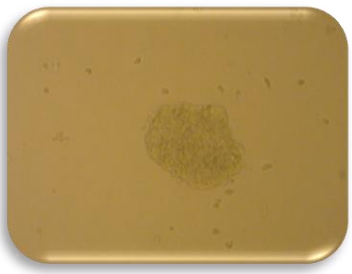
Figure 4.1 shows a trial to isolate spheres from parental MCF7 cells by using mammospheres assay. Several steps have been applied in this attempt. First of all, the MCF7 parental cells were grown to the level of over-growth of 70-80% and the total RNA was isolated at this stage. In stage 2, the spheroid is in a different shape and has formed adherent cells. In stage 3, the total RNA was extracted from spheres cells after 72 hours and 96 hours. MCF7 single cells were separated from the spheres cells and the total mRNA was isolated from these cells as well. MCF7 cells were planted in standard conditions that included normal culturing flasks and normal media and 10% of foetal calf serum (FCS). In this part, the cells clustered as a group of cells to produce spheres that are assumed to be mammospheres.

Figure 4.2 shows spheres generated from MCF7 cells grown in normal conditions (flask and media) indicating the changes during 24, 72 and 96 hours.

Spheroid after 24 hours 4 X magnification	Spheroid after 72 hours 10 X magnification	Spheroid after 96 hours 20 X magnification
		
		
		

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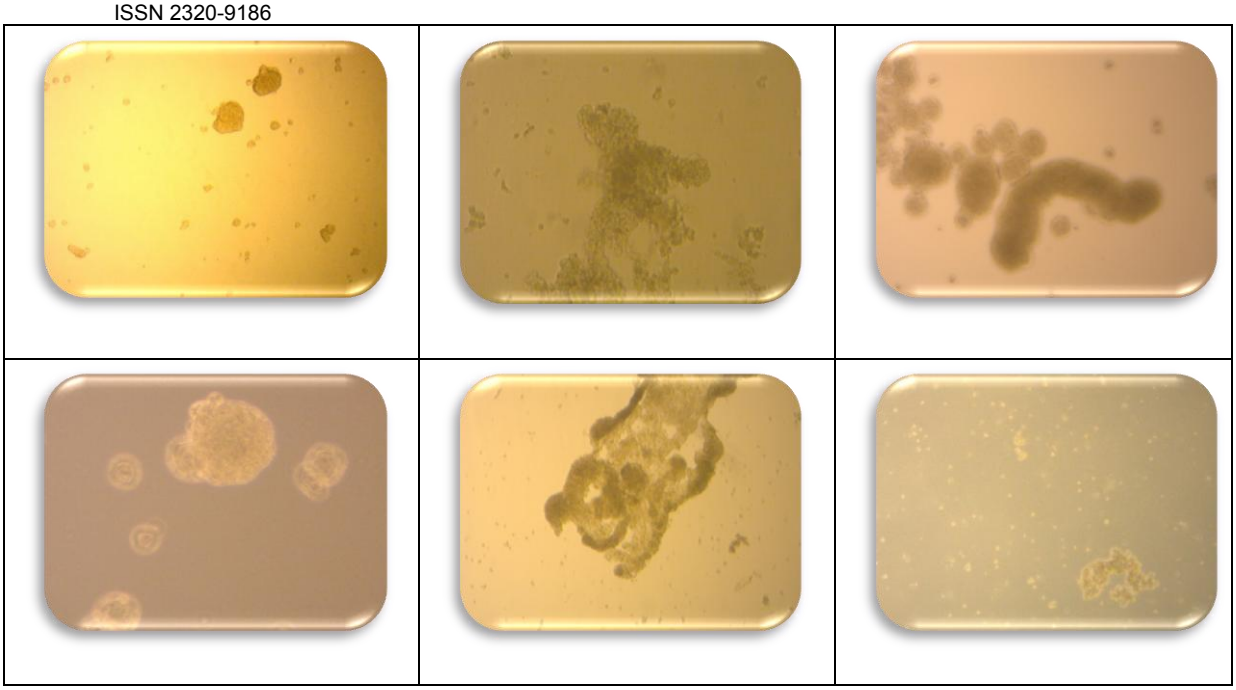

Table 4.2 Sphere formation by culturing MCF7 cells in normal flasks and normal media.

Phenotypic changes in MCF7 sphere cells are produced by the direct formation of parental MCF7 cell lines. During different times 24, 48, 72 and 96 hours under the microscope by using a camera (ZEISS, Primo Vert) in a variety of power magnifications. MCF7 cells were cultured in normal media and normal flasks for 24, 48, 72 and 96 hours.10 5cells/ml the number of cells in each large flask sizeT75. The cells were incubated at 37°c for several days and from 2-3 days the cells were regularly checked. This experiment has been done several times. [See Appendix1. for more evidence].

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4.1.2 Direct sphere formation assay of MCF7 cells by using non-adherent non- differentiate conditions (serum free media and CORNING flasks)

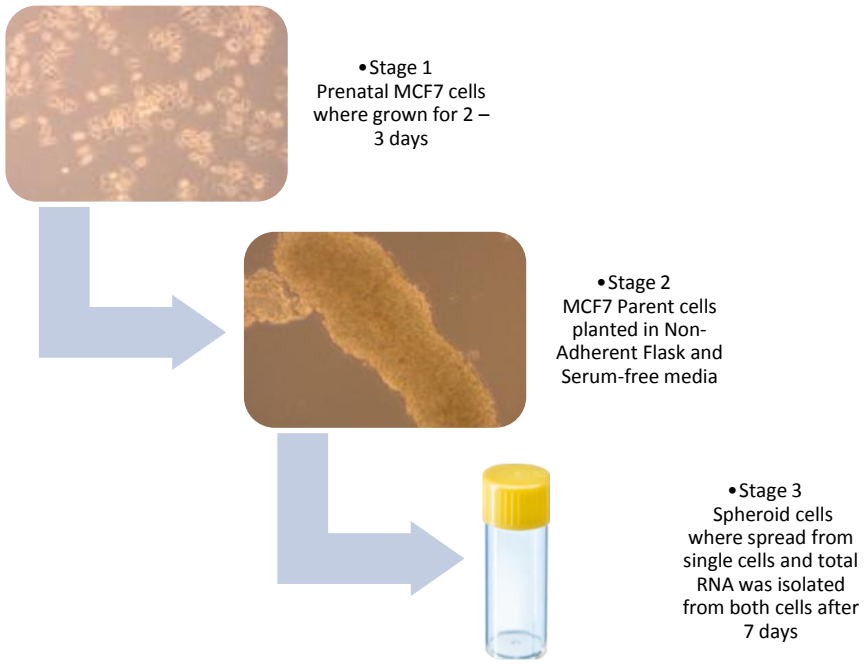

Figure 4.02 Phenotypic changes of parental, single and spheres MCF7 cells grown under non- adherent conditions

This experiment included several stages. The first phase involves MCF7 parental cells that were cultured under standard conditions for 2-3 days. In stage 2, the MCF7 parental cells were cultured under the non-adherent condition, using flasks (CORNING, USA) which are characterised as having a U shape, a vent cap and a low attachment surface. Also, serum free media was used and there was an increase in the percentage of FCS to 25% and growth factor. This was done for 96 hours.

Figure 4.3 shows an attempt to investigate mammospheres from MCF7 cells line of parental, single and sphere cells by culturing them in non- adherent conditions (new flasks CORNING and new media). In level 1, the cells were grown to a 90% confluence in normal media and the appearance of the cells were coherent with each other and formed a small tree. Total RNA was isolated from MCF7 parental, single and spheroid cells. In the second stage of this assay, the parental MCF7 cells were seeded in a non-adherent flask with 25% of FCS, free serum media. The results show a lot of large spheroids and this is proposed to be mammospheres.

Single cells were isolated from the spheres by transferring the cells from the flask to the universal tube and leaving the cells 5 minutes to settle down. Because the spheres are bigger in size and numbers, they will concentrate in the bottom of the tube and the single cells will be floating at the top of the tube.

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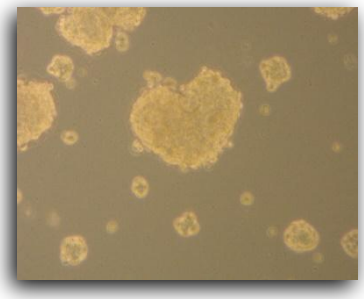
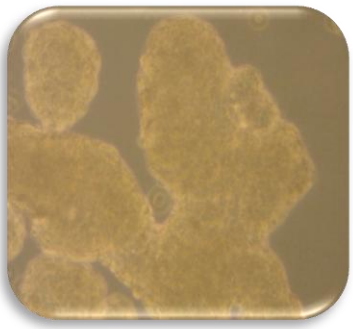
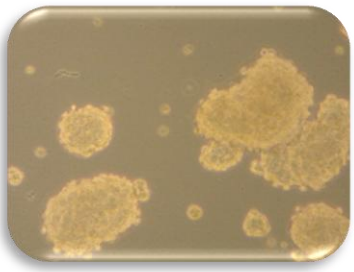
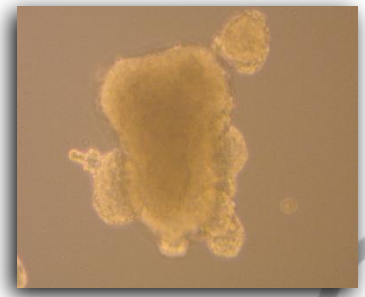
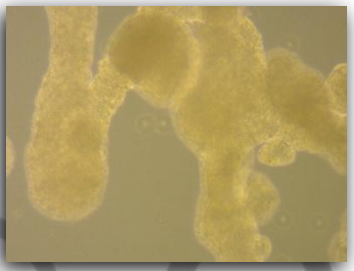
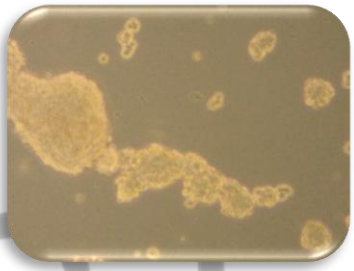
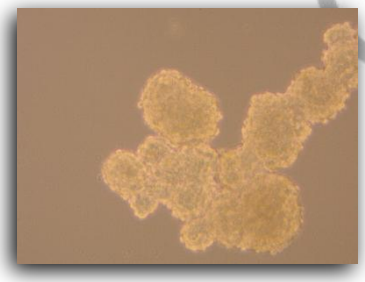
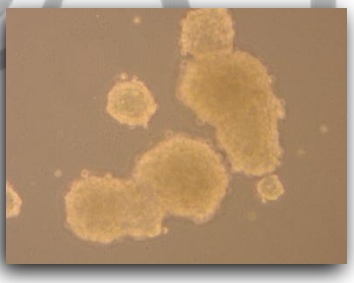
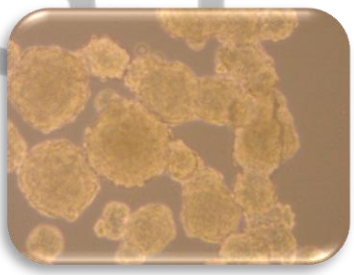
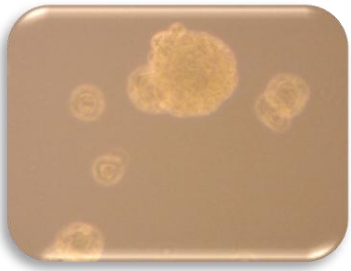
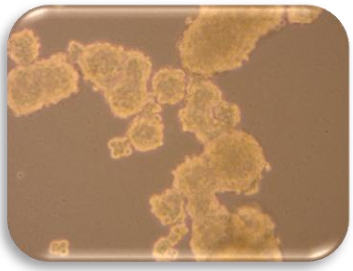
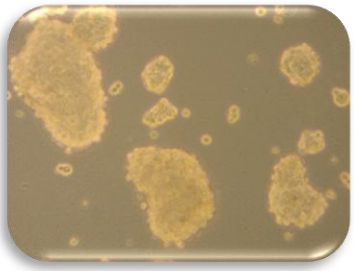
Figure 4.4 shows morphological changes of producing mammospheres by using new culturing flask (CORNING, USA) and a new media:

After 24 hours	After 72 hours	After 96 hours
		
		
		
		

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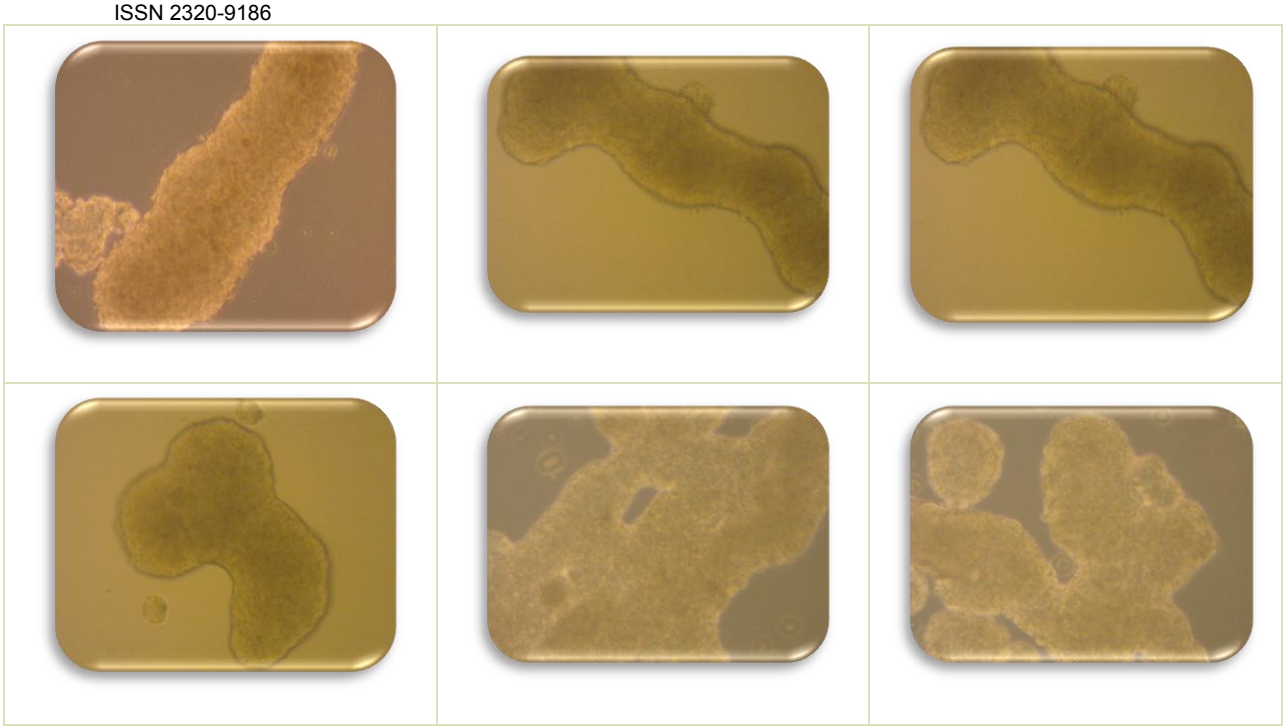

Table 4.4 indicates a comparison between the phenotype changes by direct sphere assay.

Morphological differences of producing spheres by using non-adherent conditions that include new media (serum free media, GF and 25% FCS) and Corning Flasks. These phenotypic changes in MCF7 sphere cells happen by applying direct spheres formation from parental cells. These results show that these spheres are more defined and bigger than spheres that were formed from the normal culture conditions. After one day, the sphere was very clear and big in size.

DISCUSSION

For long time the human cancer was considered as a big issue in the world wild. However, in more recent years, many investigation reported that there is highly decreased in mortality of cancer (Vera-Ramirz *et al*., 2010; Kobayshi et al.,2009). More recent studies the focus on CSCs which considers as alternative treatment therapy due to it is resistance, self-renewal and differentiation (Liu *et al*., 2015). Therefore, the current work was conducted to investigate the ideal isolation tools of BCSCs using biomarkers. This experiment includes the direct formation assay which used under different circumstances. The data shows information about the characteristics of sphere formation assay and the concept behind this procedure. This technique is used widely to understand stem cell activity and the features of self-renewal. However, several limitations might occur in this

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method, such as increasing the death of normal cells, a reduction in stem cell self-renewal and a decrease in a number of ancestor cells. Sphere assay is still a vital method for the isolation and quantification of breast cancer stem cells, in spite of these limitations (Shaw *et al*., 2012). BCSCs are used in different studies because of the importance of how breast cancer originates from it (Pham *et al*., 2012; Clevers,2011; Staveren et al.,2009). Therefore, the aim of this project was to isolate breast cancer stem cells.

A small population of tumorous breast cancer stem cells have been isolated from MCF7 cell lines using direct mammospheres assay. The CD44++ cells highly adhesive features were isolated from BCSCs (AL-Hajj *et al*., 2003). The main goal of this study was to enrich an MCF7 (CD44++) sub-population of cells, and then identify BCSC features by increasing their tumorsphere growth ability in vitro. Although there are several techniques that have been used in the past, there is a need for more specific methods and indicators, and that is the reason for this study.

Optimisation of spheroid cultures

Similar to several methods which have used in different experiments, BCSCs were cultured in two separate methods:

Producing spheroids in normal flask and normal media

In this part of the work, MCF7 cells were successfully cultured in a normal flask (SARSTEDT, Germany) with normal media with only 10% of FCS added. It is estimated that the MCF7 cells that were planted under these conditions had a different morphology when making spheres. The latter was slowly produced, one to two clusters after four to five days. The appearance of the spheroid was not very clear and it was small in size. It had a few cells and an irregular shape. There were only one, two and three spheres after 24, 72 and 96 hours respectively [Figure4.1, 4.2]. Therefore, we can say that MCF7s production of spheres in these circumstances were not in as good a condition as how we expect the isolated bulk of BCSCs to be at this stage.

Producing spheroids in a non-adherent flask and serum free media(Corning, US)

MCF7 cells were cultured in non-adherent conditions in serum free media with an increased percentage of FCS to 25% and the addition of growth factors to produce a group of cells called mammospheres. Under these conditions, it was noticed that the spheres were able to grow very quickly after 24 hours to be bigger in size and quantity and clearer than the spheroid in the normal conditions [Figure4.3, 4.4]. For this reason, BCSCs show high activity to form spheres by using non-adherent conditions compared with other methods. This might be

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because of the self-renewal abilities of CSCs. This result is similar to a study carried out by Abboodi (2014). In their findings, the clusters of cells were collected after one day (24hours) by using new media and a new flask (Corning, US). Normally the cells need four days to make spheres in ideal conditions; the images were shown under the microscope X4, X10, X20 and X40 magnification [Figure 4.3]. It is believed that spheroids are generated from single cells and this proved the ability of these cells to differentiate and initiate tumours (Ponti *et al*., 2006). Similar result were displayed by Ponti *et al*., (2005) when they cultured MCF7 cells under non-adherent conditions in serum free media with added growth factors resulted in producing spheroids. It is assumed that this formation of mammospheres might be occurred due to the presence of GF which shares CSCs in the same microenvironment. It was noticed that under these conditions using low attachment flask, non-adherent culture, MCF7 cells produce a large spheres compared with spheroid which generate from adherent conditions. Also, their observation was indicate the high quality of the morphology of spheres and quickness in producing spheres.

Wang *et al*., pointed out that serum free culture presents a crucial means to generate cancer stem cells. In their experiment they used MCF7 cells in non-adherent condition (which included using an ultralow attachment flask (Corning), DMEM/F12, 10ng/ml b-FGF, 20ng/ml of EGF) and these cells were attached to the flask and it was highly difficult to produce a sphere. This finding clarified that culturing MCF7 cells can help to enrich BCSCs in non-adherent non-differentiated circumstances to form a spheroid (Wang *et al*., 2014).

Summary

This study was aimed to eradicate the use of human breast cancer cell line(MCF7) as a model to simplify the nature of BCSCs. Different culturing methods were applied to achieve this target. Based on several kinds of literature mammospheres can be produced in breast cancer cell lines under different circumstances such as adherent flask, normal media and non-adherent, low attachment flask with serum-free media; but the results will be different in the morphology of spheres in both of the conditions. MCF7 spheres cells in CORNING flask showed higher tumorigenic than parental, single cells (Wang *et al*., 2014). Recently, mammospheres technique has been provided a relative pathway to study cancer in general and CSCs in specific in order to provide an ideal solution to clarify CSCs theory (Saadin and White, 2013).

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