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CHARACTERIZATION AND MODELING OF CELL ACTIVATION UPON TGF- β 1
STIMULATION IN A 3-D CULTURE SYSTEM

BY

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THESIS

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ABSTRACT

In this work, we explored a thick *in vitro* 3-D cell culture model with layered structure and applied transforming growth factor-beta1 (TGF- β 1) on the top of the 3-D culture to induce the conversion from normal fibroblasts to myofibroblasts and expression of a marker protein, alpha-smooth muscle actin (α -SMA). The cell response with TGF- β 1 stimulation was captured with immunofluorescence staining (IF) and confocal microscopy imaging of α -SMA in each cell embedded layer. Our results revealed the spatial-temporal profiles of the conversion from normal fibroblasts to myofibroblasts in 3-D culture upon TGF- β 1 stimulation. The conversion starting time, speed and saturation plateau are closely related to TGF- β 1 concentration. Based on these experimental results, we successfully developed a mathematical model, which incorporates diffusion of growth factors and cell activation responses to describe the dynamic cell response to growth factors across the 3-D culture depth and predict the spatial-temporal profile of the fibroblast conversion within the culture. In summary, we present here a handy experimental and computational tool for researches on tumor signaling pathways, and provide some guidance for studies on diffusion of protein factors or drugs in 3-D tissue environment.

To my family

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CHAPTER 1

INTRODUCTION

For years cancer studies have focused on the cancer cells themselves. More recently researchers have begun to investigate the role of the environment surrounding cancer cells in tumor development. Stroma is a major part of the tumor microenvironment and mainly includes extracellular matrix, fibroblasts, blood and lymphoid vessels, nerves and inflammatory cells. Stroma has shown to greatly influence tumor initiation and progression. (Wernert 1997; Liotta and Kohn 2001; Tlsty and Coussens 2006) Soluble factors such as growth factors play an important role in this process. (Seslar, Nakamura et al. 1995; Kawada, Inoue et al. 2008) However, the dynamic behavior of growth factors and other soluble factors moving in the ECM and tumors has been barely characterized in a quantitative way, partly due to the lack of a well-defined *in vitro* experimental system. The main goal of this study is to use TGF- β 1 as an example and 3-D cell cultures as a tool to characterize and model an important aspect of tumor studies, the TGF- β 1 induced cell activation in tumors.

In this chapter, the subjects pertinent to this study are introduced, including the roles of stroma and the relationship between fibroblasts and TGF- β s in cancer development, the gradient of growth factors in biological systems and *in vitro* 3-D culture systems for biological studies.

1.1 Stroma in Cancer Development

It is now increasingly clear that stroma around tumor can promote tumorigenesis and influence the tumor process instead of delaying or preventing tumor formation as it is in normal biological system. Current model of tumor-stroma interactions in cancer development is summarized in figure 1.1. In normal tissue, stroma is separated from the epithelial cells and the

underlying myoepithelial cells by basement membrane. During the carcinoma *in situ* stage, carcinoma cells promote the proliferation of transformed epithelia. More fibrillar ECM is deposited and more fibroblasts are accumulated in stroma. The basement membrane is still intact but the outline gradually becomes irregular and dysplastic cells start to appear. In the invasive carcinomas, the basement membrane is disrupted and the tumor cells spread into stroma region and form irregular areas without being confined under basement membrane. Fibroblasts in the surrounding stroma are converted into myofibroblasts, and cancer cells are mixed with myofibroblasts, inflammatory infiltrate and newly formed capillaries. In the final stage, metastatic carcinoma, cancer cells invade the dense and fibrous stroma and gradually get into the bloodstream and finally settle in distal tissues. (Kalluri and Zeisberg 2006)

As shown in the above model, fibroblasts are the key component of stroma. Some of their important functions include synthesis of ECM, regulation of epithelial differentiation and participation in wound healing. (Tomasek, Gabbiani et al. 2002; Parsonage, Filer et al. 2005) Fibroblasts can synthesize type I, type III and type V collagen and fibronectin of ECM and type IV collagen and laminin of basement membranes. (Rodemann and Muller 1991; Chang, Chi et al. 2002; Tomasek, Gabbiani et al. 2002) Furthermore, fibroblasts can regulate the differentiation and maintain the homeostasis of adjacent epithelia through the secretion of growth factors and mesenchymal-epithelial cell interactions. (Wiseman and Werb 2002)

The conversion of fibroblasts to myofibroblasts is an important step towards tumor development, and can be characterized by the expression of α -SMA (figure 1.2). In breast carcinomas, about 80% of stromal fibroblasts are thought to acquire this activated phenotype. (Sappino, Skalli et al. 1988) Recent studies indicate that myofibroblasts can provide oncogenic signals to the transformed epithelia and facilitate angiogenesis and cancer progression. This has

also been demonstrated by *in vitro* culturing of primary human breast epithelial tumor cells with fibroblasts. Fibroblasts have been observed to be converted into myofibroblasts around tumors. This conversion is strongest in the immediate vicinity of tumor cells and causes a proportional effect on the tumor spread and invasiveness. (Ronnovjessen, Petersen et al. 1995) Recent evidence also shows that in the absence of malignant epithelial cells, abnormal fibroblasts can still act as oncogenic signals and transform adjacent cells to induce different phenotypic and genomic changes of epithelial cells. (Tlsty and Hein 2001)

The involvement of myofibroblasts in the initiation of cancer has been well studied. It has been shown that introduction of myofibroblasts can induce the initiation of breast cancer within the normal tissue. (Kuperwasser, Chavarria et al. 2004) In another study, only myofibroblasts isolated from the primary tumor site can induce lesions among epithelial cells, while normal fibroblast cells cannot. (Olumi, Grossfeld et al. 1999; Orimo, Gupta et al. 2005) Similarly, tumors only form from immortalized epithelial cells in the presence of myofibroblasts but not normal fibroblasts. (Olumi, Grossfeld et al. 1999) These findings indicate that normal fibroblasts are required to maintain epithelial homeostasis, whereas myofibroblasts probably initiate and promote tumorigenic alterations in epithelial cells.

Myofibroblasts can also promote tumor progression. For example, myofibroblasts can facilitate the invasion of non-invasive cancer cell line in mice model. (Dimancheboitrel, Vakaet et al. 1994) In another study, tumors grow larger in nude mice when cancer cells are injected together with myofibroblasts than those with normal fibroblasts. (Orimo, Gupta et al. 2005) Some studies have also indicated that myofibroblasts are involved in the tumors at metastatic stage. They have been shown to promote the proliferation of cancer cells at the metastatic sites. (Olaso, Santisteban et al. 1997)

1.2 Relationship between Fibroblasts and TGF- β s in Cancer Development

Transforming growth factor- β s (TGF- β s) play a dual role in cancer development (figure 1.3). In normal tissues and during early stages of cancer, their suppressor activities are dominant. In contrast, during advanced stages of cancer development, TGF- β s favor the oncogenic activities to promote tumor development and metastasis. (Wakefield and Roberts 2002) (Kalluri and Zeisberg 2006) Both the tumor suppressor and oncogenic activities of TGF- β s are achieved through the tumor microenvironment. During tumorigenesis, it is generally accompanied by a decreased responsiveness of the tumor cell to tumor suppressors, coupled with an increased production of TGF- β s, which may promote oncogenic effects on stromal elements. (Bierie and Moses 2006; Jakowlew 2006)

In normal tissues, the anti-proliferative and pro-apoptotic response of epithelial cells to TGF- β s might limit the growth of normal epithelium, induce the apoptosis of epithelial cells and inhibit the emergence of malignant carcinomas. (Hanahan and Weinberg 2000; Siegel and Massague 2003) TGF- β s also stimulate fibroblasts to proliferate, produce ECM and induce a fibrotic response. In addition, they facilitate fibroblast-epithelial cell interactions, which further suppress cancer initiation. (Bhowmick, Neilson et al. 2004)

In carcinomas, stroma is often the main component of tumors. The amount and composition of stroma are partly dependent on the response of fibroblasts to the growth factors secreted by the cancer cells such as TGF- β s, platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2), which are all key regulators of fibroblast activation and tissue fibrosis. (Elenbaas and Weinberg 2001) Studies have shown that the conversion of fibroblasts into myofibroblasts is the most prominent stromal reaction in many carcinomas. (Ronnov-Jessen, Villadsen et al. 2002; Lewis, Lygoe et al. 2004; Micke and Ostman 2004) During this process,

fibroblasts are activated and become myofibroblasts, which are characterized by the expression of their marker protein, α -SMA. The myofibroblasts are considered to be associated with the tumors at all stages of cancer (Kalluri and Zeisberg 2006) and tumor-induced fibroblasts to myofibroblasts conversion further promotes neoplastic progression and myofibroblasts invasion. (DeClerck 2000; De Wever and Mareel 2002; Tuxhorn, Ayala et al. 2002; Zidar, Gale et al. 2002)

Results from culturing human normal breast gland fibroblasts with growth factors show that the α -SMA can be induced by TGF- β 1 in human normal breast gland fibroblasts and the effect is TGF- β 1 dose dependent. This cannot be observed with other growth factors or cytokines. The interaction between carcinoma cells and fibroblasts was studied by culturing fibroblasts with TGF- β neutralizing antibodies and breast carcinoma cell cultured medium. The results shows breast carcinoma cell cultured medium can induce the same effect as TGF- β 1 does, indicating the same role of TGF- β 1 in carcinoma.(Ronnovjessen and Petersen 1993)

The regulation of TGF- β s involves a complex signaling cascade and is summarized in figure 1.4. Both TGF- β molecules and its receptors are dimeric. The TGF- β molecules bind to two different types of serine/threonine kinase receptor: type I and type II. The type II receptor kinases are constitutively active; upon ligand binding, the type II receptors activate the type I receptors. The type I receptor kinases then activate intracellular Smad proteins. Smad2 and Smad3 are receptor-regulated Smads (R-Smads) and directly interact with type I receptors and subsequently become phosphorylated. R-Smads then form complexes with common-partner Smads (Co-Smads) such as Smad4 and migrate into the nucleus, where they interact with Smad binding element and transcription factor binding element and regulate transcription of target genes.(Miyazono 2000)

TGF- β s signaling cascade is regulated in both positive and negative ways as summarized in figure 1.5. In addition to the positive regulation described above, negative regulation also plays an important role. For example, in early embryonic development the negative regulation can inhibit or terminate signaling. (Akhurst, Lehnert et al. 1990) This can also limit the signaling range of TGF- β s and induce a gradient of ligand activities. (McDowell, Gurdon et al. 2001) The combination of the positive and negative regulation forms a balance that dictates TGF- β s signaling. Disruption of this balance might be an important cause of tumor initiation.

1.3 Gradient of Growth Factors in Biological System

Concentration gradients of soluble factors and their effects have been observed in varieties of biological systems, including cell survival (Barde 1989), proliferation (Massague, Cheifetz et al. 1992), differentiation (Massague, Cheifetz et al. 1992), and ultimately tissue structure and function (Gurdon and Bourillot 2001). Within a 3D tissue, concentration gradients might exist for any soluble component that is consumed or produced by cells. These gradients arise from the competition between diffusion and convection with cell consumption or production. The effects of concentration gradient depend not just on the presence or absence of these soluble factors, but also their quantitation, particularly their spatial arrangements and temporal sequence in biological systems. (Griffith and Swartz 2006) There are two ways of potential impact of the concentration profile on natural tissues. First, different location of the tissue might behave differently because of this concentration distribution. Second, it can induce chemotactic migration or other gradient dependent cell responses, which is often seen during tumor development.

The importance of soluble factor gradients in natural systems suggests their potential for influencing the behavior of cells embedded within *in vitro* biological systems, which usually use cells, materials, and signaling molecules to mimic natural tissue structure and function and recreate disease development. Investigating the spatial-temporal cellular response to soluble factors could be particularly important in *in vitro* biological systems.

To date, our understanding of how important soluble factor gradient and its influence on cell behaviors are in *in vitro* 3-D biological systems is still limited. The first barrier is an applicable 3-D *in vitro* system. Some researchers have utilized tools like microfluidic channels to apply gradients of soluble factors or multiple types of cells. (Wong, Perez-Castillejos et al. 2008) However, this type of controllable soluble factors gradients may be limited, as cellular behaviors can be affected by confined micro-sized environment (Ouyang, Sun et al. 2008) and stiff substrate (Engler, Sen et al. 2006). A more natural 3-D microenvironment is desirable. Another important barrier is the lack of diffusion coefficient of most important soluble factors, such as growth factors. Fluorescence-based methods have been used to determine the diffusion coefficients of growth factors. (Nauman, Campbell et al. 2007) (Stroh, Zipfel et al. 2003) However, growth factors molecules have to be conjugated with fluorescent dyes, therefore limiting the application of this technique. Availability of techniques to track and visualize gradient-induced cellular behaviors is also a barrier. Confocal microscope is most popular tool to visualize the 3-D cellular behaviors. However, the penetration is often not deep enough to capture the entire 3-D system. The maximum penetration of multiphoton confocal microscope has been reported as 1mm.(Kobat, Durst et al. 2009)

1.4 3-D Cultures for Biological Study

The majority of cell culture studies have been performed on 2-D surfaces such as well plates, tissue culture flasks and Petri dishes because of the ease, convenience, and high cell viability in 2-D culture. These conventional 2-D cell culture systems have notably improved the understanding of basic cell biology. However, nearly all biological tissue cells reside in a microenvironment of ECM consisting of a complex 3-D fibrous meshwork with a wide distribution of fibers and gaps that provide complex biochemical and physical signals. 2-D substrates are considerably limited in mimicking these complex 3-D microenvironments because of the lack of structural architecture, especially the stroma, which is the major component in tumor microenvironment and one of the major contributors to human cancer development. (Lee, Kenny et al. 2007) Furthermore, some of the cell properties are often changed in monolayer cultures. For example, normal epithelial cells in monolayers are highly plastic and express many characteristics that are also displayed by tumor cells *in vivo*. (Petersen, Ronnovjessen et al. 1992)

Recently, 3-D model systems have gained an increased popularity and are now regularly used in many studies. One of the advantages 3-D cultures have over 2-D monolayers is their well defined geometry, which makes it possible to directly relate structure to function and in turn enable theoretical analyses. Compared with conventional monolayer cultures, 3-D cultures more closely resemble the *in vivo* situation with regard to cell shape and its environment, which often determine the differentiation, proliferation, apoptosis and gene expression of the cell. They provide a well defined environment for cancer research in contrast to the complex environment of real tissues. They can better reflect the characteristics of normal and tumor cells, mimic the epithelial–stromal cell interactions and allow systematic investigation of tumor progression in a well defined and controllable 3-D environment. (Nelson and Bissell 2006)

Usually 3-D models are fibrillar substrate with pores that can support cell growth, organization and differentiation within their structure. A variety of fabrication processes and biomaterials have been developed to meet this kind of properties. An artificial 3-D model should mimic the structure and biological function of native biological system as much as possible, both in terms of chemical composition and physical structure. Depending on the specific applications, some requirements should be considered for the production of 3-D models. They should possess some appropriate mechanical and chemical environment as well as the structure matching the nature tissue. They should also be made from material with controlled biodegradability or bioresorbability and not induce any adverse response. The interconnecting pores of an appropriate size are desirable to favor tissue integration and vascularization. Finally, the fabrication process should be easy and a variety of shapes and sizes should be able to achieve. (Hutmacher, Schantz et al. 2001)

At the same time, several technologies have to be optimized before being applied to 3-D cultures. For example, the cell culture methods to deliver a precise number of cells into a 3D matrix with a homogeneous distribution are needed. The cell maintenance methods to minimize sudden change or disturbance of culture environment are also desirable. Also, the way for applying external stimuli such as mechanical stress or electrical excitation needs to be improved to better mimic the *in vivo* physical and electrical environment. Finally, a unique set of assay techniques should be developed to determine and evaluate biological performance of cultured tissues. (Lee, Cuddihy et al. 2008)

Here, we developed a unique 3-D culture system and used this system to study the cellular behavior upon the stimulation of a growth factor, TGF- β 1. We quantified the effect of TGF- β 1 in fibroblast/myofibroblast conversion, and its diffusion in the 3-D culture system.

Based on the knowledge we gathered from these studies, we have built a quantitative computational model of cell response to TGF- β 1 stimuli in a 3-D environment.

1.5 Figures

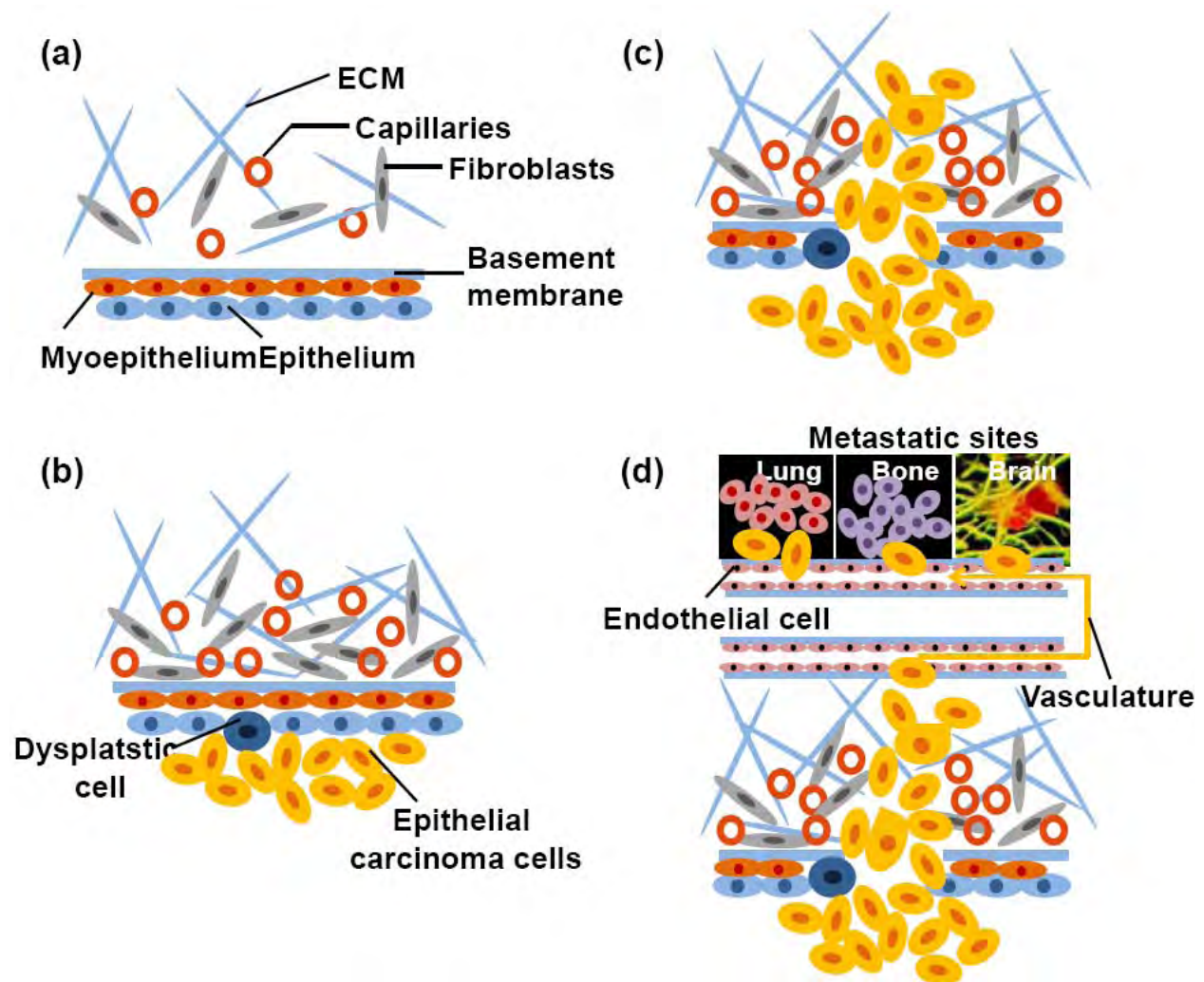


Figure 1.1 Tumor-stroma interactions in the cancer development. (a) Normal tissue; (b) Carcinoma *in situ*; (c) Invasive carcinoma and (d) Metastatic carcinoma.

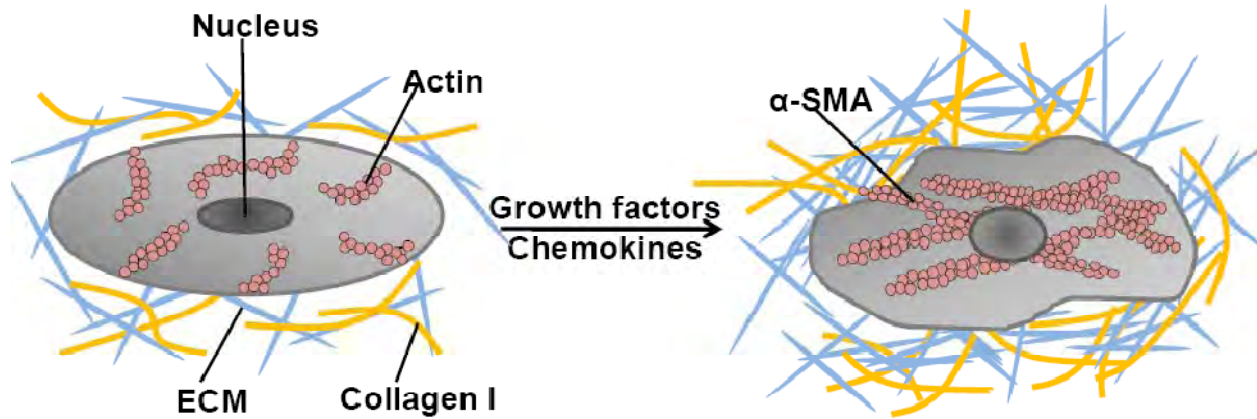


Figure 1.2 Fibroblast/myofibroblast conversion. The conversion is often characterized by expression of α -SMA and enhanced secretion of ECM protein.

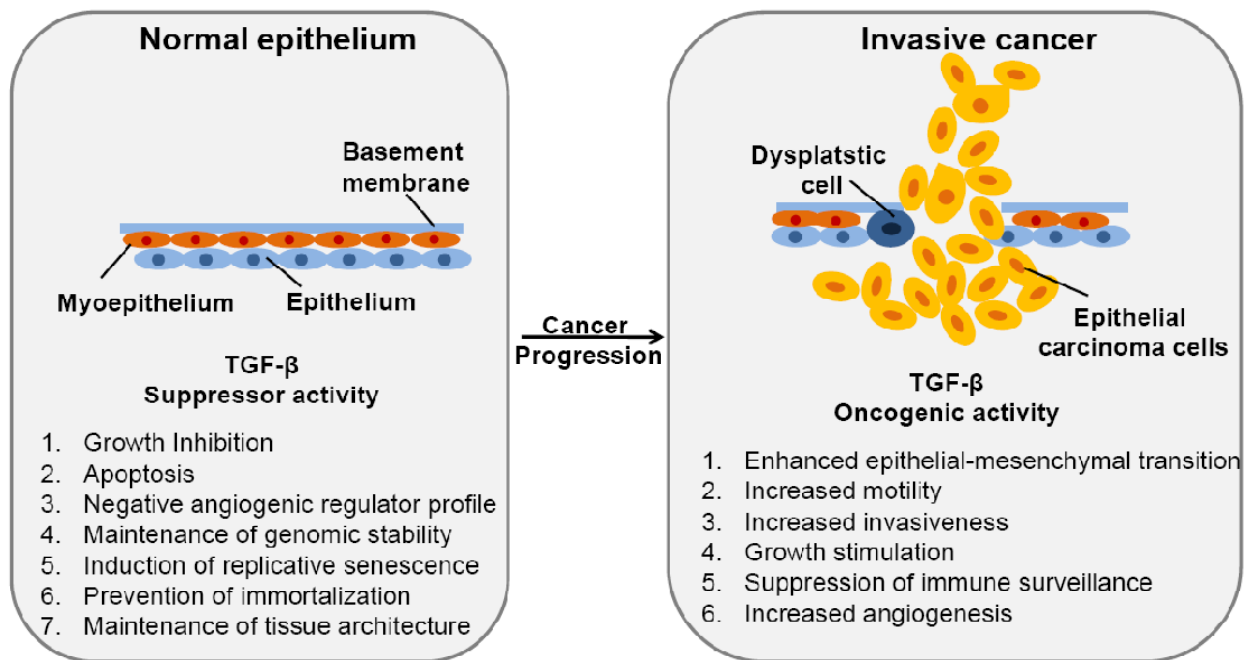


Figure 1.3 The dual role of TGF- β s in cancer development

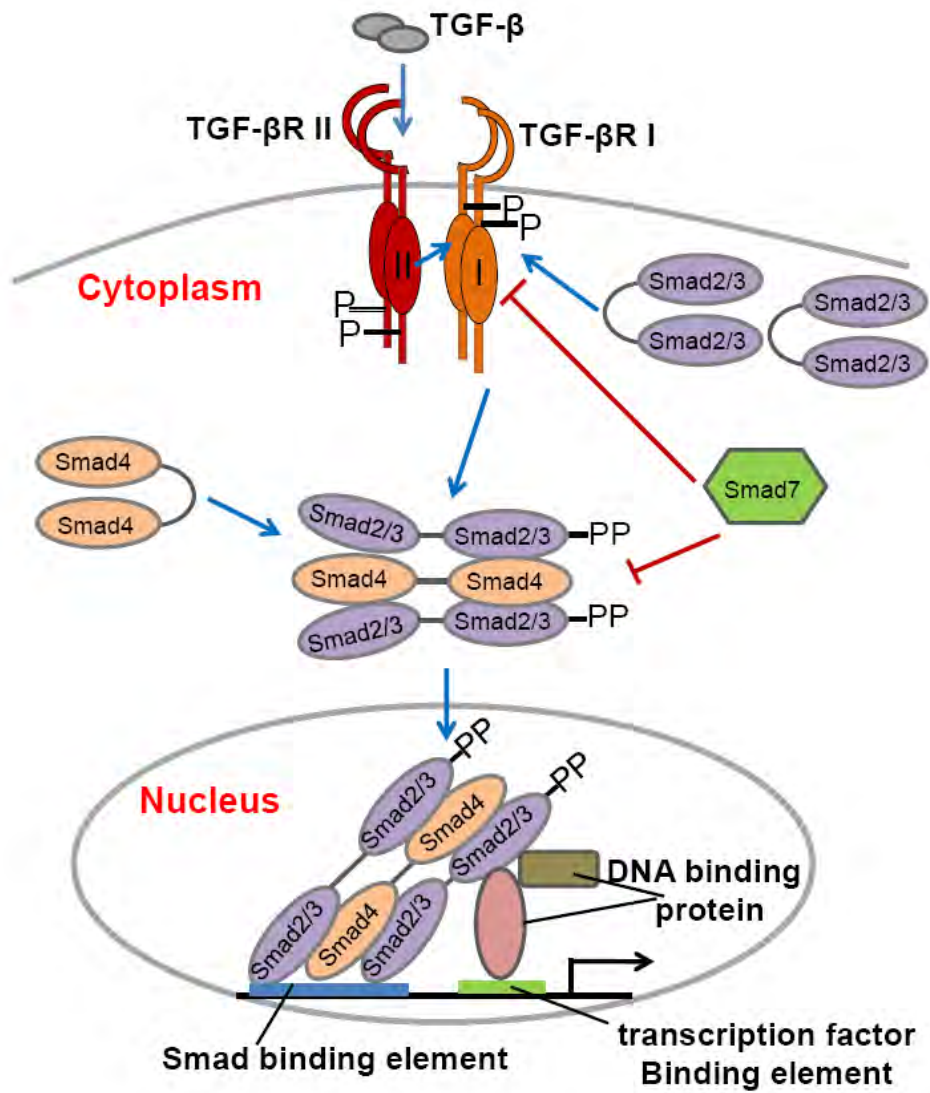


Figure 1.4 TGF- β signaling cascade (revised from (Miyazono 2000))

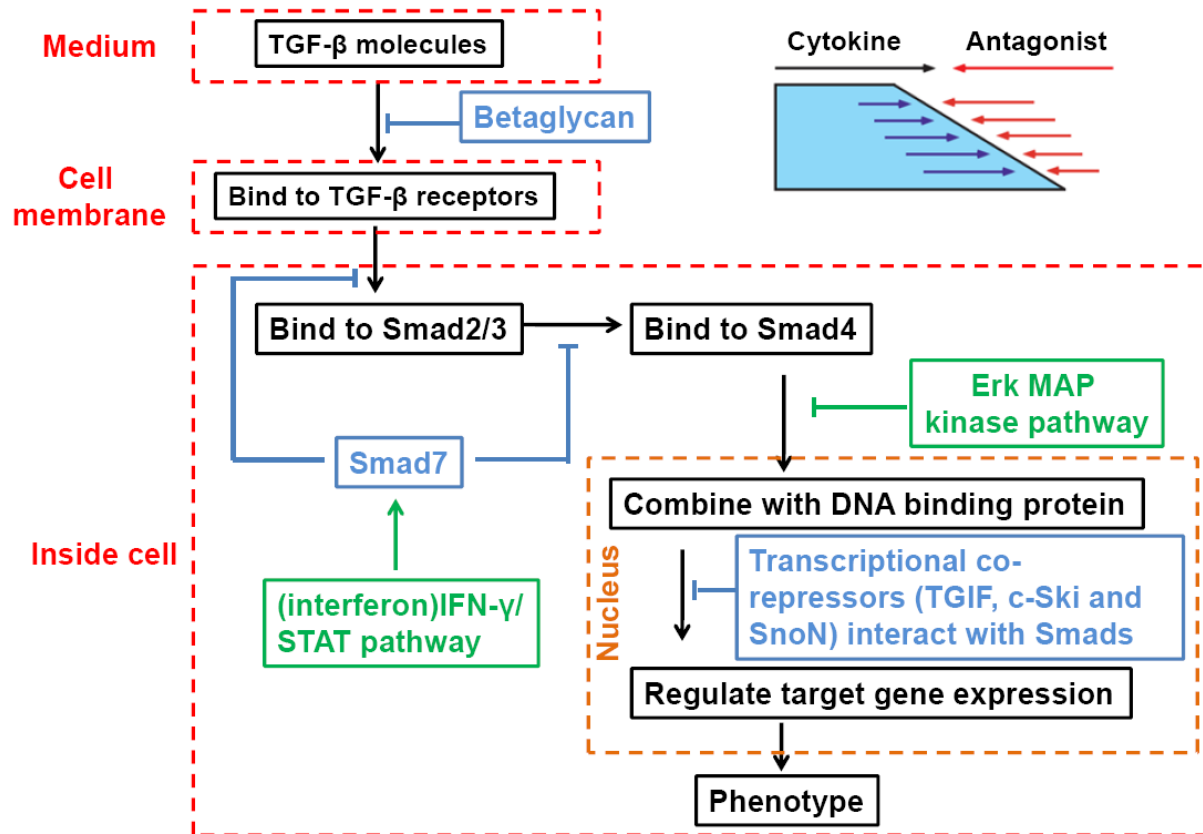


Figure 1.5 Positive and negative regulation of TGF-β signaling cascade

CHAPTER 2

OBJECTIVES

The key goal of this study is to characterize and model cell activation upon TGF- β 1 stimulation in a 3-D culture system. To achieve this goal, there are three specific objectives:

1. Develop a 3-D cell culture system, which can provide specific physiochemical characteristics of normal and cancer tissues;
2. Set up a method to characterize the spatial-temporal cellular response to growth factors in the 3-D culture;
3. Use a quantitative mathematical model to describe and predict how growth factors given at fixed concentrations diffuse into the 3-D culture, stimulate cells and induce response.

CHAPTER 3

FABRICATION OF A 3-D CULTURE SYSTEM AND CHARACTERIZATION OF CELL ACTIVATION UPON TGF- β 1 STIMULATION

In this chapter, we developed a thick 3-D cell culture model with depth of 4.5 ± 0.05 mm. It helps to avoid two problems of thin layer 3-D culture. First, thick gels can minimize the effect of stiff bottom of culture vessels, which complicates the effect of TGF- β 1 induced cell response we are interested in. It is well known that the chemical and mechanical signals are coupled in the biological system. It has been reported that mesenchymal stem cells can sense the matrix elasticity to specify cell lineage and phenotypes (Engler, Sen et al. 2006). Extracellular matrix rigidity is also indicated to regulate the structure, motility, and proliferation of glioma cells (Ulrich, Pardo et al. 2009). Therefore, decoupling the chemical responses from mechanical ones is critical in our studies of cell responses to growth factors. Second, our studies consider the diffusion effect of growth factors to mimic natural tissues, and therefore require a reasonable depth for the purposes of diffusion studies. Thick gels thus provide enough scale to study the influence of growth factor concentration gradient on cell activation.

The objectives of this chapter are threefold. First, we demonstrated that in the 2-D Petri dishes normal fibroblasts can be converted into myofibroblasts with TGF- β 1 stimulation and express α -SMA marker. Second, we fabricated a thick 3-D cell culture using collagen type I as the main scaffold, optimized the cell density and characterized the fibroblast/myofibroblast conversion upon TGF- β 1 stimulation using immunofluorescence staining and confocal fluorescence imaging. Third, we developed a novel layered structure of 3-D culture to overcome the depth limitation of confocal microscope and characterized the spatial-temporal cell response to TGF- β 1 stimulation.

3.1 Experimental Materials and Methods

3.1.1 Monolayer Cell Culture and TGF- β 1 Stimulation

Lung fibroblast cell line MRC-5 (American Type Culture Collection, Rockville, MD) was cultured in 75 cm² tissue culture flasks with Minimum Essential Medium Eagle (MEME) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep (complete medium) at 37°C with 5% CO₂. The cells were harvested from monolayer culture with 0.25% trypsin/1mM EDTA. Trypsin was neutralized with 10% FBS MEME. The cells with the 4-15th passage were used.

The effect of TGF- β 1 on MRC-5 fibroblasts was determined by culturing monolayer cells in 35 mm glass bottom dishes (MatTek Corp., Ashland, MA) with complete culture medium to 60% confluence, starving the cells overnight in 0.5% FBS/MEME and then stimulating cells with 10 ng/ml TGF- β 1 in 0.5% FBS/MEME.

3.1.2 3-D Cell Culture and TGF- β 1 Stimulation

3-D collagen matrices were prepared from collagen I (8.69 mg/ml, BD Biosciences, high concentration, rat tail). Collagen solution was diluted and neutralized with complete medium and 1N sodium hydroxide to 2.0 mg/ml to make the collagen gels without cells. Cells were added to the collagen solution to make the collagen gels with cells. To make the layer structure of collagen gels, aliquots of collagen or cell/collagen mixtures were placed in the wells of Corning 48-well culture plates. Polymerization of collagen matrices was completed in 30 min at 37°C. More layers were added with the same procedure if necessary. Complete medium was applied to the top of gels after polymerization.

To differentiate fibroblasts into myofibroblasts in collagen matrices, the 3-D matrices were incubated in complete culture medium for 24 hr, followed by overnight starvation, and then stimulated by TGF- β 1 in 0.5% FBS/MEME.

3.1.3 Diffusion of Trypan Blue in the Collagen Gel

Gels were made with phenol red free medium in a 48-well culture plate. After polymerization, trypan blue stock solution (Sigma-Aldrich) was added on the top of gels. A Canon digital camera was used to take pictures every half an hour.

3.1.4 Immunocytochemical Staining

Immunocytochemical staining (ICC) was performed on cells grown in 35 mm glass bottom dishes with 10 ng/ml TGF- β 1 stimulation for 3 days. Culture medium was subsequently removed and the cell monolayer was 3 $\times$ washed with ice-cold TBS; the cells were fixed with 4% paraformaldehyde at room temperature (RT) for 15min; 3 $\times$ 5 min/each washed with TBS at RT; permeabilized with 0.1% TX-100 in TBS for 15 min, at RT; rinsed thoroughly with TBS, 3 $\times$ 5 min/each. Following fixation, the cells were incubated with 1% BSA for 20 min, at RT; then washed 3 $\times$ TBS, 5 min/each, at RT. Subsequently, the cells were incubated with primary monoclonal α -SMA antibody (1:100, 10 μ g/ml) in TBS (1% BSA) at RT for 1 hr, followed by 3 $\times$ TBS wash, 5 min/each, at RT. Cells were then incubated in secondary antibody conjugated with HRP (Dako kit), at RT for 1 hr, followed by 3 $\times$ TBS wash, 5 min/each, at RT. DAB substrate (20 μ l in 1ml buffer, Dako kit) was used to develop the color for 5~10 min. The samples were sealed with mounting medium and taken for imaging.

3.1.5 Immunofluorescence Staining

In 2-D culture, cells were washed with ice-cold TBS and fixed with 4% paraformaldehyde at RT for 15min, followed by 3 $\times$ 5min/each wash with TBS at RT. Cells were permeabilized with 0.1% TX-100 in TBS for 15 min, and rinsed thoroughly with TBS, followed by 3 $\times$ wash in TBS 5min/each. Cells were then incubated with 1% BSA for 20 min, followed by 3 $\times$ wash in TBS 5 min/each. Cells were then incubated in primary monoclonal α -SMA antibody (1:100, 10

µg/ml) in TBS (1% BSA) at RT for 1 hr, followed by 3× wash in TBS, 5min/each. Cells were then incubated in secondary antibody conjugated with fluorescence dye (1:200) in TBS (1% BSA), at RT for 1 hr, followed by 3× TBS wash, 5 min/each. Samples were mounted with anti-photobleaching reagent, sealed with glass coverslips and then taken for imaging.

3-D collagen gels were fixed with 4% paraformaldehyde at 4°C overnight before the layers of each gel were separated. 0.15 M glycine in PBS was added to the gels for 10 min to quench the paraformaldehyde. Gels were then washed and permeabilized in 0.02% TX-100 in PBS for 15 min followed by 5% non-fat milk blocking for 2 hr. Gels were then incubated with primary monoclonal α -SMA antibody (1:100 in 1%BSA/PBS/T) overnight at 4°C and secondary antibody (1:200 in 1%BSA/PBS/T) at RT for 2 hr. Cell nuclei were stained with TO-PRO 3 (1:1000 in PBS) for 20 min before gels were mounted between two glass coverslips with anti-photobleaching reagent.

3.1.6 Confocal Microscopy Imaging

All gels were examined with a Leica SP2 laser scanning confocal microscope (Leica, Heidelberg, Germany) with Hg lamp and helium/neon laser. 488nm excitation wavelength was used for FITC and 633 nm excitation wavelength was used for TO-PRO 3. For each gel, a series of images were captured along z-coordinate (the depth of gel) in the same lateral area and the imaging step size is 3-5 µm.

3.1.7 SDS–PAGE and Western Blotting

2-D cultured cells were washed with ice-cold PBS and lysed under addition of 1% TX-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1:1000 diluted protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were incubated on ice for 10 min and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected. The total protein concentration was quantified via the

BCA protein assay reagent kit (Sigma-Aldrich), adjusted by adding appropriate amounts of lysis buffer, subsequently mixed with 2× loading buffer (Bio-Rad Laboratories), and stored at 4°C. Proteins were separated by SDS-PAGE Ready Gels (10% Tris-HCl, Bio-Rad Laboratories) with glycine-Tris running buffer in a Bio-Rad's PowerPac™ HC electrophoresis system at 100V for 60 min. A routine blotting technique was used to transfer protein to the membrane (Bio-Rad Ready Gel Blotting Sandwiches). Membranes were blocked with 5% fat free milk in Tris-buffered saline with Tween 20 (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). Proteins were detected by incubating the membranes with primary monoclonal α -SMA antibody (1:1000, 1 μ g/ml) for 1 hr at RT. After subsequent washing, horseradish peroxidase (HRP)-conjugated secondary anti-mouse (1:5000; Sigma-Aldrich) was applied for 1 hr at RT. Peroxidase activity was recorded on a Fluorchem 8900 imager using the supersignal west pico chemiluminescent substrate (Pierce). β -actin was used as a protein control.

3.1.8 Hematoxyline and Eosin Staining

Collagen gels were fixed in 4% paraformaldehyde in PBS overnight at 4°C, before processing into paraffin wax. Paraffin embedded tissue was sectioned to 10 μ m thick slices with a SHUR/Sharp™ 4060E Electronic Rotary Microtome (Triangle Biomedical Sciences) and mounted on glass slides, deparaffinized in xylene and rehydrated in a serial dilutions of ethanol (100% 3×3 min, 95% 1×3 min, 80% 1×3 min and DI water 1×5 min). They were then stained with hematoxylin for 3 min, and developed in water for 5 min. Slides were dipped into acid ethanol 8-12 times to destain and rinsed with tap water. They were then stained with eosin for 30 sec, and dehydrated in ethanol (95% 3×5 min, 100% 3×5 min) and xylene (3×15 min).

3.1.9 Automated Cell Counting

In order to count the total cell number and activated cell number, we detected red fluorescence regions (nuclei, representing total cell number) and green fluorescence regions (α -SMA, representing activated cell number) in the images from confocal microscope. Both red and green fluorescence regions in the images are brighter than background, but the level and pattern of noise vary from images to images. To handle the noise and find the candidate fluorescence objects, a 2-stage Percentile (P-tile) thresholding is applied to the images. The P-tile threshold value is determined as the intensity where the ratio of the number of pixels smaller than the intensity to the total number of pixels in the image is closest to the given percentile. (Doyle, 1962) The fluorescence we are interested in is assumed to be present in the area above the P-tile threshold value. In the first stage, the global P-tile threshold value is computed for the entire image (512×512 pixels). In the second stage, the image is divided into 16 equal-sized patches (128×128 pixels), and the local P-tile threshold values are computed for each patch. In this study, the global and local P-tiles for red fluorescence detection are 90 and 95, respectively. In green fluorescence detection, 70 and 75 are used as the global and local P-tiles, respectively. Applying 2-stage P-tile thresholding, each set of connected and selected pixels becomes the candidate object. Since the global and local threshold values may not be optimal, the candidate object may only represent a part of the true fluorescence, or it is erroneous from unwashed fluorescence dye. To obtain the remaining fluorescence region,, an iterative region growing method is used; ; the initial threshold value th_0 is the local P-tile threshold value, and at iteration i , the threshold value is updated as $th_i = th_{i-1} \pm th$ ($th = 3$). The neighboring pixels above the updated threshold value are marked as fluorescence region. It is repeated until no more pixels are found. To eliminate the artifacts, we restrict the candidate objects by their intensity and size. For each candidate object, we collect pixels (B) surrounding the object, and the intensity threshold

value is determined as $AVG(B) + STD(B)$ where $AVG(\bullet)$ and $STD(\bullet)$ represent average and standard deviation of $\bullet$. The size threshold value is computed as $0.5 * S_{median}$ where S_{median} is the median size of all candidate objects. Have detected both red and green fluorescence, we count the number of total and activated cells. The total number of cells in an image is the total number of red fluorescence objects, and the activated cell is the red fluorescence object where more than 30% of the pixels in the object are also designated to have green fluorescence.

3.2 Results and Discussion

3.2.1 Response of monolayer cultured fibroblasts to TGF- β 1 stimulation

Cell responses to TGF- β 1 stimulation in 2-D culture environment were first tested. Activated fibroblasts after TGF- β 1 stimulation were characterized by the expression of α -SMA with immunocytochemical staining, immunofluorescence staining and Western blotting. All these three methods demonstrated that expression level of α -SMA was increased with TGF- β 1 stimulation. These results confirmed that fibroblasts can be activated by TGF- β 1 in our 2-D culture (figure 3.1).

3.2.2 Diffusion of Cell Culture Medium in Thick 3-D Gels Is Not a Limiting Factor

One potential limitation of thick 3-D culture is basic nutrients delivery in the cell culture medium. We used trypan blue as a substitute to study the diffusion of culture medium from gel top to bottom (figure 3.2 (a)). Trypan blue was added on top of the gel and its diffusion into the gel was monitored. After 5.5 hr, trypan blue's concentration at the bottom of the gel started to reach the concentration of stock solution (figure 3.2 (b)), indicating a complete delivery of trypan blue across the whole gel. Molecular weight (MW) of trypan blue is 960.81, larger than that of any component in the MEME medium we used. Therefore, the molecules in the MEME medium

can generally diffuse faster than trypan blue into collagen gels and require shorter time to reach the bottom. We reasoned that even the cells at the bottom region can get nutrition from medium in a relatively short period of time after they were seeded in the 3-D collagen gels. Therefore, we no longer consider thickness as a limiting factor for our cell culture system.

3.2.3 Optimization of Cell Density

Cell density is also an important factor for 3-D cell culture. It is known that cells don't grow very well if their density is too low, yet a high density of cells will often lead to significant gel contraction. To determine the optimal cell density for our 3-D culture, MRC-5 fibroblasts at five different densities (10000, 50000, 100000, 200000, 250000, and 500000cells/ml) were cultured inside the 2.0 mg/ml collagen gels. Gel contraction and cell growth were observed and summarized in table 3.1. Cells at lower densities (10000 and 50000 cells/ml) did not grow well in collagen gels and eventually died, while cells at higher densities (250,000 and 500,000cells/ml) caused significant gel contraction over the time. Hence, 200000cells/ml was determined as the optimal density for our study and therefore used in all other experiments in this chapter.

3.2.4 Layered Structure of 3-D Cell Culture

One of the primary methods we use in our studies is fluorescence imaging detection of the cell response to TGF- β 1 stimulation. However, the penetration depth of confocal fluorescence microscope becomes a limiting factor for thick 3-D gels, whose thickness is significantly beyond our instrument's fluorescence detection range of 150 μ m. To solve this problem, we developed layered structure of thick 3-D cell cultures as an alternative (figure 3.3 (a)). Totally four layers of collagen gel were stacked and the final thickness is 4.5 ± 0.05 mm. After IF staining, each layer was placed between two pieces of glass coverslips before confocal

imaging. At this stage, the thickness of each layer was below 150 μm and information within the layer can be readily captured by confocal fluorescence microscope. Images from cells deep into the gel were successfully captured and staining of α -SMA and nuclei was very clear (figure 3.4 (a)). Cell growth in gel was also monitored. H&E staining showed normal cell growth within the thick 3-D culture (figure 3.3 (c)).

3.2.5 Characterization of Cell Response to TGF- β 1 Stimulation

TGF- β 1 induced activated fibroblasts were characterized by the expression of α -SMA, which was visualized in green with FITC-conjugated antibody. Nuclei were stained in red with TO-PRO3 fluorescence nucleus dye. The activation ratio was determined by counting the α -SMA-stained cells relative to the total number of cells, which is the same number of nuclei (equation (3-1)). Cell counting is time-consuming and sometimes subjective due to human intervention. For example, to get the activation ratio three imaging regions were needed for each TGF- β 1 treated gel layer and for each region there was about 10~20 Z-axis images. All α -SMA-stained cells and nuclei needed to be counted. At the same time, the standard of counting some cells with fainter stain often varies to different people or even different rounds of analysis by the same person. Here, we developed an automated segmentation method (See 3.1.9 Automated Cell Counting) to recognize and count two different types of staining from a confocal image (figure 3.4). It can give out the numbers of α -SMA-stained cells and total cells and then calculate the activation ratio (equation (3-1)).

$$\begin{aligned} \text{Activation ratio}, f &= \frac{\alpha\text{-SMA-stained cells}}{\text{Total number of cell nuclei}} \\ &= \frac{\text{Number of red nuclei}}{\text{Number of red nuclei} + \text{Number of blue nuclei}} \quad (3-1) \end{aligned}$$

3.2.6 Cell Response to TGF- β 1 Stimulation without Diffusion Effect

Before we could build up a mathematical model of cell response to TGF- β 1 stimulation in our 3-D environment, we separated the effect of TGF- β 1 diffusion in 3-D collagen gels from cell response to TGF- β 1. Since the chemical and mechanical environments of cells in 2-D culture and 3-D culture are different, we decided not to use the 2-D culture as a media. Instead, we assumed that the diffusion effect in the top layer of the four layers thick gel is not very significant because it is in contact with high concentration of TGF- β 1 in the stock solution. Therefore, cells can receive TGF- β 1 quickly for them to response in this layer, enabling us to ignore the diffusion effect in this layer. The results from this layer thus would be considered approximately as pure cell response to TGF- β 1 in its close vicinity in the 3-D culture. Cell responses to different TGF- β 1 concentrations (10, 1.0, 0.25, 0.15 and 0.10 ng/ml) were recorded using the methods we described. Figure 3.5 (a) shows the time-course of cell activation ratio with TGF- β 1 stimulation at different TGF- β 1 concentrations. We fitted the data with 3 parameters logistic function:

$$f = \frac{F}{1 + e^{-k_t(t-t_0)}} \quad (3-2)$$

where f is the cell activation ratio; F is the maximal cell activation ratio; t is the TGF- β 1 stimulation time; t_0 is defined as the time with a response halfway between baseline and maximum; k_t is the slope factor and defines the growth rate of the function. The curves generally show three phases: the activation lag phase, that is, the time for the TGF- β 1 bind to its receptors and initialize the signaling cascade; the log phase, in which the activation ratio begins to increase exponentially; and the plateau phase, in which the cell activation ratio becomes saturated.

Parameters in the logistic functions for different TGF- β 1 concentrations are summarized in table 3-2. Figure 3.5 (b), (d) and (e) give the plots of F , t_0 and k_t versus TGF- β 1 concentration respectively.

We can see that as TGF- β 1 concentration increases, the cell activation ratio plateau F goes up, the slope factor k_t increases and then decreases, and t_0 also goes down. In figure 3.5 (c), F as a function of TGF- β 1 concentration is fitted with the 3 parameters logistic function:

$$F = \frac{F_{max}}{1 + e^{-k_c(c-c_0)}} = \frac{0.87}{1 + e^{-17.19(c-0.15)}} \quad (3-3)$$

It also appears that as TGF- β 1 concentration is higher than 1ng/ml, the maximal cell activation ratio F gets to a plateau.

There results can be explained by the binding kinetics of TGF- β 1 and its receptors. Cells are only activated when a threshold percentage of receptors are occupied by TGF- β 1. The increase of TGF- β 1 concentration results in higher ratio of cells that reach that threshold and get activated, which leads to the increase of plateau value F . Similarly, the kinetics of binding determines that the time for cells to reach the threshold is reduced when more TGF- β 1 molecules are available, which explains why t_0 goes down and the slope factor k_t increases initially.

The balance of TGF- β 1 positive and negative regulations may also play a role. Positive regulation can come from TGF- β 1 molecules binding to their constitutively active type II receptors, which lead to the activation of its downstream signal pathway and subsequent increase of cell activation. (Wrana, Attisano et al. 1994) On the contrary, negative regulation may also occur through various mechanics, involving various TGF- β 1 receptor inhibitors from both outside of cells, primarily medium and extracellular matrix (Andres, Stanley et al. 1989;

Saharinen, Hyytiainen et al. 1999) and from inside, primarily cytoplasm and nuclei. (Miyazono 2000) The positive regulations are more likely the dominant factors until TGF- β 1 concentration is very high, when the negative regulations can overpower positive ones, as the results have shown.

Due to the statistical nature of TGF- β 1/receptor binding, some receptors cannot get enough TGF- β 1 molecules to activate cells, which explain why the plateau cell activation ratio value gets closer but never reaches 100% as TGF- β 1 concentration increases. This might also partly be contributed from the negative regulations. When the concentration is over 1ng/ml, the plateau value no longer gets closer significantly towards 100%, which explains the F.

To test the reproducibility of experiments, three independent experiments with 0.25 ng/ml TGF- β 1 stimulation were conducted. After fixation, the top layers of the collagen gels were stained; three regions in each layer were imaged. The activation ratio in each layer was first calculated, then the ratios from three top layers were averaged and standard error was calculated. The result was summarized in figure 3.5(f). It shows low standard error of the mean associated with the measurements carried out in three independent collagen gels, indicating a good reproducibility of our experiments.

3.2.7 Cell Activation Ratio as a Function of Collagen Gel Depth

As we have discussed earlier, diffusion of growth factors is likely to play a role. We hypothesized that the cell activation is a function of collagen gel depth due to the diffusion effect. We took advantage of the layered structure of collagen gel to measure the activation ratio at different depths. In the figure 3.5 (a), the cell activation starts to reach saturation as TGF- β 1 concentration is over 0.25 ng/ml. If TGF- β 1 solutions of higher concentration such as 10 or 1.0 ng/ml are added on the top of gel, the local concentration inside the gel is likely to reach a level

higher than the saturation concentration of 0.25 ng/ml, and thus masking the difference across the depth of the gel. Therefore, 0.25 ng/ml TGF- β 1 was used to measure the cell response to TGF- β 1 stimulation in the thick collagen gels. As we can see from figure 3.5 (a), the cell activation is insignificant before 30 hours of 0.25 ng/ml TGF- β 1 stimulation. So in this experiment we only collected and studied the stimulated samples at 36, 42, 48 and 60 hr respectively. The cell activation ratio in each layer was calculated. Figure 3.6 shows the cell activation ratio as a function of time in each layer.

Data in the figure 3.6 are the mean from all cells in the three imaging areas independently chosen in each layer. Collagen gels became much thinner after they were placed between two pieces of glass coverslips for confocal microscopy imaging. We considered that in this step there is no significantly relative cellular position change, and reconstructed the compressed gel to its original thickness to measure the cell activation ratio as a function of gel depth (figure 3.7). Depth = 0 is the boundary of TGF- β 1 solution and collagen gel. Cells in the region near TGF- β 1 stock solution started to be activated after 36 hr. Cells inside the gel and near to the bottom were activated later. Cell activation ratio gradually increased over the time.

3.3 Conclusion

In this chapter, we have developed a 3-D cell culture system that can promote fibroblast/myofibroblast conversion by TGF- β 1 stimulation. Cell density, cell culture medium, and cell culture condition were optimized. We have characterized the spatial-temporal cell activation profiles in the 3-D collagen gel upon TGF- β 1 stimulation with IF staining and confocal microscopy imaging. Our results revealed that the conversion from normal fibroblasts

to myofibroblasts in 3-D culture is both time and gel depth dependent, and the conversion starting time, speed and saturation plateau are all closely related to TGF- β 1 concentration.

3.4 Figures and Tables

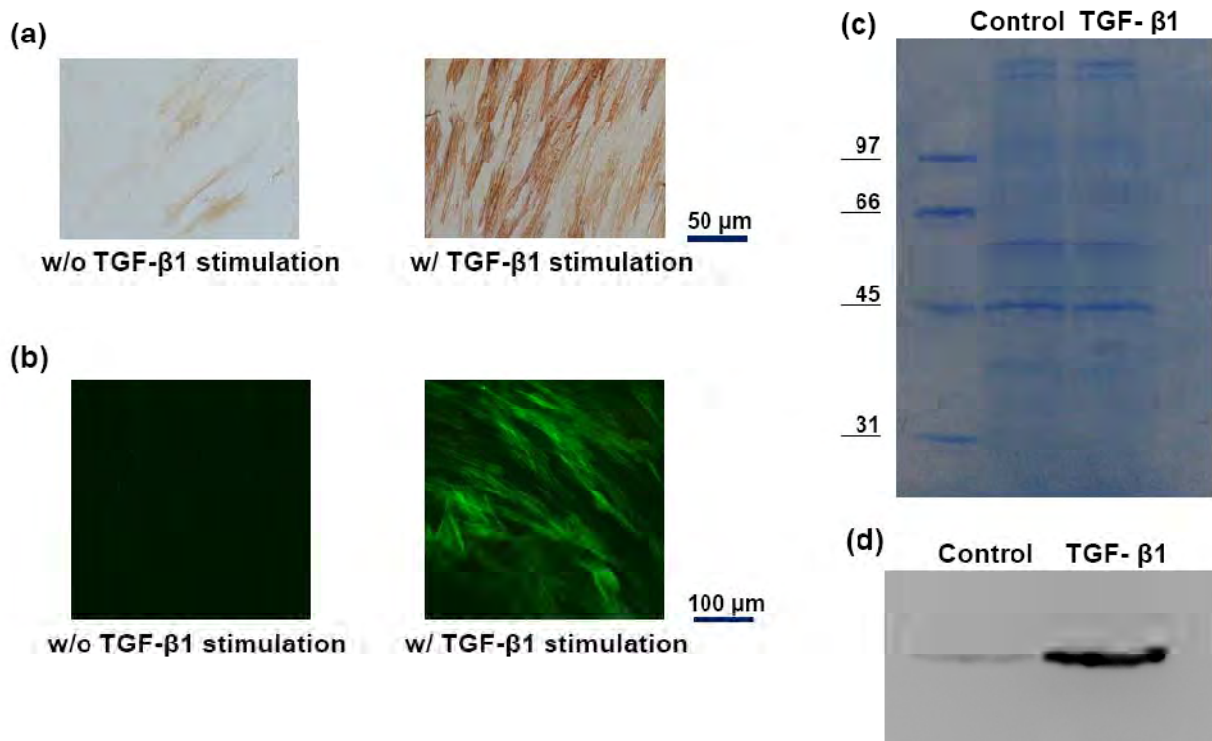


Figure 3.1 Response of monolayer cultured fibroblasts to TGF- β 1 stimulation. (a) Microscopy images of cells with immunocytochemical staining (20 $\times$). α -SMA is shown in brown; (b) Confocal fluorescence microscopy images of cells with immunofluorescence staining (20 $\times$). α -SMA is shown in green. (c) SDS-PAGE analysis of monolayer cultured fibroblasts. Lane 1, MW marker; Lane 2, diffusate of cells w/o TGF- β 1 stimulation; Lane 3, diffusate of cells w/ TGF- β 1 stimulation. The result shows that there is same amount actin in the diffusates w/ and w/o TGF- β 1 stimulation. (d) Western blotting analysis of monolayer cultured fibroblasts. It shows that the amount of α -SMA in the sample w/ TGF- β 1 stimulation is much higher than it in the sample w/o TGF- β 1 stimulation.

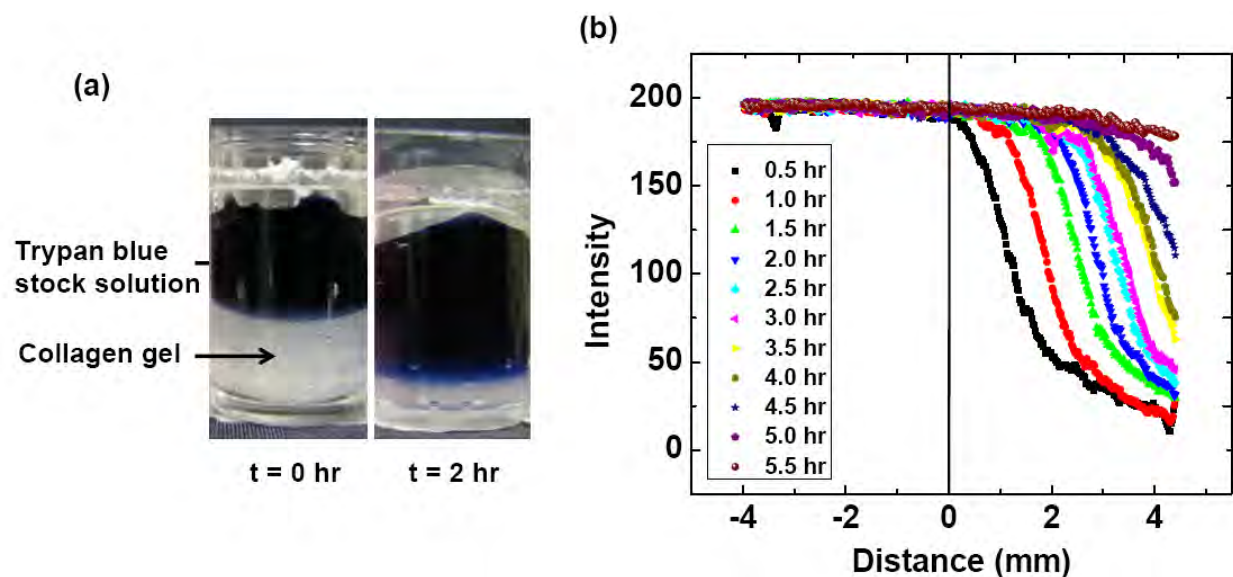


Figure 3.2 Diffusion of trypan blue in the collagen gels. (a) Picture of trypan blue diffusion, captured by a digital camera; (b) The y axis is the intensity of blue pixel in the digital camera captured pictures, corresponding to the concentration of trypan blue. The x axis is the diffusion distance ($x=0$, the boundary between trypan blue stock solution and collagen gel; $x < 0$, the stock solution region; and $x > 0$, the collagen gel region).



Figure 3.3 Schematic of layered structure of 3-D cell culture. (a) Layered collagen gel with MRC-5 lung fibroblasts embedded; (b) Bottom view of 3-D cell culture in the wells of 48-well-plate; (c) H&E stained collagen gel with cells embedded.

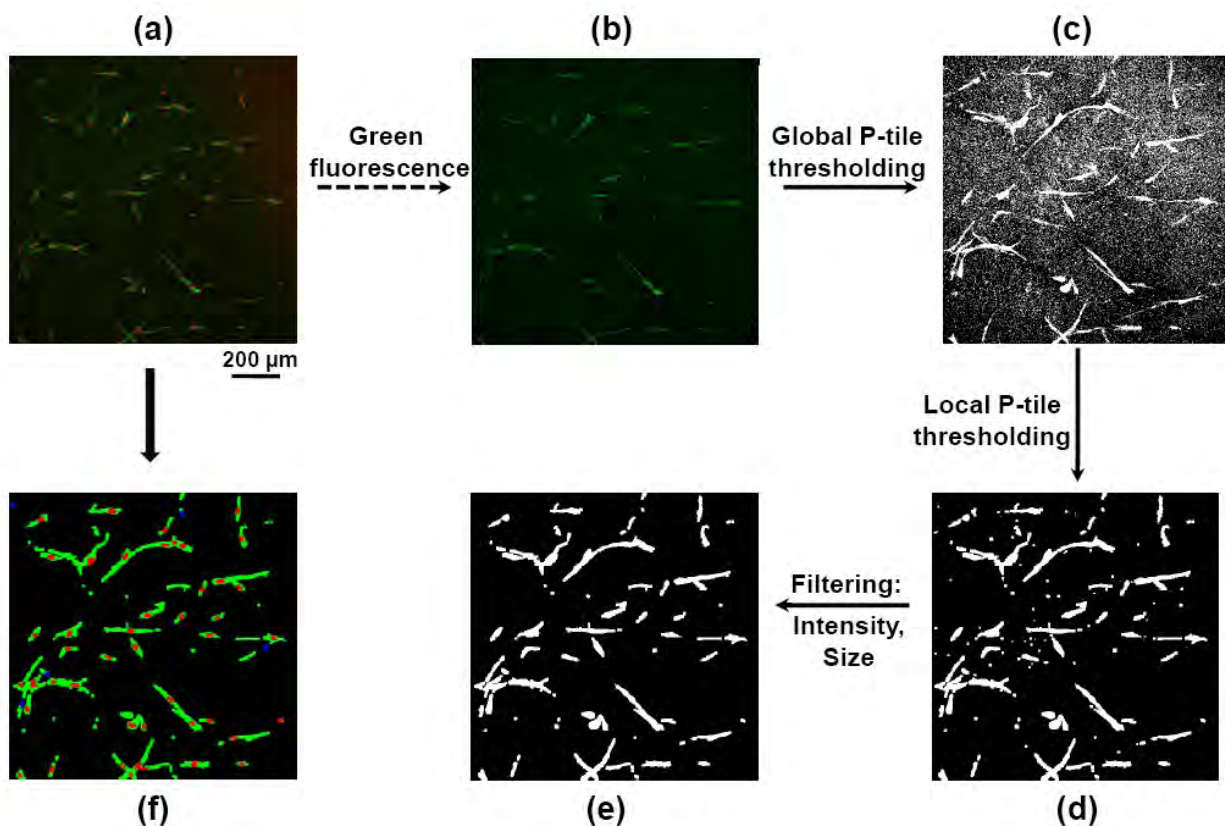


Figure 3.4 Automated cell counting and cell activation ratio calculation. (a) Confocal fluorescence microscopy image (20 $\times$). α -SMA is shown in green and nuclei are in red. Cells with green are activated. (b)-(e) show the automated segmentation method applied to a green fluorescence image. The same method was used for red fluorescence images (not shown here). (b) Input image of green fluorescence. (c) Global P-tile thresholding. (d) Local P-tile thresholding. (e) Intensity and size restriction. (f) Computer generated image for automated cell counting. α -SMA is shown in green; nuclei are in red for cells with α -SMA and in blue for cells without α -SMA.

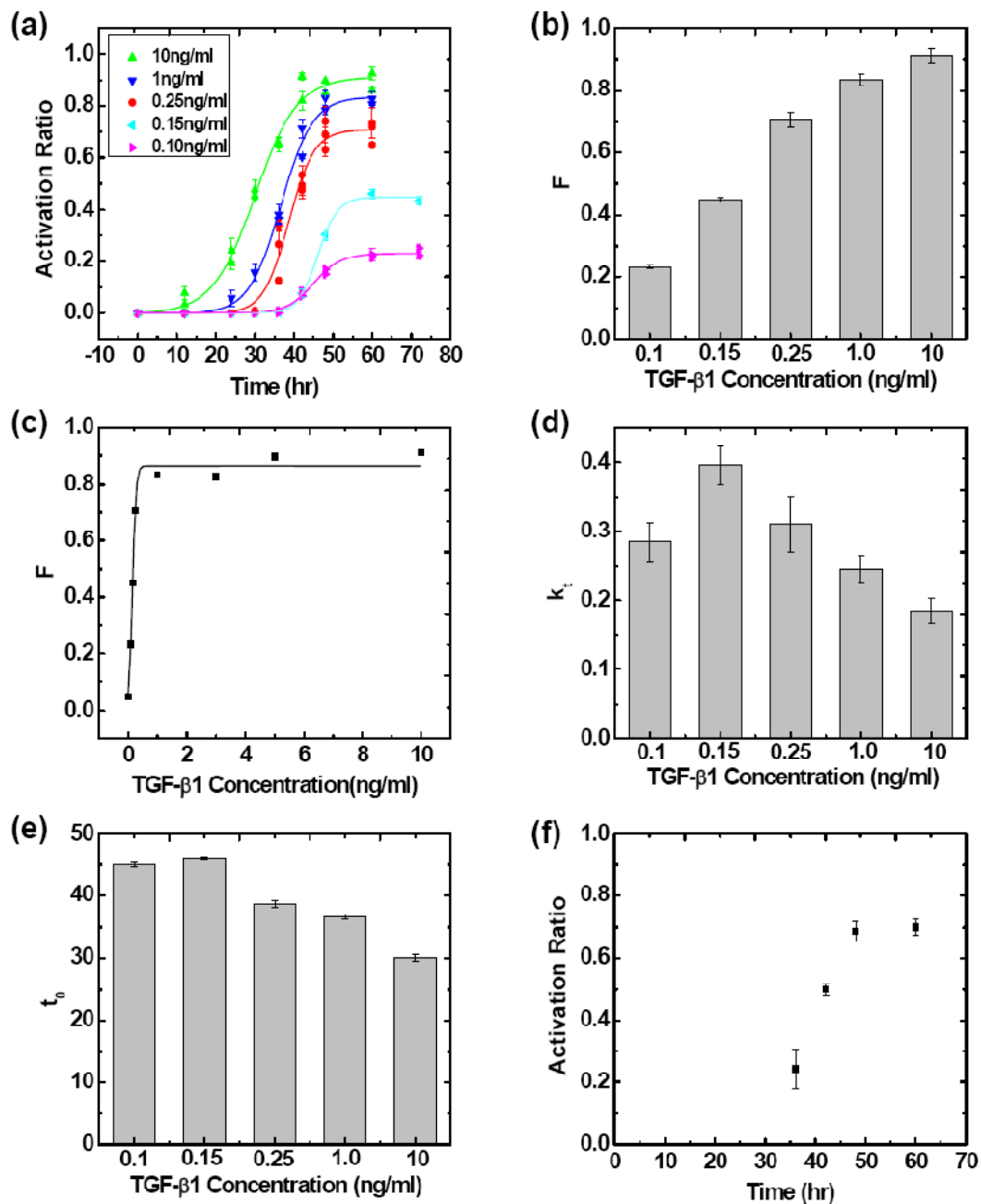


Figure 3.5 Cell responses to TGF-β1 stimulation without its diffusion effect. (a) Cell activation ratio as a function of time. 10, 1.0, 0.25, 0.15 and 0.10 ng/ml TGF-β1 was used to stimulate MRC-5 fibroblasts respectively. Data are calculated as the mean of three regions in each layer with standard error of the mean. Experimental data are fitted with 3 parameters logistic function. (b) Activation ratio plateau F as a function of TGF-β1 concentration. (c) F as a function of TGF-β1 concentration fitted with 3 parameters logistic function. (d) k_t as a function of TGF-β1 concentration. (e) t_0 as a function of TGF-β1 concentration. Error bars in (b), (d) and (e) are standard error reported by Originpro 8.0. (f) The reproducibility of experiments. Data are calculated as the mean of three independent gels with standard error of the mean.

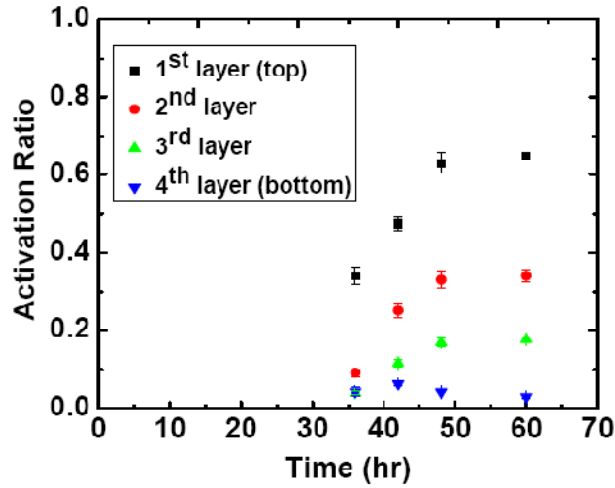


Figure 3.6 Cell activation as a function of time in each collagen gel layer. Data are calculated as the mean of three regions in each layer with standard error of the mean.

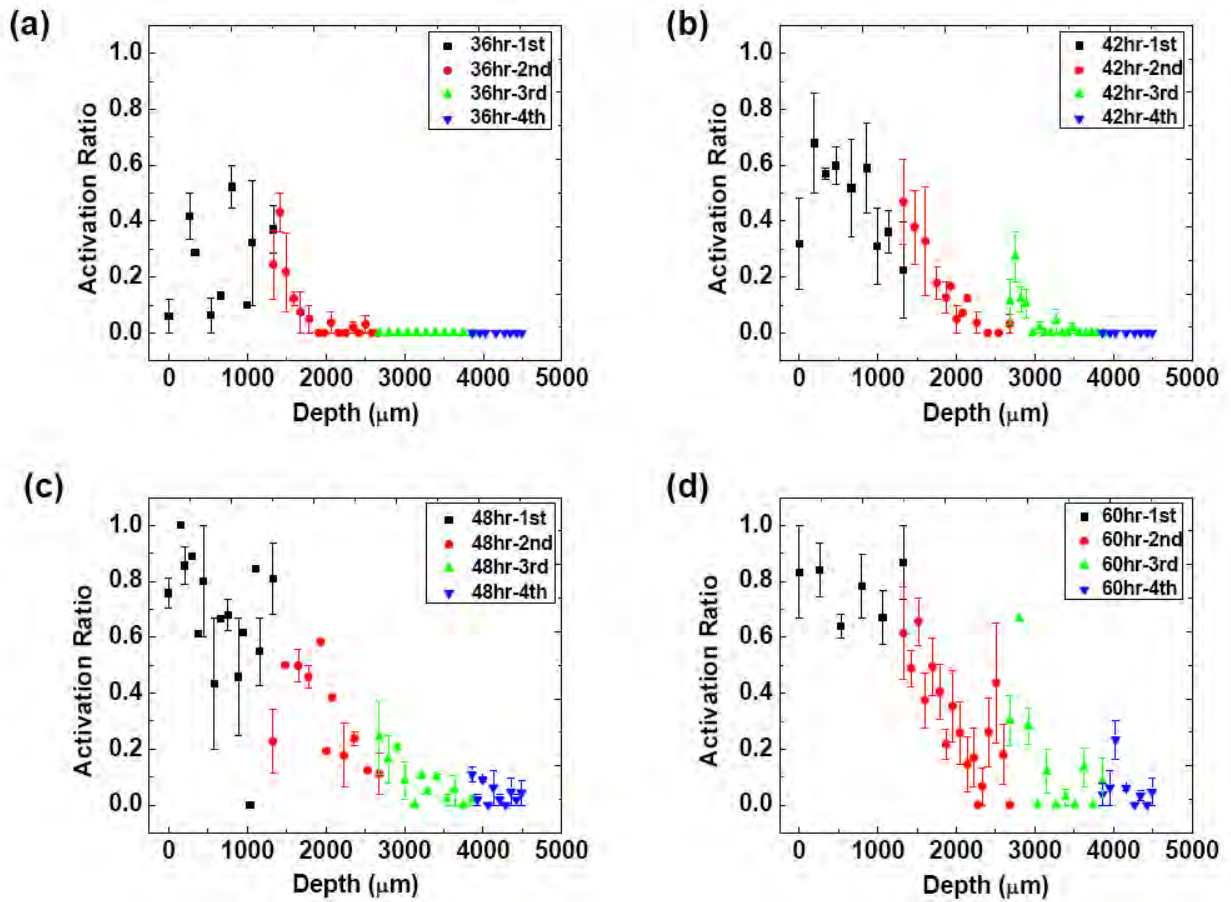


Figure 3.7 Time course of cell activation as a function of collagen gel depth. (a) at 36hr; (b) at 42hr; (c) at 48hr and (d) at 60hr.

Table 3.1 Gel morphology with different cell densities

Cell density (number/ml)	10,000	50,000	100,000	200,000	250,000	500,000
with TGF- β 1 stimulation	N/A*	N/A	N/A	N/A	at 36 hour, 60% of original size; at 60 hour, 40% of original size	at 36 hour, 50% of original size; at 60 hour, 30% of original size
without TGF- β 1 stimulation	N/A	N/A	N/A	N/A	at 36 hour, original size; at 60 hour, 80% of original size	at 36 hour, 80% of original size; at 60 hour, 50% of original size

*: N/A means there is no gel contraction.

Table 3.2 Parameters in the logistic functions for different TGF- β 1 concentrations

TGF- β 1 concentration (ng/ml)	F	SE of F	k_t	SE of k_t	t_0	SE of t_0
0.1	0.231	0.005	0.284	0.028	45.063	0.400
0.15	0.449	0.006	0.397	0.028	46.102	0.214
0.25	0.707	0.024	0.311	0.040	38.651	0.526
1	0.834	0.019	0.246	0.020	36.626	0.405
10	0.913	0.022	0.185	0.018	30.080	0.550

CHAPTER 4

TGF- β 1 DIFFUSION AND CELL ACTIVATION MODEL

The process of TGF- β 1 stimulation in thick 3-D cultures is a complicated system, including the diffusion of TGF- β 1 molecules from the external medium into the gel, TGF- β 1 molecules binding to their receptors and its down-regulation and finally translocation to the nuclei to regulate target gene expression and other subsequent events. At first look, it seems like there are a large number of parameters involved in the system. In this chapter, we simplified this complicated 3-D cell activation and response system to a “cue-response” system, which allowed us to predict how soluble factors provided at a constant concentration in the medium outside diffuse into the 3-D culture and reach individual cells to induce the cell response. We separated the 3-D cell activation and response system into three individual steps: (1) TGF- β 1 diffusion in a 3-D environment; (2) seeding cells in the 3-D environment; and (3) cell response to TGF- β 1 in its immediate vicinity.

4.1 TGF- β 1 Diffusion in a 3-D Environment

In the chapter 3, we added TGF- β 1 solution on top of a homogeneous gel. TGF- β 1 diffusion in this system can be simplified to 1-D diffusion along gel depth. According to Fick’s second law, the process for TGF- β 1 diffusion in the collagen gel can be expressed as

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial z^2} \quad (4 - 1)$$

where D is the diffusion coefficient independent of the TGF- $\beta 1$ concentration c in the gel at distance z at a given time t . The initial conditions are

$$c(z, 0) = \begin{cases} 0, & 0 \leq z < h \\ c_0, & z \geq h \end{cases} \quad (4-2)$$

where h is the thickness of collagen gel. The boundary conditions are

$$\begin{aligned} \frac{\partial c(z, t)}{\partial z} &= 0, \quad \text{at } z = 0 \\ c(z, t) &= c_0, \quad \text{at } z = h \end{aligned} \quad (4-3)$$

We looked the collagen gel in the well as a semi-infinite cylinder and with an impermeable boundary at $z=0$, all the diffusion occurs in the direction of positive z . To solve equation (4-1), the concept of reflection at the boundary was introduced. (Crank, 1975) We consider the solution for the negative z to be reflected in the plane $z=0$ and superposed on the original distribution in the region $z>0$. Thus the solution to equation (4-1) is

$$c(z, t) = c_0 \sum_{n=0}^{\infty} \left[(-1)^n \left[\operatorname{erfc} \left(\frac{(2n+1)h - z}{2\sqrt{Dt}} \right) + \operatorname{erfc} \left(\frac{(2n+1)h + z}{2\sqrt{Dt}} \right) \right] \right] \quad (4-4)$$

Figure 4.1 (b) shows the concentration distribution of TGF- $\beta 1$ in a defined 3-D environment.

4.2 Seeding Cells in the 3-D Environment

Cells are randomly seeded in the 3-D environment with uniform distribution. The density of cells seeded is based on experiments. X and y dimensions are same as the size of confocal microscope images with 20× objective. The dimension of z axis is the thickness of collagen gel (Figure 4.1 (c)).

4.3 Cell Response to TGF- β 1 without Diffusion Effect

The input of this step is based on the experimental results of cell activation ratio time-course without considering TGF- β 1 diffusion effect in the section 3.2.6. Percentage of activated cells is modeled as a 3 parameters logistic function of time and TGF- β 1 concentration. Equation (3-3) was used to obtain the maximum percentage of activated cells as a function of TGF- β 1 concentration as showed in figure 4.1 (d)(1). Also the data in figure 3.5 (d) and (e) were included to get percentage of activated cells as a function of time as showed in figure 4.1 (d)(2). We can then generate the cell activation time course curves with any concentration TGF- β 1 stimulation.

4.4 Simulation

As shown in figure 4.1, with the consideration of TGF- β 1 diffusion effect on cell activation in the 3-D environment, the three steps mentioned above were used as input of the model to simulate the spatial-temporal cell response. The only unknown parameter in this model is the diffusion coefficient D of TGF- β 1. The model was run with a series of D values for 0.25 ng/ml TGF- β 1 stimulation and then the χ^2 was calculated. For the simulation of each D value, the gel was divided into 40 slices along the z direction; the cell activation ratio in each slice was output. Figure 4.2 gives three simulation results with different D values and χ^2 as a function of D.

χ^2 indicated that as D in the range from 4.5×10^{-8} to $1.8 \times 10^{-7} \text{ cm}^2/\text{s}$ the simulation outputs can match the experimental results reasonably well. In this range we selected $D = 1.35 \times 10^{-7} \text{ cm}^2/\text{s}$, because it gave the smallest χ^2 . We then ran the model 10 times to get the average simulation curves. Figure 4.3 shows that the simulation curves can fit the experimental data for all other three time points with the exception of the data in the top 1500 μm gel at 36 hr.

4.5 Model Prediction and Experimental Confirmation

$D = 1.35 \times 10^{-7} \text{ cm}^2/\text{s}$ was used to predict the results from 0.50 and 0.15 ng/ml TGF- β 1 solutions. Activation ratio of cells in the 3rd layer with 0.50 ng/ml TGF- β 1 stimulation and 2nd layer with 0.15 ng/ml TGF- β 1 stimulation were measured with immunofluorescence staining and confocal imaging. Figure 4.4 shows that with 0.50 ng/ml TGF- β 1 stimulation the experimental result matches with the simulation result for all four time points and figure 4.5 shows that with 0.15 ng/ml TGF- β 1 stimulation the experimental result at 60 and 72 hr is consistent to simulation output but the experimental result at 42 and 48 hr is a little lower than the simulation output.

4.6 Conclusion

In this chapter, We have simplified the complicate 3-D cell activation and response system into three individual steps: (1) TGF- β 1 diffusion in a 3-D environment; (2) seeding cells in the 3-D environment; and (3) cell response to TGF- β 1 without diffusion effect and successfully built up a mathematical model to describe the time course of conversion across the 3-D culture depth and predict the spatial-temporal conversion within the cultures.

4.7 Figures

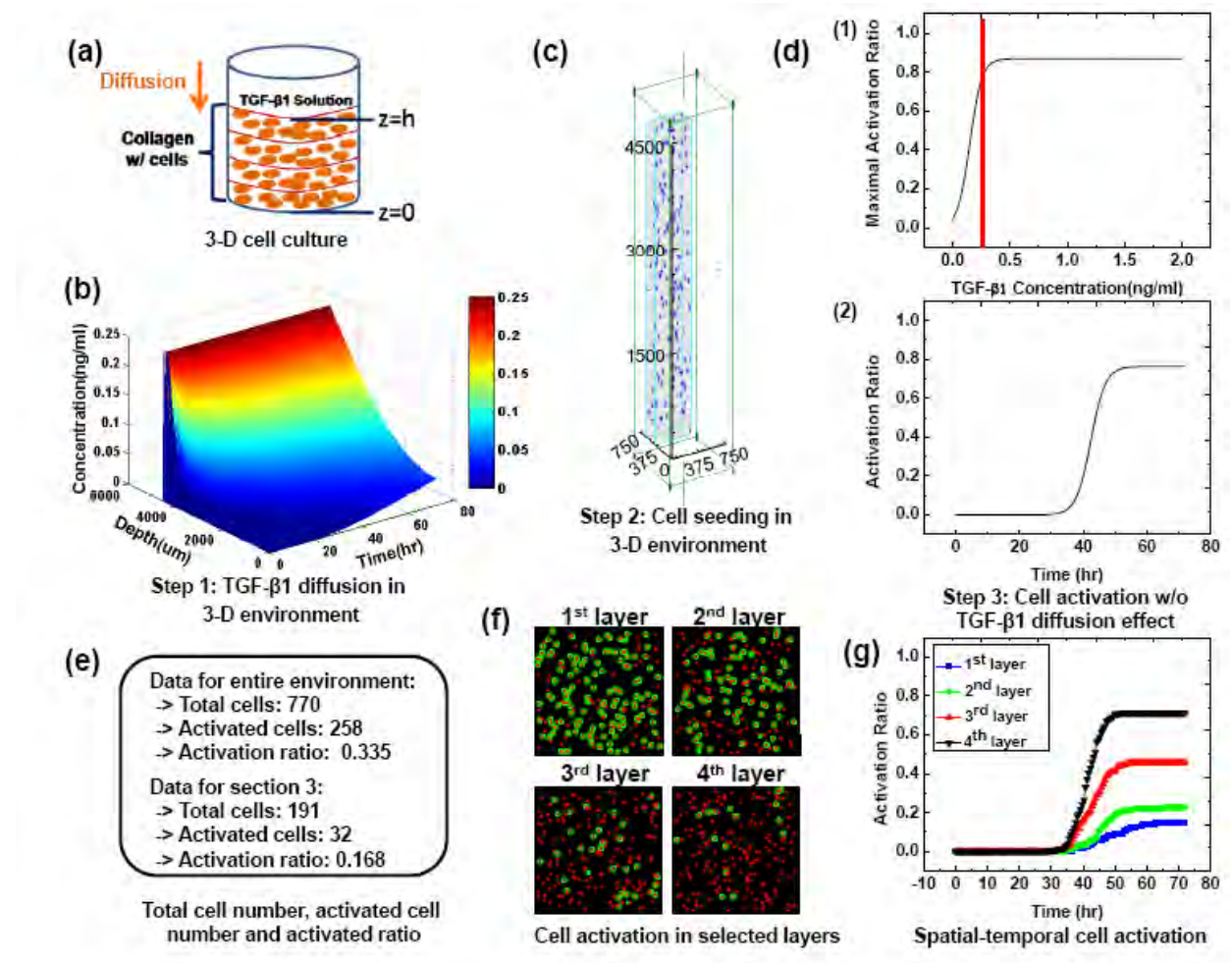


Figure 4.1 Schematic of cell activation model. (a) Schematic of 3-D cell culture and TGF-β1 diffusion. (b), (c) and (d), input of the model includes TGF-β1 diffusion in a 3-D environment, cell seeding in the environment, and dynamic cell activation without TGF-β1 diffusion effect respectively. After running the stimulation, we can get cell number and activation ratio in entire environment and selected layers (e), 2-D images for each layer (f) and spatial-temporal cell activation curves (g).

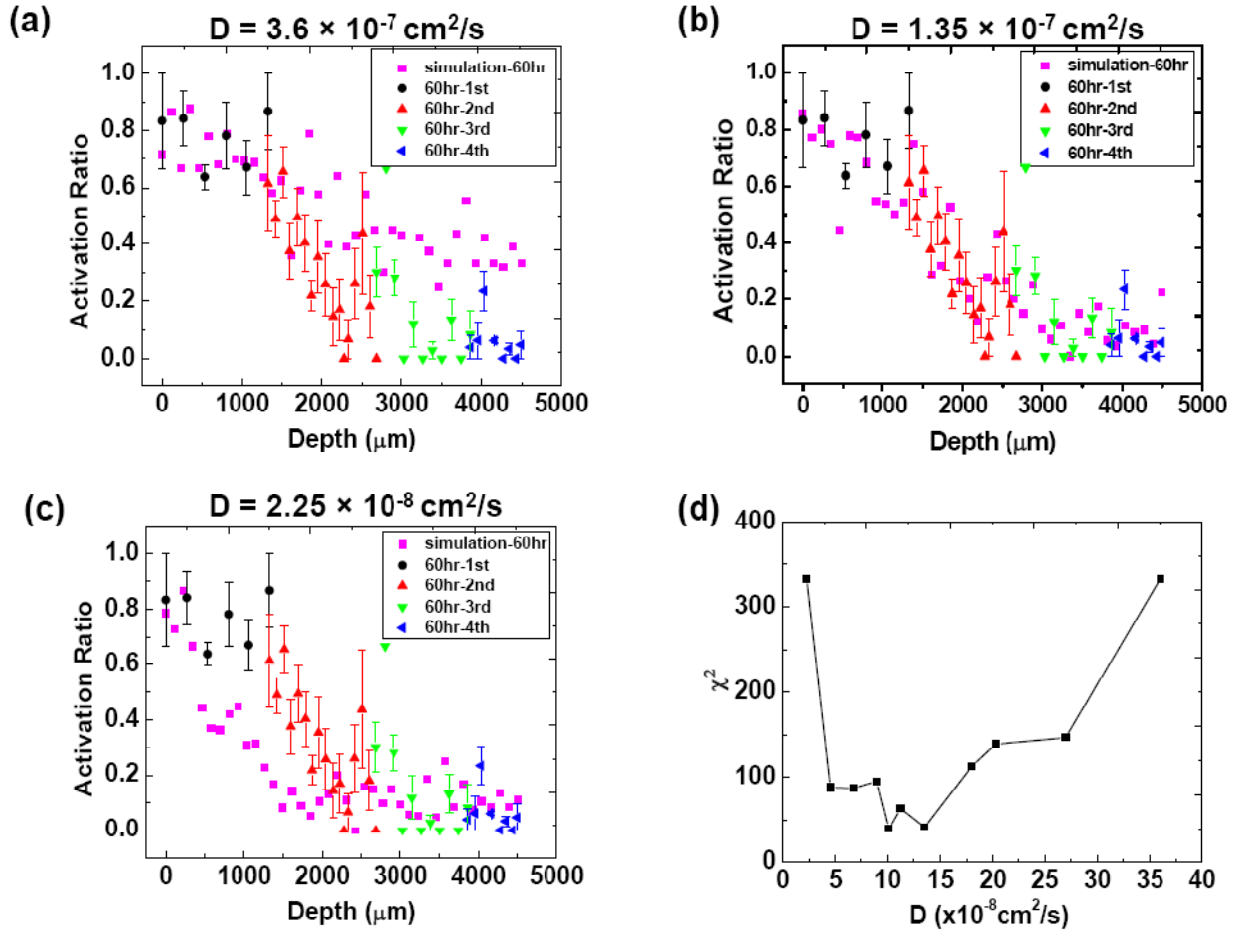


Figure 4.2 Simulation results from one run for 0.25 ng/ml TGF- β 1. (a) Simulation result is higher than experimental result with $D = 3.6 \times 10^{-7} \text{ cm}^2/\text{s}$; (b) Simulation result is close to experimental result with $D = 1.35 \times 10^{-7} \text{ cm}^2/\text{s}$; (c) Simulation result is lower than experimental result with $D = 2.25 \times 10^{-8} \text{ cm}^2/\text{s}$; (d) χ^2 as a function of TGF- β 1 diffusion coefficient D . Note: for all activation ratio vs. depth curves, the order of depth was reversed that the bottom of gel is $z=h$ and the boundary between gel and medium is $z=0$. This reversion was also adopted in figure 4.3, 4.4 and 4.5.

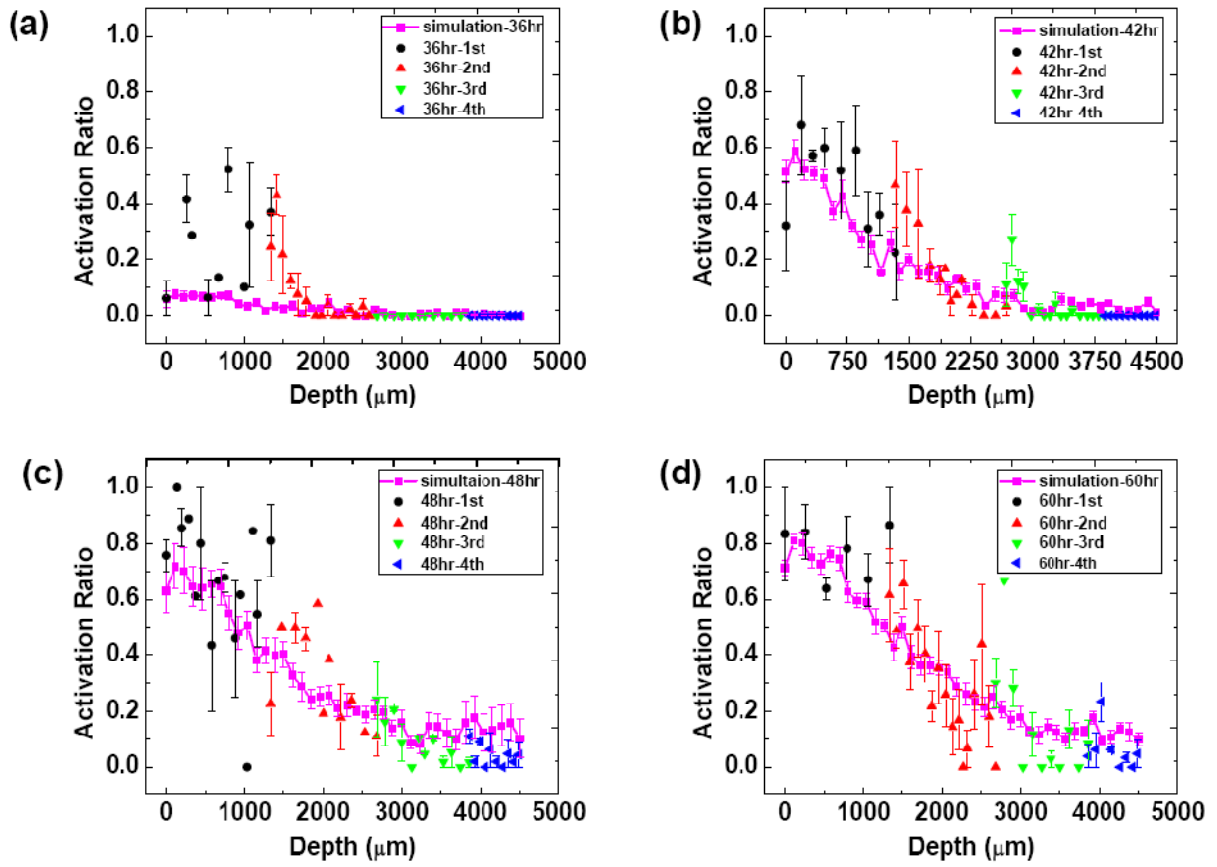


Figure 4.3 Averaged simulation results for 0.25 ng/ml TGF- β 1. (a) 36hr; (b) 42hr; (c) 48hr and (d) 60hr.

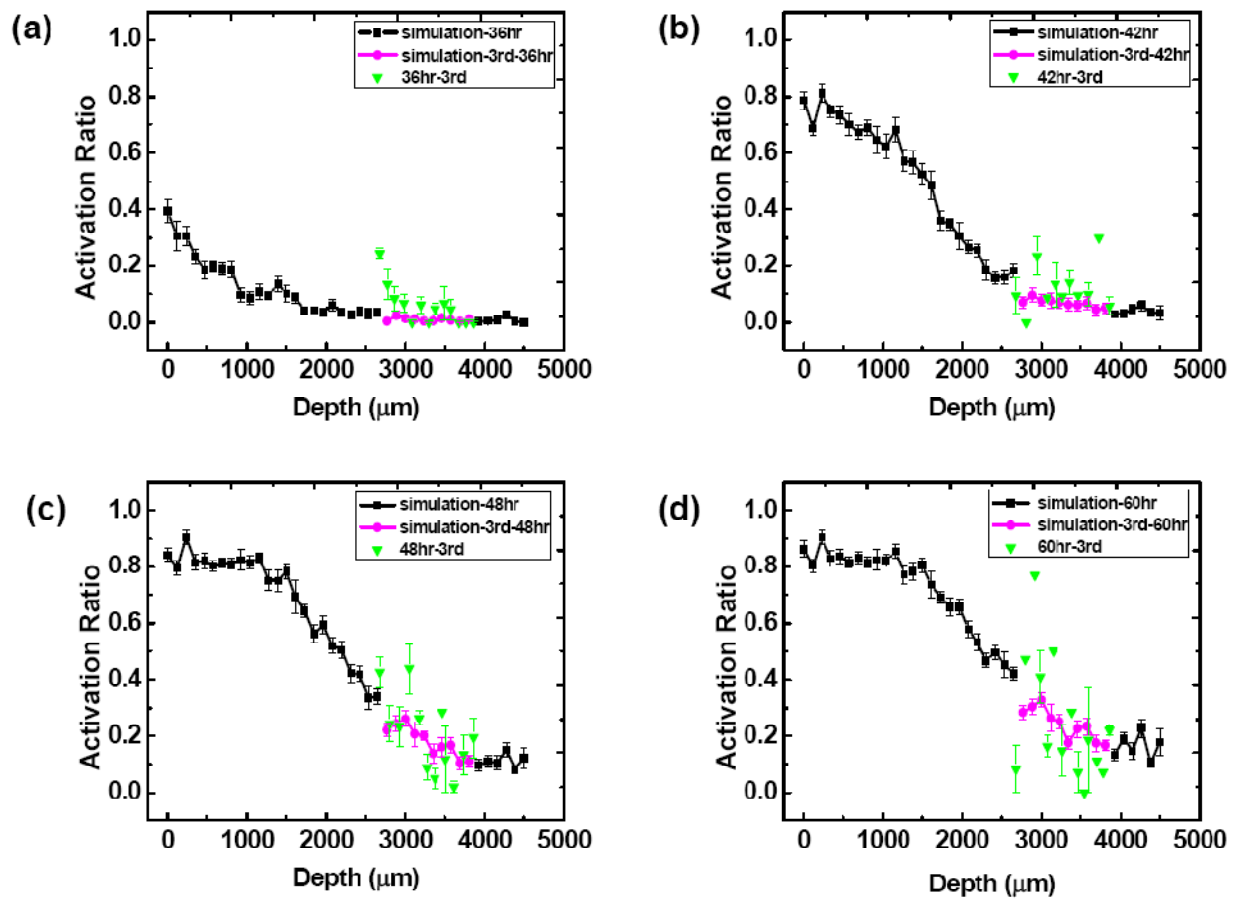


Figure 4.4 Averaged simulation results for 0.50 ng/ml TGF- β 1. (a) 36hr; (b) 42hr; (c) 48hr and (d) 60hr.

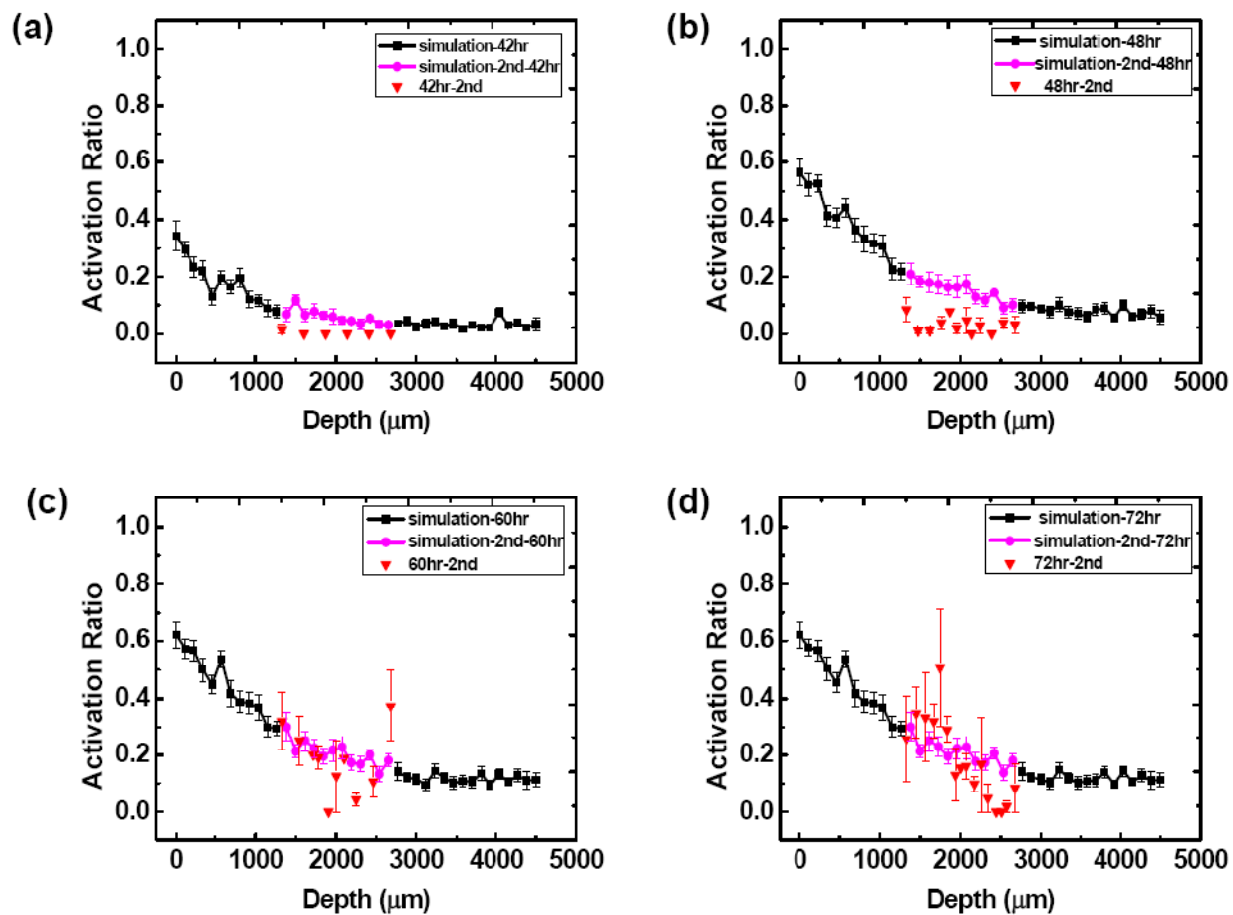


Figure 4.5 Averaged simulation results for 0.15 ng/ml TGF- β 1. (a) 42hr; (b) 48hr; (c) 60hr and (d) 72hr.

CHAPTER 5

MULTIMODALITY IMAGING OF 3-D CULTURE

In this chapter we report multimodality imaging of 3-D culture. Chemical and structural changes of the cell response to TGF- β 1 stimulation were detected by Fourier transform infrared spectroscopy (FT-IR). The structure of 3-D culture was also observed by ultrasound imaging.

5.1 Experimental Materials and Methods

5.1.1 Preparation of 3-D Cell Culture for FT-IR Imaging

Each 3-D cell culture gel was fixed with 4% paraformaldehyde at 4°C overnight at a specified time, followed by gradient dehydration in 70%, 95% and 100% ethanol. The sample was then cleared with a 1:1 xylene/ethanol mixture and then with neat xylene. Subsequently, the sample was embedded in paraffin following standard histological procedure. Embedded tissue was sectioned to 5 μ m slices with a SHUR/Sharp™ 4060E Electronic Rotary Microtome and placed on appropriate slides.

5.1.2 FT-IR Imaging: Data Acquisition and Processing

Samples for IR imaging were placed on 1 mm thick BaF₂ substrates and dehydrated with hexane. IR spectroscopic images of tissue sections were collected using the Perkin-Elmer Spotlight 400 imaging spectrometer. A spatial pixel size of 6.25 μ m and a spectral resolution of 4 cm⁻¹ were employed, with 4 scans averaged for each pixel over the entire mid-infrared range. An undersampling ratio of 2 with respect to the He-Ne laser was used in recording the interferogram. Norton-Beer medium apodization was employed with a zero fill factor of 2 during the Fourier transform. An IR background image was acquired with 120 scans co-added at a location on the substrate where no tissue was present. A ratio of the background to tissue spectra was then

computed to remove substrate and air contributions to the spectral data. All further computation was done using programs written in-house in ENVI/IDL.

5.1.3 Preparation of 3-D Cell Culture for Ultrasound Imaging

In this experiment, the total thickness of collagen gels and complete medium on top was 9 mm and the depth of the 48 well-plate was cut to 9 mm as shown in figure 5.1 (a). This way the ultrasound transducer can directly touch the medium and keep close to the 3-D gels. As described in section 3.1.2, 3-D collagen gels without cells were prepared from collagen I stock solution diluted and neutralized by complete medium and 1N sodium hydroxide to 2.0 mg/ml (figure 5.1 (b)). Cells were added to make the collagen gels with cells (figure 5.1 (c), (d) and (e)). To make the layer structure of collagen gels, aliquots of collagen or cell/collagen mixtures were placed in the wells of Corning 48-well culture plates. Polymerization of collagen matrices was completed in 30 min at 37°C. More layers were added with the same procedure if necessary (figure 5.1(c) and (d)). The cell cluster shown in figure 5.1 (e) was made by injected cell/collagen mixture into polymerized collagen gel. Complete medium was applied to the top of gels after polymerization.

5.1.4 Ultrasound Imaging: Data Acquisition and Processing

A Vevo 2100 system (VisualSonics Inc. , Canada) was used to acquire B-mode images using a MS550S (32–56 MHz) linear-array transducer excited at a center frequency of 40 MHz. A 100% excitation power on the transmitting end and a 63 dB gain on the receiving end were adopted. Images were acquired with high line density setting on the system. Lateral width of the image is 14.3 mm and depths from 5 to 15 mm axially are presented. The images were scaled to the dynamic range of 60 dB. Images were internally processed and provided to the user via a USB port. A Ziplock bag filled with castor oil below the samples was used in order to

suppress the reflection from the bottom surface on which samples were sitting, which proved to be an effective technique.

5.2 Results and Discussion

5.2.1 FTIR Imaging: Chemical and Structure

While structural characterization of tissue and tissue models is routinely accomplished by structural imaging, a biochemical characterization typically requires destruction of the structure, thereby losing spatially specific information. A combination of biochemical and structural knowledge is often helpful and is enabled by the emerging fields of chemical imaging and microscopy. Among the prominent approaches is vibrational spectroscopic imaging, including both Raman and infrared imaging. FT-IR imaging is useful for the analysis of tissue biopsies and has a well-developed instrumentation. Here, we used FT-IR imaging to study the structural and chemical changes of cells from TGF- β 1 stimulation. Samples from 2-D and 3-D cultures in the presence of TGF- β 1 were compared with those without TGF- β 1 stimulation using FT-IR imaging (Figure 5.2). While there are not apparent structural changes, there are a few noticeable differences across the spectra, indicating possible chemical changes due to the overexpression of α -SMA proteins, among many other biochemical changes resulted from TGF- β 1 stimulation. More analysis will be needed before more concrete conclusions can be drawn.

5.2.2 Ultrasound Imaging: 3-D Culture Structure

Ultrasound imaging was used to characterize 3-D culture. Figure 5.3 shows the B-mode images of 3-D cultures. In the 3-D gel without cells (figure 5.3 (a)), we can only see bright spots and multiple lines. These bright spots are bubbles and the multiple lines are formed because of “ringing” effect which happens when ultrasound pulse interacts with bubbles. In 3-D samples

with cells (figure 5.3 (b), (c) and (d)), the gel/cell regions are clearly isolated from the gel regions and the shapes and positions of gel/cell regions are noticeably monitored. The thin stripe on the top of the round region in image 5.3 (d) is from a thin layer gel/cell. It formed as the needle was taken out from the gel and the unpolymerized gel/cell mixture ran out of the channel and spread on the top of gel.

5.3 Conclusion

In this chapter, we used different imaging methods to study our 3-D culture. We have demonstrated that both FT-IR imaging and ultrasound imaging are suitable tools for this study. Preliminary results have shown some promise in using multimodal imaging to study 3-D cultures, including the structural and chemical changes resulted from TGF- β 1 stimulation.

5.4 Figures

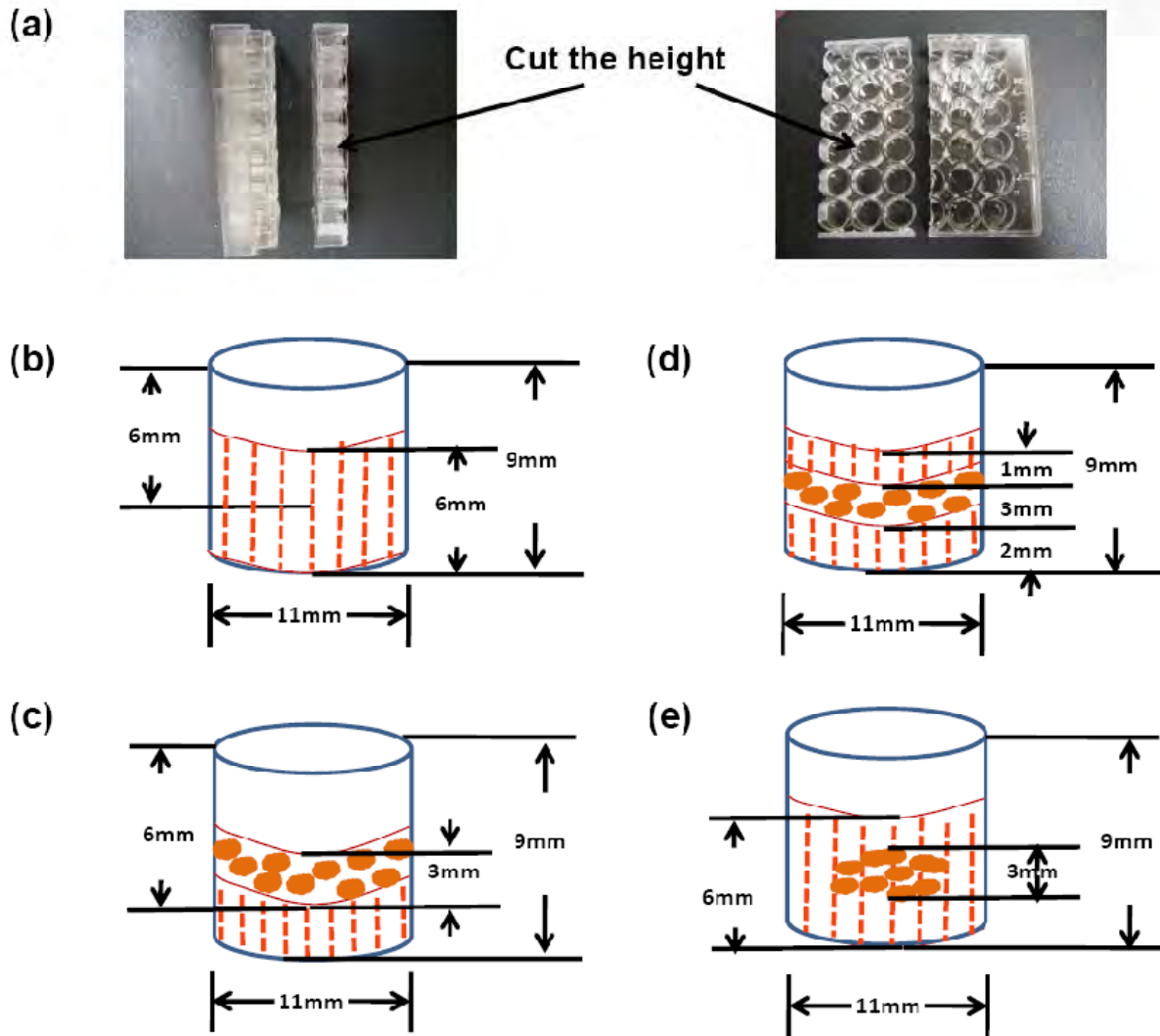
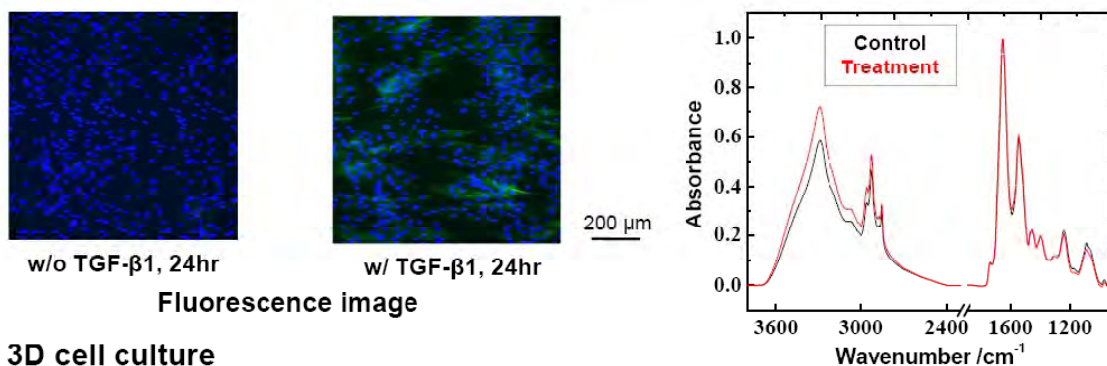


Figure 5.1 Schematic of ultrasound samples. (a) Cut 48-well-plate. (b), (c), (d) and (e) are four structures of 3-D gels: gel without cells, gel with cells as the top layer and gel without cells as the bottom layer, gel with cells as middle layer in the gel without cells and gel with cells forming as a ball inside gel respectively.

(a) 2D cell culture



(b) 3D cell culture

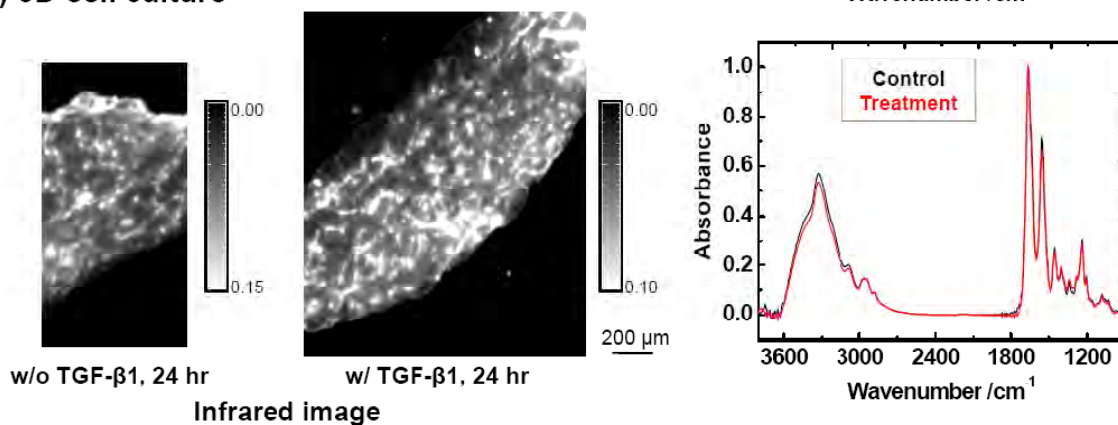


Figure 5.2 FT-IR spectrum and images. (a) Immunofluorescence images and FT-IR spectrum of 2-D cell culture (b) Infrared absorbance images and FT-IR spectrum of 3-D cell culture.

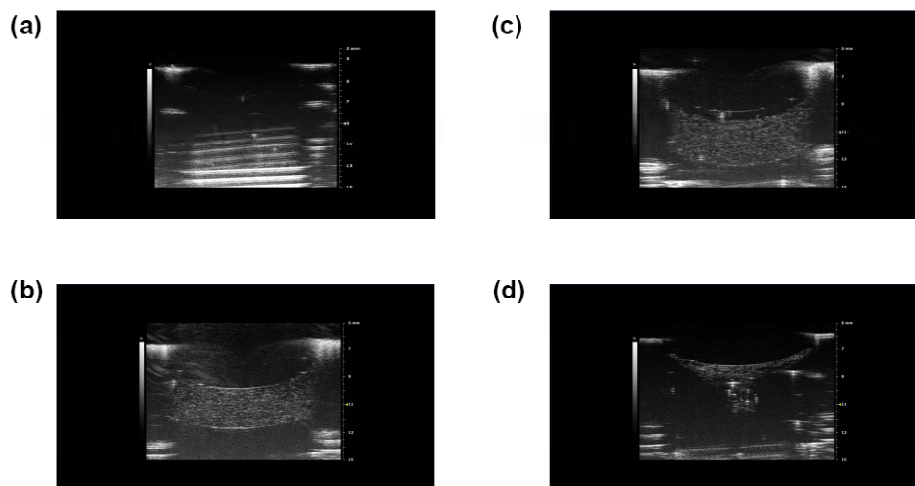


Figure 5.3 B-mode images of 3-D cultures. (a), (b), (c) and (d) are corresponding to the configurations of (b), (c), (d) and (e) in figure 5.1, respectively.

CHAPTER 6

CONCLUSION

In this project, we developed a thick *in vitro* 3-D cell culture model with layered structure and quantitatively studied TGF- β 1 induced cell spatial-temporal activation in cancer development. TGF- β 1 applied on top of the 3-D model can diffuse into the model and convert the embedded normal fibroblasts into myofibroblasts, which are characterized by the expression of α -SMA. Our results revealed the spatial-temporal profiles of TGF- β 1 induced fibroblast/myofibroblast conversion in 3-D culture and the dependence of conversion starting time, speed and plateau level on TGF- β 1 concentration. Based on these experimental results, we successfully developed a mathematical model, which considers the diffusion of soluble factors and the induced signaling cascade that activates cells, to describe the dynamic cell response to growth factors across the 3-D culture depth and predict the spatial-temporal conversion within the culture. The result should shed new light on the understanding of soluble factors concentration gradient induced cellular behavior in natural biological system and give guidance on the dosage of soluble factors applied to *in vitro* biological system with desired influences. In addition, we used both FT-IR imaging and ultrasound imaging to study the effect of TGF- β 1 stimulation on cells in 2-D and 3-D culture. These results have shown some promise in using multimodal imaging to study 3-D cultures, including the structural and chemical changes resulted from TGF- β 1 stimulation. In summary, we present a good experimental and computational tool for researches on tumor signaling pathways, and provide some guidance for studies on protein factor or drug diffusion in 3-D tissue environment.

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APPENDIX A: GFP DIFFUSION IN COLLAGEN GELS

To our best knowledge, there is no literature so far to report the diffusion coefficient of TGF- β 1 in collagen gels. Fluorescence-based methods have been used to determine the diffusion coefficients of growth factors.(Nauman, Campbell et al. 2007) (Stroh, Zipfel et al. 2003) Growth factors molecules have to be conjugated with fluorescent dyes for this kind of studies. However, there is no commercial fluorescent dyes conjugated TGF- β 1. Considering that the molecular weight of GFP (~ 28 kd) is similar to that of TGF- β 1 (~ 25 kd), we used GFP as a substitute for TGF- β 1 to measure the diffusion coefficient of GFP in collagen gel and used this value as a reference for diffusion coefficient of TGF- β 1.

A piece of polydimethylsiloxane (PDMS) film was sealed with a piece of glass coverslip. Two chambers were formed by cutting off two pieces of PDMS blocks as showed in figure A.1 (a). The smaller PDMS block was taken out from the chamber and filled with collagen mixture. After polymerization of collagen mixture, the device was placed on the confocal microscope stage. The bigger PDMS block was taken out from the chamber right before the experiment to add GFP solution. Images area was selected at the boundary corner between GFP solution and collagen gel as shown in figure A.1 and images were captured every 10 min. Green fluorescence intensity in the region of interested (region in the red dash line in figure A.1 (b)) was calculated with ENVI and plotted as a function of distance. The boundary between GFP solution and collagen gel was defined as distance $z=0$; the distance in the GFP region was negative and in the collagen region was positive. In the figure A.1 (c), the fluorescence intensity was normalized as c/c_0 .

In this work, the diffusion of GFP solution in this system is simplified to 1-D diffusion from the GFP solution/collagen gel boundary into the gel. According to Fick's second law, the process for GFP diffusion in the collagen gel can be expressed as

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial z^2} \quad (4-1)$$

where D is the diffusion coefficient independent of the GFP concentration c in the gel at distance z at a given time t. The initial conditions are

$$c(z, 0) = \begin{cases} 0, & 0 < z \leq h \\ c_0, & -d_0 \leq z \leq 0 \end{cases} \quad \text{at } t = 0 \quad (A-1)$$

where h is the length of collagen gel and equal to 5 mm; d_0 is the length of GFP solution and equal to 12 mm. The boundary conditions are

$$\frac{\partial c(z, t)}{\partial z} = 0, \quad \text{at } z = h \quad (A-2)$$

Since there is no diffuses through the gel bottom, to solve the equation (4-1), the concept of reflection at the boundary is introduced. (Crank, 1975) We consider the solution can be reflected in the plane $z = h$ and superposed on the original distribution in the region $z \leq h$. Thus the solution to equation (4-1) is

$$c(z, t) = \frac{1}{2} c_0 \sum_{n=-\infty}^{\infty} \left[\operatorname{erf} \left(\frac{2nh - z}{2\sqrt{Dt}} \right) + \operatorname{erf} \left(\frac{-2nh + z + d_0}{2\sqrt{Dt}} \right) \right] \quad (A-3)$$

When $n=0$, there is no reflections at the boundary and got D equal to $1.4478 \times 10^{-6} \text{ cm}^2/\text{s}$ at 38 min. Second and higher-order terms add little.

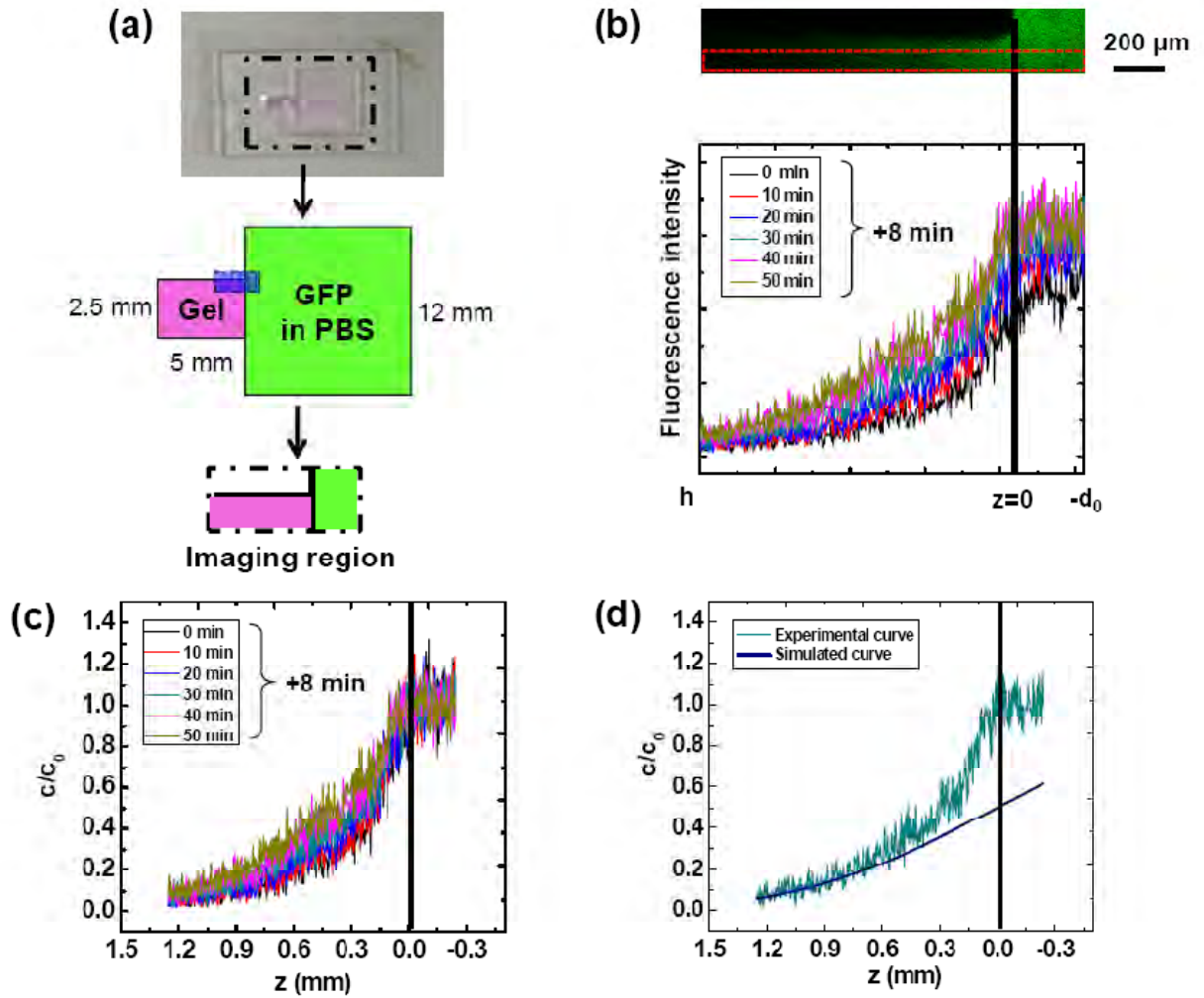


Figure A.1 Diffusion of GFP solution in the collagen gel. (a) Design of the experiment; (b) Confocal image of the GFP diffusion in the collagen gel and green fluorescence intensity as a function of distance. The 8 min is the time gap between adding GFP solution to the chamber and capturing the first image with confocal microscope. (c) c/c_0 as a function of distance. (d) Simulated GFP diffusion curve at 38 min.

APPENDIX B: FUTURE WORK OF CELL ACTIVATION MODEL

In the section 4.1 “TGF- β 1 Diffusion in a 3-D Environment”, we mentioned in the TGF- β 1 diffusion and cell activation model the initial conditions are

$$c(z, 0) = \begin{cases} 0, & 0 \leq z < h \\ c_0, & z \geq h \end{cases} \quad (4 - 2)$$

and the boundary conditions are

$$\begin{aligned} \frac{\partial c(z, t)}{\partial z} &= 0, \quad \text{at } z = 0 \\ c(z, t) &= c_0, \quad \text{at } z = h \end{aligned} \quad (4 - 3)$$

Though the model has produced good simulation results and predictions, we are considering (1) amount of TGF- β 1 solution is limited (in this project, it is 300 μ l, d_0 is equal to 3.1 mm) instead of unlimited; (2) as TGF- β 1 solution diffuses into gel, the medium in the gel comes out and diffused into TGF- β 1 solution to dilute the TGF- β 1 concentration. These can cause the initial and boundary conditions to be changed.

The initial conditions are then

$$c(z, 0) = \begin{cases} 0, & 0 < z \leq h \\ c_0, & h \leq z \leq h + d_0 \end{cases} \quad \text{at } t = 0 \quad (B - 1)$$

where h is the thickness of collagen gel. The boundary condition is

$$\frac{\partial c(z, t)}{\partial z} = 0, \quad \text{at } z = 0 \quad (B - 2)$$

In the next step, we will focus on solving the Fick’s second law equation with the conditions mentioned above, changing the first step of the model, running simulation and making predictions.

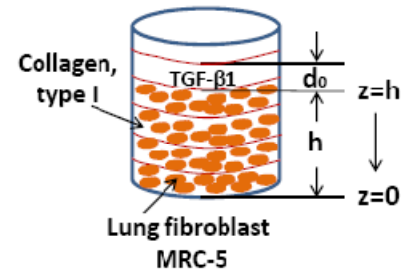


Figure B.1 Schematic of 3-D cell culture and TGF- β 1 diffusion

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Jing Xu graduated from Tianjin University, China in 2001 with a Bachelor of Science degree in Polymer Materials and in 2003 with a Master of Engineering degree in Biomedical Engineering. Then she came to United States and completed a Master of Science degree in Materials Science and Engineering from Clemson University in 2006.