To Study the effects of 3D scaffold cultured CAFs on Stemness of Breast Cancer Epithelial Cells

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ABSTARCT

Approximately 12 % women worldwide develop a tumor in the breast tissues which may be invasive and therefore cause impairment of different organs. Breast Cancer results in about 14% of Cancer related death fallacies. Cancer Associated Fibroblasts (CAFs) play an important role in cancer metastasis as they aid in proliferation and progression of cancer. It has been shown that studying CAFs in-vitro by culturing them in petri dishes causes deterioration in their cancer promoting properties. This calls for a need to prepare 3D tissue scaffolds suitable for providing an in-vivo like atmosphere to the CAFs culture. For this purpose, we have prepared and analyzed fibers formed by electrospinning of Poly-caprolactane solution. After analyzing fibers formed at different working conditions, we could optimize the conditions at which most suitable fibrous tissue scaffolds could be formed. CAFs were found to retain their properties in respect to expression of α– Smooth Muscle Actin (α-SMA) when they were cultured in such fibrous 3D tissue scaffolds. This project was to investigate whether they also retain their property to enhance Epithelial to Mesenchymal Transition (EMT), and thereby cause stemness in Breast Cancer Epithelial Cells.

Keywords: Electrospinning, α– Smooth Muscle Actin (α-SMA), Immunocytochemistry

INTRODUCTION

Cancer involves a group of diseases caused due to abnormal cell growth, wherein the cells have a potential to invade in other body parts. Abnormal proliferation of cells can result in development of tumors which may be benign or malignant. Benign tumors remain constrained to the place where they form and are not cancerous, while malignant ones can invade the nearby tissues and even spread to other organs and cripple their function. Such tumors are cancerous and this phenomenon of formation of secondary tumors is known as metastasis [1].

Cancer metastasis is aided by the process of Epithelial to Mesenchymal transition (EMT) [2].In this process, the well-polarized epithelial cells get converted to non-polarized mesenchymal cells and thereby exhibit enhancement of properties like invasion, motility and drug insensitivity [2].This triggers the reversion of cells to cancer stem cells (CSC), which retain cancer initiating potential and self-renewal capability [27]. Being drug resistant, CSCs thrive in the body even after chemotherapy. Having cancer initiating potential, the presence of CSC increases the probability of relapse of tumor. CSCs were first identified in Acute Myeloid Leukemia in 1997 [3] and later identified in several types of solid tumors like brain, breast, colon, ovary, pancreas and melanoma. Their proportion in a tumor depends on the type and the grade of the tumor.

Breast cancer is the most common type of invasive cancer among females, worldwide. Moreover, it is the second leading cause of cancer related deaths among women [4]. Majority of breast cancers are carcinomas originating from the cells lining the milk forming duct of the mammary glands. The mammary glands consist of a branching ductal unit having interlobular fat and fibrous tissues. The mammary epithelia include two layers of epithelial tissue- the inner layer or the luminal epithelium and the outer layer or the basal epithelium. Depending on the type of cells where breast cancer originates, it may be classified as luminal or basal. When these cells go through loss of senescence and thus immortalization, a tumor is formed.

The interaction between the epithelial tissue and supporting stroma determines the functional properties of the tissues. The stroma of the mammary tissue is made up of extra cellular matrix (ECM) components like basement membrane and interstitial matrix and the cellular components like fibroblasts, adipocytes and vasculature. The extra cellular matrix is known to influence the cell-cell and cell-matrix interaction of the breast tissue [5]. Basement membrane (BM)is ECM connecting epithelial and connective tissue of the breasts. Breakage of BM allows cells to invade the underlying ECM and move into the surrounding tissue. This marks the progression of breast cancer towards invasive stage [6]. Presence of an intact basement membrane denotes non-invasive carcinoma.

Breast cancer metastasis follows a cascade starting with local invasion of the surrounding tissue, spreading into the blood or lymphatic vessels and ending with dissemination of tumor cells to distal organs [7]. In all these processes, Cancer Associated Fibroblasts (CAFs) play a vital role. CAFs are tumor-associated fibroblasts with myofibroblast like phenotype [8]. In normal tissues, fibroblasts are predominant cell type in connective tissue stroma and are responsible for deposition of fibrillar ECM. Similarly, CAFs are present in tumor stroma but they are functionally and phenotypically different from normal fibroblasts. They have spindyloid appearance and can be differentiated from normal fibroblasts by the presence ofα-smooth muscle actin. They are perpetually activated cells which neither revert to normal phenotype and nor undergo apoptosis and thereby play a central role in tumor-stromal interaction [9].

CAFs have been shown to play a major role in proliferation and progression of cancer. CAFs aid tumor progression by production of growth factors and chemotactic factors, angiogenesis factors, and matrix metalloproteases [10]. The up-regulation of these factors is directly linked with increase in stem cell related gene expression of cancer cells [11].

The traditional way of studying the properties of cells was by isolating them from the required tissues and growing them on petri dishes as monolayer cultures. Although it is a convenient method, there are a few limitations associated with it. In petri dishes, the cells get a 2D environment and become flat, lose their native morphology, and there is a loss of cell-cell and cell-ECM interaction [12]. A decrease in cell-ECM interaction implies reduction in the cell signaling via ECM. The ECM, directly or through proteins like integrins, is responsible for signaling cells for various functions including cell proliferation, differentiation, immune responses etc. [13] When there is decrement in cell signaling, as is the case of cells cultured in petri dishes, there is a change in cell's activity and therefore cells behave in a different way as compared to how they behave in-vivo.

To compensate for these changes, researchers have developed approaches to culture cells in 3- dimension. A 3D matrix is more effective in mediating cell-cell adhesions and therefore it provides an accelerated rate of acquisition of in-vivo characteristics [12].Such potentials have led to advancement in using 3D models for tissue culture. A widely used strategy is to culture cells and implant them in 3D matrix scaffold as either single cell or as tissue-like aggregates.

In any 3D model, the microenvironment of the cells has a great influence on the properties of the cells. Both, the composition and the stiffness of the extra cellular matrix surrounding the cells have major effects on cell signaling and behavior [14].

PRIOR WORK

As mentioned earlier, CAFs show increased expression of α-SMA as compared to its expression in normal fibroblasts. α-SMA is widely used as a marker to identify CAFs in tumor stroma [15]. Formation of CAFs has many consequences on tumor development, growth and metastasis. Cancer associated fibroblasts have been reported to produce a variety of growth factors and cytokines like TGFb1, HGF, IL-1, IL-6 etc. that contribute to ECM remodeling and foster cancer cell invasion. ECM remodeling and stiffening has important implications on tumor progression [16]. There exists a correlation between stiffness of tissues and invasiveness of tumors.

CAFs are responsible for developing a stiff ECM which drives epithelial cells into an invasive and proliferation phenotype [17].

The lab I work in was interested in studying the biology of cancer and cancer associated fibroblasts. The major drawback of doing experiments related to CAFs in vitro was that CAFs tend to lose their activity in terms of α-SMA expression by the end of around 10 passages when they are cultured in petri dishes [18]. However, whenthese cells were cultured in fibrous poly-caprolactane (PCL) scaffolds, it was observed that they regain their activity in terms of α-SMA expression. Along with it, the actin and nuclear staining in these cells showed that the cells were aligned. Also, an increased secretion of desmoplastic ECM i.e. the presence of type 1-collagen and fibronectin was observed. All these changes suggested that culturing CAFs in 3D fibrous PCL scaffolds increased their cancer associated activity. Based on this preliminary observation, this study was aimed to understand the effect of scaffold cultured CAFs on the stemness of breast cancer epithelial cells.

OBJECTIVE

1. To fabricate fibrous PCL scaffolds by electrospinning to culture CAFs.
2. To compare the relative expression of stem cells marker on MDA-MB-231 (Breast cancer epithelial) cells when they are cultured in 2D and 3D CAF conditioned media.
   The following are the stemness markers whose expression was planned to be studied. We will be looking at one of the following markers
   - Nanog [25]
   - Bmi [26]

MATERIALS AND METHODS

1. Preparation Of Nanofibrous Scaffolds Of Polycaprolactane By Electrospinning:

Electrospinning is a technique which produces fibers of nanometer range from liquid solutions, using electrical forces [19]. The liquid solution, containing the desired polymer in an appropriate solvent, is taken in a syringe which is subjected to voltage in range of tens of kVs. A collector is grounded and placed at certain distance from the nozzle. The polymer solution injects out from the syringe and gets accelerated towards the collector, thereby depositing fibers on the collector [20].

In this process, the electric field induces an electric charge on the liquid surface which is held at the end of capillary tube due to its surface tension. On increasing the electric field, the electrical forces reach a critical value after which they overcome the surface tension and eject the solution from the tip of the needle, known as Taylor cone [21]. In the space between capillary tip and collector, rapid whipping of jet occurs, which leads to evaporation of solvent, leaving just the polymer behind [22].

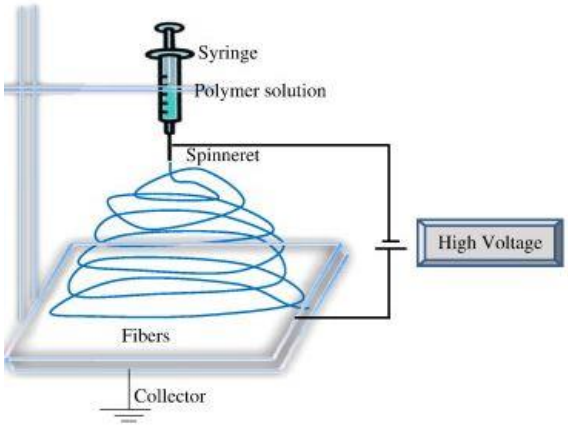

Figure 1: Schematic Diagram of set up of Electrophoresis apparatus. Source: N. Bharadwaj et.al. 2010

10%, 12% and 15% w/v solution of PCL (average molecular weight 80,000 g/mol, Sigma Aldrich) were prepared in Chloroform. The polymer solution was loaded in a 2 ml syringe with a 23-gauge (0.6 x 25mm) stainless steel needle fitted to a syringe pump. A high voltage was applied to the needle, which was placed at a fixed distance from the collector. The collector was Aluminum foil that was grounded. Working conditions like flow rate, working distance and the voltage were systematically varied to generate uniform, smooth and non-aligned fibers without the formation of beads. The fiber mat was sputter-coated with Au and fiber morphology was assessed by scanning electron microscopy (ESEM Quanta 200, FEI)

2. Prior Background work - Preparation Of Sample And Control For Analysis Of Cell Markers:

CAFs were cultured in fibrous PCL scaffolds for 7 days. Media was conditioned in these cells for 12 hours and then the media was frozen for later use. Later, the conditioned media was thawed and added in a 1:1 ratio to MDA-MB-231 cells. After culturing the latter for 48 hours, they were taken for further analyses. As a control, CAFs were cultured in 2D Tissue Culture Polystyrene surfaces (TCPS) and their condition media was added in 1:1 ratio to a similar culture of MDA-MB-231 cells for 48 hours.

3. Checking For Markers Using Immunocytochemistry:

Immunocytochemistry is a method for identification of antigens in tissues/cells by stimulatingspecific antigen-antibody interaction, and staining the antibody with a visible marker [23]. The antigen is allowed to bind to its specific antibody while the latter is stained by a secondary antibody. The secondary antibody specifically binds to the primary one and produces a visible signal when seen in fluorescence microscope.

Cells were fixed with 3.7% formaldehyde for 15 minutes and permeabilized with 0.2% TritonX-100 for 5 minutes. Formaldehyde causes crosslinking of proteins and formation of their hemiacetal derivatives. Fixation preserves the cellular morphology and the chemical composition and the relative positions of the components of the cells. Most antibodies, being large molecules, are impermeable in the cells. The cells, therefore, need to be permeabilized to allow the entry of antibodies.

Incomplete fixation can leave free aldehyde groups in the cells which can either inactivate the proteins or bind non-specifically to them. To prevent this, these cells were blocked for 45 minutes with PBS containing 0.2%FSG and 0.01% Tween 20. They were then incubated with primary antibodies (1µg/ml) for overnight at 4°C. Nanog and Bmi(Cell Signaling Technology) were respectively the primary antibodies used. The cells were then stained with 1:200 dilution of Cy3 anti rabbit secondary antibodies for 45 minutes at room temperature.1µg/ml of Hoecsht 33342 (Sigma Aldrich) for 5 minutes at room temperature was used to stain

the nucleus of the cells. The samples were imaged visualized using an epi-fluorescence microscope (Olympus IX71). Ki67 (Abcam) and Vimentin(Sigma) were initially used for staining to learn the procedure.

4. Isolation Of RNA Using Trizol Method

RNA isolation involves a series of steps in which the cells are first lysed under conditions such that the integrity of RNA is maintained while the proteins and DNA get denatured and other cellular components disrupted. This is done by using Trizol (TRI Reagent) [24], which is a mixture of guanidine thioacyanate and phenol. Once the cells are homogenized, Chloroform is added to this solution. Phenol-Chloroform mixture completely dissolves the cellular components which on hard centrifugation separate into aqueous and organic phases. The aqueous layer contains RNA, the middle layer contains white precipitous DNA and the bottom layer has the rest of the organic material.

RNA is then precipitated out of the aqueous phase using iso-propanol followed bycentrifugation anddissolved in Molecular Biology treated water for further processing.

The RNA was quantified using a UV-Vis spectrophotometer (NanoDrop2000, Thermo Scientific). The quality of RNA was assessed by the 260/280 ratio as an estimate of nucleic acid to protein levels, and the 260/230 ratio, an estimate of the nucleic acid to salts and organic compounds. RNA samples with 260/280 and 260/230 ratios of 1.8 to 2.2 were used for downstream applications. Additionally, the samples were run on 1% agarose gel to assess the integrity of the RNA. Samples which were not degraded were taken for further analysis.

RESULTS AND DISCUSSION

1. Fabrication of fibrous PCL scaffolds by electrospinning:

Poly-Caprolactane (PCL) is a semi crystalline aliphatic poly-ester which is being used in tissue engineering due to its properties like biocompatibility, slow degradation and non-toxic degradation by-products [28]. Moreover, its ability to blend easily with organic solvents helps in modifying its properties according to the requirement of the tissues [29].

To culture cancer associated fibroblasts, fibrous PCL scaffolds were to be prepared by electrospinning. In body, CAFs are present in a fibrous matrix [33], therefore fibrous scaffolds are most appropriate to mimic the in-vivo atmosphere. The working conditions i.e. the concentration, working distance and the flow rate were varied and the fibers were observed using Scanning Electron Microscope.This was done so as to get a uniform matrix of fiber diameter of 1 micron which could be later used as scaffolds for culturing CAFs.

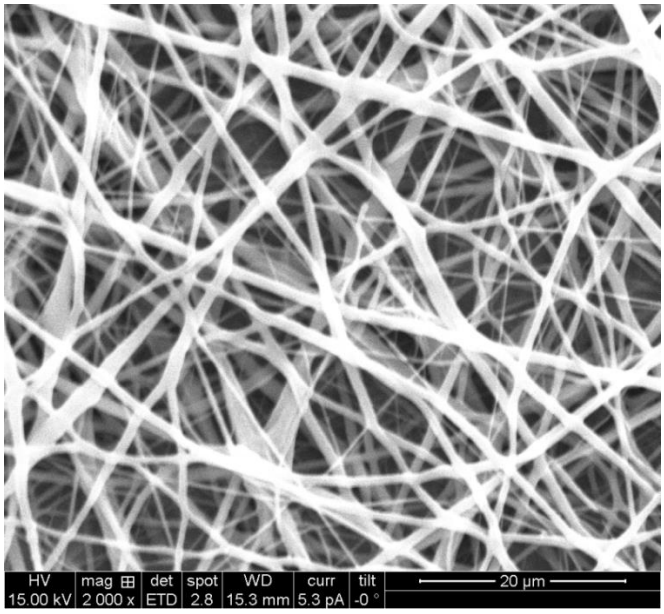

Figure 2: SEM micrographs of electrospun fibers

Initially, we tried with the following working conditions

PARAMETER	**WORKING CONDITION**
Concentration	12%
Flow Rate	10cm
Applied Voltage	0.4ml/hour
Distance between nozzle and collector	10 KVs

On viewing the fibers in Scanning Electron Microscope (Figure 2), we could see that the fibers were of varying diameter, they were not suitable for culturing cells as it would have resulted in uneven growth of cells. The working parameters were altered to achieve a uniform mat of fibers.

Different fiber diameters may be a result of splitting of primary jets into multiple jets [30]. This occurs during the travelling of jet from nozzle to the collector and it might get reduced by increasing the flowrate of the jet. Increasing the flow rate also increases the fiber diameter [31].It has also been observed that if splitting of fibers does not occur, a higher viscosity of solution results in higher fiber diameter [30]. The viscosity of a solution increases with increase in its concentration; therefore, a higher concentration will result in larger fiber diameter. When higher voltage is applied, an increase in potential difference ejects more fluid in a jet thus increasing the fiber diameter [32].

With a different set of working conditions, the fibers were again electrospun and viewed using SEM.

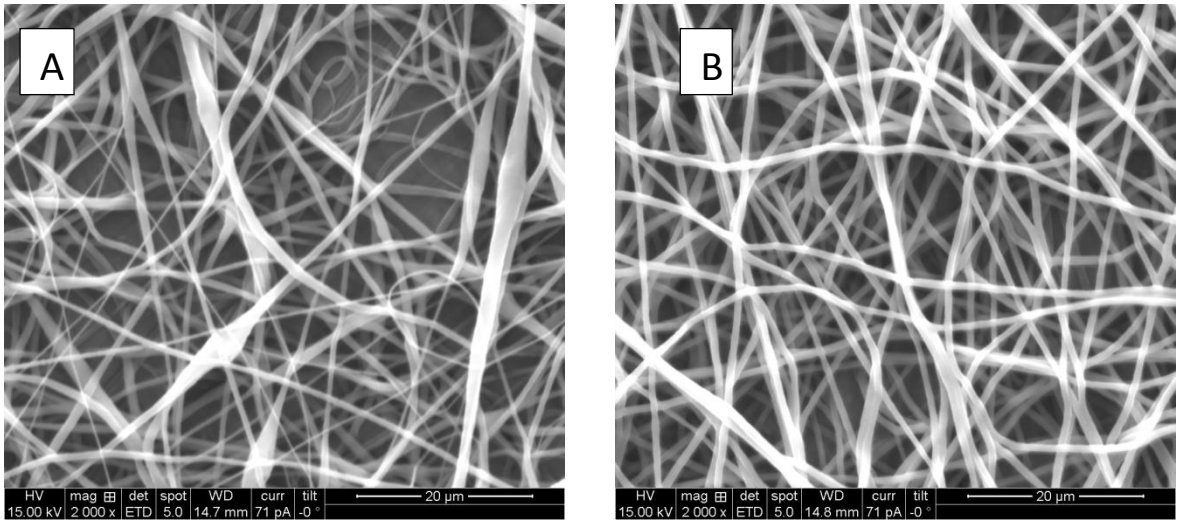

Figure 3: SEM micrographs of electrospun fibers (A)- 12% PCL in TFE, flowrate 0.6ml/hour (B)- 15% PCL in TFE, Flowrate 0.4ml/hour.Magnification- 2000x

PARAMETER	**WORKING CONDITION – A**	**WORKING CONDITION – B**
Concentration	12%	15%
Flow rate	0.6 ml/hour	0.4 ml/hour
Applied Voltage	10 KVs	10KVs
Working Distance	10 cm	10 cm

As can be observed, the fibers in figure 3(B) showed uniform diameter and less splitting as compared to the fibers in figure 3(A). But the diameter observed was smaller than required. To get the diameter of 1 micron range, fibers were again spun for a different set of working conditions.

Figure 4 shows the SEM micrographs of fibers formed by using 15%PCL in TFE. As can be seen, they showed good qualities in terms of uniformity of diameter and no formation of beads. The diameter observed was also of acceptable range. Therefore, these fibers can further be used as scaffolds to culture Cancer Associated Fibroblasts.

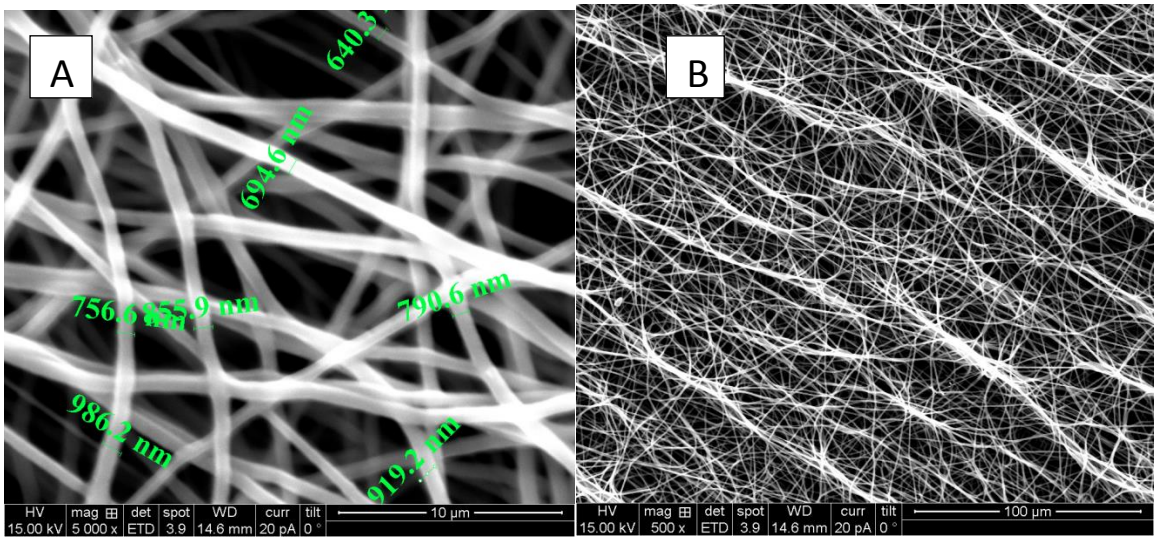

Figure 4: SEM micrographs of electrospun fibers, 15% PCL in TFE, Flowrate- 0.6ml/hr, working distance- 12cm, Voltage applied- 10KV at 2000x (A) and 500x (B) magnification respectively

PARAMETER	**WORKING CONDITION**
Concentration	15%
Flow Rate	12cm
Applied Voltage	0.6ml/hour
Distance between nozzle and collector	10 KVs

2. Immunocytochemistry To Check For The Presence Of Stemness Markers

As mentioned, initially cells were immuno-stained with anti-Ki-67 and anti-Vimentinantibodies to learn the technique. Vimentins are class-III intermediate filaments found in mesenchymal cells. It is attached to various cell organelles like nucleus, endoplasmic reticulum, and mitochondria, either laterally or terminally. Ki-67, on the other hand, is a nuclear protein associated with cell proliferation and is known to be present inside the nucleus.

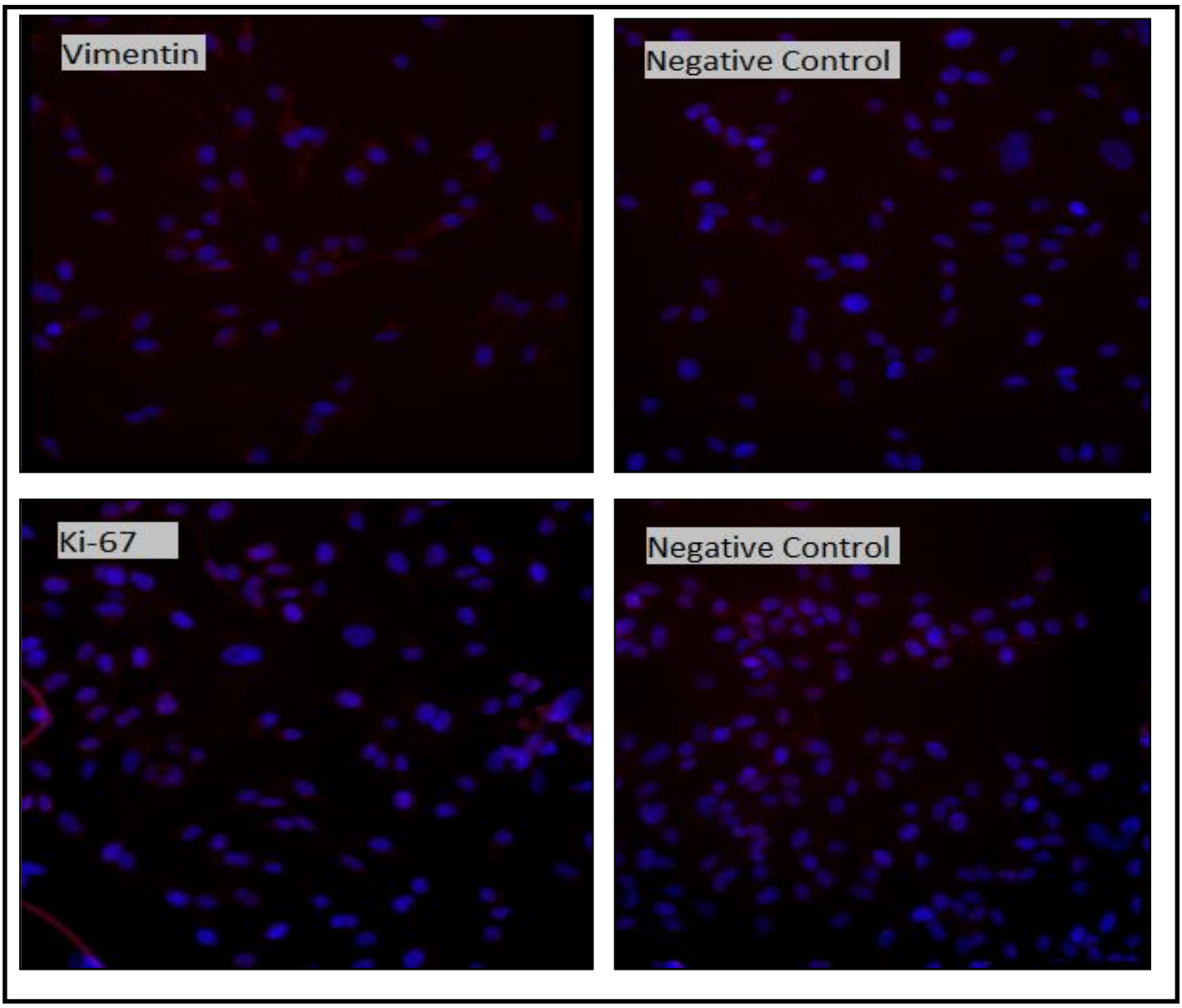

Figure 5: MDA-MB-231 cells stained by Vimentin and Ki-67. Nuclei were counterstained using Hoecsht.

When the staining was viewed in fluorescence microscope (Figure 5), it was observed that there had been mixing of antibodies. Ki-67 showed presence of marker outside the nucleus and some positive signal could be observed on the negative control. The reason for this could have been mishandling of samples and therefore, experiments were performed carefully and in more stringent conditions. Moreover, the acquisition of images was not optimal which is evident from the faint signal and hence required improvement.

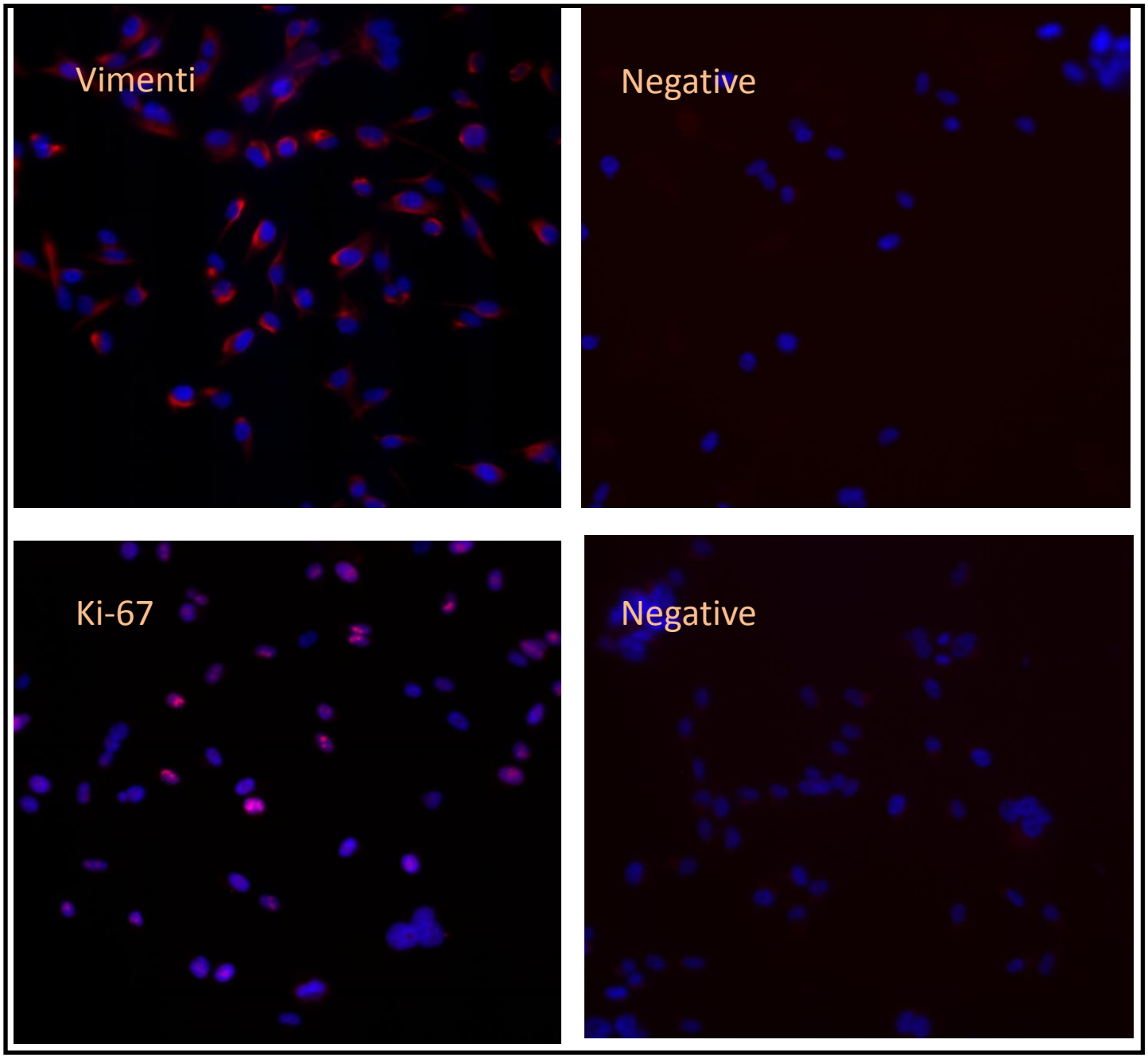

Figure 6: Vimentin and Ki-67 staining on MDA-MB-231 cells. Nuclei were counterstained using Hoecsht.

Figure 6 shows a marked difference between the staining and the negative control. As we can see, Ki-67 has been stained inside the nucleus and Vimentin seems to be present outside it. This is in accord with what has been shown in literature.

Prior to the actual experiment, anti-Bmi-1 and anti-Nanogantibodies were used on MDA-MB-231 cells to make sure the appropriate staining was obtained. Figure 7 shows the results of the staining performed with these antibodies. The staining was similar to as observed in the literature. Absence of signal from the negative control confirmed the specificity of the signal.

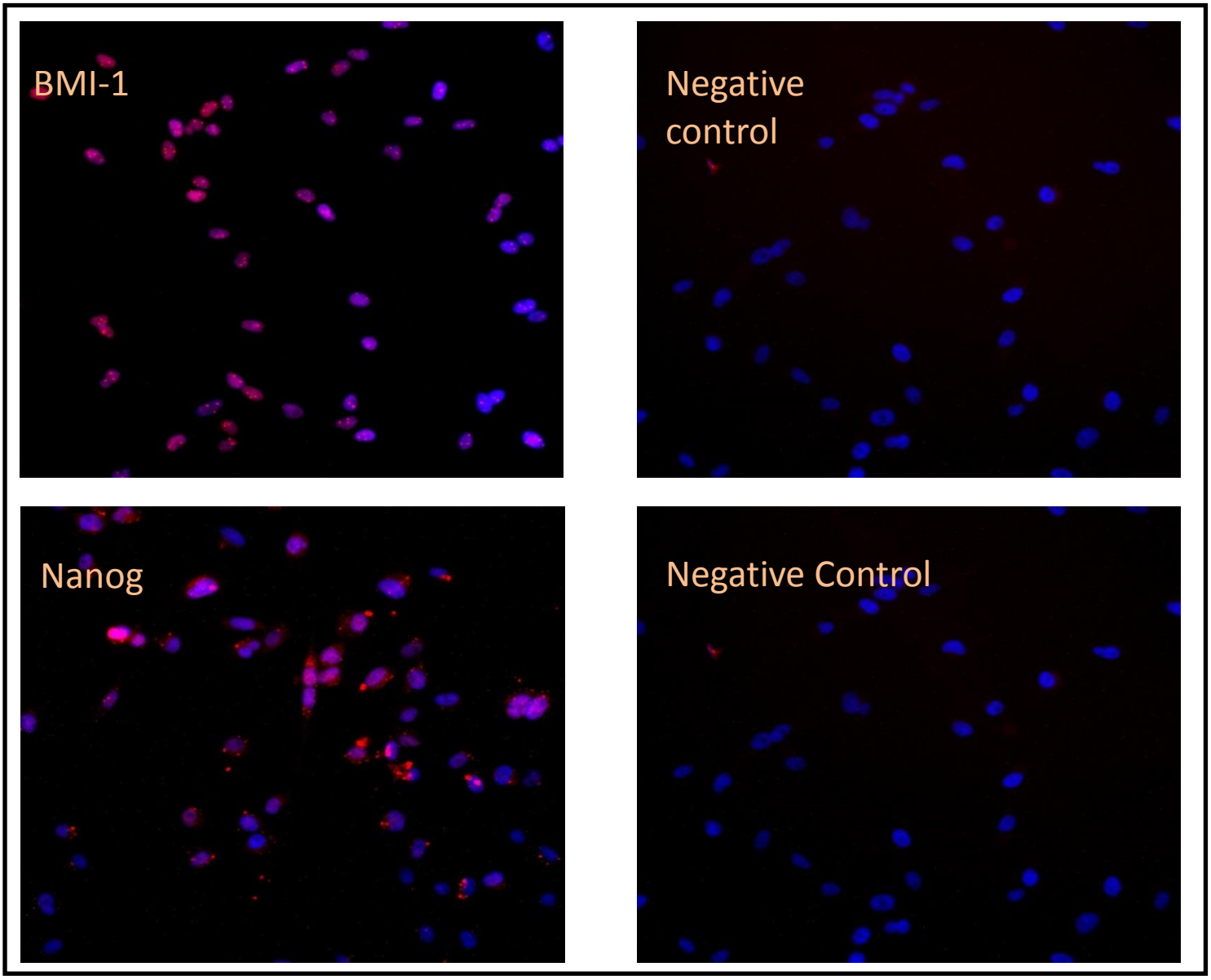

Figure 7: Nanog and Bmi-1 staining on MDA-MB-231 cells. Nuclei were counterstained using Hoecsht.

To compare the relative expression of Bmi-1 on MDA-MB-231 cells cultured in 2D and 3D CAF conditioned media, staining of these cells was done by the same protocol as given above. Figure 8 shows the images obtained.

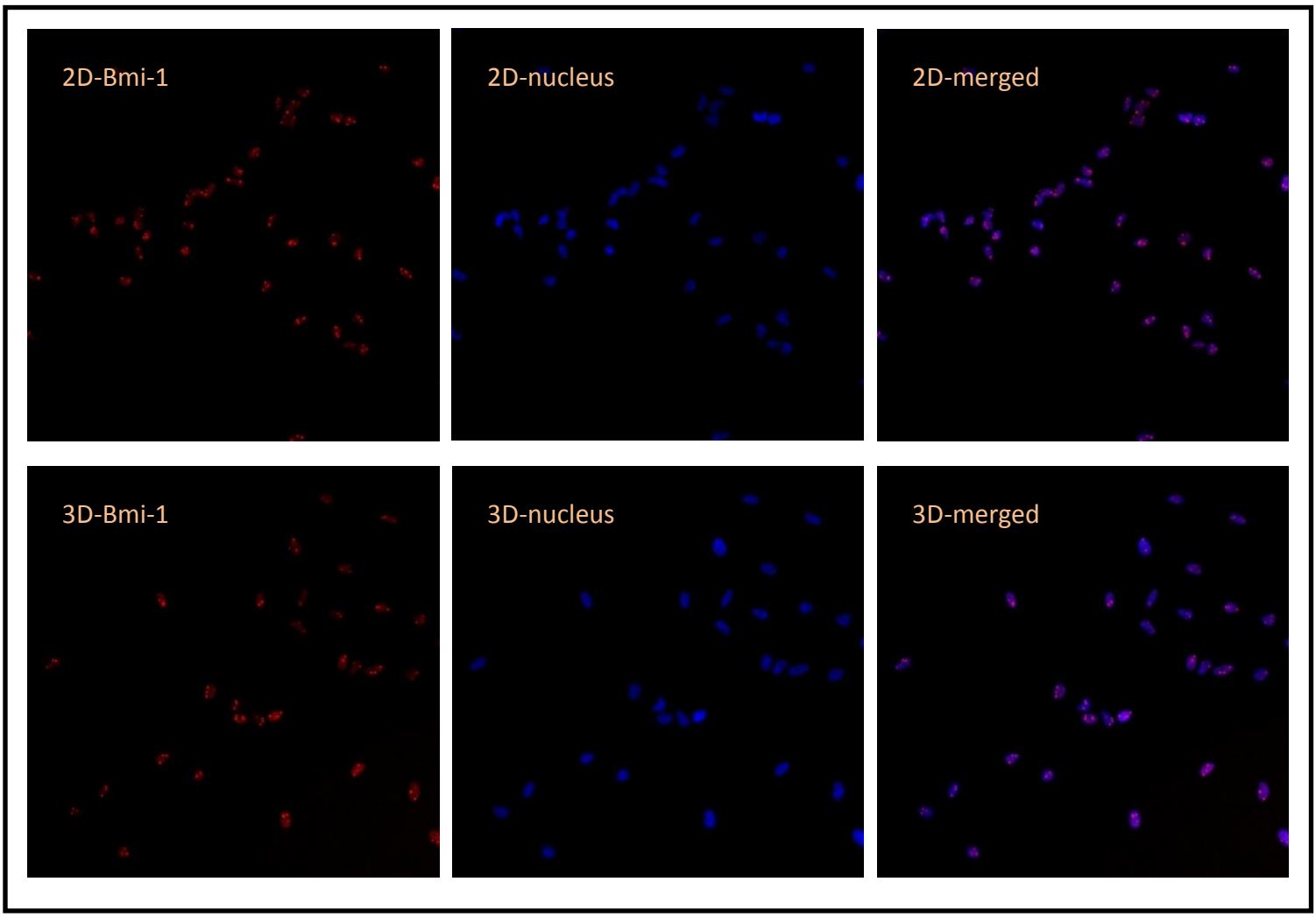

Figure 8: MDA-MB-231 cells cultured in 2D and 3D CAF conditioned media.

MDA-MB-231 cells grown in 3D CAF conditioned media were expected to show a higher expression of Bmi-1. But, as can be seen in figure 8, there was no profound difference in the expression of Bmi-1.The conditioned media was collected after 12 hours of exposure to CAFs which could be one of the reasons that it failed to increase the stemenss of breast cancer cells. Conditioning the media for longer periods of time could improve the effects.

Additionally there could have been loses of growth factors on storage and cycles of freeze-thaw which could have also affected the results. Moreover, these cells were cultured in 1:1 ratio of CAF conditioned media and theirfreshmedia. This dilution must have further decreased theamount of growth factors present.

To amend these results, the experimented could be repeated using freshly isolated CAF conditioned media or a more concentrated conditioned media.

3. RT-PCR to detect the presence of stemness markers

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) is a widely used technique to quantify mRNA. In this process, the expression of a particular gene is evaluated by finding the amount of mRNA of that protein present in the cells. The steps involved in this process are shown in figure 9. For this procedure, initially the RNA needs to be isolated from the cells. This was done by using Guanidine method [33], as given above. Initially, MDA-MB-231 cells were taken in a 60 mm dish and RNA was isolated from them. This was just to get an understanding of the procedure involved.

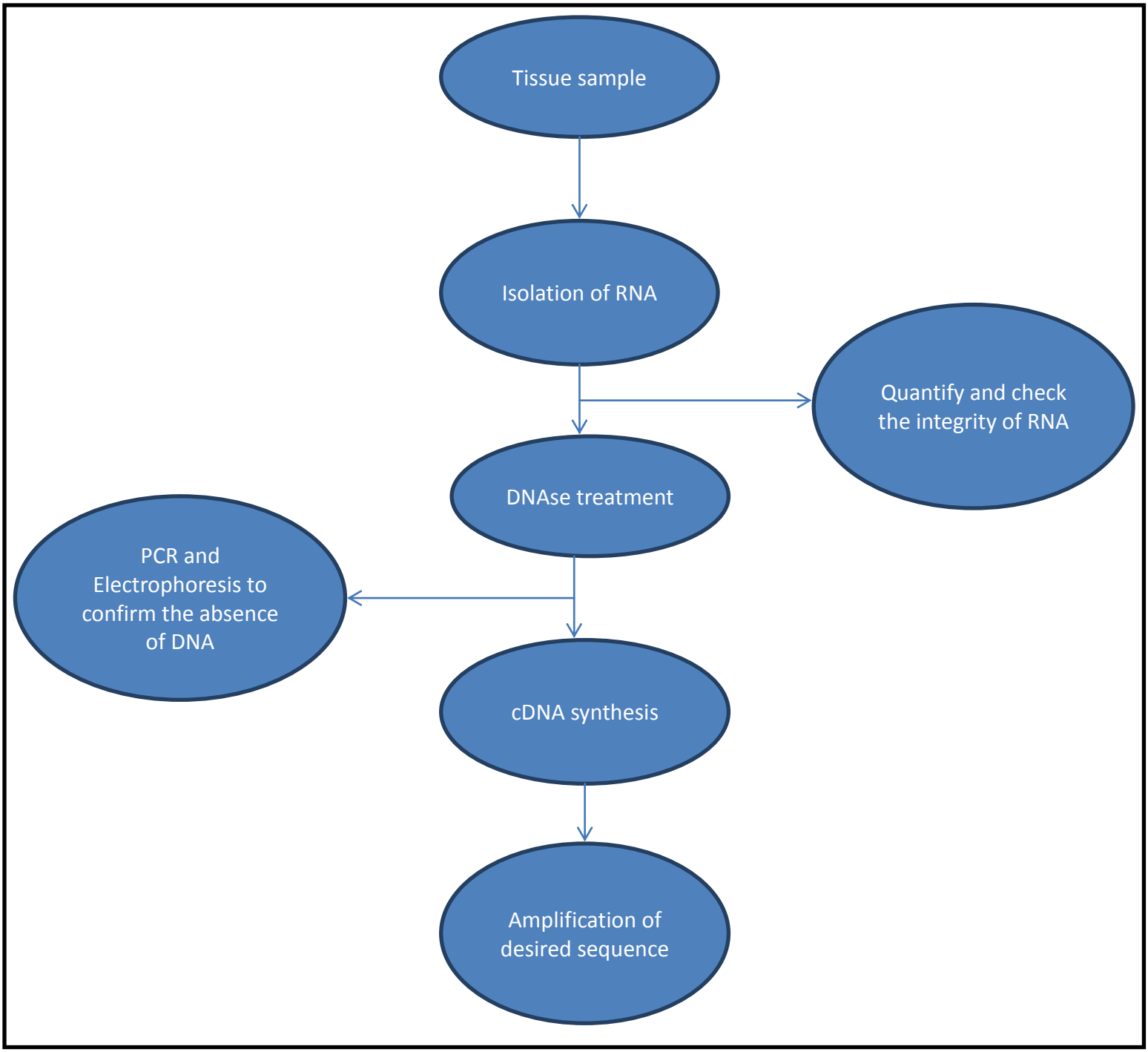

Figure 9: Steps to be followed for Reverse Transcriptase Polymerase Chain Reaction

The quantification of isolated RNA was done using UV-Vis spectrophotometer (NanoDrop2000, Thermo Scientific) and then its integrity was checked by performing electrophoresis on a 1.8% agarose gel.

The results were as follows

PARAMETER	VALUE
Concentration	1732.9 ng/µl
260/280 ratio	2.06
260/230 ratio	2.27

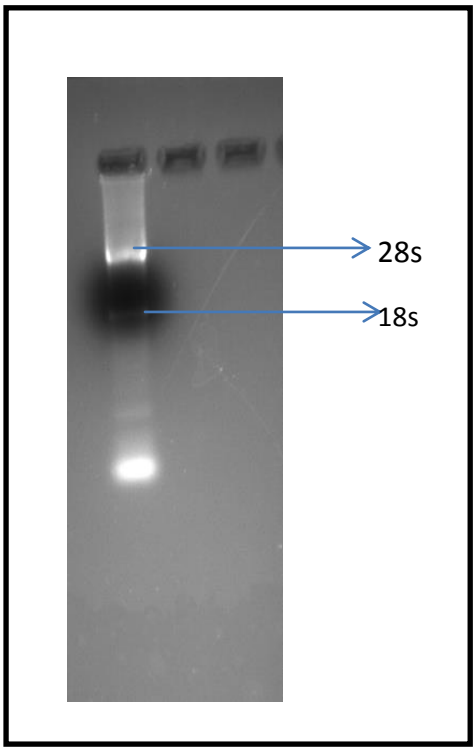

Figure 10: Electrophoresis on 1.8% Agarose Gel

The results showed that the RNA thus obtained was good in quantity as well as quality and we could proceed further with the experiment.

This RNA was then treated with DNAse enzyme so as to degrade any residual DNA left in the sample. PCR was performed on a part of resulting sample using β2m primers and then the amplified sequence was checked by electrophoresis on 1%agarose gel. This was to confirm that no DNA was left in the sample.

Reagent	Final Concentration
dNTPs	200µM
Forward Primer	0.2µM
Reverse Primer	0.2µM
10X Taq Buffer	1X
Taq Polymerase	1 unit

Table: Reaction mix for PCR optimization

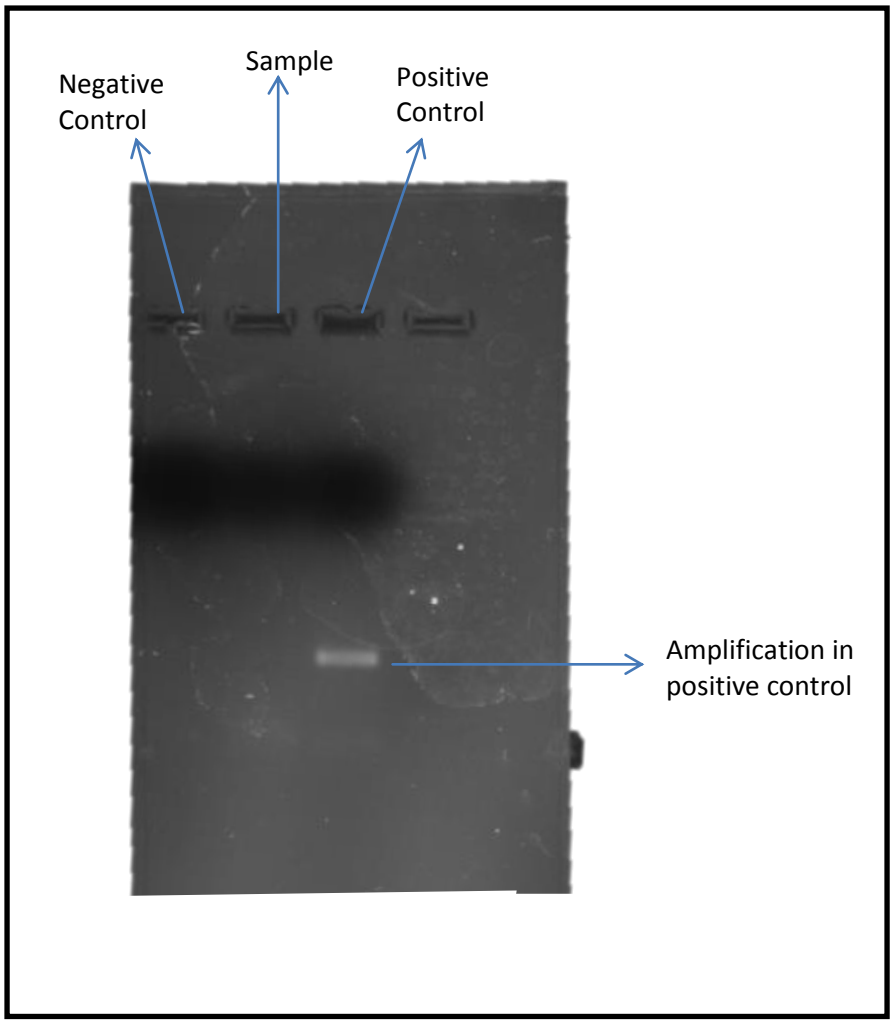

Figure 11: Electrophoresis on 1% agarose gel

This shows that the sample had no genomic DNA left. For practice, the same experiment was now performed using two samples of MDA-MB-231 cells.

After performing RNA isolation, when these cells were checked by UV-Spectrophotometer and by electrophoresis on 1.8% agarose gel, we got the following results

SAMPLE	**CONCENTRATION**	**260/280**	**260/230**
Sample 1	732.2ng/µl	2.00	2.09
Sample 2	337.5ng/µl	1.96	1.86

Although, the quantity and the 260/280 and 260/230 ratio showed that the yield was good, but the gel picture (Figure 12) shows a shear indicating that the RNA might have degraded. This can give erroneous results; to prevent which, RNA isolation was again performed.

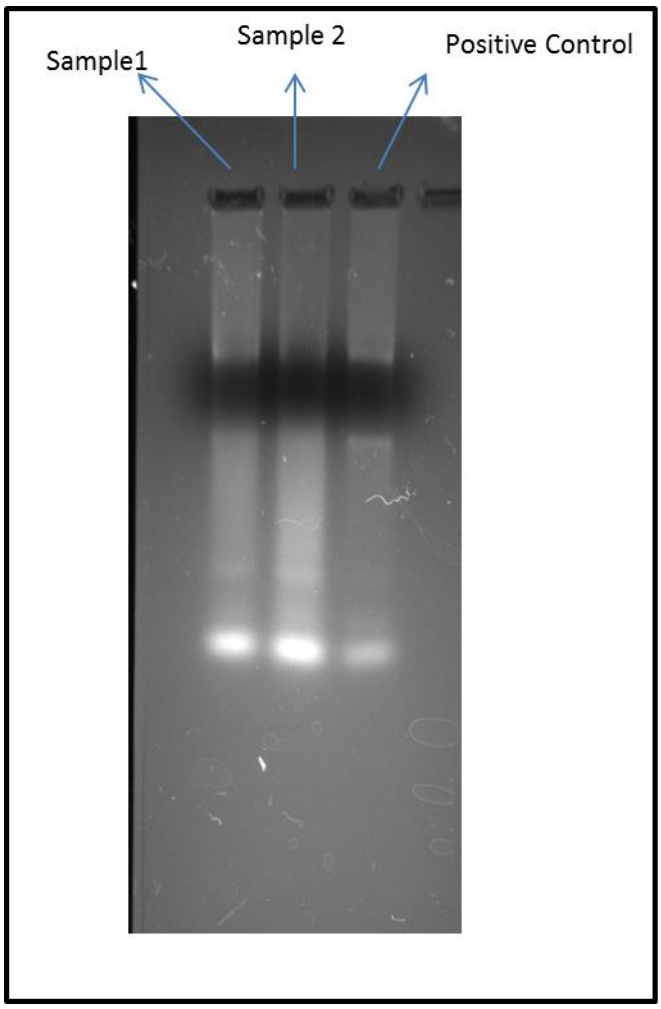

Figure 12: Electrophoresis on 1.8% Agarose Gel

Next we wanted to compare the gene expression of stem cell markers in MDA-MB-231 cells cultured in 2D and 3D CAF conditioned media. Therefore, these similar experiments were performed using these samples.

On isolation of RNA, both the samples were analyzed for their concentration and integrity. It was observed that the concentration of RNA was lesser as compared to those of previously analyzed samples. The reason for this decrement could be a decreased confluency of the samples taken.

SAMPLE	**CONCENTRATION**	**260/280**	**260/230**
BC cells in 2D CAF conditioned media	156.4ng/µl	2.02	1.76
BC cells in 3D CAF conditioned media	167.4ng/µl	2.07	1.86

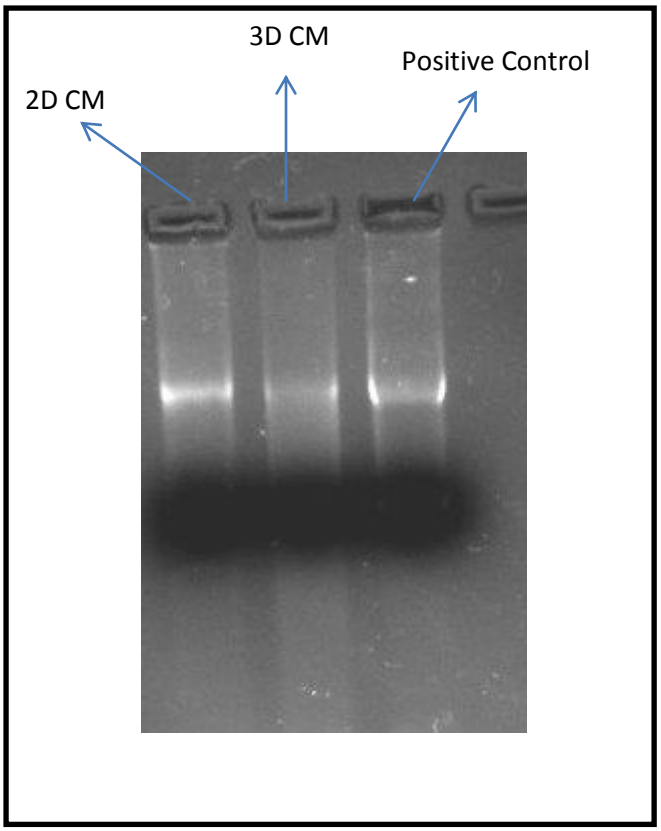

Figure 13: Electrophoresis on 1.8% Agarose Gel of MDA-MB-231 cells cultured in 2D and 3D CAF conditioned media

RNA has been isolated and has not degraded and therefore, it can be used to study the gene expression of various stem cell markers by quantitative RT-PCR. This RNA can hence be used for further studies.

CONCLUSION

Although the Breast Cancer Epithelial Cells cultured in 2D and 3D CAF conditioned media could not be completely analyzed for the stemness markers, optimization of fibers and isolation of RNA have been performed, both of which can be further used to carry out the experiment.

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