Impact of extracellular matrix stiffness on cancer cell function and stem cell fate

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Here, I guarantee that all the work in this dissertation was accomplished by me except indicated annotation and hasn't been previously published or submitted to obtain any other degrees in any other universities. And I also declare that this thesis is fully written by me and the contents are absolutely original except indicated citations.

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Abbreviations

ALDH1	Acetaldehyde dehydrogenase 1
ALP	Alkaline phosphatase
APES	3-Aminopropyltriethoxysilane
APS	Ammonium persulfate
Baf A1	Bafilomycin A1
BCSC	Breast cancer stem cell
Blebbist	Blebbistatin
C3	C3 transferase
DCDMS	Dichlorodimethylsilane
CMA	Chaperone-mediated autophagy
CSC	Cancer stem cell
CTX	Cyclophosphamide
dPG	Dendritic polyglycerol
EB	Embryoid body
eMSC	ectodermal Mesenchymal stem cell
EMT	Epithelial-mesenchymal transition
FN	Fibronectin
HCC	Hepatocellular carcinoma
HSPG	Heparin sulphate proteoglycans
iPSC	induced pluripotent stem cell
Lat.A	Latrunculin A
LIF	Leukemia inhibitory factor
MET	Mesenchymal-epithelial transition
mESC	Mouse Embryonic stem cell
MMP	Metalloproteinase
mPSC	mouse Pluripotent stem cell
MSC	Mesenchymal stem cells
NBCC	Normal breast cancer cells

NSPC	Neural stem/progenitor cells
NCSC	Neural crest stem cell
PA	Polyacrylamide
PCL	Polycaprolactone azide
PDMS	Polydimethylsiloxane
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PGs	Proteoglycans
PR	Progesterone receptor
ROCK	Rho-associated protein kinase
RGD	Arginine-Glycine-Aspartate
TEMED	Tetramethylethylenediamine
TNBC	Triple-negative breast cancer

Table of content

1 Introduction

The extracellular matrix (ECM) is an acellular structural construction that exists within all the tissue and organs of the organism, which plays a fundamental role participating in organ development and tissue homeostasis. So far, two main types of ECM are recognized according to their constituent and location [Figure 1-1], the interstitial connective tissue matrix mainly consists of collagen I, fibronectin, proteoglycans (PGs) and elastin to provide structural support for surrounding cells; and the basement membrane, a specialized ECM mainly constituted by collagen IV, laminins and heparin sulphate proteoglycans (HSPGs) for separating the epithelium with its surrounding stroma [1]. The function of ECM is not only to provide physical support as a structural scaffold for keeping the integrity and intactness of surrounding cells and tissues but also to present both biochemical and biomechanical signals in order to guide cell functions and maintain tissue homeostasis [Figure 1-2, ref 2].

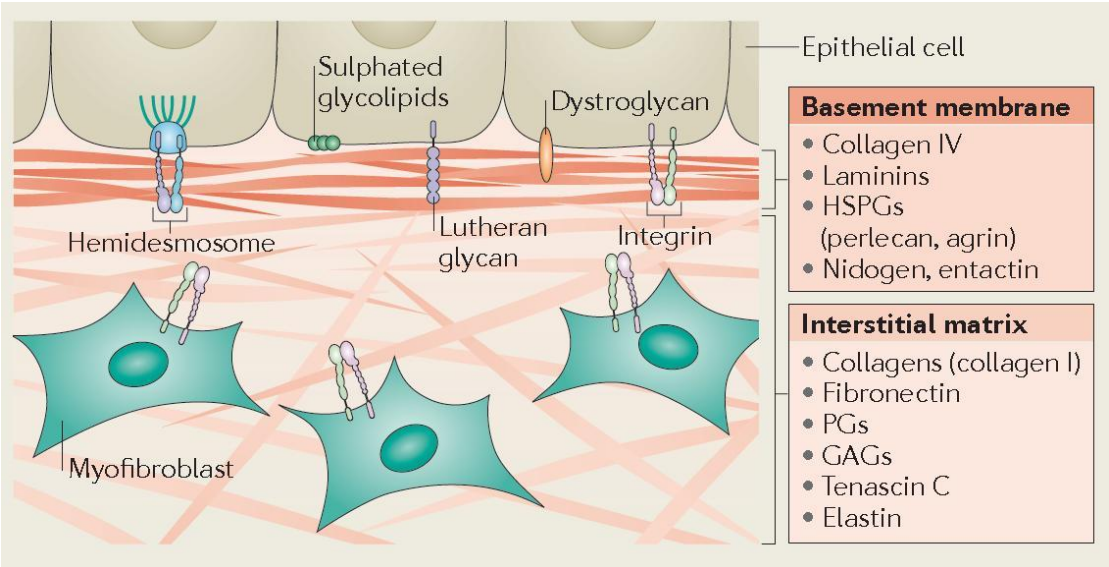


Figure 1-1. Two main types of extracellular matrix (ECM) in mammalian tissues. Figure reprinted from ref. [1]. Copyright © 2014 Macmillan Publishers Limited.

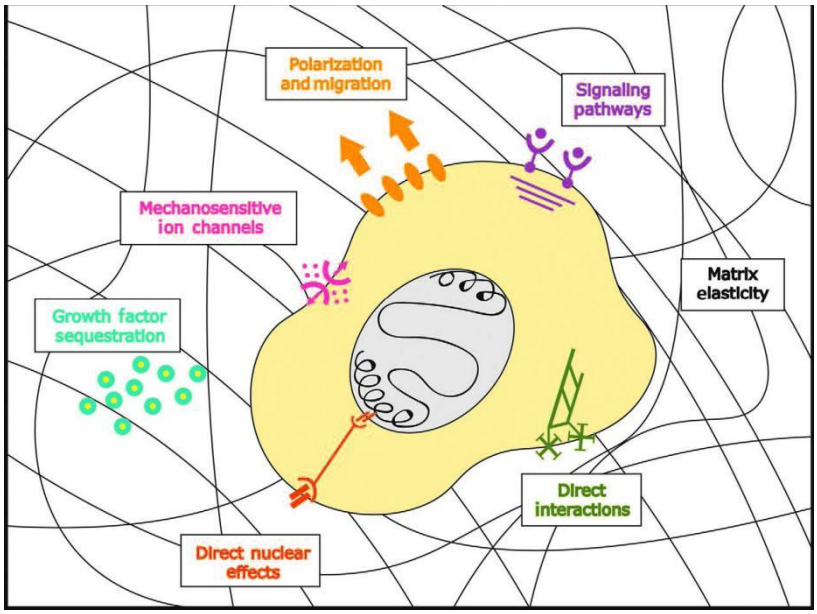


Figure 1-2. Various biochemical and biomechanical signals presented in the extracellular matrix (ECM). Figure reprinted from ref. [2]. Copyright © 2018 Elsevier Ltd.

Moreover, ECM is tissue-specific, i.e., the physical topological structure, biochemical composition and mechanical characteristics of the ECM are different in different tissues and organs [3]. During the development of organisms, the ECM keeps highly dynamic and undergoes constantly remodeling by continuously cell-ECM interactions also called reciprocal dialogue [Figure 1-3], which are influenced by these physical, chemical and biomechanical cues including but not limited to specific three-dimensional architecture, different compositions, specific enzymolysis, growth factors and transmembrane receptors mediated signal transduction, elasticity-mediated mechanotransduction [4]. Mechanical signals and other stimuli transmit through the ECM components into cells, as a response, cell, in turn, initiates gene transcription and post-translational modifications such as secreting ECM proteins into their microenvironment to accelerate matrix remodeling. In one word, the homeostasis of extracellular matrix (ECM) mediated by cell-ECM interaction and remodeling is crucial for the embryogenesis, tissue morphogenesis as

well as the structural integrity and normal physiological function of each organ. And perturbations of ECM composition and structure or dysregulation of ECM mechanics, such as increased matrix stiffness, will compromise the normal development process and result in severe physiological diseases including fibrosis and cancer [Figure 1-4].

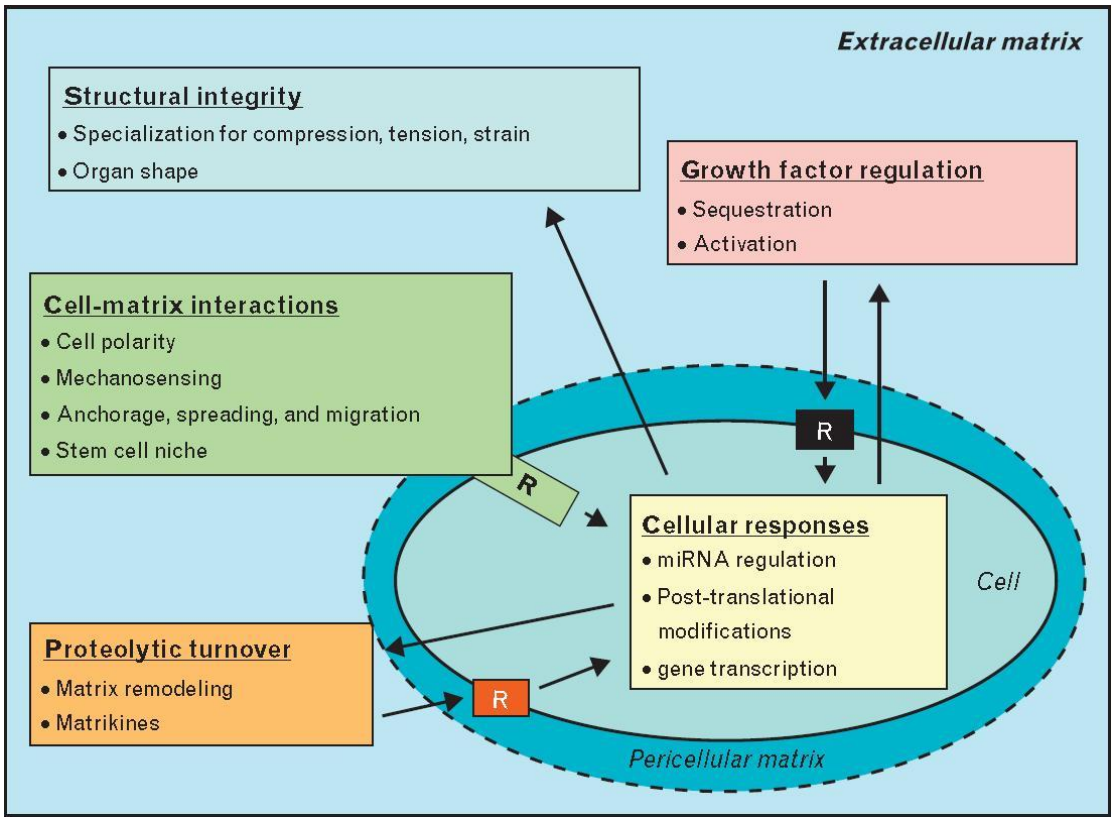


Figure 1-3. Reciprocal dialogue between cells and extracellular matrix (ECM). Figure reprinted from ref. [3]. Copyright © 2012 Lippincott Williams & Wilkins.

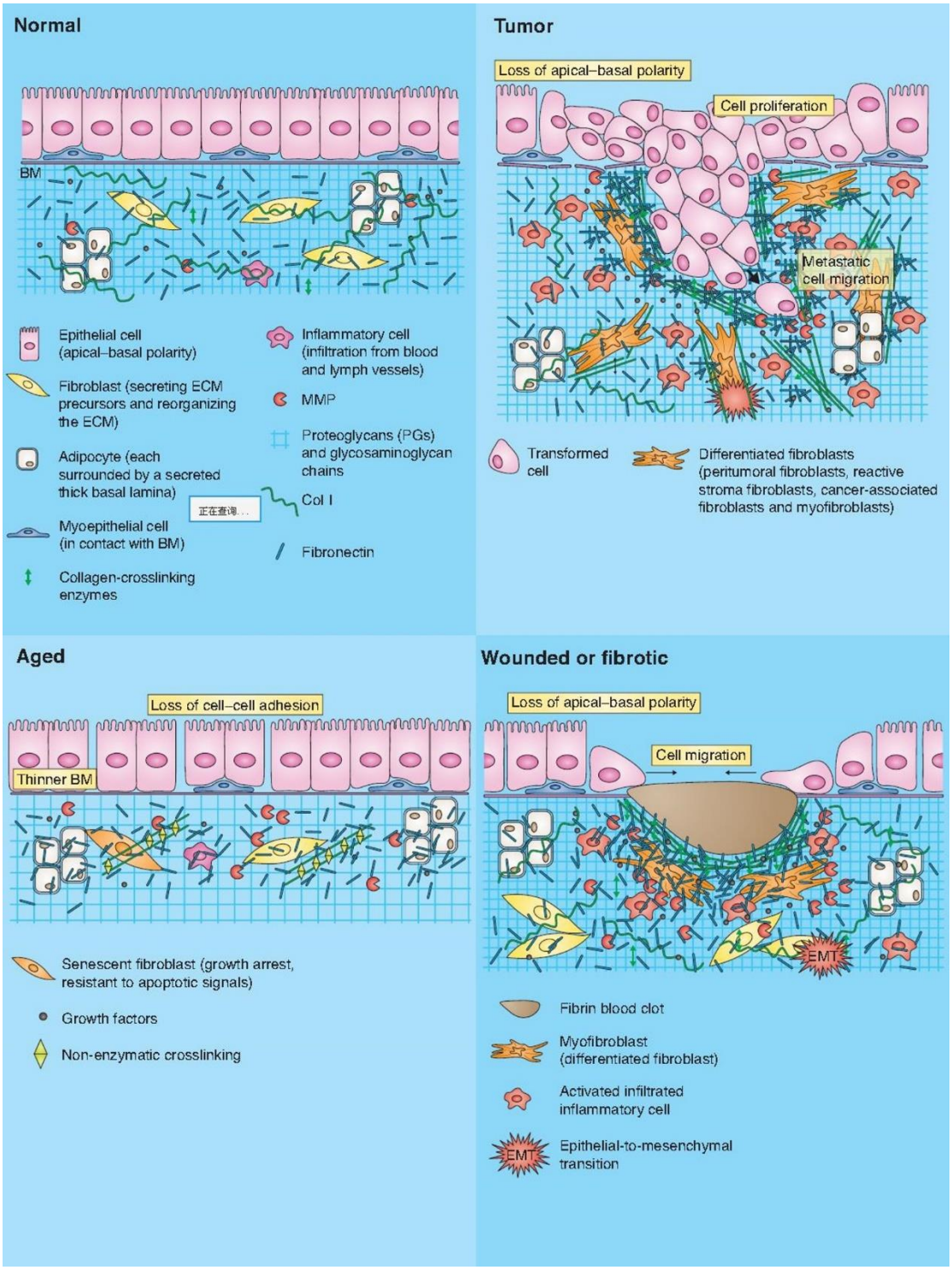


Figure 1-4. Normal and abnormal extracellular matrix (ECM) structure. Figure modified from ref. [5]. Copyright © 2010 The Company of Biologists Ltd.

1.1 Extracellular matrix stiffness regulate cancer cell function and progression

As above said, ECM is tissue-specific, which is meaning that each tissue or organ has an ECM with a particular composition, architecture structure, topology, elasticity and viscoelasticity characteristics that are gradually formed during the organ development [5]. Among which, matrix elasticity or stiffness is the most intuitive and macroscopical property. Indeed, the stiffness of matrices throughout different tissues and organs of our body varies over a huge range [Figure 1-5] from very soft fat, mammary or brain (0.01-0.5 kPa) to quite stiff articular cartilage and bone (950 kPa-11500MPa) [6-10].

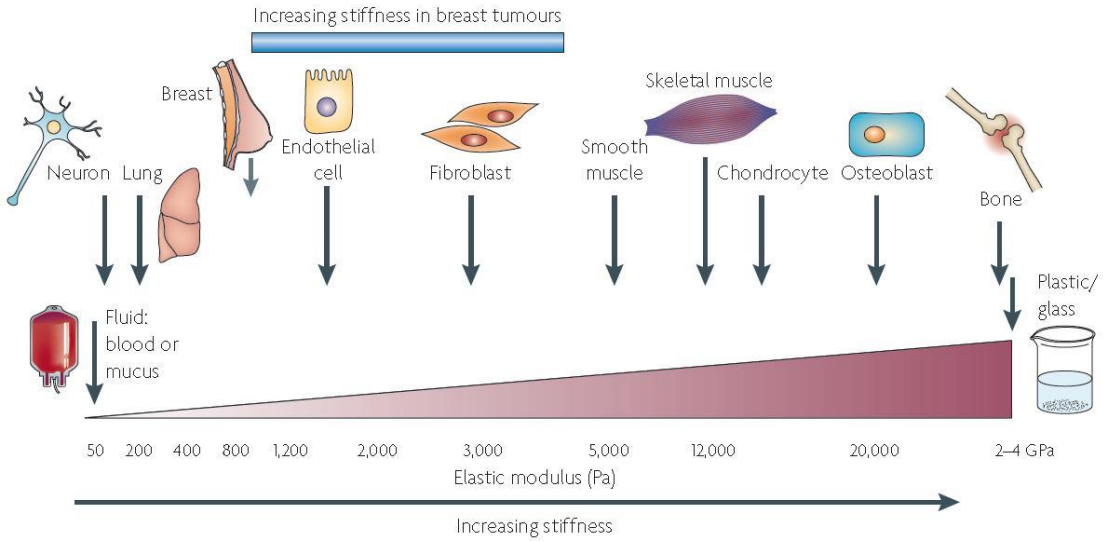


Figure 1-5. Different tissue elasticity in the human body. Figure adapted from ref. [9]. Copyright © 2009 Macmillan Publishers Limited.

Aberrant alterations in tissue or matrices rigidity are generally accompanied by unpredictable organ pathological dysfunction and disease [1]. For example, breast

tumors normally are firstly discovered by palpation of stiffer bossing hiding in the compliant chest via hand touching before the following determinant of pathological and genetic techniques, which are supported by scientific evidence that stromal matrix adjacent to the tumor and tumor itself are much stiffer than the normal mammary tissue probably due to elevated collagen deposition [11, 12]. More than that, the biomechanical environment provided by no matter naïve extracellular matrix of organism tissue or synthetic artificial matrix such as scaffold and hydrogel is suggested to be a crucial role in many aspects of tumorigenesis and progression [13].

1.1.1 Matrix stiffness significantly influences cellular behaviours including morphology, motility and proliferation

First of all, the most distinct influence of matrix stiffness acting on cells is morphology changes. As early as in 1992, Petersen and his colleagues distinguished the different behaviours of human breast epithelial cells in a soft environment. Human normal breast mammary cell was found to grow in a monolayer manner on tissue culture plates [Figure 1-6] but be able to form a hollow lumen surrounded by polarized epithelial luminal cells on soft basement membrane gels [14]. However, breast carcinoma cells were not able to form that kind of structure under the same condition [14]. In addition, great response to matrix stiffness was also observed in fibroblasts by better cell stretch and spreading was presented on stiff collagen-coated PA hydrogels [15]. However, at the same time, cell motility was reduced on stiff substrates. Moreover, another study found an interesting phenomenon which is that fibroblasts prefer to migrate to a stiffer area, termed “durotaxis”. And similar behavior was also observed in pancreatic stellate cells [16]. What’s more, Wei-hui et al also found that, on stiff substrate, cells move away from each another while on soft substrate, cells gather together to form tissue-like structure due to reduced adhesion and contractility [17].

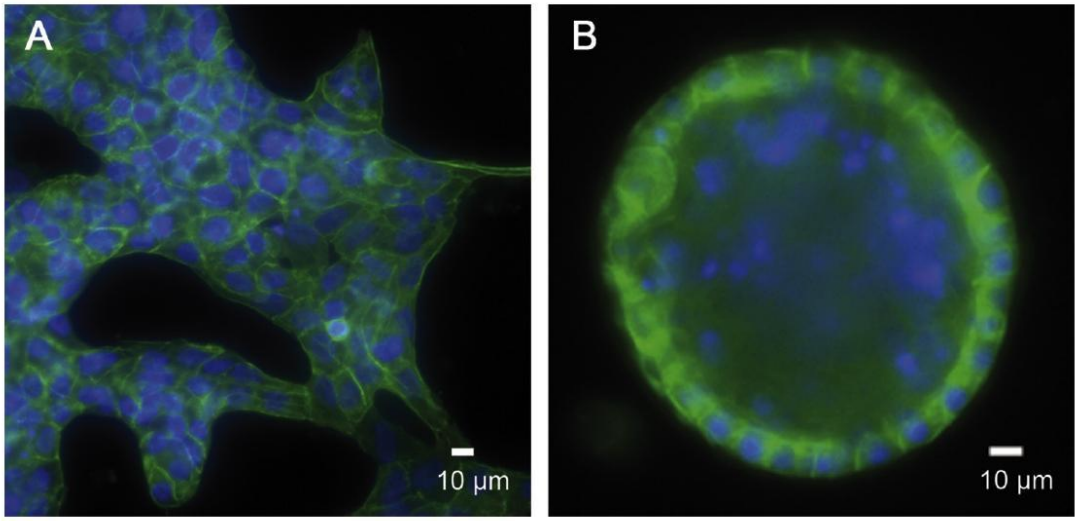


Figure 1-6. Human mammary epithelial cells growing on tissue culture plate (A) and soft basement membrane substrate (B). Figure reprinted from ref. [13]. Copyright © 2015 Elsevier Ltd.

Beyond the acting of substrate stiffness on migration, Ghosh et al further found that, compared with soft substrate, fibroblasts preferentially chose to proliferate on stiffer one [18]. This indicates that rigid substrate promotes the proliferation of fibroblasts which was also described in another report [19]. Of course, not just fibroblasts, many other cell types were also involved in the matrix stiffness-dependent proliferation regulation. For instance, both mouse and human mammary epithelial cells exhibited elevated proliferating potential on stiff matrix with high density of collagen and the FAK-Rho-ERK signal pathway was proved to be involved in this mechanoregulation of matrix density-induced stiffness [20]. Another example, compared with soft substrates, human vascular endothelial cells still showed high proliferation ability on rigid substrates though fewer cells were attached [21]. A more valuable study further proved that increased matrix stiffness facilitated neovessel growth and branching during the tumor angiogenesis by enhancing metalloproteinase (MMP) activity [22]. Similar research in lung cancer cells also confirmed this concept [23], which indicates

tumor matrix stiffness as a feasible target for clinical cancer therapy in the near future. Given the fact that the solid tumors are usually stiffer than the surrounding normal tissue [24], it is not surprising that matrix stiffening is involved in regulating proliferation of many different cancer cell types.

1.1.2 Matrix stiffness affects the chemosensitivity and the stemness characteristics of cancer cells

Another remarkable and interesting point involved in the mechanical regulation in cancer cells is stemness characteristics and related chemosensitivity. It is a well-known perspective that the tumor bulk is made up of not equal cell community but heterogeneous cell populations [25]. Within the solid tumor, a small minority of cells exhibiting stem cell characteristics of both self-renewal and differentiating abilities are so-called cancer stem cells (CSCs) and account for sustaining tumorigenesis [26]. Liu J and Tan Y found that much more and larger melanoma tumor spheroids which expressed upregulated stem cell markers were generated in soft fibrin gels compared with stiffer gels [27]. Additionally, soft environment was also reported to promote stem cell characteristics but meantime suppress proliferation and drug resistance in HCC [28]. However, conflicting evidence showed that HCC cells exhibited higher stem cell markers and greater self-renewing ability on stiff substrate [29], which indicated that how precisely matrix stiffness act on specific cancer stemness still remains unclear. The microenvironment of tumor tissue is believed to play a critical role in tumorigenesis including not only initiation, progression, and metastasis but also chemotherapeutic responses and drug resistance. However, up to now, only very little research focuses on the relationship between extracellular matrix and cancer cell chemosensitivity. Schrader et al first reported that the enhanced chemotherapeutic resistance to cisplatin caused by increasing matrix stiffness in hepatocellular carcinoma cells [28]. In addition, Shin et al explored the systematic variations in chemosensitivity regulated by matrix stiffness in myeloid

leukemia. [30]. Recently, one latest study on Biomaterials found that the chemotherapeutic response of breast cancer cells on soft hydrogel matrices was distinctly weak than that of on stiff substrates [31]. However, the related mechanism involved in the regulation of matrix stiffness on chemotherapeutic response still remains mysterious.

1.1.3 Autophagy may be involved in the mechanical regulation of cancer cells

Besides the impact of matrix stiffness on tumor aggression, metastasis and even tumor chemosensitivity [28], what's really interesting is that autophagy probably is also involved in the mechanotransduction. Increased autophagy level was detected in normal mammary cells along with the increasing matrix stiffness [32]. Though the related mechanism remains unclear, this research gives us a hint that autophagy may be also involved in the mechanical regulation of cancer cells. Autophagy is a natural, evolutionary conserved process mediating the degradation of cellular unnecessary or dysfunctional components, a physiological self-cleaning function, which has been proved to be an important mediator in the occurrence and progression of many kinds of cancer types in both promoting and suppressing ways [33, 34]. According to the different ways that cytoplasmic contents enter lysosomes, autophagy can be classified into three different forms: macroautophagy, microautophagy and chaperone-mediated autophagy [35, 36]. Macroautophagy is the main type, so the term "autophagy" commonly means macroautophagy unless otherwise specified. Besides the normal mammary cells, another research found that stiff substrate-induced increased chaperone-assisted autophagy in smooth muscle cells [37]. Nevertheless, the response of autophagy to matrix stiffness in many cancer cells is still under mysterious veils.

1.2 Extracellular matrix compliance and 3D microenvironment influence stem cell fate

In the last decade, along with the increasing founding of naturally derivative or synthetic hydrogels [38], the interactions between extracellular matrix (ECM) microenvironment and cells or tissues built by hydrogel are drawing increasing attention of more and more scientists. Specifically, cells can feel and respond to various physical, chemical and mechanical signals from the hydrogel-based microenvironment [39]. Therefore, the fates of the cell, especially the stem cell, are greatly influenced by the specific characteristic of the hydrogel such as elasticity, viscoelasticity, mechanical forces, nanotopography, porous structure, and so on. Among various properties, the compliance of hydrogel, which is determined by the hydrogel elasticity and further determines the rigidity of the substrate, was plentifully proved by many studies to greatly impact the self-renewal ability [40, 41] and differentiation potentials of stem cells [42, 43].

1.2.1 Extracellular matrix stiffness regulates self-renewal abilities of stem cells

In the naive environment of organism bodies, stem cells normally reside in a specific site known as niche, which is constituted by extracellular matrix, surrounding supportive cells, soluble molecule signals and so on, determines the stem cell fate such as whether should remain quiescent, activate to divide or initiate differentiation [Figure 1-7, 44-47]. Among those various factors that may disturb the niche homeostasis, extracellular matrix is believed to be a central element of the stem cell niche environment, and to play a significant role in the regulation of stem cell behavior such as proliferation, migration, or apoptosis [48, 49]. Beyond the cell focal adhesions [50], morphology or shape [51], spreading [52] are influenced by ECM stiffness; matrix stiffness can also regulate the self-renewal status of stem cells.

An interesting study ten years ago found that soft polyacrylamide substrates with the properties of bone marrow could keep human mesenchymal stem cells (MSCs) quiescent [53]. What’s more important, these quiescent MSCs could reboot the cell

cycle again when reseeded on a rigid substrate and also exhibited the abilities to adipocytes and osteoblasts when cultured in the respective induction medium. This gives us a hint that the hydrogel mimicking the elasticity of the bone marrow niche could keep hMSC at a quiescent state without impairing their proliferation ability and multilineage differentiation potential, so that holds MSCs as a reservoir for quite long period. Coincidentally, a recent study presented an in vitro bone marrow niche model which is made up of very soft type I collagen gel, in this model they could generate multicellular MSC spheroids that remain resting state until regenerative need appeared and then were capable to differentiate into osteoblasts, chondrocytes and adipocytes [54]. More than that, compliant hydrogel matrices with the similar physiological elasticity of muscle tissue were also reported to promote the self-renewal of muscle stem cells in culture, and even engraftment, niche repopulation and muscle regeneration in vivo [55].

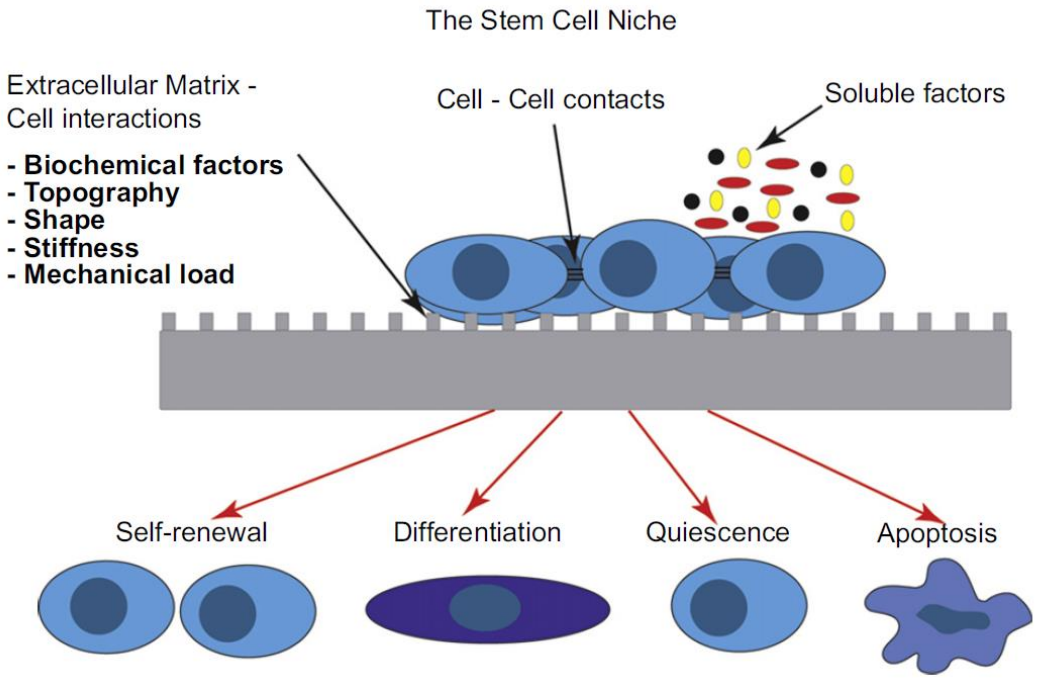


Figure 1-7. Multifactorial niche environment affects stem cell fate. Figure reprinted from ref. [47]. Copyright © 2012 Elsevier Ltd.

In the case of hematopoietic stem cells, tropoelastin coated plates with higher extensibility could produce much more hematopoietic stem and progenitor cells than glutaraldehyde cross-linked or truncated tropoelastin coated plates which had reduced elasticity [56]. What's more amazing, one notable study from Tzahor lab reported that compliant matrices induced the dedifferentiation and cell cycle re-entry of cardiomyocyte accompanied with loss of differentiation markers [57]. The author explained that the compliant substrates resulted in the myoskeletal disorganization, which led to a round morphology acquisition and finally facilitated cytokinesis and cell division. In stem cell mechanobiology studies that try to clarify the influence of matrix stiffness on stem cell fate determinations, most of them mainly focus on the forward differentiation processes. This is the first experimental evidence showed that the differentiated cardiomyocytes could be induced to dedifferentiate by compliant environment and shift to a less-differentiated stage, which further validated the prominent role of extracellular matrix environment on stem cell decision.

Beyond the adult stem cells, the effects of ECM stiffness on the self-renewal abilities of pluripotent stem cell have also been investigated. For instance, culturing mESCs on soft matrices that mimicking the inherent elasticity of mESCs, resulted in undifferentiated homogeneous colonies even without exogenous LIF factor [58], suggest that soft naive environment contribute to maintain the pluripotency and self-renewal abilities of mESCs by generating low cell-matrix tractions. Similarly, other studies also reported the soft polyacrylamide substrates or nanofilms were able to preserve the stemness of mESCs by enhancing the expressing levels of Oct4 and Nanog or suppressing the late epiblast stage genes [59, 60]. However, in the case of human induced pluripotent stem cells, the soft environment was reported to facilitate the neurogenic or ectodermal differentiation instead of maintaining the self-renewal [61, 62], which indicates the different mechanical response between mouse and human pluripotent stem cells. Taken together, on one hand all these evidence

demonstrate that the mechanical property of the naïve environment is crucial for early embryonic development, on the other hand, furthermore investigations are needed to explore the different response between mPSCs and hPSCs in order to update our understandings to mammalian embryonic development as well as better directing to the further biomaterial design and production.

1.2.2 Extracellular matrix stiffness directs the differentiation of stem cells

Similar to self-renewal maintenance, stem cell differentiation is also an indispensable or even dominant aspect in stem cell mechanical regulation related studies. The very early experimental evidence in 1979 first observed the phenomenon that soft collagen substrates influenced the differentiation of mouse mammary epithelial cells [63, 64]. And recent study also reported the effect of matrices stiffness on human mammary epithelial progenitors. Increased matrix stiffness promoted the myoepithelial differentiation of these multipotent progenitors and meanwhile suppressed luminal differentiation and progenitor maintenance [65]. However, the effect lost with age because aging impairs YAP activity and nuclear translocalization.

Such stiffness-dependent differentiation also appeared in other progenitors and stem cells. For instance, by culturing mouse myoblasts on collagen patterned polymer gels with varied elasticity, myosin and actin fibers formed only on the gels with elasticity similar to normal muscle [66]. In neonatal rat cardiomyocytes, the action potential duration was longest when cells were grown on hydrogels with similar elastic modulus to normal myocardium [67]. In terms of adult neural stem cells, substrates resembling stiffness of naïve brain tissue resulted in improved levels of neuronal-specific markers and further produced more neurons compared with stiffer hydrogels [68]. In a similar study, soft surfaces with elastic modulus no more than 1 kPa facilitated the neuronal differentiation of neural stem/progenitor cells (NSPCs) while stiff surfaces with elastic modulus more than 7 kPa supported oligodendrocyte differentiation [69].

Beyond that, various mechano-dependent differentiations of pluripotent stem cells have also been widely demonstrated. Mouse embryonic stem cells (mESCs) cultured on soft PDMS substrates were reported to tend to mesendoderm differentiation, while stiff substrates reinforced the osteogenic differentiation [70]. Similarly, in human embryonic stem cells (hESCs), enhanced Wnt-dependent mesoderm differentiation was induced on compliant hydrogel matrix, while the self-renewal was not affected [71]. During this process, Src-mediated ubiquitination of E-cadherin contributed to the initiation and reinforcement of mesoderm differentiation by raising the β-catenin transcriptional activity. Conversely, Thomas et al reported that increased alginate-based capsule stiffness enhanced hESC differentiation toward mesendoderm but meanwhile inhibited pancreatic lineage differentiation [72]. Interestingly, in other stories, polyacrylamide substrates characterized by brain-mimicking stiffness was shown to promote human and mouse pluripotent stem cell differentiating toward neurogenic lineage [73, 74]. Another more detailed study showed that soft substrates promoted neuroepithelial transformation while suppressing neural crest conversion of hPSCs. They further found that soft environment inhibited YAP/TAZ-dependent Smads phosphorylation and nuclear translocation as well as promoting Lats-dependent YAP phosphorylation, hence finally resulted in far more production of functional motor neurons than stiff substrates.

As one of the most famous adult stem cell population, mesenchymal stem cells attract dominated the attention of scientists in the fields of biomaterials-based stem cell mediated regenerative therapies, extracellular matrix derived mechanotransduction and mechanics-mediated differentiation regulation of stem cells. In a remarkable study, Engler et al reported that human mesenchymal stem cells cultured on soft collagen-covered polyacrylamide substrates with the softness of brain tissue (0.1-1 kPa) differentiated into neuron, adipose and chondrocyte lineage; on intermediate substrates mimicking muscle tissue (8-17 kPa) developed into myoblast lineage; and on rigid substrates resembling bone tissue (25-40 kPa) displayed a

osteoblastic phenotype [Figure 1-8, 75, 76]. Similar differentiation trends to special lineage induced by elastic property of the extracellular matrix were also confirmed by a large number of studies on various substrates [77-82]. Beyond that, even human neural crest stem cell (NCSC)-derived ectodermal MSCs (eMSCs) also preferred adipogenic and chondrogenic differentiation on soft substrates [83].

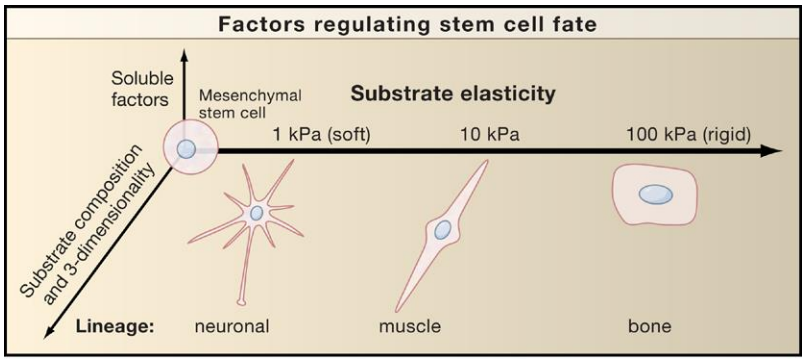


Figure 1-8. Substrate elasticity regulates hMSC differentiation. Figure reprinted from ref. [75]. Copyright © 2006 Elsevier Ltd.

In addition, the possible molecular mechanisms implicated in the stiffness-mediated mechanical regulation in stem cell lineage decisions were also described. Jing et al demonstrated that soft matrices promoted integrin internalization through caveolae/raft-mediated endocytosis, which finally resulted in the induction of neural lineage [84]. Another line of evidence from Joe and his colleague proved that knockdown of lamin-A promoted soft matrix induced adipogenesis while overexpression of lamin-A enhanced stiff matrix mediated osteogenesis [85], which suggested that lamin-A reinforced matrix elasticity-mediated MSC differentiation. On the other hand, a challenging perspective indicated that the difference of protein tethering and anchoring densities resulted from different matrix stiffness instead of the stiffness itself probably is the true regulator of mechanics-mediated stem cell differentiation [86]. Soon, this interesting viewpoint was refuted by another scientist with the evidence that by adjusting the linker density, the greatly varying protein tethering didn’t affect osteogenic and adipogenic lineage differentiation of hMSCs on

respective elastic substrates. And also, hMSCs still stick to the differentiation trends to adipogenesis and osteogenesis when cultured on both soft and stiff matrices with constant density of cell adhesion ligand, RGD peptide [87]. Taken together, these works demonstrate that matrix stiffness directing the differentiation process of stem cells was not affected by protein tethering and anchoring.

1.2.3 3D environment and matrix stiffness regulate stem cell fate

As we all know, life is 3D. Cells in organisms reside in a 3D environment constructed by its surrounding extracellular matrix and neighboring cells. However, almost all the in vitro studies in cell biology are carried out on a flat, stiff, 2D surface cell culture system such as polystyrene plate/dish and glass coverslip. Due to the convenient manipulability, the traditional 2D cell culture system contributed greatly to the cell-based biological studies. Even though, because of lack of proper structural architecture and crucial features present in vivo environment such as continual biochemical biomechanical cues, spatially stereoscopic organization of actin cytoskeleton as well as complicated original interaction with neighboring cells, the plain 2D cell culture system fail to imitate the natural morphology and original function of various cells especially stem cells in realistic in vivo environment. To overcome the limitation of 2D culture platform on stem cell-mediated translational medical research and clinical therapy, varieties of excellent natural or artificial biomaterial-based 3D culture systems have been exploited to achieve expansion, self-renewal maintenance and differentiating induction of human in the last decade, such as, hyaluronic acid hydrogel [88], PEG-vinylsulfone hydrogel [89,90], alginate/chitin-composed microfiber scaffold [91,92], PNIPAAm-PEG hydrogel [93], alginate-Matrigel cross-linked 3D network [94].

Given the fact of the significant influence of matrices stiffness on stem cell fate control, it is not difficult to infer that the control of matrices stiffness on the self-renewal abilities and differentiation of stem cell also remains in the 3D

environment [95-99]. Notable, beyond the biomechanical and biochemical signals from the stem cell niche, matrix remodeling is also critical for the self-renewal of stem cell. Christopher et al found that matrix remodeling of 3D hydrogel leads to hydrogel degradation by promoting cadherin-mediated cell-cell contacting and the activation of β-catenin, thereby maintains the stemness of neural progenitor cells in the 3D environment [100]. In addition, 3D environment, for the first time, was recently reported to enhance somatic cell reprogramming to iPSC by promoting mesenchymal-to-epithelial transition and epigenetic remodeling [101], which is a brand new discovery in biomechanics field and will open up a new avenue of 3D reprogramming and gene editing related study [102].

2 Scientific Goals

2.1

Breast cancer, according to the newest data, is the most frequent cancer mainly occurs in female, with the second-highest mortality rate. However, the exact mechanisms involved in the mammary tumorigenesis and progression, as well as invasion and metastasis, have not been fully demonstrated yet. Though some certain breast cancer subtypes have a good prognosis after surgical excision, hormonotherapy, chemotherapy or combination therapy, some other breast cancer subtypes still have a very high fatality rate, such as triple-negative breast cancer (TNBC). As a highly dynamic, complex scaffold supporting cells and tissues which resides throughout the organism, the extracellular matrix (ECM) plays a remarkable role not only in the normal tissue and organ development but also in the progression of many diseases, for instance, cancer. The main purpose of this part is to explore the influence of perturbation of ECM homeostasis, such as altered matrix elasticity also called stiffness on breast cancer cell functions, particularly autophagy activation.

As an evolutionarily conserved physiological process, autophagy exists in all the eukaryotes and is involved in the development of many cancer and other diseases. The correlation between extracellular matrix stiffness and autophagy activation is barely demonstrated in breast cancer cells as well as in other kinds of cancer cells. It is of great significance to disclose the mysterious veil of autophagy activation under diverse mechanical environment, for enriching our knowledge in the understanding of tumor initiation and progression and discovering potential targets in clinical therapy. In this thesis, we will employ fibronectin-coated polyacrylamide hydrogel with tunable elasticity property as substrates to explore autophagy activation under nutritional deprivation conditions. On the other hand, we also want to analyze the chemotherapeutic response of breast cancer cells in different elastic environment. In addition, alterations of CSC population in common breast cancer cells that on varied

elastic substrates will be also introduced. According to the CSC theory, exist of CSC is the main cause of cancer recurrence due to their extraordinary tolerance to anticancer drugs, therefore, survived from chemotherapy. So the study on matrix stiffness regulated chemosensitivity and CSC maintenance is also very meaningful for illuminating the molecular mechanism of tumor recurrence and metastasis and developing better clinical chemotherapy.

2.2

Since the 21st century, increased human life span and aging trends of the population have brought great demands on regenerative medicine and the immense development of biomedical materials. To achieve the expected therapeutic effect, tremendous amounts of high-quality undifferentiated stem cells are needed. However, by the current 2D surface-based cell culture method, it is not easy to generate so many qualified cells in a short time due to the contact inhibition of monolayer. What's more important, after undergoing in vitro amplification, stem cells sometimes display an abnormal morphology and aberrant function when transplants into the patients. The most likely cause is that the traditional 2D culture platform fails to build a better physiologically similar in vivo environment because of the lack of 3D architecture structure, hence lead to the alterations not only in cellular phenotypes but also even in gene transcription pattern. To overcome the multiple limits of the 2D culture system and produce enough satisfying cells, developing new 3D culture methods becomes a promising solution and urgent mission.

As one of the indispensable categories of biomaterials, hydrogels have been well explored due to its tremendous potency in tissue and organ engineering and clinical therapy. The main goal of the part is to try to develop a totally chemically defined 3D hydrogel platform for iPSC expansion and self-renewal maintenance by taking advantage of highly biocompatible polyethylene glycol (PEG) polymer and dendritic polyglycerols (dPGs) polymer. After that, we further aim to explore the impact of

hydrogel-based 3D environment on stem cell growth and fate determination. As previously reported, the 3D environment generates some changes in cellular gene transcription thereby regulate the self-renewal abilities and differentiation of stem cells. By adjusting the concentration and ratio of PEG and dPG polymers, different hydrogels with tunable elasticity are employed to optimize the most suitable hydrogel system for iPS expansion and self-renewal, and to explore the potential mechanism involved in the stem cell mechanics regulation.

3 Extracellular matrix stiffness regulates breast cancer cell chemosensitivity, stemness and autophagy

3.1 Preparation of polyacrylamide hydrogel substrates

To investigate the regulation of matrices stiffness on cancer cells behaviors and functions, polyacrylamide hydrogel substrates were produced on the basis of the Current Protocols in Cell Biology [Figure 3-1, ref 103]. Briefly, the coverslips were treated with plasma, and then NaOH solution was spread on its surface to let it dry by evaporation. Next, 3-Aminopropyltriethoxysilane (APES) was applied to the semi-transparent NaOH film to react. The coverslips were washed twice with Milli-Q water to rinse off unreacted APES. Incubate coverslips under glutaraldehyde solution for half an hour and then remove the solution to leave coverslips naturally air dry. Prepare mixture solution with desired acrylamide and bis-acrylamide concentrations followed by degassing to exhaust dissolved oxygen. After adding APS and TEMED solution and adequate mixing, proper volume of hydrogel solution was dropped onto the dichlorodimethylsilane (DCDMS) treated glass slides and immediately covered by amino-silanated coverslips. After leaving the hydrogel to polymerize for half an hour, carefully uncover the top coverslips which covalently binding with the PA gel and transfer the PA gel conjugated coverslips to petri dish or plate. After rinsing twice with Milli-Q water, the PA gel substrates are ready to use or store. Before seeding cells, the PA gel substrates are treated with sulfo-SANPAH for a short time under 365 nm UV light and then coated by fibronectin (FN) protein solution overnight. Expose the FN coated PA gel substrate under UV light for sterilization before seeding cells.

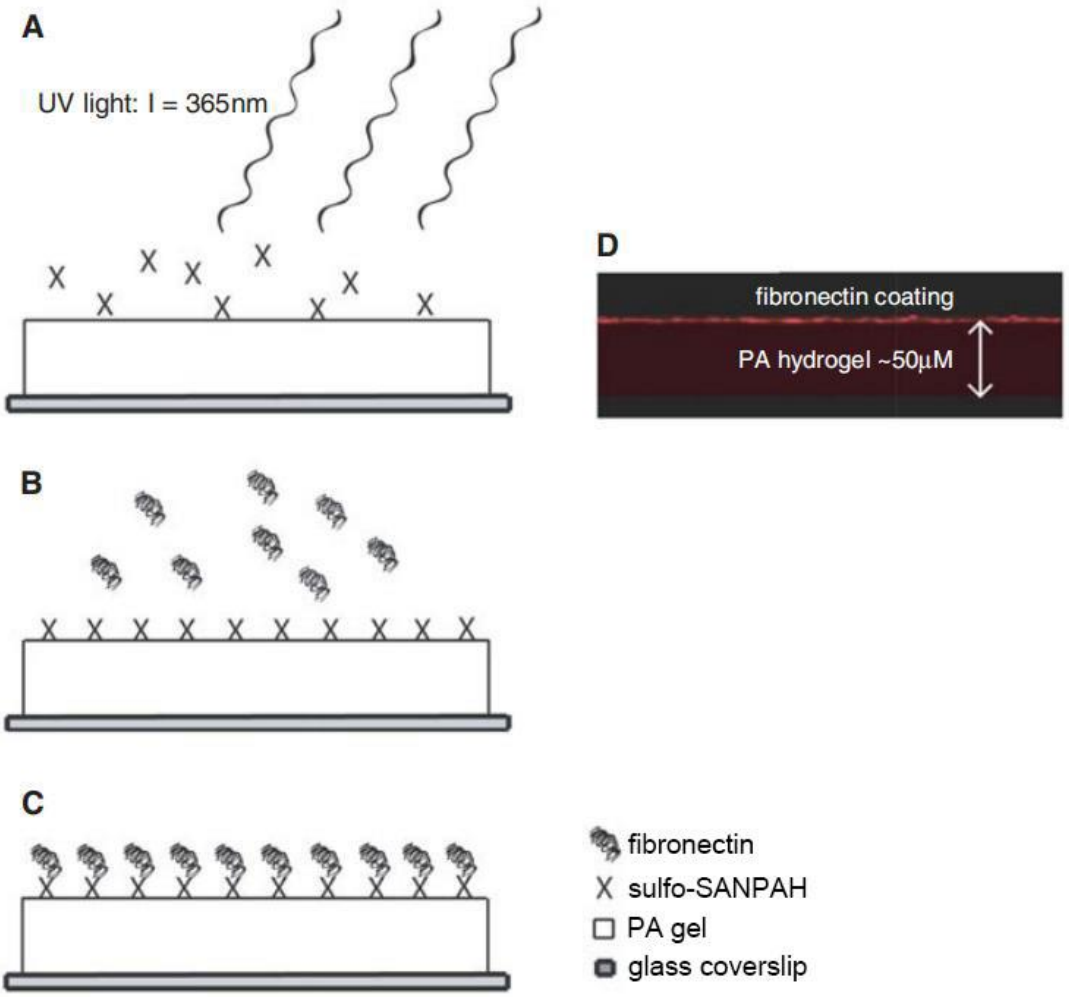


Figure 3-1. Coating process of polyacrylamide substrates. Figure reprinted from ref. [61]. Copyright © 2010 John Wiley & Sons, Inc.

In this project, three group of PA hydrogel substrates were settled, which I named soft (3% acrylamide, 0.06% bis-acrylamide, ~0.48 kPa), median (5% acrylamide, 0.15% bis-acrylamide, ~4.47 kPa), stiff (10% acrylamide, 0.3% bis-acrylamide, ~34.88 kPa) conforming to the classic protocol [103]. The elasticity properties of the PA hydrogel substrate were characterized by testing the shear modulus (G) of hydrogel with a Kinexus rheometer (Malvern Instruments, UK) [Figure 3-2]. Young's modulus (E) was calculated on the basis of material Poisson ratio (ν) which links the shear modulus to Young's modulus: $E = 2*G(1+\nu)$ [104, 105].

Group	Acrylamide% + Bis-acrylamide %	Elastic modulus in Ref. [61]	Elastic modulus by rheological test	
			Shear modulus (G)	Young's modulus (E) (calculated by E = 2*G(1+ν))
Soft	3% + 0.06%	480 Pa	75.82 Pa	~ 220 Pa
Median	5% + 0.15%	4.47 kPa	767.74 Pa	~ 2.3 kPa
Stiff	10% + 0.3%	34.88 kPa	12.83 kPa	~ 38 kPa

Table 3-1. Elasticity of different PA substrates.

3.2 Extracellular matrix stiffness influences the morphology, spreading and proliferation of breast cancer cells

To detect the difference of breast cancer cells on elastic matrices with tunable stiffness. Firstly we cultured breast cancer cell MCF-7 on soft, median, and stiff fibronectin-coated polyacrylamide substrates and observed the cellular changes in morphology. From the phase-contrast images and fluorescent staining results of the actin cytoskeleton, we found that cells exert immense difference among three different substrates [Figure 3-2, 3-3]. Cells on 'stiff' substrate displayed an irregularly polygonal epithelioid morphology which looked more similar to that on glass coverslip. Meanwhile, cells on the 'median' substrate presented also an irregular but with a little round shape and retained less pseudopodium than that of stiff substrate. Whereas, cells on the 'soft' substrate acted quite different, which were growing as round spheres with different sizes.

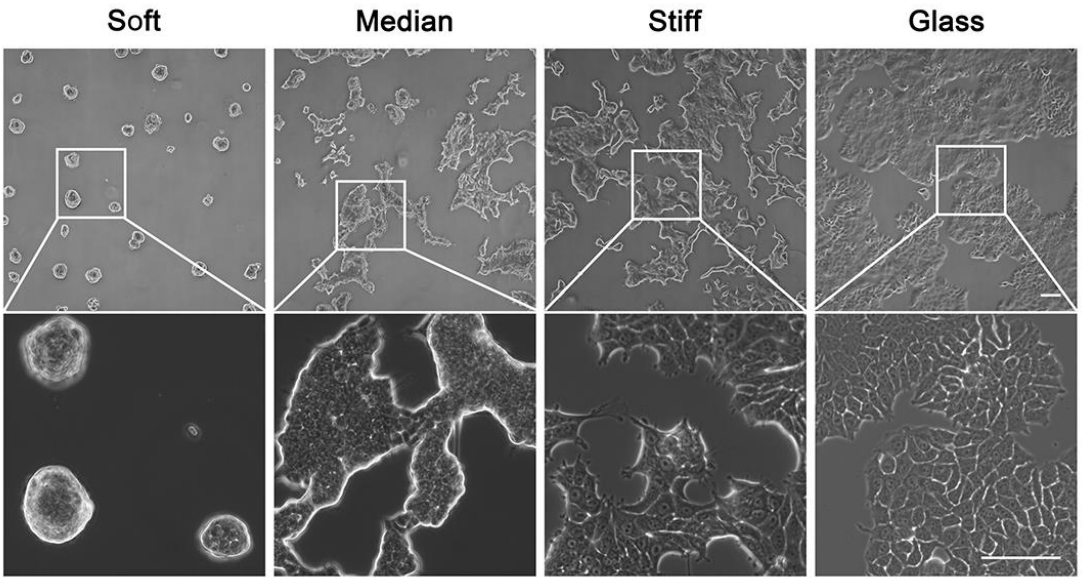


Figure 3-2. Phase-contrast images of breast cancer cell MCF-7 cultured on soft, median, stiff PA substrates and coverslips for 3 days. (Scale bar = 100 μm)

As a key factor in communications with the extracellular matrix environment, cell spreading is known to regulate cell proliferation, apoptosis and other functions in tissue development [106, 107]. Since Pelham RJ and Wang YL firstly found that cell spreading was facilitated on rigid substrates but suppressed on flexible substrates in epithelial cells and fibroblasts [108], many other cell types were also reported to follow the same rule. Without exception, cell spreading in MCF7 was also greatly affected by matrix stiffness [Figure 3-3]. Cells barely spread on a quite soft environment while spread very well on stiff substrate, and the spreading area was increased along with the increasing matrices stiffness [Figure 3-4].

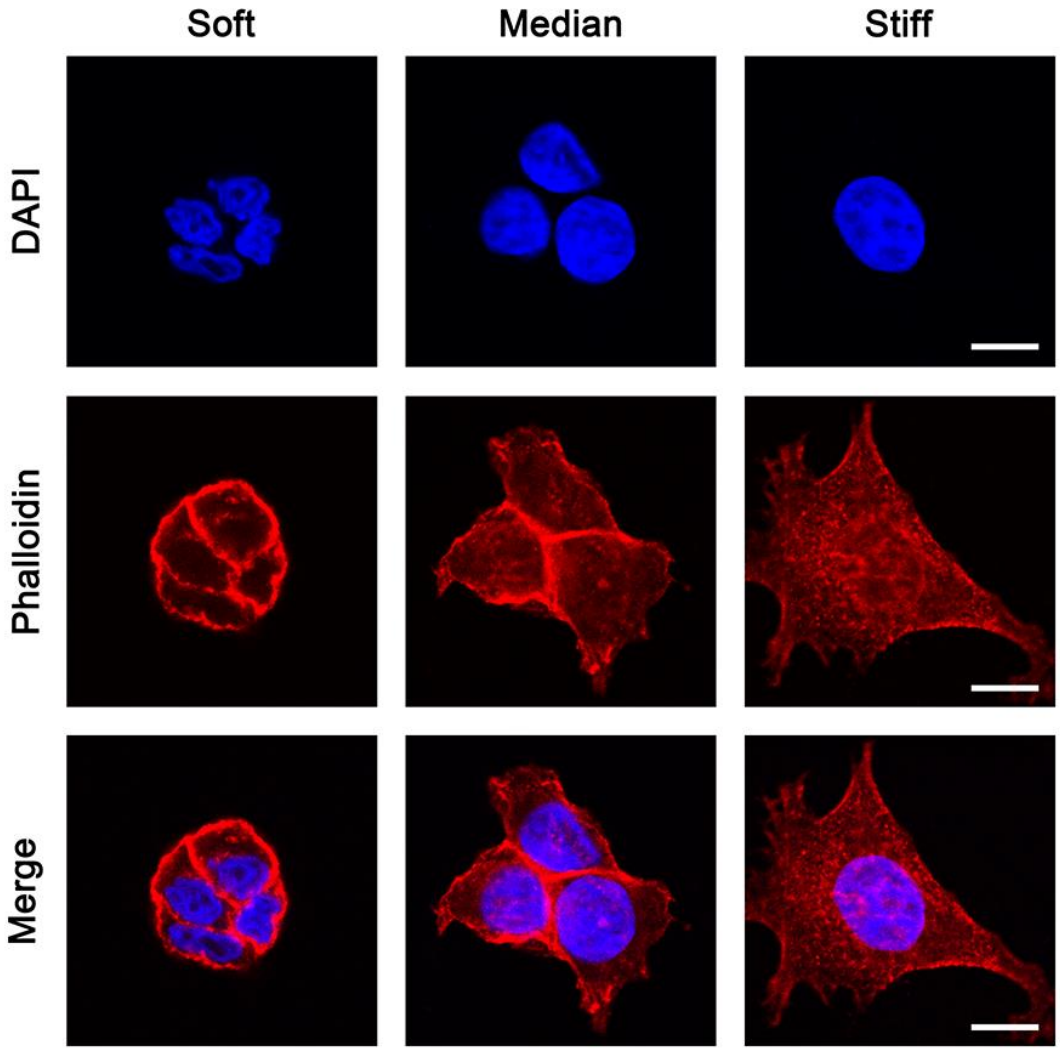


Figure 3-3. The morphology, stress fiber and actin cytoskeleton of breast cancer cell line, MCF-7 growing on different PA substrates. (Scale bar = 10 μm)

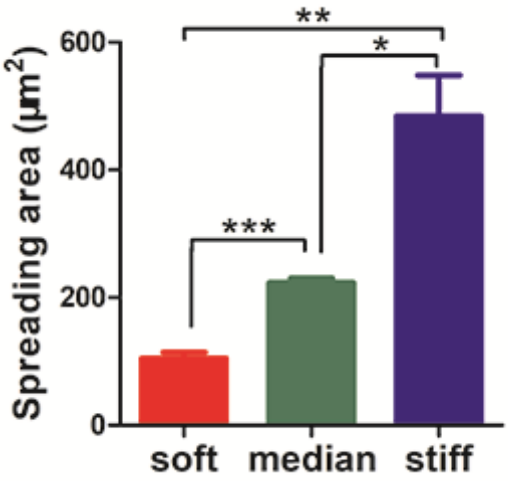


Figure 3-4. Cell spreading area increases along with increasing matrix stiffness. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

In addition, we also found that cell grew faster on stiff matrix and slower on soft one which indicated that increasing matrix stiffness promotes breast cancer cell proliferation [Figure 3-5], which is consistent with the results in hepatocellular carcinoma cells [28], lung cancer cells [109, 110], colorectal cancer cells [111], and glioblastoma cells [112, 113].

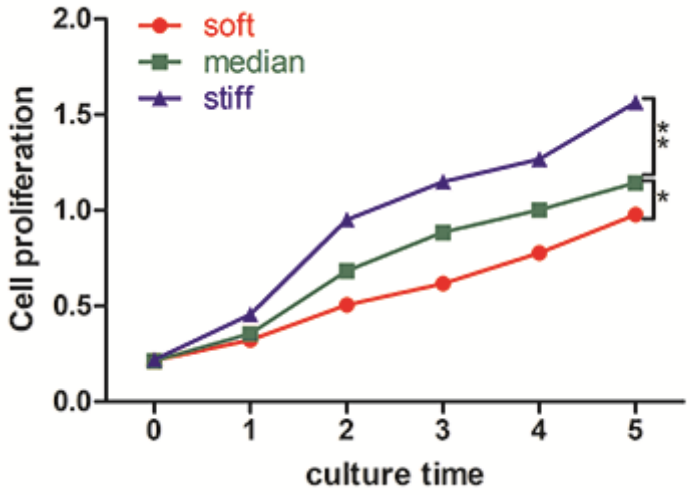


Figure 3-5. Increasing matrix stiffness promotes the proliferation of MCF-7 cells. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

3.3 The effect of matrix stiffness on chemotherapeutic response to anticancer drugs in breast cancer cells

Tumor development is a multifactorial process regulated by interactions between cells and adjacent cells or the surrounding extracellular matrix. The surrounding

extracellular matrix which also called tumor microenvironment is crucial to tumor cell proliferation, metastasis, and chemotherapeutic efficiency. To explore the effect of matrix stiffness on chemotherapeutic response, breast cancer cell lines MCF-7, MDA-MB-231 and T-47D were cultured on different PA substrates for two days and then treated with the most commonly used anticancer drug in breast cancer cells, doxorubicin for 24h. And the results showed that cells cultured on stiff substrate displayed lowest relative viability (compared with untreated groups) while on soft substrate displayed highest relative viability, in another words, breast cancer cells exhibited highest chemotherapeutic response on stiff substrate and lowest chemotherapeutic response on soft substrates [Figure 3-6, 3-7]. This demonstrated that the cellular chemotherapy response also known as chemosensitivity increased along with increasing matrix stiffness.

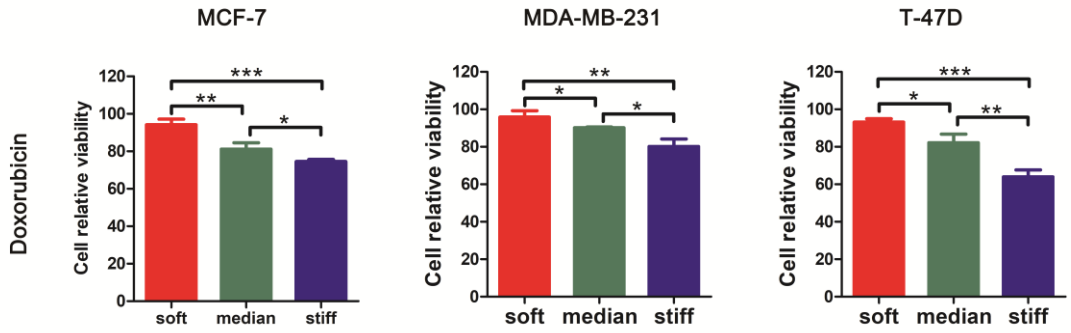


Figure 3-6. Chemotherapeutic response to doxorubicin in different breast cancer cells. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

We also employed another common drug used in a variety of cancer types, cisplatin to test the matrix stiffness-mediated cellular chemosensitivity in breast cancer cells. And similar results were observed, which further confirmed the concept that compliant extracellular matrix could decrease the chemosensitivity in breast cancer cells.

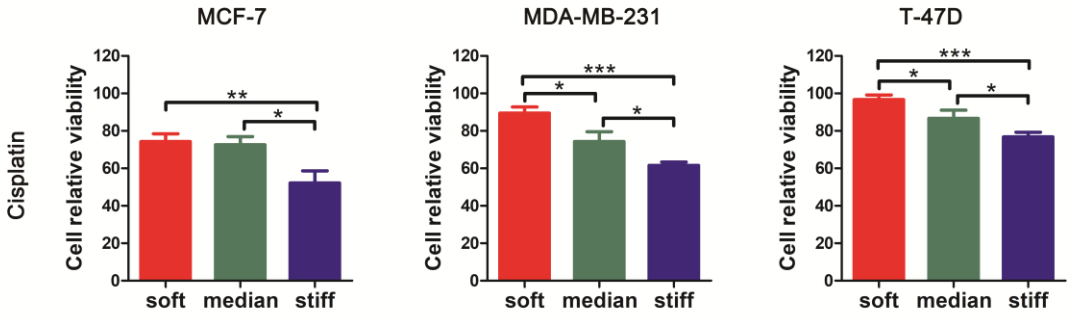


Figure 3-7. Chemotherapeutic response to cisplatin in different breast cancer cells. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

However, it should be noted that the increasing chemosensitivity along with increasing matrix stiffness is drug-specific. Only doxorubicin and cisplatin could trigger the stiffness dependent chemosensitivity in breast cancer cells, while the chemotherapeutic response to CTX was independent of stiffness [Figure 3-8].

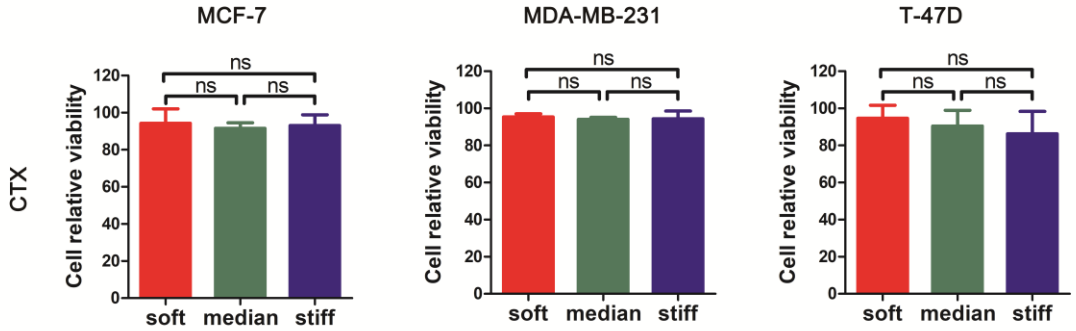


Figure 3-8. Chemotherapeutic response to cyclophosphamide (CTX) in different breast cancer cells. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

3.4 Matrix stiffness impact BCSC population maintenance in breast cancer cell lines

More and more oncologists agree that one indispensable member of the critical culprits responsible for chemotherapeutic resistance and tumor recurrence is cancer stem cells [114]. To investigate the effect of matrix stiffness on the stemness characteristics of breast cancer cells, the cells were seeded and cultured on soft, median and stiff PA substrates for several days and then flow cytometry was performed to analyze the ALDH1+ cell population which is identified as breast cancer stem cells (BCSCs) population [115]. The results showed that the percentage of ALDH1+ cells was reduced when matrix stiffness increased [Figure 3-9].

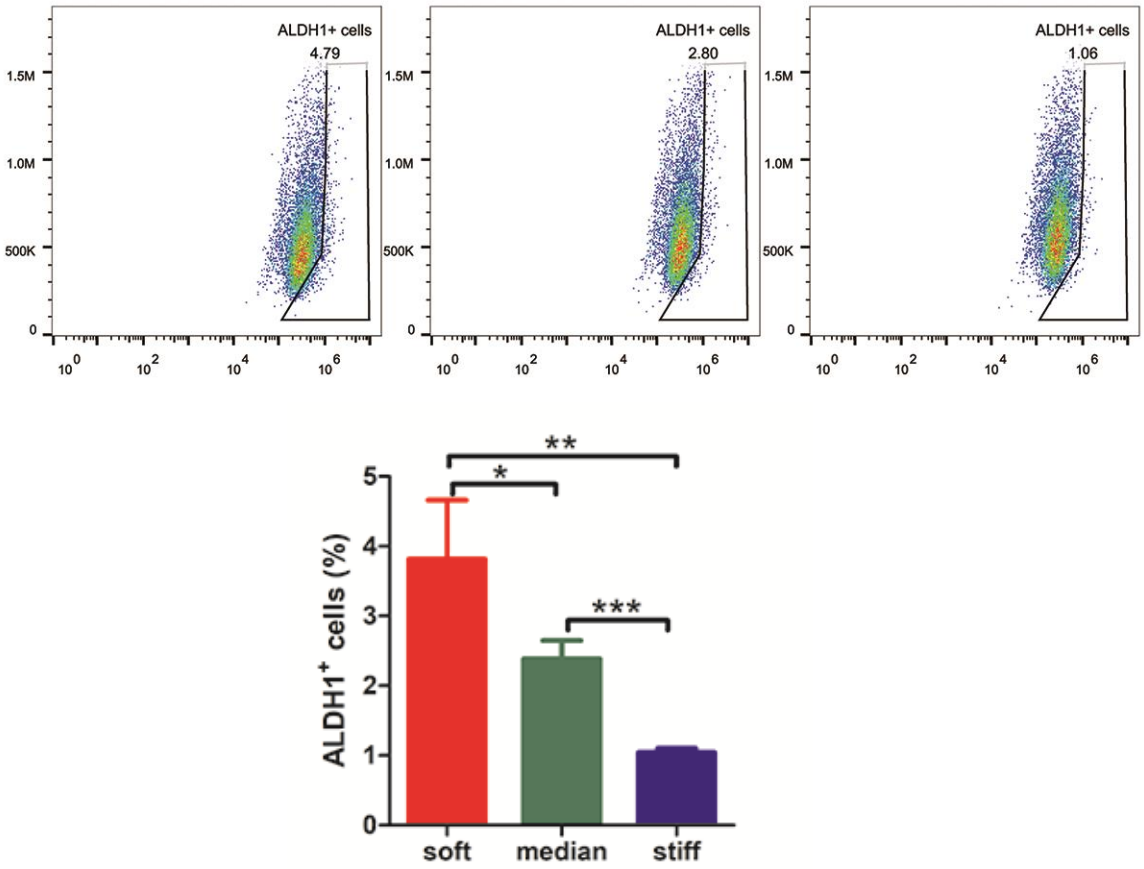


Figure 3-9. BCSC population analysis by flow cytometry in MCF-7 cells cultured on PA substrates for 3 days. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

More than that, to further confirm the breast cancer stem cell response to the matrix stiffness, we carried out mammosphere (also called tumorsphere) formation assay which is considered to be the classic method to assess the activity of BCSCs [116, 117]. Seminal work demonstrated that cells can acquire mechanical memory from their underlying substrate [118, 119]. Based on this fact, we cultured breast cancer cells on PA substrates with different stiffness for 5 days to allow cell form mechanical memory, and then harvested the cells; trypsinized into single cells and reseed in ultra-low attachment plate in a very light concentration to generate mammospheres which considered to be enriched in BCSCs. Consistent with flow cytometry result of CSC marker, more mammospheres were formed by normal breast cancer cells from soft substrate while less mammospheres were formed by cells from stiff environments [Figure 3-10, 3-11], which indicated that soft or compliant environment is beneficial for BCSCs maintenance.

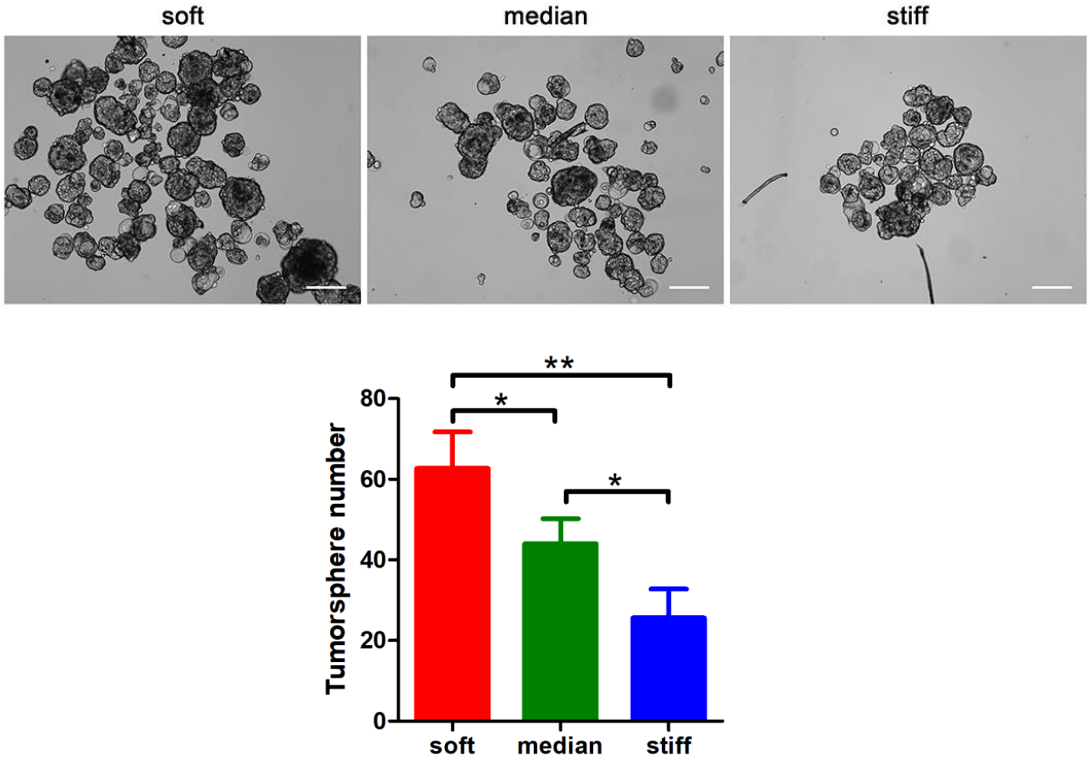


Figure 3-10. BCSC population enrichment in MCF-7 after cultured on PA substrates for 5 days. (Scale bar = 200μm)(*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

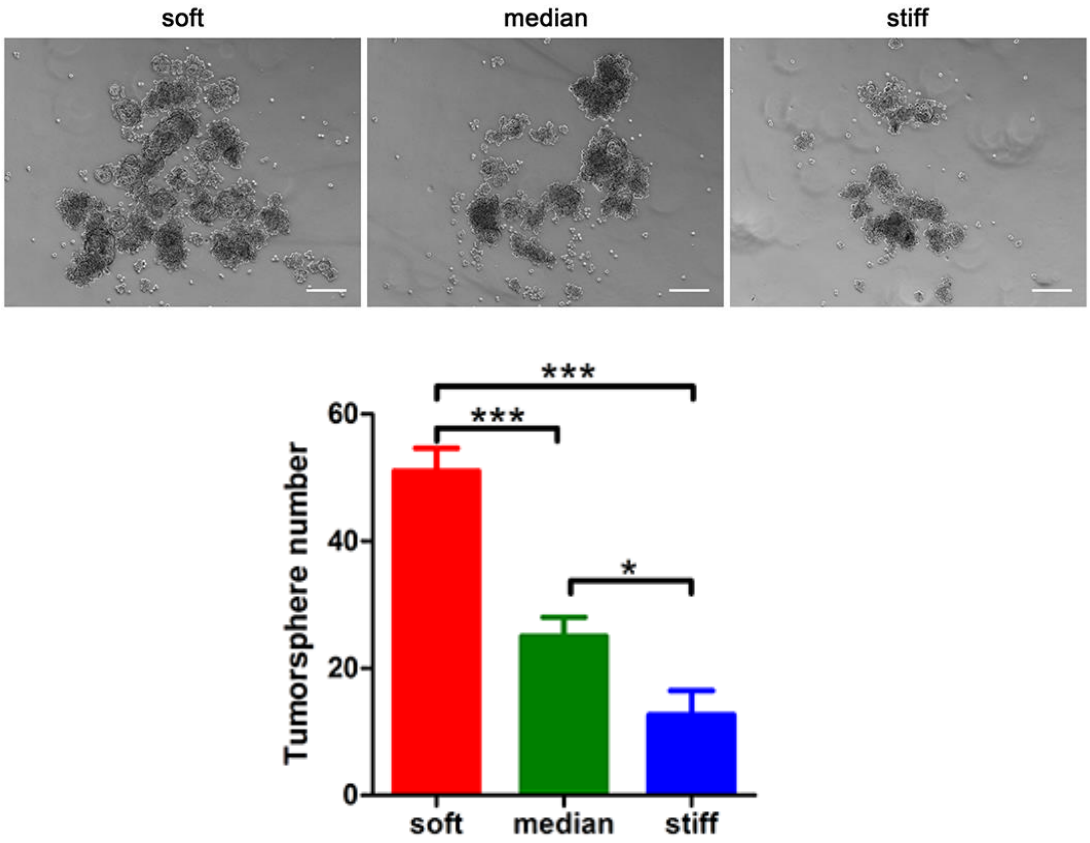


Figure 3-11. BCSC population enrichment in MDA-MB-231 after cultured on PA substrates for 5 days. (Scale bar = 200μm)(*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

As mentioned above, as the central mediator of mechanotransduction, YAP is affected by substrate stiffness [120]. Meanwhile, YAP is able to maintain stemness in cancer cells by acting as a downstream effector of Sox2 [121, 122]. To answer the question that whether YAP is involved in the maintenance of BCSCs by matrix stiffness, siRNA of YAP was employed both during the culture period of breast cancer cells on PA substrates and BCSCs enriching process. The results showed that knockdown of the YAP factor reduced ALDH1+cells populations in all three groups and the difference among these groups was alleviated [Figure 3-12]. On the other hand, the numbers of mammospheres formed by three different cell populations were

altered by YAP knock down in a more complicated way. Knock down of YAP decreased the number of mammospheres formed by cells that cultured on soft matrices, whereas increased the number of mammospheres formed by cells that cultured on rigid matrices [Figure 3-13], which finally lead to the elimination of different mammosphere forming ability of three different cell populations. This further indicated that the difference of mammosphere forming ability induced by matrix stiffness was alleviated by the knock down of YAP factor.

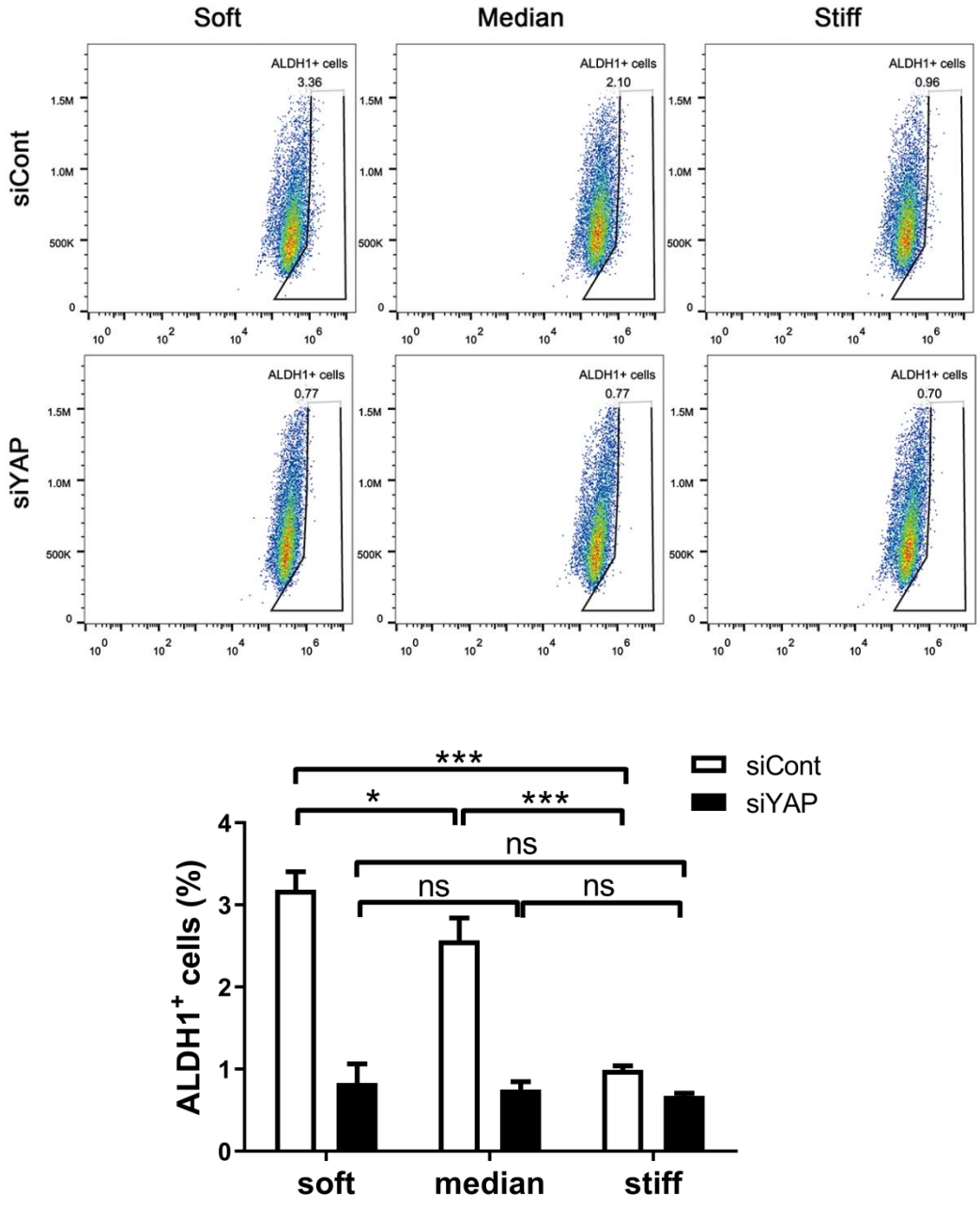


Figure 3-12. BCSC population analysis by Flow Cytometry in siYAP treated MCF-7

cells cultured on different PA substrates for 5 days. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p < 0.05$, ns indicates $p > 0.05$)

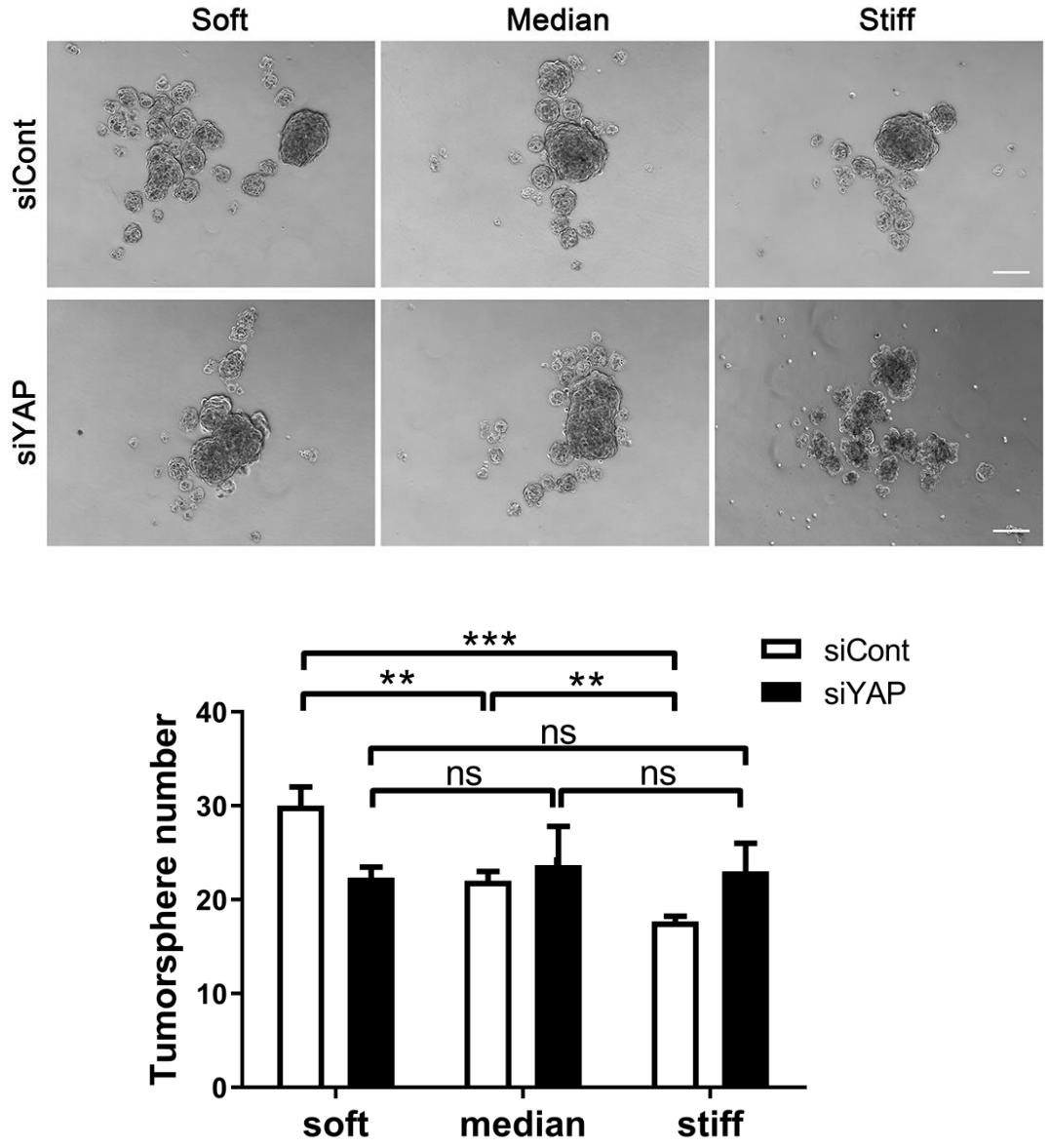


Figure 3-13. BCSC population enrichment in siYAP treated MCF-7 after cultured on PA substrates for 5 days. (Scale bar = 200 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

3.5 Matrix stiffness regulate nutrient deprivation induced autophagy activation in breast cancer cells

As an evolutionarily conservative cellular process in eukaryotes, autophagy remains quite low basal level under normal physiological condition [123], and only to be activated when it has to be, like oxidative stress, nutrient starvation, infection and so on. To activate and increase autophagy up to a detectable level, after culturing breast cancer cells MCF-7 on different PA substrates for several days, EBSS buffer was employed to create a starvation environment accompanied by Bafilomycin A1 (Baf A1) inhibiting fusion of autophagosomes and lysosomes to block autophagic flux. After starvation treatment for 2 h, we observed the expression of LC3B in MCF-7 cells using fluorescent immunostaining. LC3B is the most widely used marker of autophagosome, and from the fluorescent staining results, we found that cells on stiffer substrate generated more autophagosome while on softer substrate generated less autophagosome [Figure 3-14, 3-15].

Beyond that, we also detected the level of LC3B-II protein by western blot. LC3 protein has two forms, LC3-I and LC3-II. LC3-I protein is cytosolic and will be conjugated to phosphatidylethanolamine (PE) to form PE-conjugated LC3, termed LC3-II, which will be recruited to autophagosomal membranes to form autophagosome when autophagy is activated. Hence, the amount of LC3-II or the ratio of LC3-II/LC3-I reflects the activated autophagy level. From the band result, we knew that LC3B-II also increased along with the increasing matrix stiffness [Figure 3-16], which proposed that elevated autophagy was induced by increasing matrix stiffness.

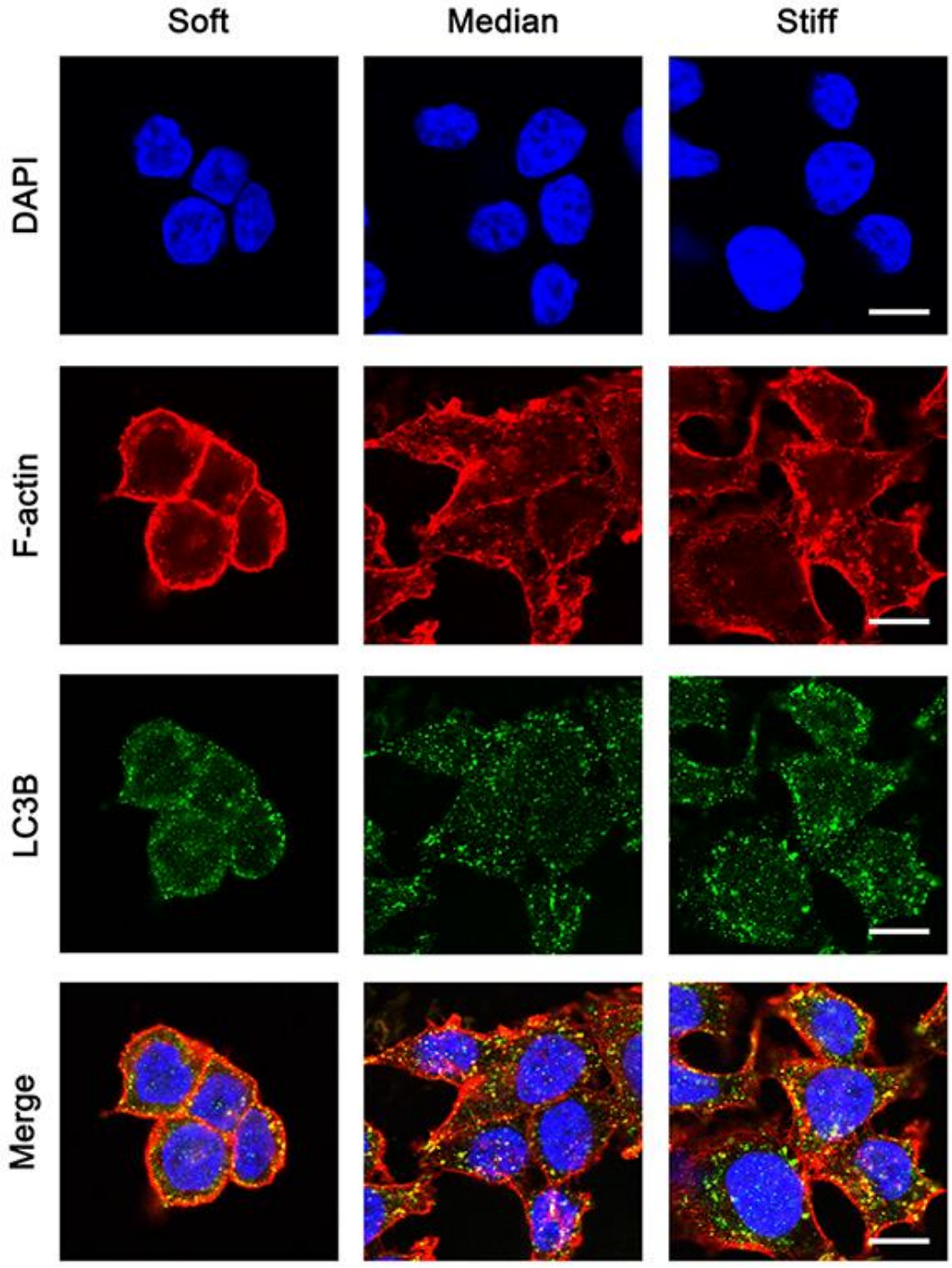


Figure 3-14. Fluorescent images of autophagosome in MCF-7 cells cultured on different PA substrates. (Scale bar = 10 μm)

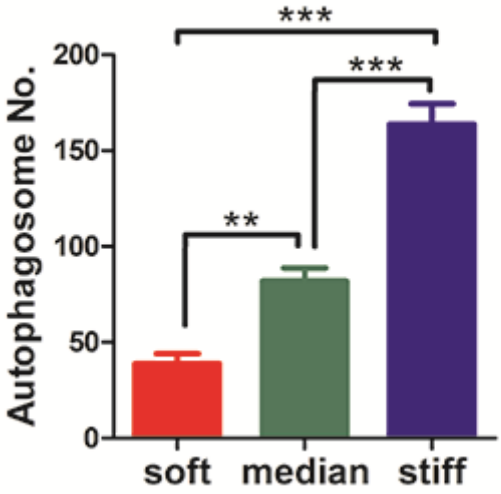


Figure 3-15. Generation of autophagosome in MCF-7 cells cultured on different PA substrates. (*** indicates $p < 0.001$, ** indicates $p < 0.005$)

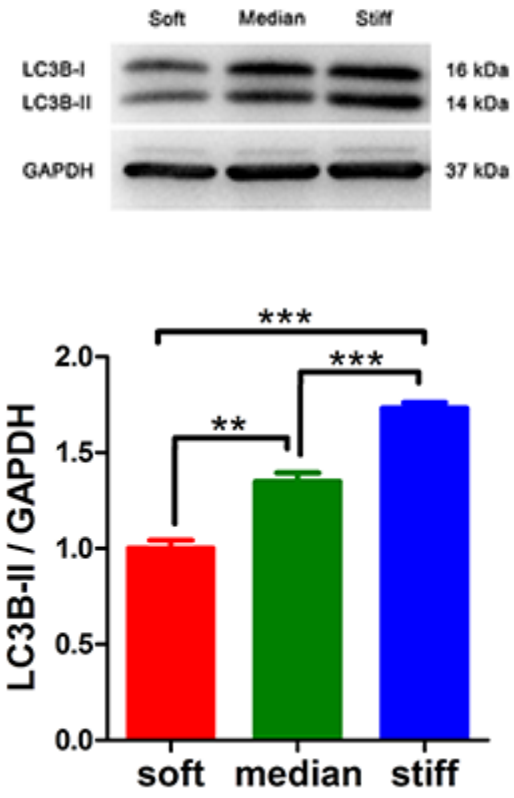


Figure 3-16. The expression of LC3B in MCF-7 cells on different PA substrates and coverslip (untreated group). (*** indicates $p < 0.001$, ** indicates $p < 0.005$)

In another more malignant cell line, MDA-MB-231, which is estrogen receptor (ER), progesterone receptor (PR) and HER2 negative and regarded as in vitro model of Triple-negative breast cancer (TNBC), the same phenomenon was observed: more autophagosomes (Figure 3-17, 3-18) and higher LC3B-II level (Figure 3-19) on more rigid substrate. This further proved the concept that stiff extracellular matrix environment generates higher level of starvation-induced autophagy.

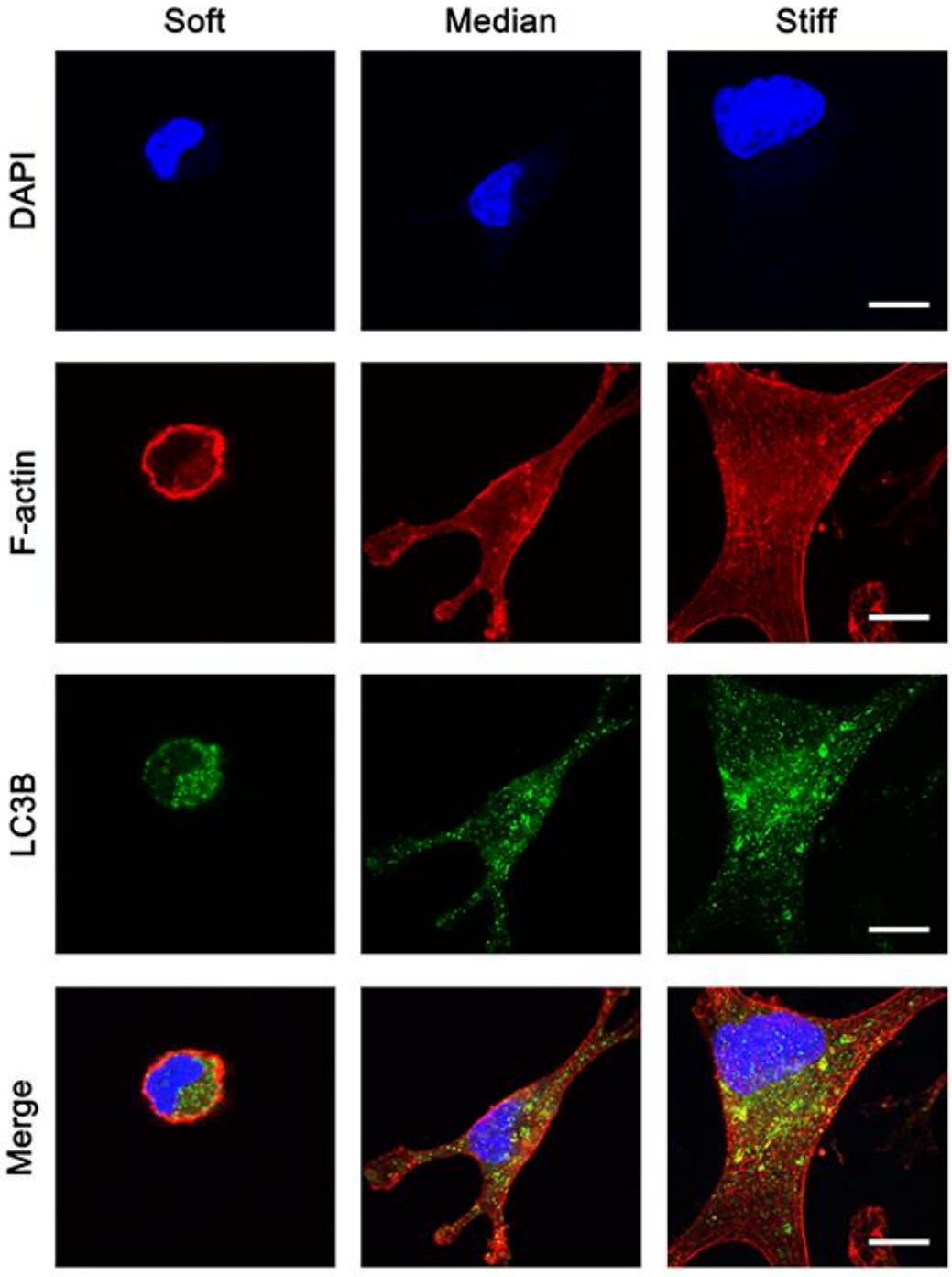


Figure 3-17. Fluorescent images of autophagosomes in MDA-MB-231 cells cultured on different PA substrates. (Scale bar = 10 μm)

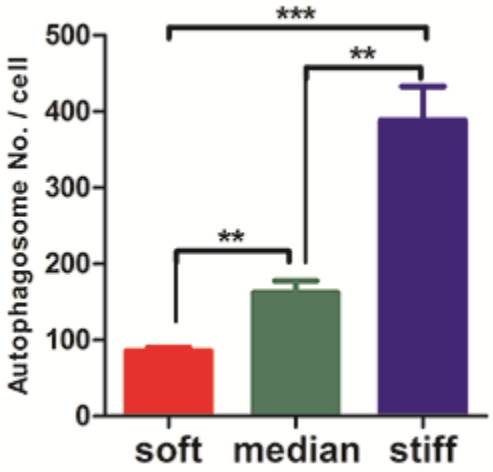


Figure 3-18. Generation of autophagosome in MDA-MB-231 cells grew on different PA substrates. (*** indicates $p < 0.001$, ** indicates $p < 0.005$)

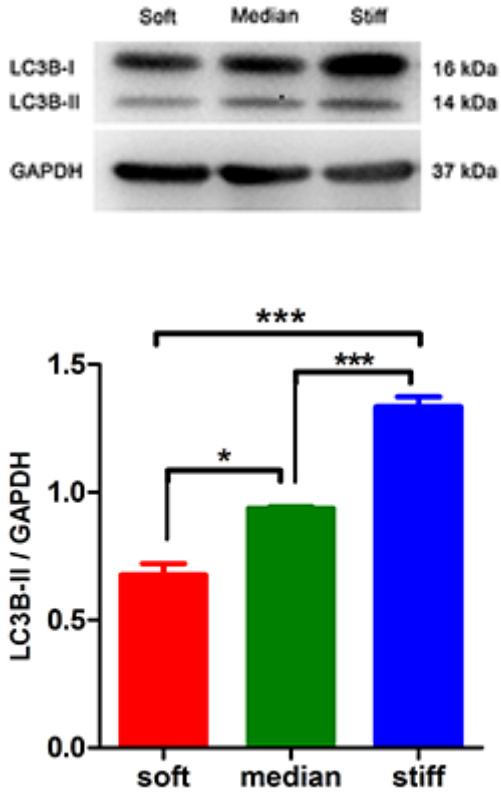


Figure 3-19. The expression of LC3B in MDA-MB-231 cells on different PA

substrates. (*** indicates $p < 0.001$, * indicates $p < 0.05$)

Matrix stiffness is known to affect stress fiber formation [120] and modulate actin cytoskeletal tension [76, 124], which was suggested to be involved in the initiation of starvation-induced autophagy [125]. Given this, to further confirm the regulation of matrix stiffness on autophagy, we cultured MCF-7 cells on glass coverslips for several days. F-actin inhibitor Latrunculin A (Lat.A) and non-muscle myosin inhibitor blebbistatin (Blebbist) were added during the period of autophagy induction process to disturb the actin cytoskeleton tension and stress fiber formation. The results showed that cells treated with Lat.A or Blebbist displayed a much smaller spreading area than the control group [Figure 3-20, 3-21], and furthermore the treatment of Lat.A or Blebbist reduces the amount of autophagosome [Figure 3-20, 3-22].

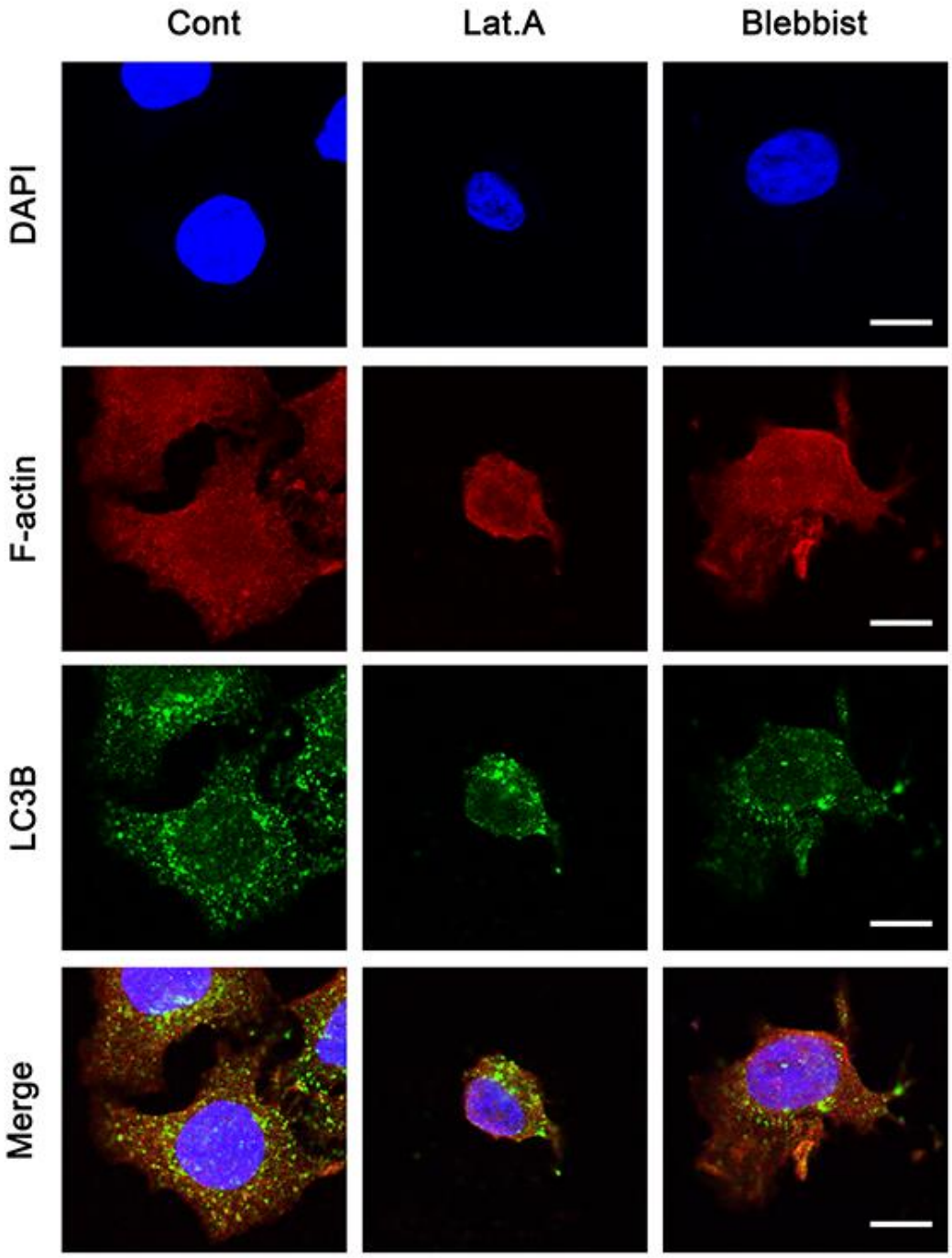


Figure 3-20. Fluorescent confocal image of autophagosome in MCF-7 cells treated by Lat.A (F-actin inhibitor) and Blebbist (non-muscle myosin inhibitor). (Scale bar = 10 μm)

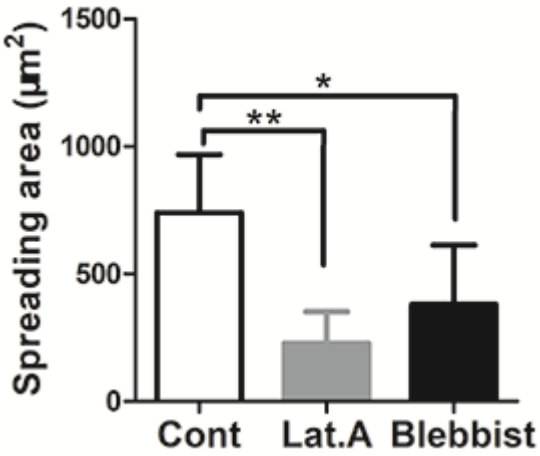


Figure 3-21. Spreading area of MCF-7 cells treated by Lat.A and Blebbist. (** indicates $p < 0.005$, * indicates $p < 0.05$)

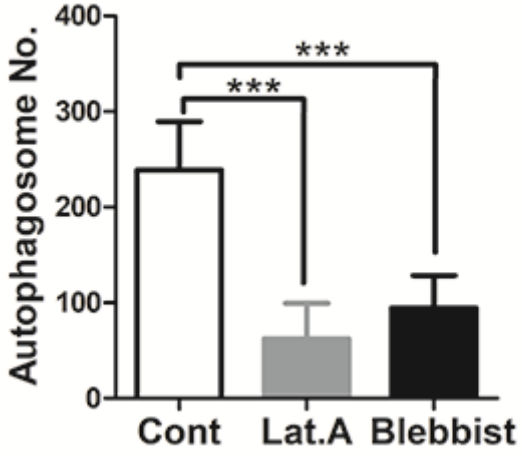


Figure 3-22. Quantification of autophagosome numbers in Lat.A and Blebbist treated MCF-7 cells. (*** indicates $p < 0.001$).

Even more important, when MCF-7 cells culturing on different elastic substrates were treated by these two inhibitors, the trend of increasing autophagy which resulted from increasing matrix stiffness was eliminated due to the inhibition of actin cytoskeleton and actomyosin tension [Figure 3-23, 3-24]. These results indicate that stable and intact actin cytoskeleton and stress fiber was required for autophagy activation.

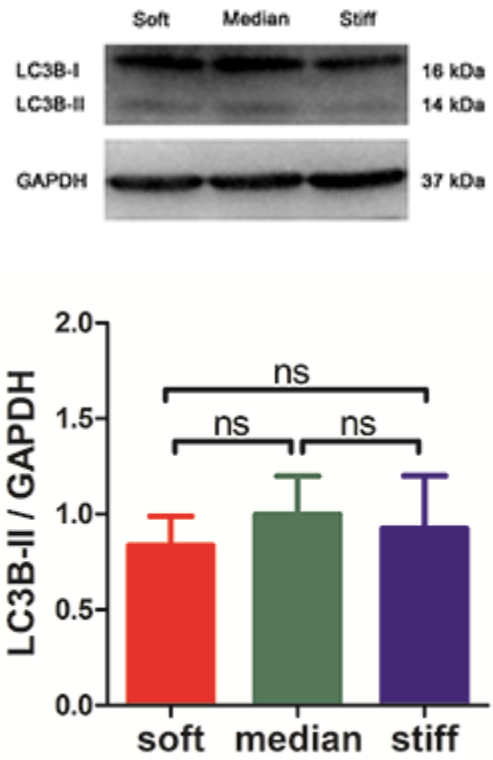


Figure 3-23. The expression of LC3B in MCF-7 cells treated by Lat.A. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

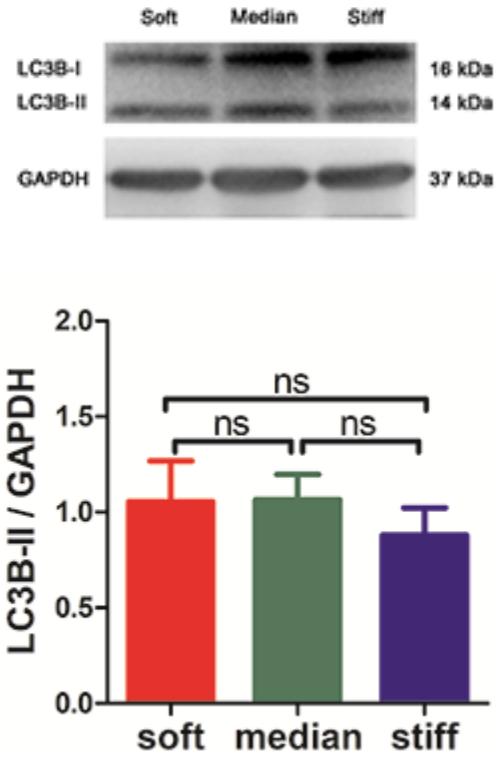


Figure 3-24. The expression of LC3B in MCF-7 cells treated by Blebbist. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

3.6 YAP is involved in the regulation of matrix stiffness on autophagy

As an ancient process preserved in all eukaryotic cells, the regulation of autophagy is complicated and multifactorial. And YAP, one of the core elements of the Hippo signal pathway, seems to be involved in autophagy activation. In contact-inhibited noncancerous cells, repressed YAP/TAZ activity was reported to result in the depolymerization of F-actin stress fibers which finally impair autophagosome formation [30]. In this study, to assess the role of YAP in the matrix stiffness regulated autophagy, siRNA of YAP was employed during the culturing of breast cancer cells on PA substrates including autophagy induction section. As we expected, knockdown of YAP expression not only reduced the amount of autophagosome [Figure 3-25] but

also downregulated the level of LC3B-II [Figure 3-26] in breast cancer cells cultured on PA gels regardless of matrix stiffness. However, interestingly, knockdown of YAP didn't eliminate the increasing trend of autophagy unexpectedly [Figure 3-25, 3-26]. Taken together, these data demonstrate that the regulation of matrix stiffness on autophagy activation is independent of YAP.

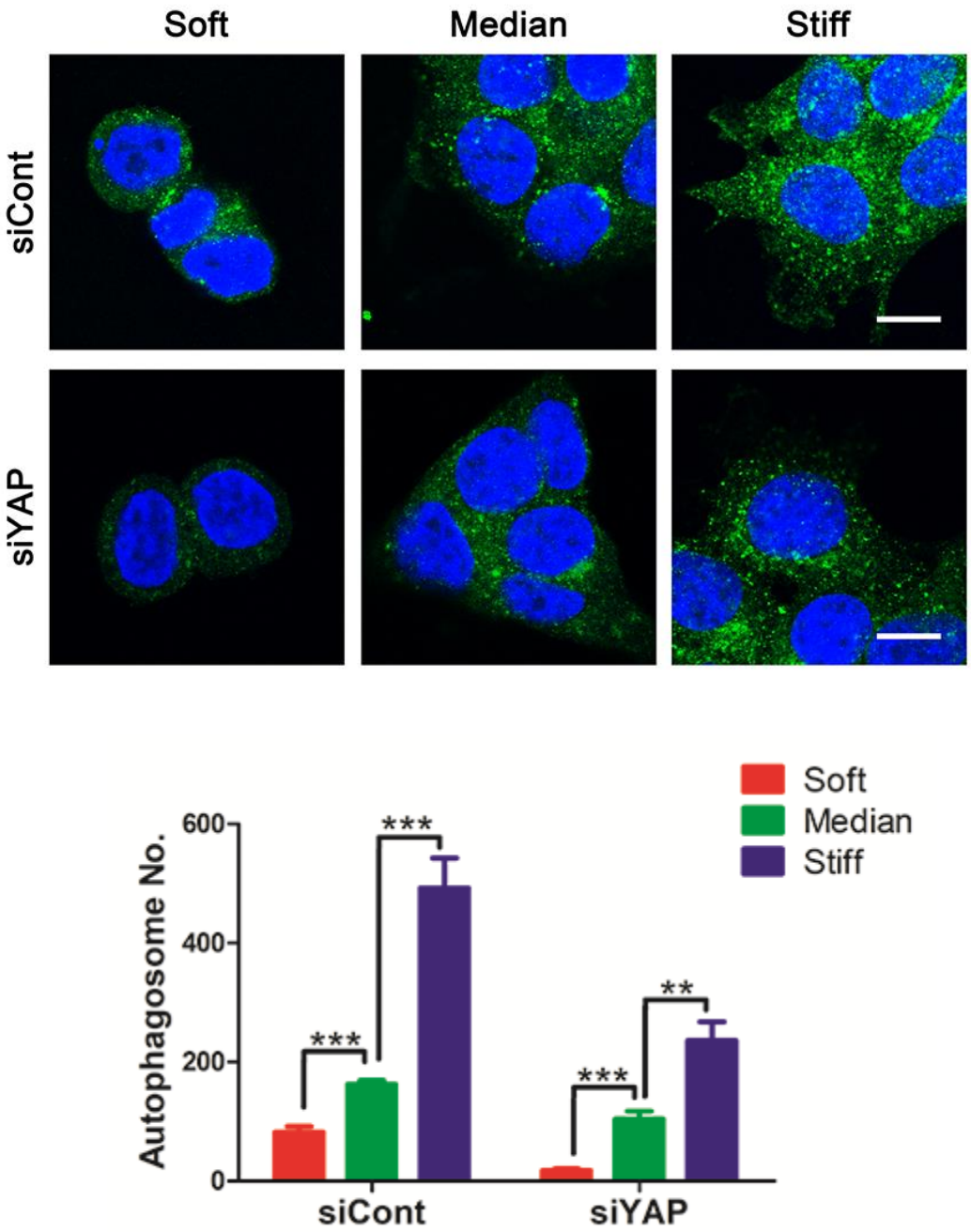


Figure 3-25. Fluorescent images of autophagosome in siYAP treated MCF-7 cells

cultured on different PA substrates. (Scale bar = 10 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$).

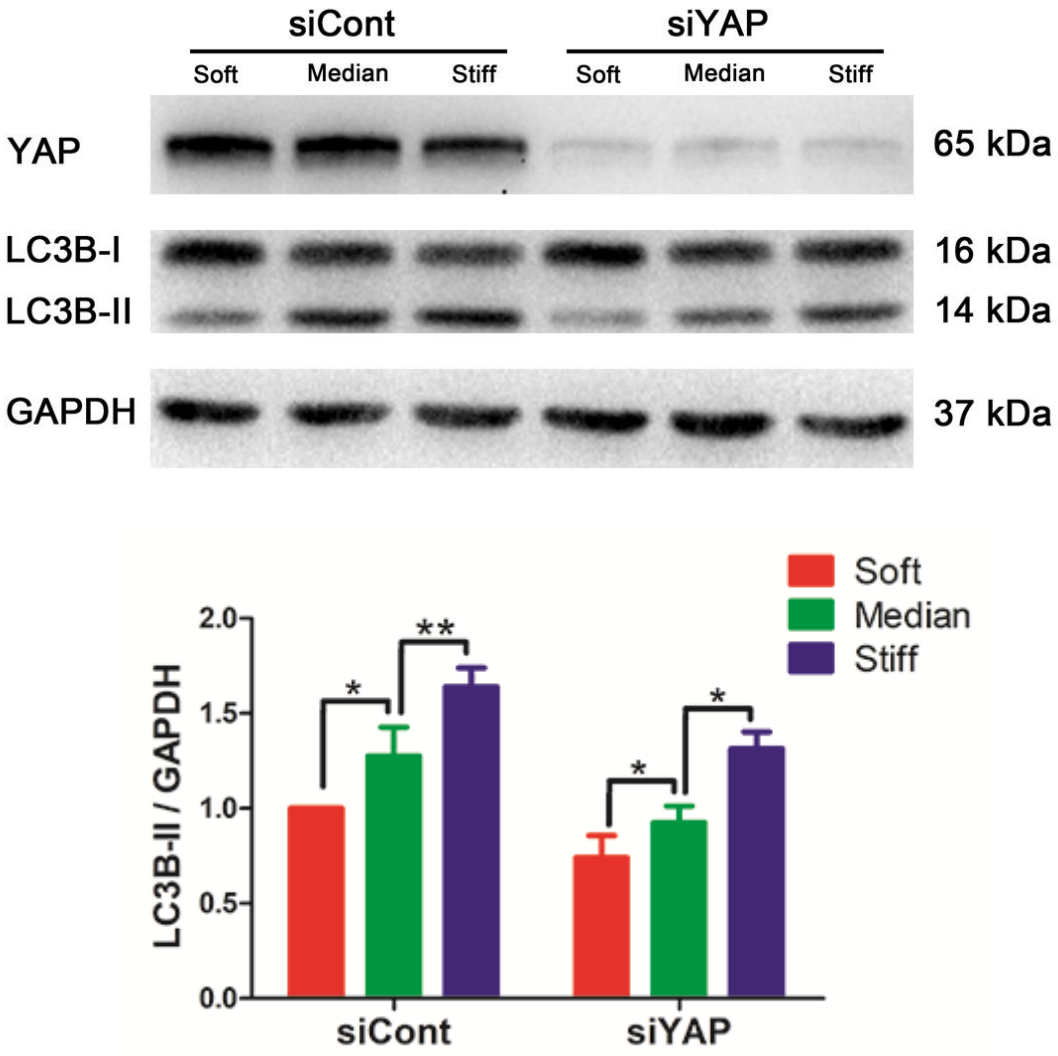


Figure 3-26. The expression of YAP and LC3B in siYAP treated MCF-7 cells cultured on different PA substrates. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$).

3.7 The influence of matrix stiffness on YAP subcellular localization in BCSC and normal breast cancer cells

Consider the results that YAP is a key mediator for soft substrate facilitating BCSCs maintenance but not indispensable for high matrix stiffness promoting autophagy activation. We urgently want to figure out the related mechanism which should be responsible for the functional differences of this conserved gene in breast cancer cells. To address this question, we enriched BCSCs in ultra-low attachment plate firstly, and then mammospheres that are riched in BCSCs were collected and trypsinized into single cells to replate on different PA substrates. By immunofluorescent staining of YAP, we found that nearly all of YAP located in the nucleus on rigid substrate while almost all of YAP located in the cytoplasm on soft substrate [Figure 3-27]. The percentage of nuclear YAP increased along with the increasing matrix stiffness [Figure 3-27]. In contrast, when directly culturing normal breast cancer cells on PA substrates, YAP nuclear translocation was not affected by matrix stiffness [Figure 3-28]. The results suggest that matrix stiffness regulates the stemness characteristics in breast cancer cells by controlling YAP nuclear translocation.

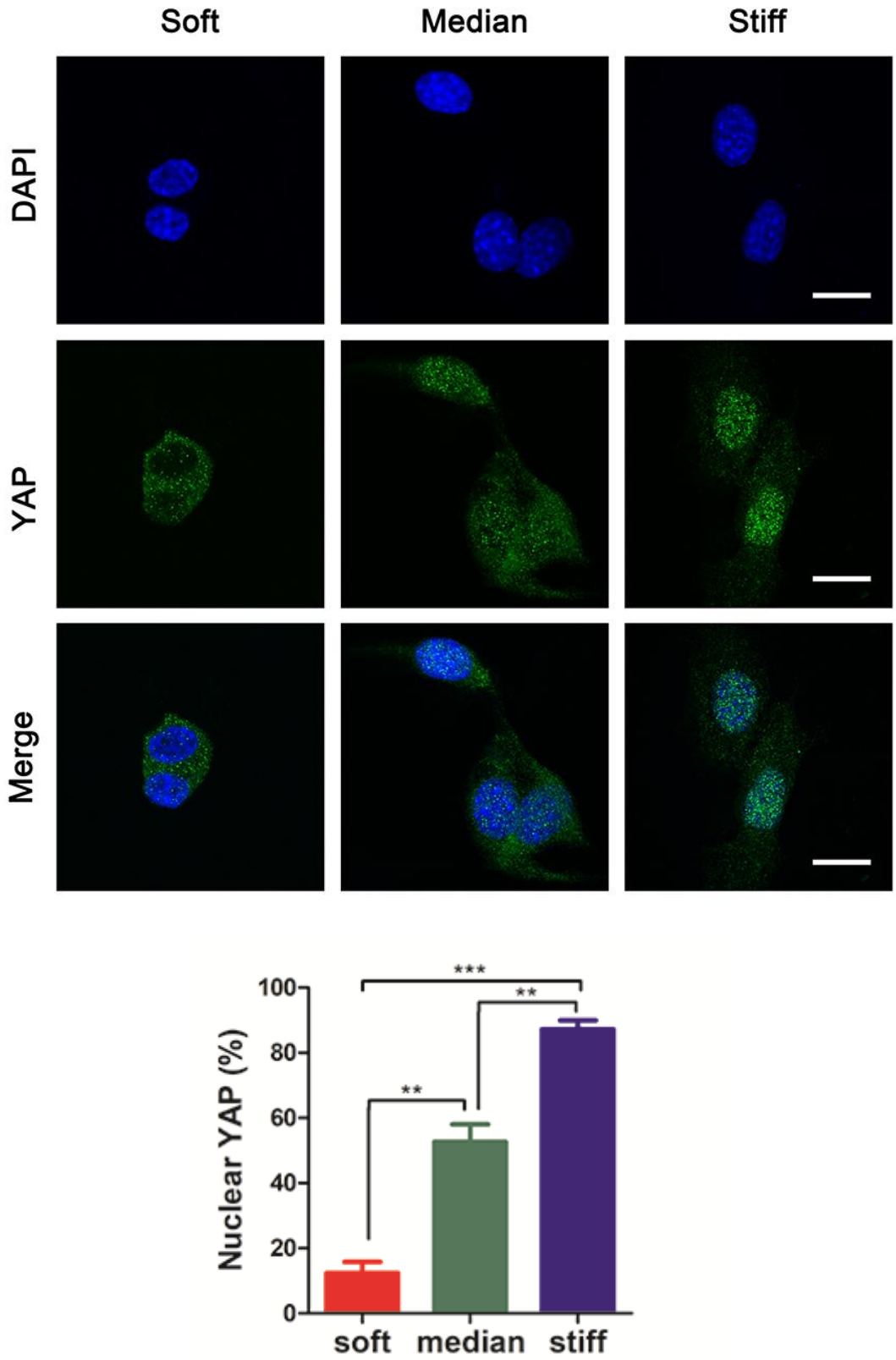
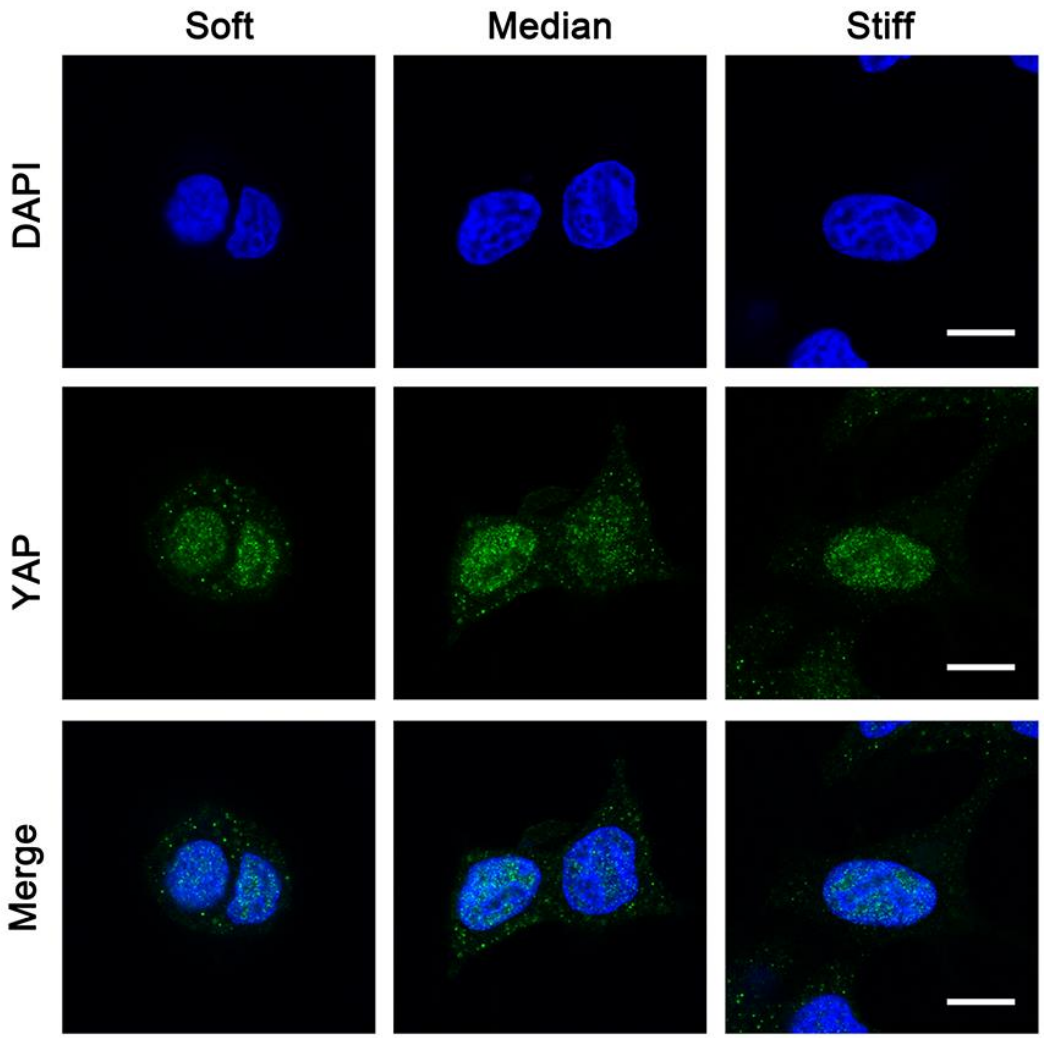


Figure 3-27. Fluorescent confocal image of YAP expression in breast cancer stem cells on soft, median and stiff PA substrates. (Scale bar = 10 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$).



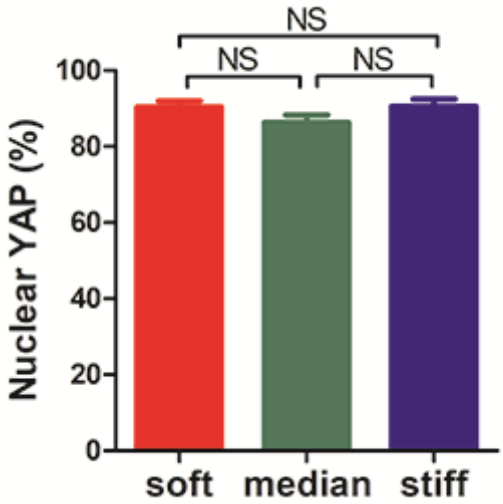


Figure 3-28. Fluorescent confocal image of YAP expression in normal breast cancer cells on soft, median and stiff PA substrates. (Scale bar = 10 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$).

3.8 Potential signal pathway that involved in the regulation of autophagy by matrix stiffness

As YAP is unnecessary in the regulation of matrices stiffness on autophagy, so which should be responsible for this regulation and what kind of role does it play in this process still remain unclear. To figure out the truth, we employed some inhibitors of classical signaling pathways. Firstly, the small GTPase Rho is considered to be a critical mediator of the actin cytoskeleton [126, 127], so the specific inhibitor of Rho C3 transferase (C3) was used to inhibit Rho-dependent cytoskeletal tension. And the result showed that C3 was able to suppress the increasing autophagy on stiff substrate [Figure 3-29], which suggested that Rho is a key mediator of cytoskeletal tension-dependent autophagy activation.

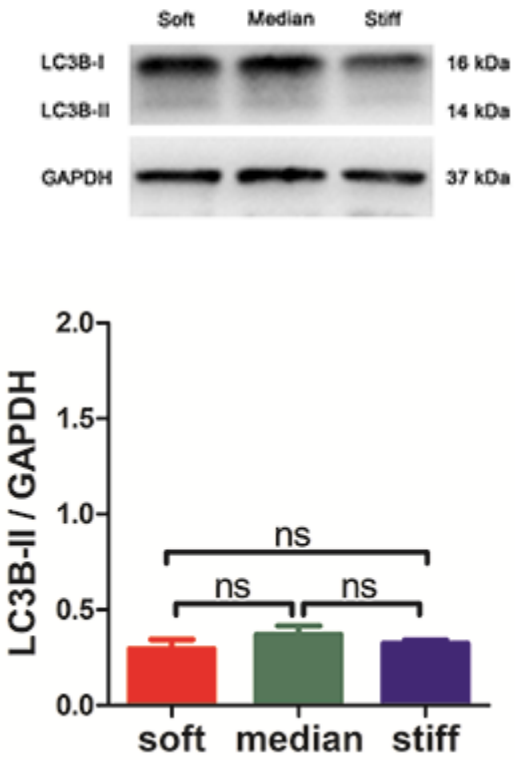


Figure 3-29. The expression of LC3B in MCF-7 cells treated by C3. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

The second inhibitor we used was Y27632, which is the specific inhibitor of ROCK, which of the full name is Rho-associated protein kinase. As the most important downstream effector of Rho [128, 129], ROCK is believed to be implicated with a wide range of different cellular functions [130], especially behaviors that based on actin organization, including but not limited to cancer cell migration and invasion [131], stress-fibre formation [132], cellular contractility [133], cell-cell adhesion [134], cell cycle control [135], and so on. The inhibition experiment showed that Y27632 was also able to eliminate the increasing trend of autophagy between different substrates [Fig 8A-C]. This strongly suggests that Rho/ROCK signal pathway is involved in the mechanical regulation of ECM on autophagy.

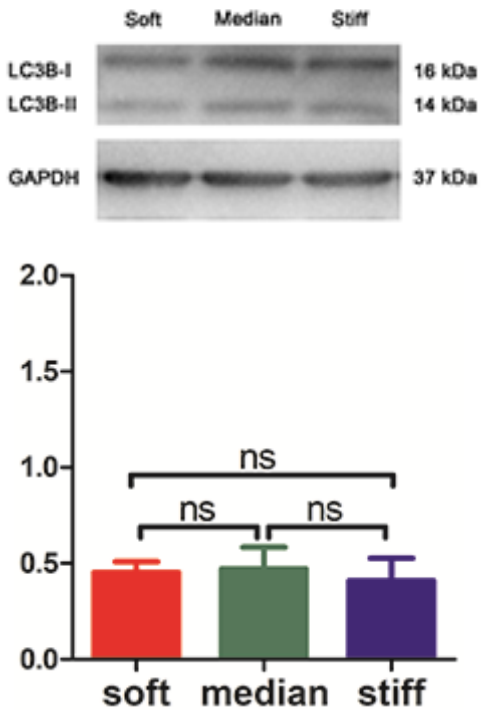


Figure 3-30. The expression of LC3B in MCF-7 cells treated by Y27632. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

Besides, it should be noted that Provenzano, Paolo P., et al found that ERK act as a key mediator of many transcriptional events response to mechanical signals such as

matrix stiffness in mammary epithelial cells [136]. ERK, the full name of which is extracellular signal-regulated kinase, as a subfamily of MAP kinase, is also believed to participate in various cellular physiological functions such as adhesion, proliferation, cytokinesis and so on [137]. And more evidence show that ERK activation is greatly influenced by extracellular mechanical signals [138, 139]. In keeping with this notion, we found that inhibition of ERK can also abolish the increasing autophagy responded to increased matrix stiffness [Fig 8A]. Furthermore, the phosphorylation of ERK can be inhibited by Rho inhibitor C3 and ROCK inhibitor Y27632 [Fig 8D], which indicated that Rho-ROCK-ERK signal pathway is involved in the mechanical regulation of extracellular matrix on autophagy.

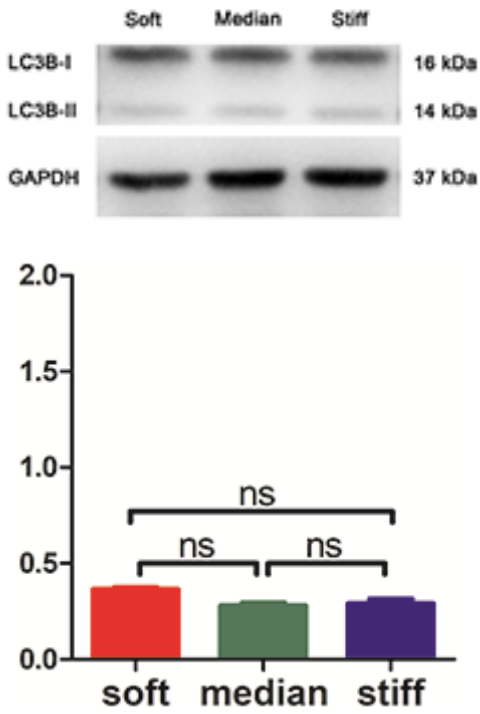


Figure 3-31. The expression of LC3B in MCF-7 cells treated by FR180204. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$, ns indicates $p > 0.05$)

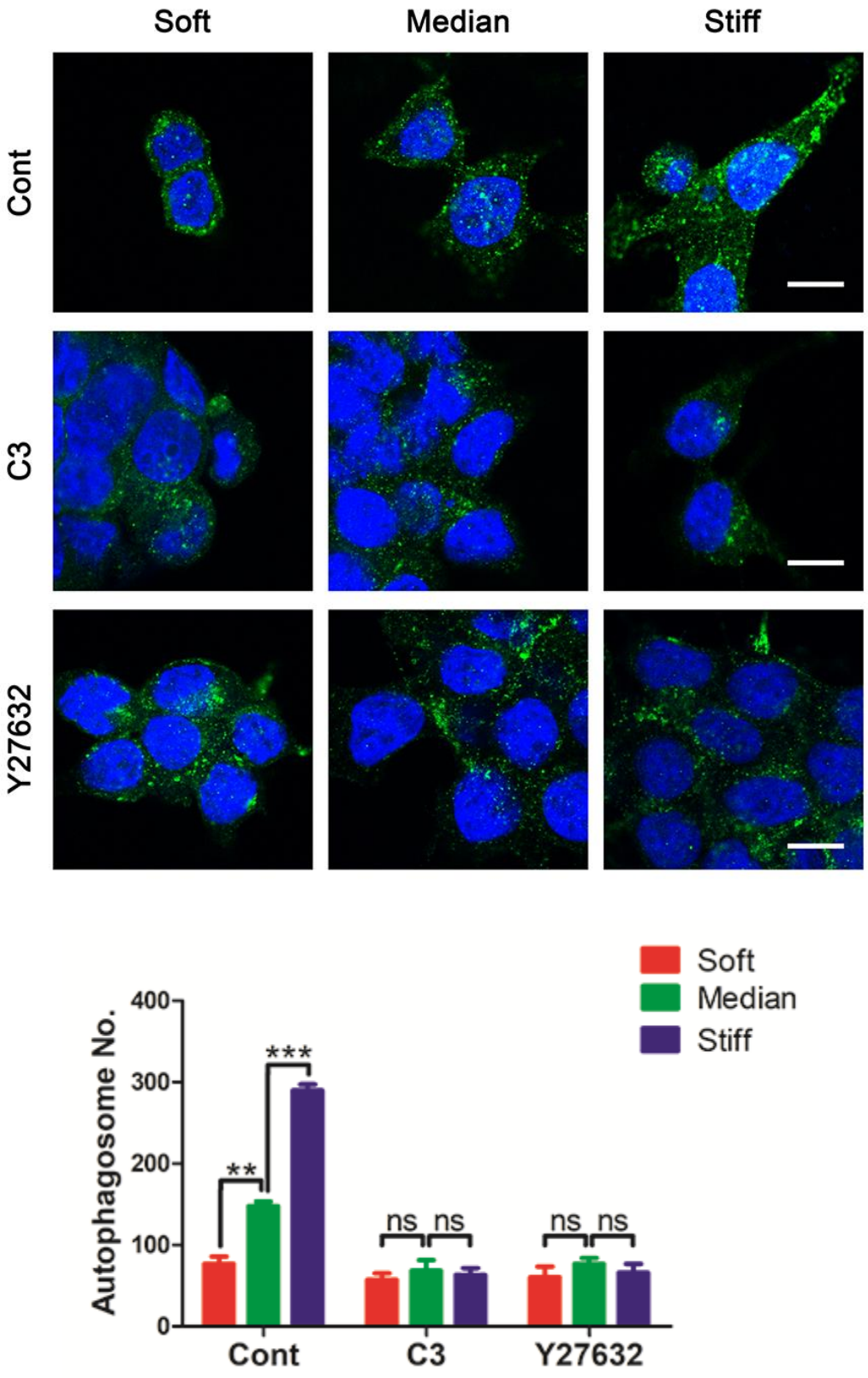


Figure 3-32. Fluorescent image of autophagosome in C3 and Y27632 treated MCF-7 cells. (Scale bar = 10 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$, ns indicates $p > 0.05$).

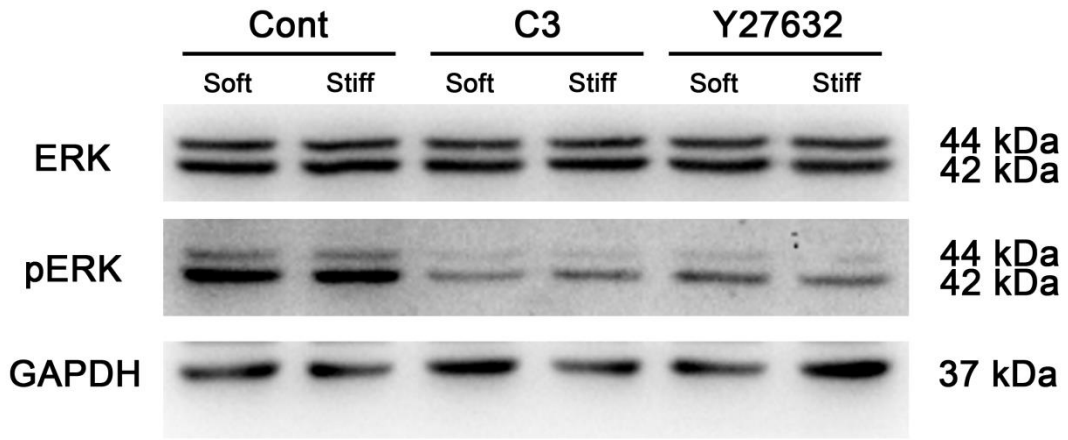


Figure 3-33. The expression of phosphorylated ERK in C3 or Y27632 treated MCF-7 cells on soft and stiff substrates.

4 Fully chemically defined 3D microgels for iPSCs encapsulation and expansion

This part is a cooperation project together with my colleague Wanjun Liang.

4.1 Production of dPG-PEG-PCL microgels and iPS encapsulation.

To build a more physiological 3D hydrogel system similar to in vivo stem cell niche, two polymers of polyethylene glycol - polycaprolactone azide (PEG-PCL-N3, 100 mg/ml, synthesized by Wanjun Liang) and dendritic polyglycerol cyclooctyne (dPG-DIC, 100 mg/ml, synthesized by Wanjun Liang) were employed for cross-linking to form hydrogel. Utilizing microfluidics technology, iPS single cells suspension containing 16% density gradient medium (to present cell aggregation) were mixed together with these two polymers (with the ratio of PEG-PCL-N3 : dPG-DIC = 2 : 1) at room temperature and went through a microchannel inside a microfluidics chip to form microgels with the size of ~200 μm in diameter. The microgels containing iPS cells were collected and then transferred to a six-well cell culture plate.

4.2 dPG-PEG-PCL microgels support iPS survival and expansion.

To assess whether the hydrogel can support pluripotent stem cells survival and expansion, iPS cell encapsulated microgels were cultured in complete plus clonal grade medium for up to 14 days. By Live&Dead staining, we found that iPS cells survived very well and had great viability (Figure 4-1) in the microgels. And the encapsulated iPS cells grew into round regular cell spheroids, which also be known as embryoid bodies (EB).

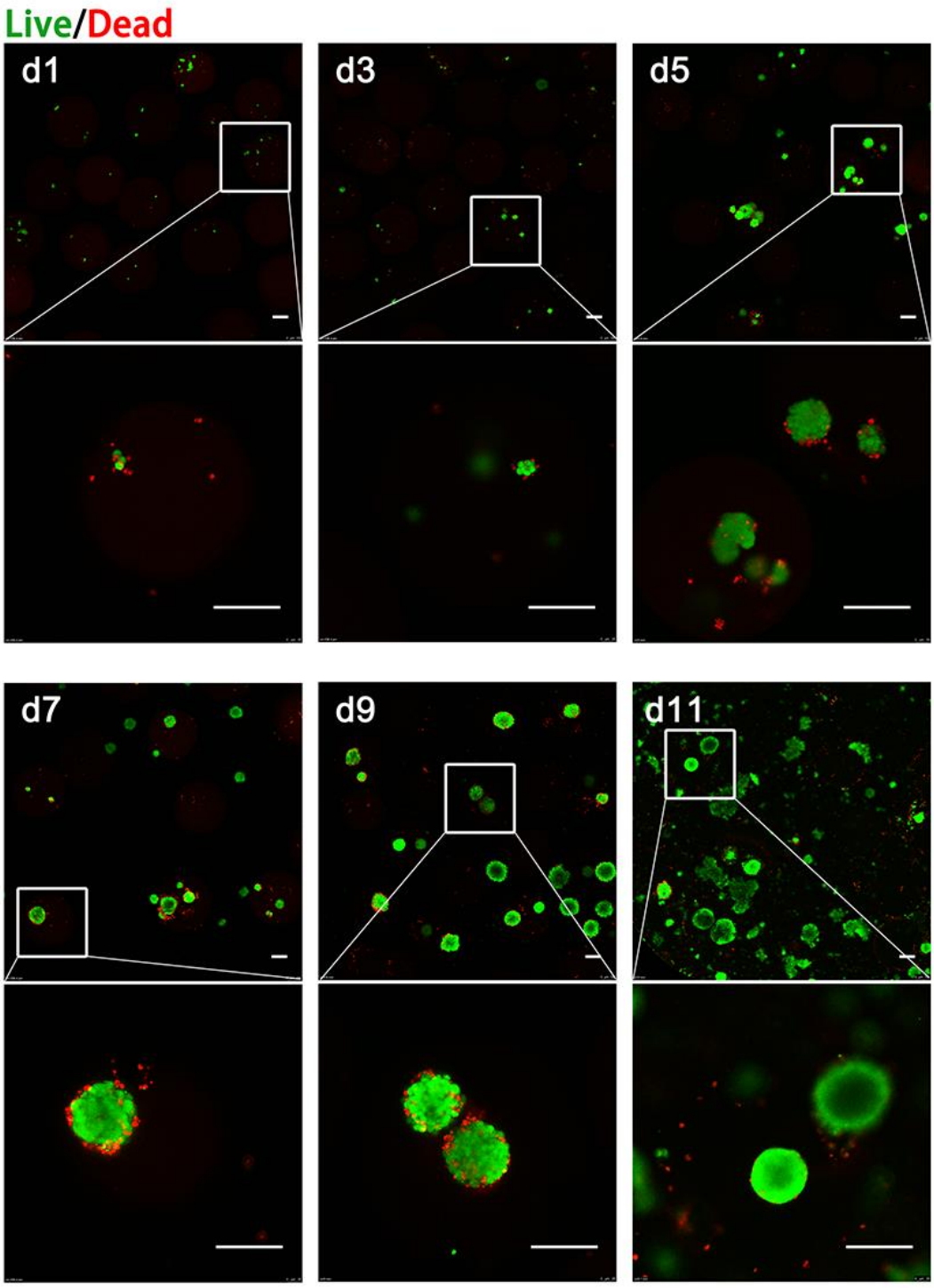


Figure 4-1. Live & Dead staining showed high cell viability during the whole culture period. (Scale bar = 100 μm)

As a three-dimensional multicellular spheroid usually formed when the pluripotent stem cells are cultured in suspension, in which consists of ectodermal, mesodermal and endodermal tissues, embryoid body has been regarded as a means to assess the pluripotency of pluripotent stem cells and a routine approach to induce the differentiation of pluripotent stem cells to different cell lineages [140, 141]. To determine whether the cell spheroids were formed by big cell aggregate or grew from single cells or small clusters, we isolated single microgels and traced daily. By monitoring the growth process, we proved the overall period that the cells grew from a single cell or small cluster, at least not big cell aggregate, and became bigger and bigger and finally grew out of the microgel (Figure 4-2) due to the degradability of the hydrogel.

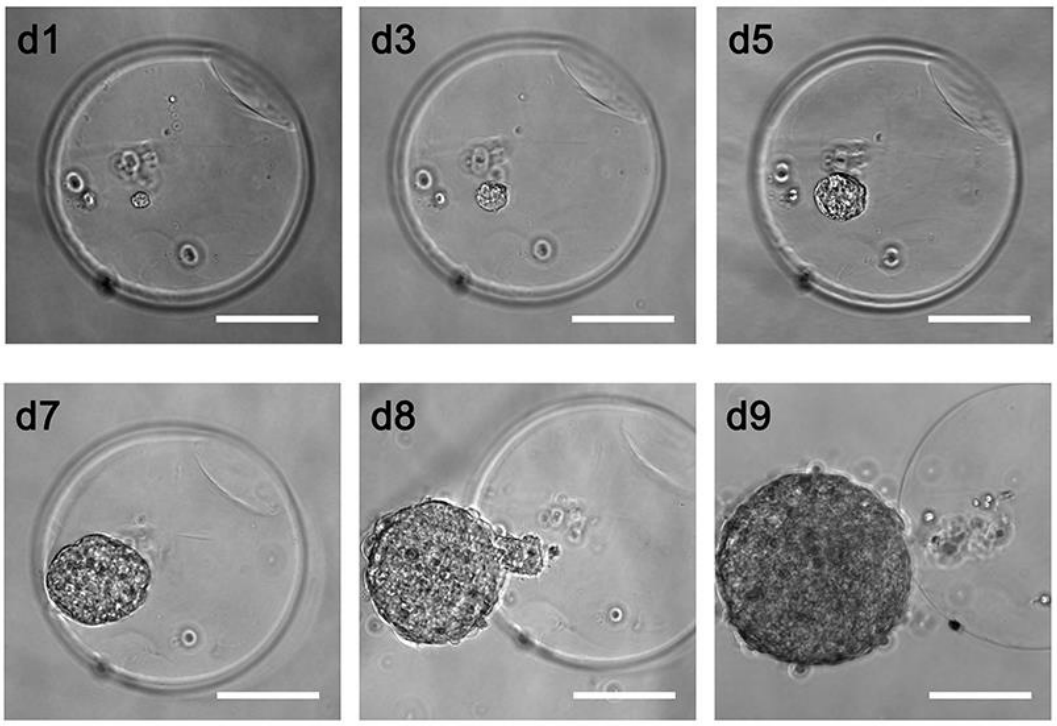


Figure 4-2. Single microgel tracing to monitor the growth of cell spheroid in the hydrogel during the culture period. (Scale bar = 100 μm)

It is well known that, among dozens of factors that affect the cell behavior in the 3D environment, the cell spheroid size is regarded as an important parameter to influence the proliferating abilities and lineage-specific differentiation of pluripotent stem cells [142, 143]. Too large spheroid will lead to not only impaired proliferation, increased apoptosis due to insufficiently transport of nutrients and growth factors, oxygen exchange, metabolic waste elimination [144, 145], but also unexpected differentiation result from the ratio change of the three germ layers cells and spatial signaling alteration from cell-cell interaction or cell-environment interaction [146]. The cell spheroid numbers formed in each microgel varies from 1 to 8 and decreased along with the culture time (Figure 4-3), which further proved the cell spheroids could grow out of the microgel when they were too big. We also measured the diameter of the cell spheroids (Figure 4-4) and found that cell spheroids formed in the microgels were smaller than that of in suspension culture (Figure 4-5).

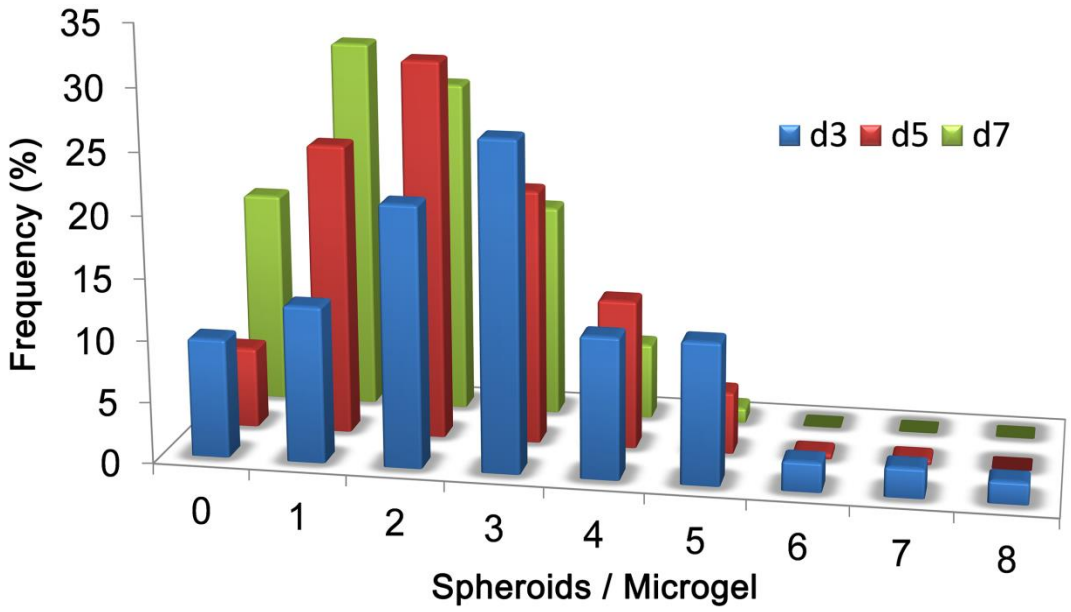


Figure 4-3. The frequency of the spheroid numbers formed in a single microgel at different culture times.

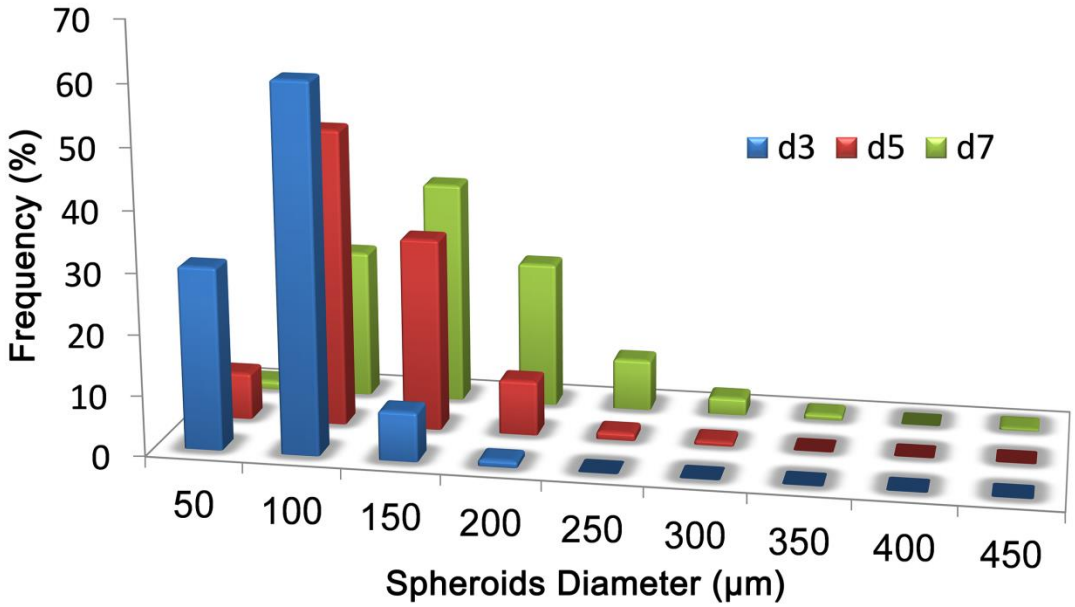


Figure 4-4. The size distribution of cell spheroids in 3D microgels at different culture times.

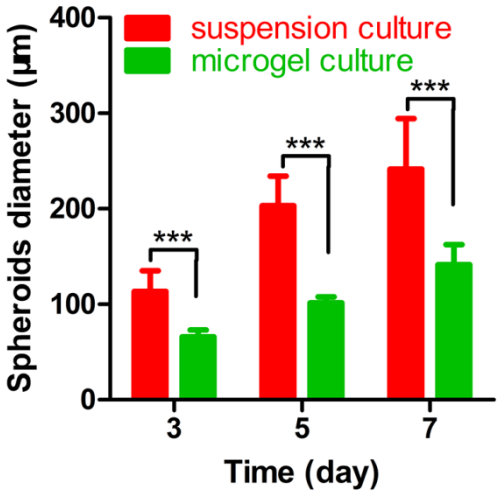


Figure 4-5. The differences in spheroid size between microgel culture and suspension culture. (*** indicates $p < 0.001$)

Beyond that, the results also indicated that iPS cells in microgels kept high proliferation ability (Figure 4-6) during the culture time and showed a better expansion rate than that of suspension culture (Figure 4-7). Although static suspension culture system is the most widely used way to generate iPS spheroid, the disadvantages of traditional suspension culture include uncontrolled spheroid size and shape, agglomeration of spheroids into large irregular masses and limited cell expansion rates which were proved by our results have been complained by many researchers [147].

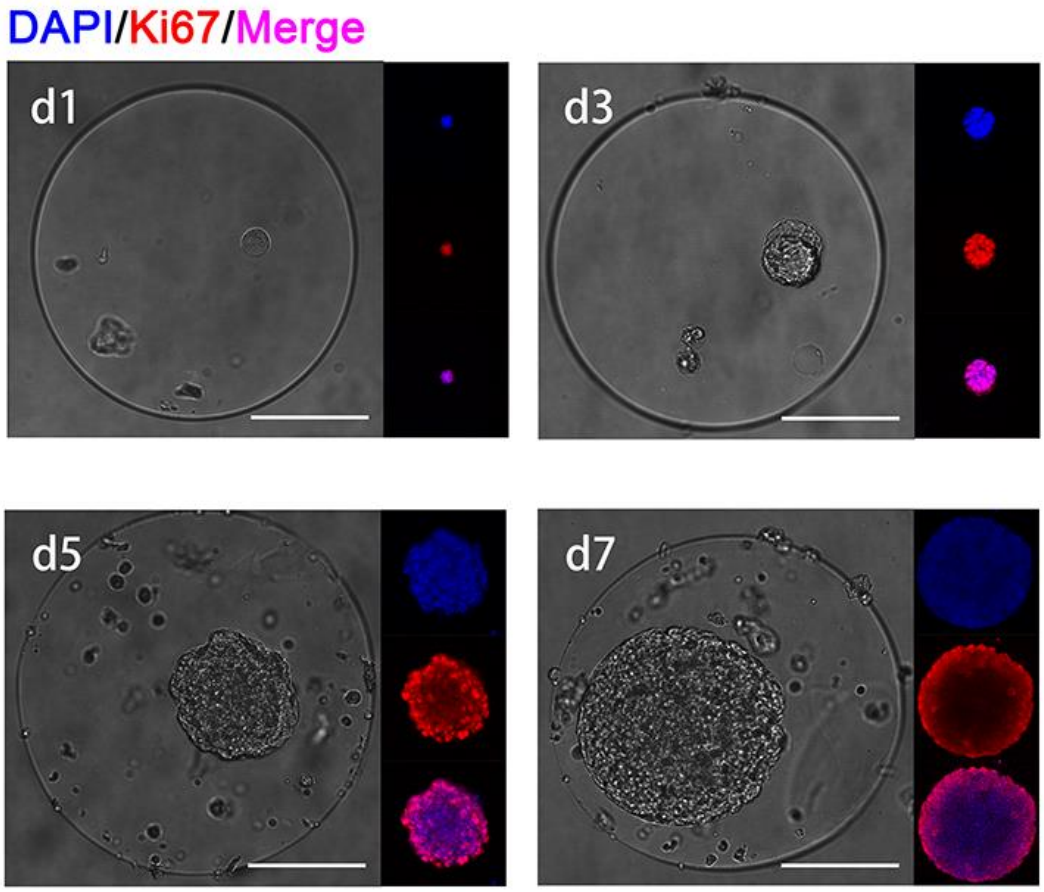


Figure 4-6. dPG-PEG-PCL microgels maintained high expression of proliferation marker, Ki-67, at different culture times. (Scale bar = 100 μm)

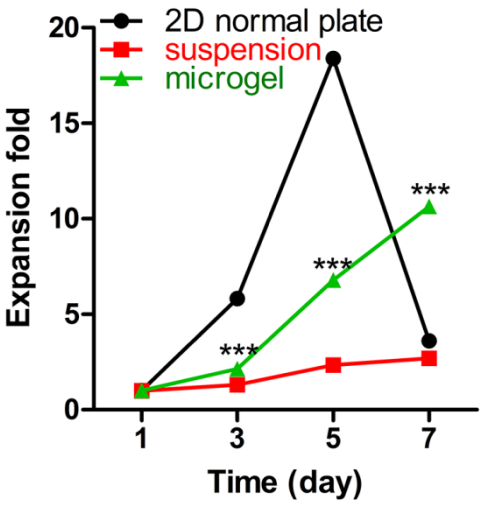


Figure 4-7. iPSCs show a better expansion rate in microgels than that of suspension culture and traditional 2D culture. (*** indicates $p < 0.001$)

4.3 **Optimal cell concentration for encapsulated iPSC growth in dPG-PEG-PCL microgels.**

Many factors can affect whether you can acquire enough high-quality spheroids among which, the cell density must be the most important one. Previous study has proved that too low cell seeding density wasn't enough to form spheroids [148] while too high seeding density lead to spheroids agglomeration, which means separate spheroids merged and formed a larger spheroid, therefore the spheroid formation efficiency was decreased and the spheroid quality was reduced [149]. To optimize the suitable cell concentration for the encapsulated iPS culture in dPG-PEG-PCL microgels. IPS cells with three different concentration of 2×106, 1×106, 5×105 cells /ml were involved in the microgel fabrication process. The cellular growths were observed continuously during the culture time and the results showed that the more cells encapsulated in the microgels, the faster iPS cells grew and the more cell

spheroids formed (Figure 4-8).

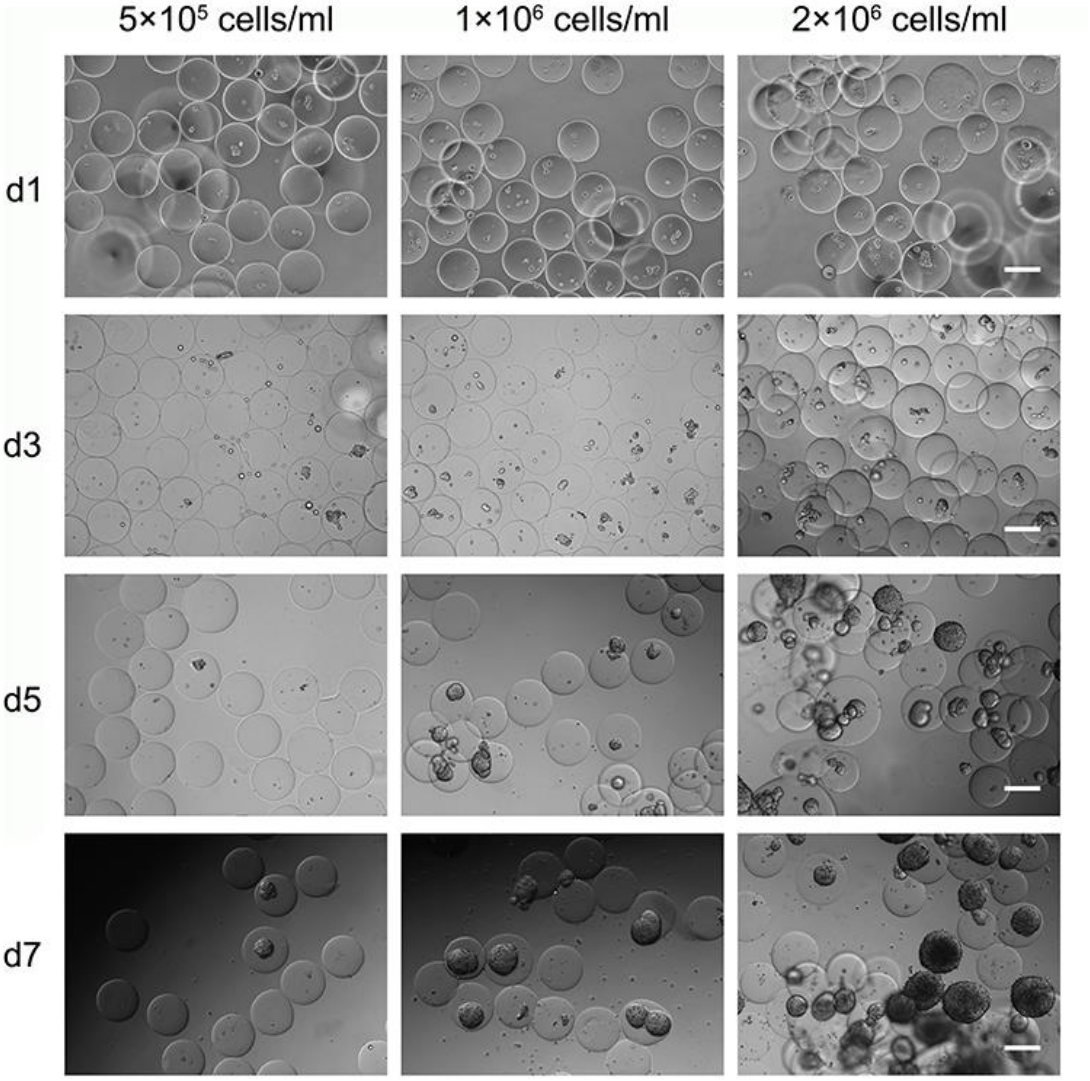


Figure 4-8. Phase-contrast images showed the morphologies of microgels containing spheroids inside with different cell seeding density (2×10^6, 1×10^6, 5×10^5 cells /ml, respectively) at different culture times. (Scale bar = 200 μm)

However, too high concentration may lead to the aggregation and fusion of iPS spheroids and the generation of super big spheroids which would be extruded out of the microgels after 5 days' culture. And this also very well explained the decrease expansion rate and smaller spheroid diameter at d7 under a concentration of 2×106 cells / mL than that of 1×106 cells / mL (Figure. 4-9, 4-10).

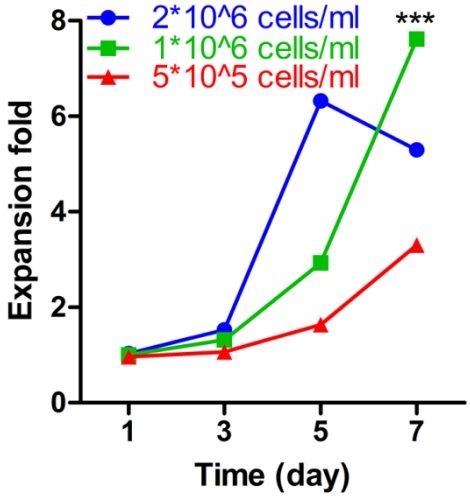


Figure 4-9. iPSC achieved a better expansion rate with a cell seeding density of 1×10^6 cells /ml than the other cell density. (*** indicates $p < 0.001$)

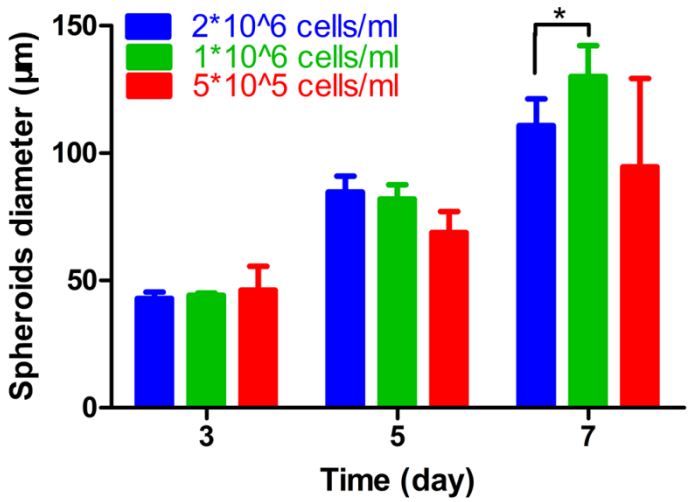


Figure 4-10. The size of cell spheroid in 3D microgels was influenced by the seeding density. (* indicates $p < 0.05$)

Notably, too low cell concentration led to very little iPS spheroid (Figure 4-11) which indicates that certain high cell concentration is needed to form embryoid bodies during the microgel fabrication by microfluidics technology. Conclusion that cell concentration of 1×106 cells / mL was the best density for iPS cells to survive in the microgels, to grow in a great expansion rate, and to form more and enough high-quality spheroids while avoiding the spheroids agglomeration.

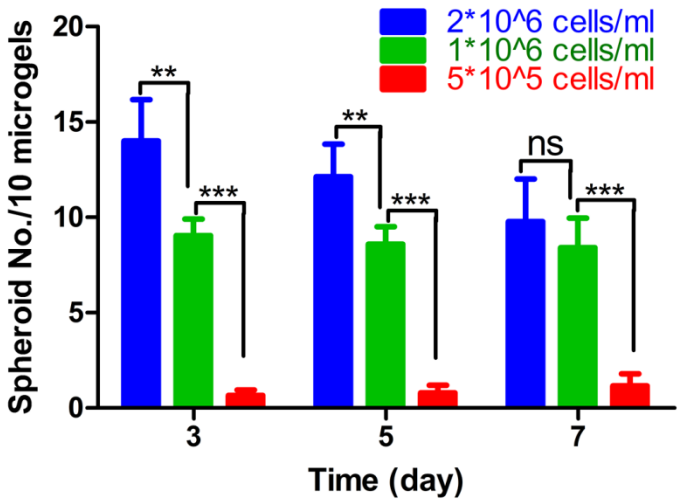


Figure 4-11. More cell spheroids were formed in microgels with higher cell seeding density. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, ns indicates $p > 0.05$)

4.4 **Mechanical property of microgels affects iPS expansion and embryoid body formation in the 3D environment.**

The phenomenon of iPS spheroids grew out from the microgels after culturing for several days remind us that maybe the hydrogels were too soft to restrain the spheroids inside the microgels. So next we prepared two stiffer hydrogels by increasing the concentration of these two polymers to determinate the better hydrogel

with proper elasticity suitable for iPS survival and expansion. Three kinds of hydrogels with different elasticity of 0.69 kPa, 1.24 kPa, and 12.6 kPa were prepared to encapsulate the iPS cells for 3D culture. Apparently, soft environments were more welcome for the iPS survival, and softer the hydrogels were, much better the iPS cells survived (Figure 4-12).

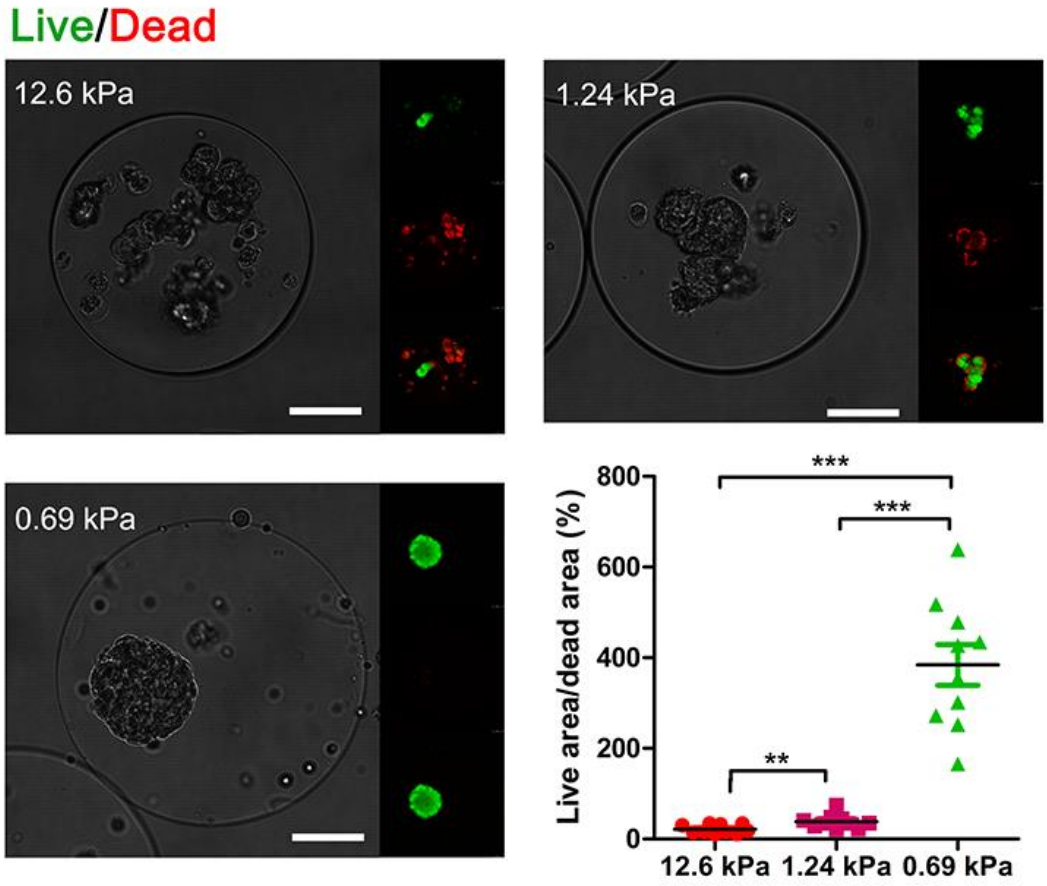


Figure 4-12. Live&Dead staining showed better survival of iPSC in soft microgels than stiff ones. (Scale bar = 50 μm) (*** indicates $p < 0.001$, ** indicates $p < 0.005$)

In addition, much more and better quality spheroids were formed in the soft hydrogels (Figure 4-13) which indicate that it was much more difficult for iPS cells to gather together with each other to form clusters and then grow into spheroids in stiff

environment even enough cells were encapsulated in the microgels (Figure 4-13, 4-14). We can also imagine that the iPS cell almost didn't expand in the stiff microgels (Figure 4-15) because of the lack of proliferation ability (Figure 4-16).

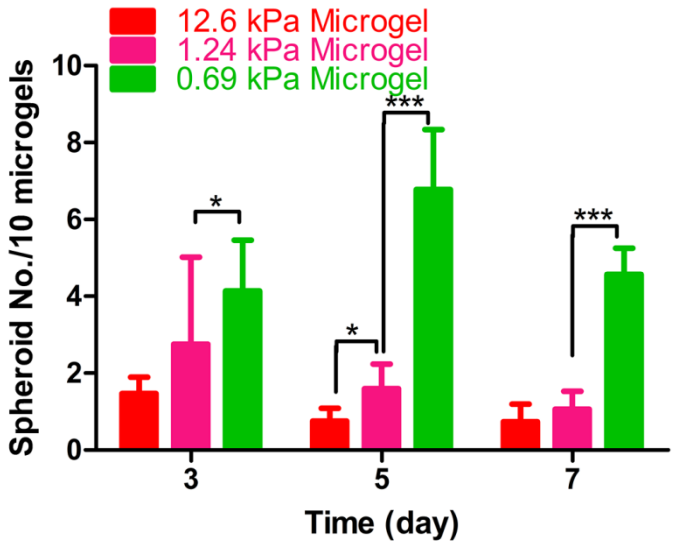


Figure 4-13. Diameter of EB formed in different dPG-PEG-PCL microgels with different elasticity. (*** indicates $p < 0.001$)

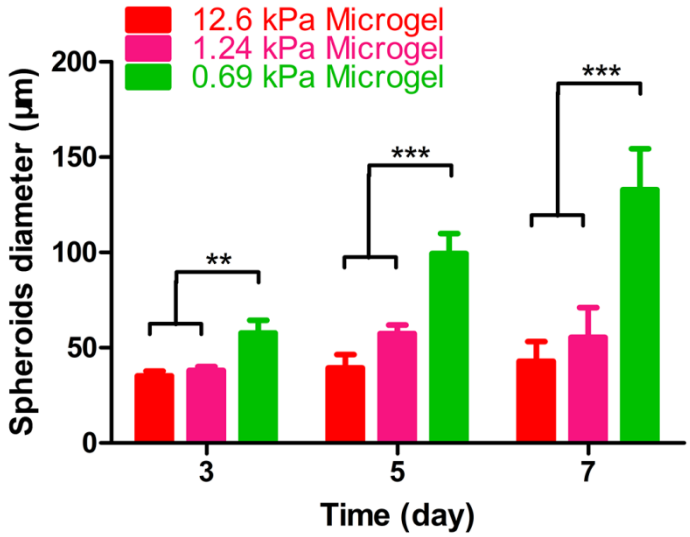


Figure 4-14. Diameter of EB formed in different dPG-PEG-PCL microgels with different elasticity. (*** indicates $p < 0.001$)

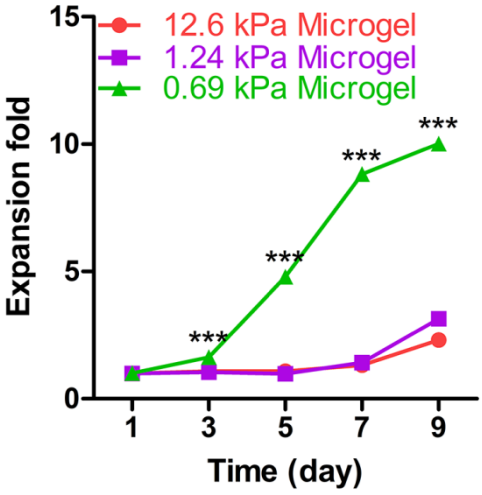


Figure 4-15. Proliferation of iPSCs in different dPG-PEG-PCL microgels with different elasticity. (*** indicates $p < 0.001$)

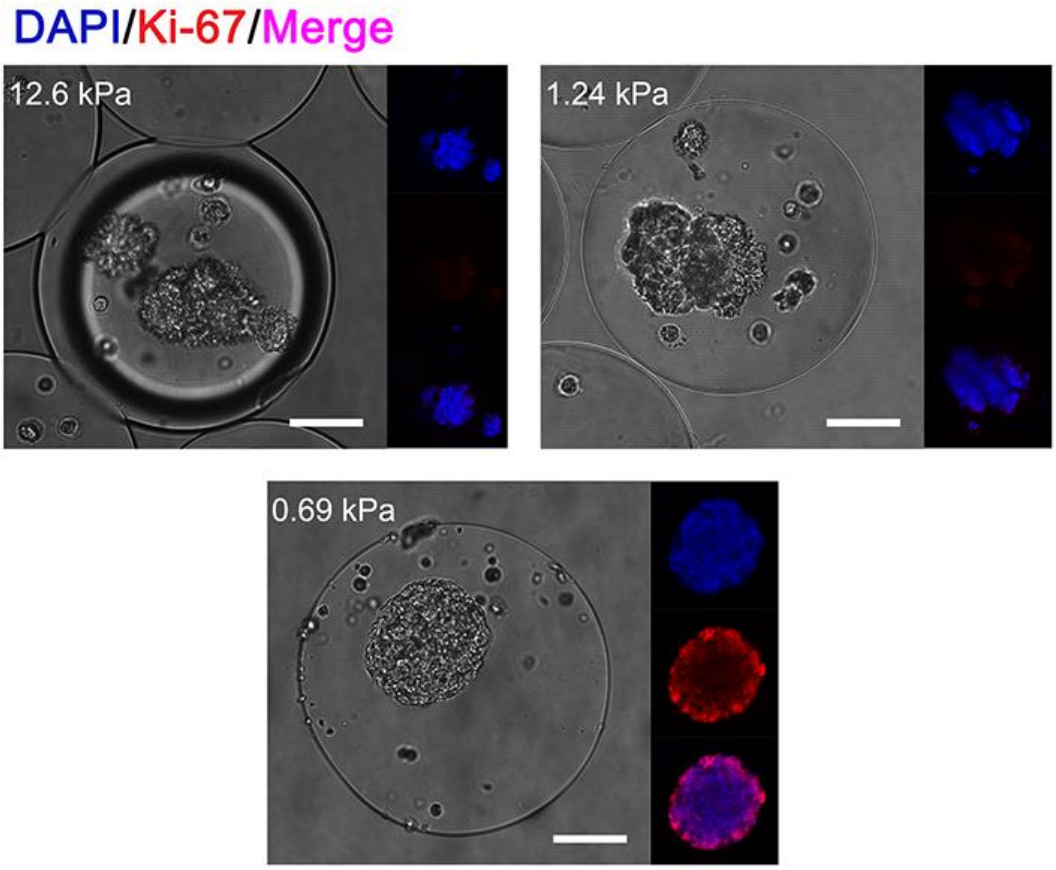


Figure 4-16. Expression of proliferation marker, Ki-67, in dPG-PEG-PCL microgels with different elasticity at different culture times. (Scale bar = 100 μm)

4.5 **dPG-PEG-PCL microgels maintain the pluripotency of iPS in the 3D environment.**

To achieve the requirements of clinical application, undifferentiated iPS cells must be acquired in vitro. To assess whether the pluripotency can still be maintained in iPS cells within the dPG-PEG-PCL hydrogel environment, a series of pluripotency-related tests were performed. First, we detected the expression of alkaline phosphatase (ALP), which is considered to be a marker of high pluripotency in both human and mouse pluripotent stem cells [150]. The result showed that continuously high expressions of alkaline phosphatase were detected until d5, but with a little decrease on d7 (Figure 4-17), indicated that undifferentiated state of iPS cells were maintained in the microgels.

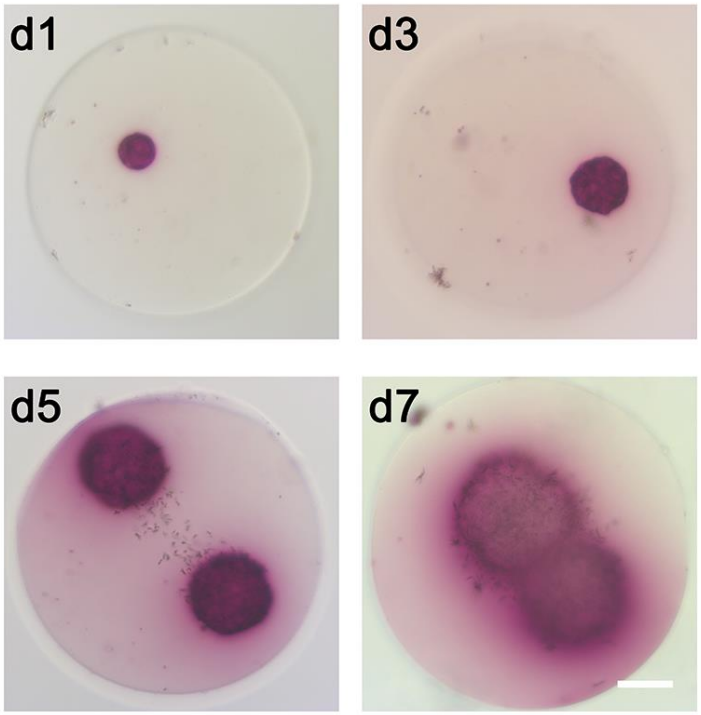


Figure 4-17. Alkaline phosphatase staining of cell spheroids inside the microgels. (Scale bar = 50 μm)

In addition, we also measured the expression of other pluripotent markers by immunofluorescent staining method. And the results showed that the dPG-PEG-PCL hydrogel-produced iPS cell spheroids presented high levels of Nanog, Sox2, Oct4 and SSEA1 (Figure 4-18, 4-19).

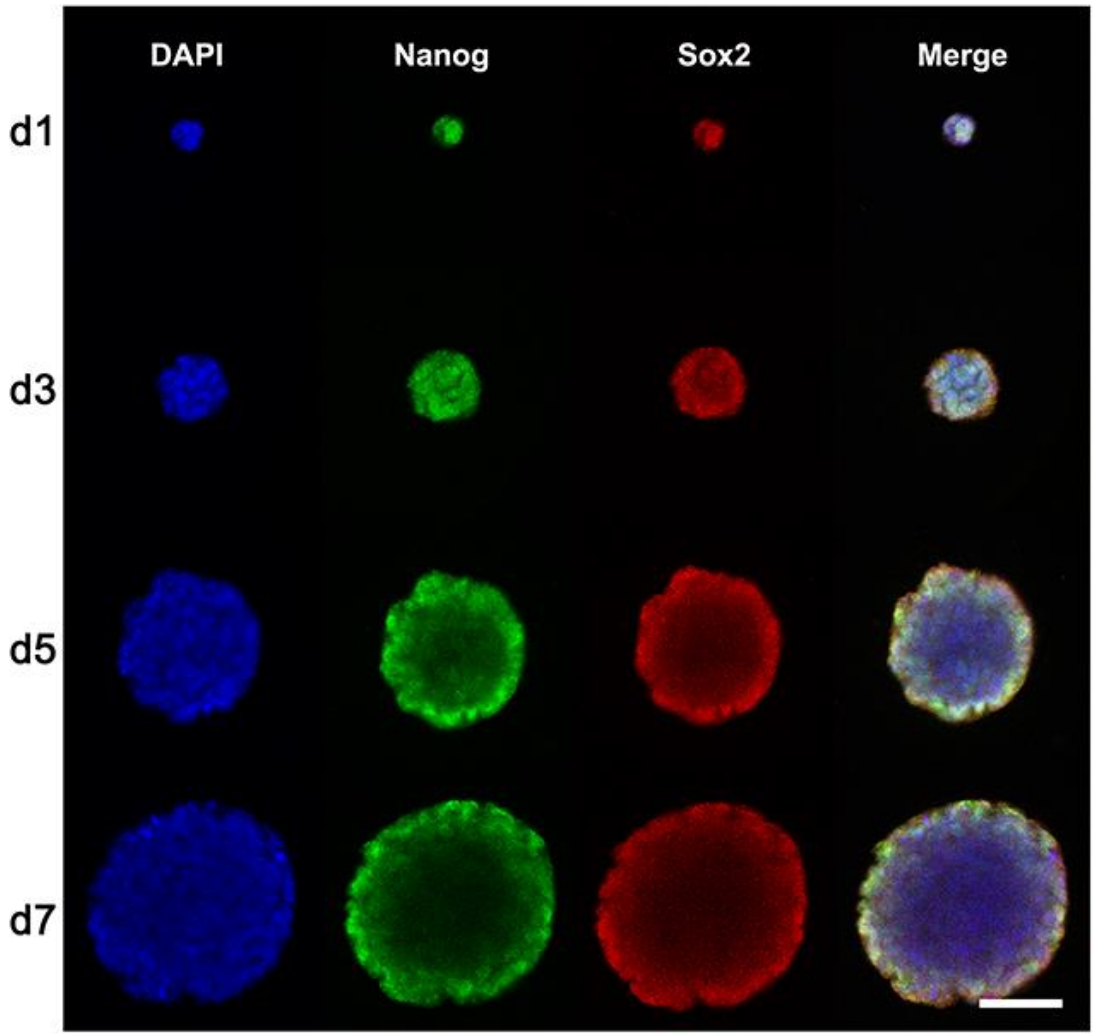


Figure 4-18. Immunofluorescent staining of Nanog and Sox2 in microgels at different timepoints. (Scale bar = 100 μm)

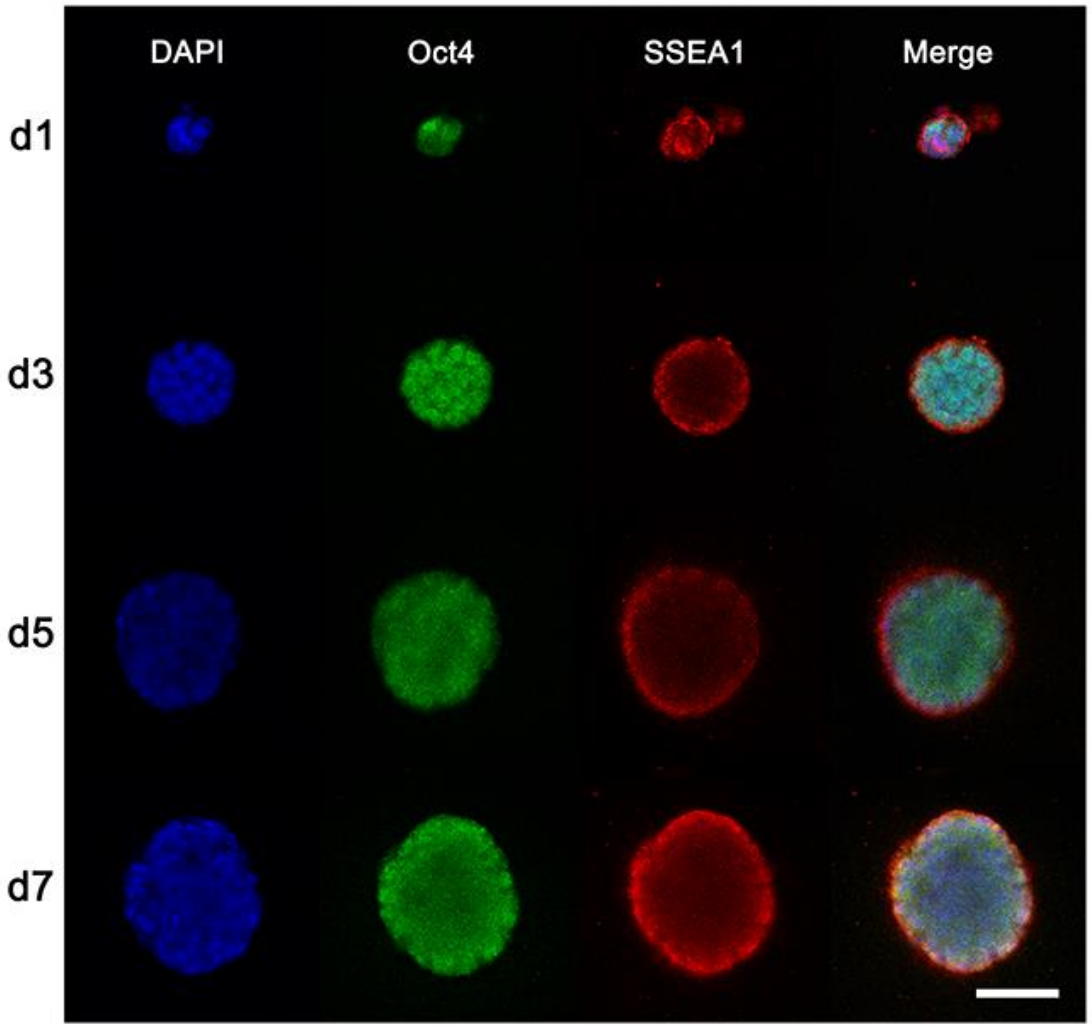


Figure 4-19. Immunofluorescent staining of Oct4 and SSEA1 in microgels at different timepoints. (Scale bar = 100 μm)

Beyond that, we compared the difference of pluripotency between iPS cells in suspension culture and of which in dPG-PEG-PCL hydrogel. The results showed that the expressions of Nanog in suspension and in hydrogel were similar and remained stable during the culture period (Figure 4-20). However, the expressions of Oct4 both in suspension and hydrogel were decreased along with culture time, but the decreasing trend in dPG-PEG-PCL hydrogel was slower than that of in suspension

culture (Figure 4-20). On the other hand, the expressions of Sox2 had a little unexpected increase both in suspension and in the hydrogel, but there was no difference between suspension and hydrogel culture (Figure 4-20). The core dynamics pluripotency network formed by Nanog, Oct4, and Sox2 were first described in 2006 [151, 152] and proved by many researchers [153, 154]. Although, temporal and spatial heterogeneity of expression, Nanog is still widely regarded as a gatekeeper to control pluripotent stem cell fate in response to signals from internal gene regulation network and external microenvironment [155, 156]. And the stable expression of Nanog in the above indicated that the dPG-PEG-PCL hydrogel could maintain the pluripotency of iPS cells in the 3D environment during the culture time.

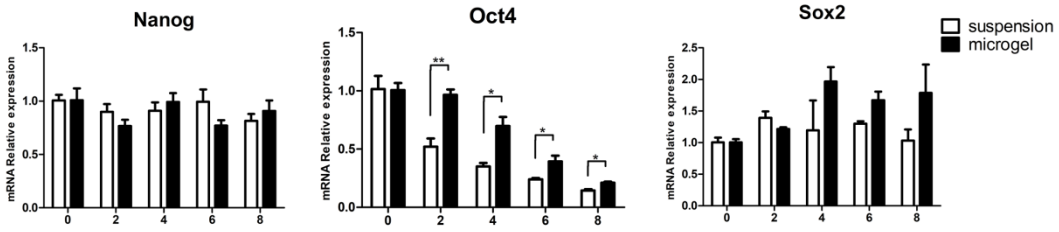


Figure 4-20. qPCR analysis of pluripotent markers in iPSCs cultured in microgels and suspension culture. (*** indicates $p < 0.001$, ** indicates $p < 0.005$, * indicates $p < 0.05$)

5 Methods and materials

Fabrication of polyacrylamide hydrogel substrates

Polyacrylamide hydrogel substrates were fabricated according to the Current Protocols in Cell Biology [103] with some modification. The first step is to prepare amino-silanated coverslips and chloro-silanated glass dishes or slides.

To prepare amino-silanated coverslips, new coverslips with proper size were treated with plasma first, then 0.1 M NaOH solution were spread on the entire surface of the plasma-treated side. Then the coverslips were heated to ~80 degrees until the solution was evaporated to dry. Repeat the evaporation process once by applying Milli-Q water to form a uniform semi-transparent NaOH film. Next, a proper amount of 3-Aminopropyltriethoxysilane (APES) was spread on the NaOH film to react for 5 min inside the fume hood. After that, the coverslips were washed with Milli-Q water for 2~3 times to ensure no unreacted APES remained on the both side of coverslips. Then the coverslips were incubated in a 0.5% glutaraldehyde solution in PBS for half an hour at room temperature to react. In the end, the glutaraldehyde solution was removed and the coverslips were wiped with bibulous tissue gently and transferred to new dishes to dry naturally. The new prepared amino-silanated coverslips were viable for 2 days but recommended to be used immediately.

To prepare chloro-silanated glass dishes or slides, a proper amount of dichlorodimethylsilane (DCDMS) was spread on the entire surface of new glass dishes or slides to react for 3~5 min inside the fume hood. After that, the dishes and slides were wiped with bibulous tissue gently and washed with Milli-Q water to remove excess DCDMS. Then the chloro-silanated glass dishes or slides were ready to use.

When amino-silanated coverslips and chloro-silanated glass dishes or slides were well prepared, the second step is to polymerize polyacrylamide hydrogel on the coverslips. The acrylamide and bis-acrylamide solutions with desired concentrations were mixed together uniformly and then degassed for 30 min by vacuuming to

remove oxygen that dissolved in the mixture solution. Then an appropriate amount of 10% APS and TEMED solution was added to the mixture for hydrogel polymerizing. After that, proper volume (5 μl – 150 μl according to the size of coverslip) of the mixture was immediately dropped on the treated side of chloro-silanated glass dish or slide and then the amino-silanated coverslip was quickly covered on the droplet with treated side down. Around half an hour later, the top coverslip with covalently bonded hydrogel was uncovered and transferred to a new Petri dish for rinsing with the hydrogel-coated side up. After rinsing for 2~3 times with Milli-Q water, the hydrogel substrates were ready to use or store at 4 degrees for months.

Before seeding cells on the hydrogel substrates, the final step is to coat fibronectin on the hydrogel surface. After being transferred to a new culture plate, the hydrogels substrates were covered by 0.2 mg/ml sulfo-SANPAH solution and exposed in the 365 nm UV light for 10 min. Then the hydrogel substrates were rinsed with 50mM HEPES for 2~3 times to remove excess sulfo-SANPAH solution. Next, a proper amount of 10 ug/ml fibronectin in 50mM HEPES was spread on the surface of the hydrogel. The plate was incubated at 4-degree fridge overnight and then transferred to 37-degree cell incubator for 1 hour on the next day. After sterilization under UV light for 30 min, the fibronectin-coated PA hydrogel substrates were ready to use.

Cell culture

Human breast cancer cell MCF-7, MDA-MB-231, T47-D were respectively obtained from ATCC, Sigma, ATCC and cultured in complete DMEM, DMEM/F12, RPMI 1640 medium (Thermofisher, USA) supplemented with 10% FBS (Merck Millipore, Germany), 1% P/S, 1% glutamine. The cells were split every 3-5 days with 0.05% trypsin-EDTA (Thermofisher, USA) when 90% cell confluency achieved. For desired research, cells were harvested and reconstructed into single cell suspension and then reseed on sterilized PA hydrogel substrate.

Mouse iPS cell line PhiC31 was obtained from System Biosciences (Catalog# SC211A-1) and maintained on laminin (Cultrex, #3400-010-01) coated plate with complete clonal grade medium (Merck Millipore, #SF001-500P) containing GSK3β

inhibitor. The medium was changed daily and the cells were split every 3-4 days with accutase (Merck Millipore, #SCR005).

For traditional suspension culture, ips cells were trypsinized with accutase for around 3 mins into single cells and then reseeded on uncoated plate with defined concentration. The medium was changed daily by gently centrifuging the cell spheroids into the bottom of the tubes and carefully aspirating the supernatant until ready to use.

For 3D hydrogel culture, ips cells were trypsinized with accutase into single cells and then mixing with density gradient medium (Sigma, #D1556) to prevent the cell aggregation formation. Then the PEG-PCL-N3 polymer solution, dPG-DIC polymer solution, and iPS cell suspension mixed together and went through a microchannel inside a microfluidics chip to generate microgels with size of ~200 μm in diameter. The microgels containing iPS cells were harvested and filtered through 100 um cell strainer (Corning, #352326) to remove unencapsulated cells. And then the cell contained microgels were reseeded on a normal plate. The medium was changed daily by carefully inclining the plate and aspirating the supernatant without disrupting the floating microgels. The cell contained microgels were imaged by a normal or fluorescent microscope at the specified time point and the cell spheroids' number and diameter were counted and measured by Image J software.

Cancer stem cell enrichment and tumorsphere formation

To enrich cancer stem cells, normal cancer cells were harvested and resuspended into single cells with mammosphere-forming medium (DMEM/F12 medium supplement with 1/50 B27, 4 ug/ml heparin, 20 ng/ml EGF, 20 ng/ml bFGF) and then seeded in Corning Ultra-Low attachment cell culture flasks or plates (Sigma-Aldrich, Germany) at a density of 1000-5000 cells/ml. Allow the cells cultured in 37℃ CO2 incubator for 7-10 days to form mammospheres. To passage, mammospheres were collected and disassociated with 0.05% trypsin-EDTA at 37℃ for 5-10 minutes to obtain single cell suspension which was reseeded in Ultra-Low attachment flasks in the same way. For

the desired experiment, mammospheres were harvested and trypsinized into single cells to reseed on PA hydrogel substrates.

Cell viability assay

The viability of the iPS cells cultured in microgel was measured by the Live/Dead Viability Kit (Thermo, #L3224). The microgels were collected and washed with PBS, and then freshly prepared staining solution was added. After incubation for 30 mins at room temperature, microgels were imaged by confocal microscopy (Leica SP8). Live cells were marked by green-fluorescent calcein-AM while red-fluorescent ethidium homodimer-1 indicating dead cells.

Proliferation assay

The expansion rate of the iPS cells growing in the microgel was measured by Cell Counting Kit - 8 (Sigma, #96992). After harvest, the same amount of microgels were seeded in 24-well plates and cultured for several days. At the indicated time point, one-tenth volume of CCK-8 reagent of the cell culture medium was added to each well of the plate and then incubated the plate in the incubator for 1-4 hours. After incubation, the supernatants were transferred to 96-well plate and the absorbance was measured at 450 nm by a microplate reader.

Immunofluorescent staining

The cell contained microgels were collected and washed with PBS at the specified time point. Then the microgels were fixed with 4% PFA for 15-30 mins followed by permeabilization for 10-20 mins (bigger cell spheroids need longer time). After washing with PBS for 3 times, the microgels were blocked with 10% goat serum at 37 ℃ for 30 mins to cover nonspecific sites. Then microgels were incubated with defined primary antibodies (anti-Nanog, 1:400, Abcam, #ab80892; anti-Oct4, 1:400, Abcam, #ab19857; anti-SOX2, 1:1000, Abcam, #ab97959; anti-SSEA1, 1:200, ThermoFisher, #MA1-022; anti-Sox2, 1:400, CST, #4900; anti-Ki-67, 1:400, CST, #9129) at 4℃ overnight. The second day microgels were washed by PBS for 3 times

and then incubated with second antibodies (Goat anti-Rabbit IgG H&L (Alexa Fluor 488, 1:400, Abcam, #ab150077; Goat anti-Mouse IgG H&L (Cy5), 1:1000, Abcam, #ab6563) at 37℃ for 1 hour. The microgels were washed by PBS again and then stained with DAPI at room temperature for 10-20 mins. At last, microgels were imaged by confocal microscopy.

ALP staining

ALP staining was performed to confirm the pluripotency of iPS cells using the Alkaline Phosphatase Detection Kit (Merck Millipore, #SCR004) following the instruction. Briefly, the microgels were collected and washed by PBS, and then fixed by 4% PFA for very short time followed by PBS washing. Next, enough stain solution was added to the tube and then incubated protected from light at room temperature for 15 mins. After 3 times' washing by PBS, the images were observed and acquired by color microscope

ALDH1 Assay and Flow cytometry

Cells were harvested after culturing on PA hydrogel substrates for several days to determinate ALDH1 activity with the ALDEFLUOR Kit (STEMCELL Technologies, USA) according to the instruction manual. Briefly, a fluorescent non-toxic ALDH1 substrate, BODIPY-aminoacetaldehyde, was added into the single cell suspension and then incubated for 45 mins. Diethylaminobenzaldehyde (DEAB), a specific ALDH1 inhibitor, was added to the control tube as the negative control. After incubation, cells were measured by BD Accuri C6 Flow Cytometry (BD, USA) to analyze ALDH1-bright (ALDH1+) cells.

Western blot

Western blot was performed according to the previous protocols [157], briefly, cell total protein was extracted from cells that were cultured on PA substrates for desired days with or without treatment by employed RIPA (Thermofisher, USA) buffer to the

cell pellets. The concentrations were measured by the Pierce BCA Protein Assay Kit (Thermofisher, USA). An equal amount of protein was loaded in 8%-12% SDS-PAGE gel for electrophoresis and then transferred to new PVDF membrane with desired size. After incubation in 5% BSA for 3 hours to block unspecific sites, the membrane was incubated with specific primary antibodies (LC3B, YAP, GAPDH) at 4℃ overnight. The next day, after rinsing three times by washing buffer, the membrane was then incubated with HRP-conjugated 2^{st} antibodies at room temperature for 1 hour followed by rinsing three times. Last, the membrane was reacted with SuperSignal Chemiluminescent Substrate (Thermofisher, #34080) and imaged by ChemiDoc MP imaging System (Bio-Red, USA).

Real-time PCR analysis

After culturing on PA substrates for several days, cell total RNA was extracted by Trizol reagent (Thermofisher, USA) and then reverse transcripted into cDNA by QuantiTect Reverse Transcription Kit. Real-time PCR reaction was performed by PowerUp SYBR Green Master Mix (Thermofisher, USA) in PikoReal Real-Time PCR device with respective primers (hGAPDH-F: GCAAGAGCACAAGAGGAAGAG, hGAPDH-R: AAGGGGTCTACATGGCAACT, hCTGF-F: AGGAGTGGGTGTGTGACGA, hCTGF-R: CCAGGCAGTTGGCTCTAATC, hANKRD1-F: AGTAGAGGAACTGGTCACTGG, hANKRD1-R: TGGGCTAGAAGTGTCTTCAGAT, Thermofisher, USA). The data were acquired by PikoReal Software 2.2 and analyzed by the Comparative Ct method (ΔΔCt). The value of 2^-ΔΔCt were analyzed with the GraphPad Prism 5 software by the unpaired Student's t-test method.

Summary and Outlook

Generally, in this thesis, we studied the effect of diverse mechanical environments including different extracellular matrix stiffness and varied hydrogel elasticity on the behaviors and functions of cancer cells and stem cells. Specifically, two topics are included in this thesis. In the first topic, the impacts of 2D substrate stiffness on the cancer stem cell (CSC) maintenance, chemotherapeutic response and autophagy activation in breast cancer cells was investigated. While in the second topic, we focused on the impact of the 3D environment and hydrogel elasticity on the proliferation and self-renewal ability of mouse iPS cells. Taken together, no matter culturing on 2D substrates or encapsulating in 3D hydrogels, the mechanical environment provided by extracellular matrix indeed makes a great influence on the phenotypes and gene expressions in either cancer cells or stem cells.

In the first project, to study the impact of extracellular matrices stiffness on breast cancer cell functions, we used FN coated polyacrylamide hydrogels as substrates to culture breast cancer cells. By adjusting the ratio and concentration of acrylamide and bis-acrylamide, we fabricated three different elastic substrates with elasticity of ~0.48 kPa, ~4.47 kPa, ~34.88 kPa, which I named “soft” ”median” ”stiff” substrate. First, we showed the diversity of cellular morphology of MCF-7 cells on different substrates, which is consistent with the previous studies. Cells spread widely on stiff substrate while barely spread on soft one, which may be the main cause of increasing proliferating capacity along with the increased stiffness. Next, we found the different chemotherapeutic response of breast cancer cells on different substrates. Breast cancer cells showed enhanced chemosensitivity to doxorubicin and cisplatin but not cyclophosphamide when matrix stiffness increased. To figure out the main reason for matrix stiffness-dependent different chemotherapy response, we detected the stemness characteristics of breast cancer cells on different substrates by analyzing the changes in the cancer stem cell (CSC) population. And we found that the CSC population

became smaller and smaller when matrix stiffness increased. On the other hand, cells cultured on stiff substrate generated less tumorspheres in the CSC enrichment experiments. These data indicated that the soft substrate could maintain the CSC population in breast cancer cells.

Another focus of this project is autophagy. By creating an environment of nutrient deprivation, we found that the level of activated autophagy was highest on stiff substrate and lowest on soft one, in other words, along with the increasing matrix stiffness, autophagy increased. And interestingly, the increased autophagy was suppressed when actin cytoskeleton and stress fiber was disturbed by adding F-actin inhibitor or non-muscle myosin inhibitor. These results demonstrated integrated actin cytoskeleton tension is required for autophagy activation. We also involved YAP in the matrices stiffness-mediated autophagy regulation. The knockdown of YAP greatly reduced the autophagy levels in all group, however, the increasing trend of autophagy along with increasing stiffness remained unexpectedly. This indicated that the regulation of autophagy by matrix stiffness is independent of YAP. To further confirm this discovery, we next detected YAP nuclear translocation on different substrates and found that in normal breast cancer cells, almost all the YAP located in the nucleus, in another word, YAP nuclear translocation was not influenced by stiffness. In contrast, in breast cancer stem cells (BCSC) YAP nuclear translocation was inhibited on the soft environment which indicated that YAP is a crucial factor in the regulation of the BCSC population. This concept was also proved by the result that the knockdown of YAP eliminated the difference of CSC population among different substrates.

Last but not the least, the increasing trend of autophagy induced by increasing stiffness could be eliminated by Rho inhibitor, ROCK inhibitor and ERK inhibitor, indicated that Rho-ROCK-ERK signal pathway could be involved in the regulation autophagy by matrix stiffness. But more evidence is needed to strengthen this viewpoint in the future. In addition, the relevance between chemotherapy sensitivity and autophagy activation on different substrates is also worth exploring, which was

missing from this project.

In the second project, a chemical defined hydrogel based on polyethylene glycol (PEG) and dendritic polyglycerols (PGs) was manufactured to build a more physiological 3D environment. Utilizing microfluidic chip, mouse iPS cells were encapsulated in microgels of 200 μm in diameter. Multicellular spheroids also called embryoid bodies were formed inside the microgels which were floating in the culture medium. Comparing with traditional suspension culture, more but smaller embryoid bodies with high proliferative capacity were generated within the microgels which lead to a better expansion curve. Further detection showed that microgel encapsulated iPS cells had equivalent or better pluripotency compared with that in traditional suspension culture. By increasing the concentration of polymers, more stiff hydrogels with low elasticity were generated but accompanied by rapidly decreased cell viability and proliferative ability as well as the amount of embryoid body. In this project, we wanted to explore the effect of hydrogel elasticity on the proliferative capacity and self-renewal ability of iPS cells. However, the extremely low cell survival rate made this impossible. In the future, more work is needed to generate hydrogels with proper elasticity.

Zusammenfassung

In der vorliegenden Arbeit untersuchten wir die Auswirkungen verschiedener mechanischer Umgebungen wie unterschiedlicher Steifigkeiten der extrazellulären Matrix und unterschiedlicher Hydrogelelastizität, auf das Verhalten und die Funktionen von Krebszellen und Stammzellen. Dabei wurden in dieser Arbeit zwei Themen behandelt: Im ersten Projekt wurde der Einfluss der 2D Substrat-Steifigkeit auf die Erhaltung von Krebs-Stammzellen (CSC) untersucht, sowie das Ansprechen auf Chemotherapeutika und die Aktivierung der Autophagie in Brustkrebszellen. Im zweiten Projekt hingegen beschäftigten wir uns mit dem Einfluss der 3D-Umgebung und der Hydrogelelastizität auf die Proliferation und Selbsterneuerungsfähigkeit von induzierten pluripotenten Stammzellen (iPS) der Maus. Zusammengefaßt konnten wir zeigen daß, unabhängig davon ob sie auf 2D-Substraten kultiviert oder in 3D-Hydrogelen eingekapselt werden, die mechanischen Eigenschaften der extrazellulären Matrix in der Tat einen großen Einfluss auf die Phänotypen und die Genexpression in Krebszellen oder Stammzellen haben.

Um den Einfluss der Steifigkeit der extrazellulären Matrix auf die Funktionen von Brustkrebszellen zu untersuchen, verwendeten wir im ersten Projekt mit Fibronektin (FN) beschichtete Polyacrylamid-Hydrogele als Substrate, um Brustkrebszellen zu kultivieren. Durch Einstellen der Konzentration und des Verhältnisses von Acrylamid zu Bisacrylamid wurden drei verschieden elastische Substrate mit einer Elastizität von ~0,48 kPa, ~4,47 kPa, ~34,88 kPa hergestellt, die ich als "weiches" "mittleres" "steifes" Substrat bezeichnet habe. Zunächst haben wir die unterschiedlichen Morphologien von MCF-7 Zellen auf verschiedenen Substraten untersucht, welche mit vorherigen Studien übereinstimmten. Zellen breiteten sich auf dem steifem Substrat weit aus, während sie sich auf weichem Substrat kaum ausbreiteten. Dieses Verhalten ist vermutlich die Hauptursache für die Zunahme der Proliferationskapazität mit zunehmender Steifigkeit des Substrates. Als nächstes beobachteten wir die

unterschiedliche Reaktion von Brustkrebszellen auf chemotherapeutische Agenzien auf verschiedenen Substraten. Mit zunehmender Matrixsteifigkeit zeigten Brustkrebszellen eine erhöhte Chemosensitivität gegenüber Doxorubicin und Cisplatin, jedoch nicht gegenüber Cyclophosphamid. Um den Hauptgrund für die von der Matrixsteifigkeit abhängige unterschiedliche Reaktion auf Chemotherapeutika herauszufinden, haben wir die Stammzelleigenschaften von Brustkrebszellen auf verschiedenen Substraten durch Analyse der Veränderungen in der Population der Krebsstammzellen (CSC) ermittelt. Wir stellten fest, dass die CSC-Population mit zunehmender Matrixsteifigkeit kleiner wurde. Andererseits wurden in CSC-Anreicherungsversuchen mit auf steifem Substrat kultivierten Zellen weniger Tumorsphäroide gebildet. Diese Daten zeigten, dass das weiche Substrat die CSC-Population in der Brustkrebszellinie aufrechterhalten konnte.

Ein weiterer Schwerpunkt dieses Projektes war die Autophagie. Durch die Erzeugung einer Umgebung mit Nährstoffmangel stellten wir fest, dass der Grad der aktivierten Autophagie bei Zellen kultiviert auf steifem Substrat am höchsten und auf weichem Substrat am niedrigsten war, d.h. mit zunehmender Steifigkeit der Matrix nahm die Aktivierung der Autophagie zu. Interessanterweise wurde die erhöhte Autophagie unterdrückt, wenn das Aktin-Zytoskelett und die Ausbildung von Stressfasern durch die Zugabe von F-Aktin Inhibitoren oder nicht-Muskel-Myosin Inhibitoren gestört wurde. Diese Ergebnisse zeigten, dass eine intakte Actin-Zytoskelett-Spannung für die Aktivierung der Autophagie erforderlich ist. Weiterhin haben wir YAP in die Regulation der Matrixsteifigkeits-vermittelten Autophagie einbezogen. Ausschaltung von YAP verringerte den Grad der Autophagie in allen Gruppen erheblich, jedoch blieb der zuvor beobachtete Trend der steigenden Autophagie mit zunehmender Matrix-Steifigkeit unerwarteterweise bestehen. Dies deutet darauf hin, dass die Regulation der Autophagie durch die Matrixsteifigkeit unabhängig von YAP ist. Um diese Entdeckung weiter zu bestätigen, untersuchten wir als nächstes die YAP-Kerntranslokation auf verschiedenen Substraten und stellten fest, dass in normalen Brustkrebszellen fast das gesamte YAP im Kern lokalisiert war, d.h.

die YAP-Kerntranslokation wird nicht von der Steifigkeit beeinflusst. Im Gegensatz dazu wurde in Brustkrebs-Stammzellen (BCSC) die YAP-Kerntranslokation in einer weichen Umgebung gehemmt, was darauf hinweist, dass YAP ein Schlüsselfaktor bei der Regulation der BCSC-Population ist. Dieses Konzept wurde auch durch den Befund gestützt, dass der Unterschied der CSC-Population zwischen verschiedenen Substraten durch Ausschaltung von YAP beseitigt wurde.

Nicht zuletzt konnte der durch zunehmende Matrix-Steifigkeit hervorgerufene Steigerung der Autophagie durch Rho-, ROCK- und ERK-Inhibitoren eliminiert werden, was darauf hindeutet, dass der Rho-ROCK-ERK-Signalweg an dieser Regulation beteiligt sein könnte. Es sind jedoch weitere zukünftige Untersuchungen erforderlich, um diese Beteiligung zu untermauern. Darüber hinaus ist es wert, den Zusammenhang zwischen der Sensitivität gegenüber Chemotherapeutika und der Aktivierung der Autophagie in Abhängigkeit von den Substrateigenschaften zu untersuchen, was in diesem Projekt fehlte.

Im zweiten Projekt wurde ein chemisch definiertes Hydrogel auf der Basis von Polyethylenglykol (PEG) und dendritischen Polyglycerine (PGs) hergestellt, um eine 3D-Umgebung zu schaffen die eher den physiologischen Bedingungen entspricht. Unter Verwendung eines Mikrofluidik-Chips wurden Maus-iPS-Zellen in Mikrogelen mit einem Durchmesser von 200 µm eingekapselt. Im Innern der im Kulturmedium schwimmenden Mikrogele bildeten sich mehrzellige Sphäroide, auch Embryoidkörper genannt. Im Vergleich zur traditionellen Suspensionskultur bildeten sich mehr, aber kleinere Embryoidkörper mit hoher Proliferationskapazität in den Mikrogelen, was zu einer besseren Expansionskurve führte. Weiterhin konnten wir zeigen, dass in Mikrogelen eingekapselte iPS-Zellen im Vergleich zu herkömmlichen Suspensionskulturen eine äquivalente oder bessere Pluripotenz aufwiesen. Durch Erhöhen der Polymerkonzentration wurden steifere Hydrogele mit geringer Elastizität erzeugt, deren Verwendung jedoch zu einer rasch abfallenden Viabilität und Proliferationsfähigkeit der Zellen sowie einer geringeren Menge an Embryoidkörpern

führte. In diesem Projekt wollten wir den Einfluss der Hydrogelelastizität auf die Proliferationsfähigkeit und Selbsterneuerungsfähigkeit von iPS-Zellen untersuchen. Die extrem niedrige Überlebensrate der Zellen machte dies jedoch unmöglich. Zukünftige Arbeiten sind daher erforderlich, um Hydrogele mit der richtigen Elastizität zu erzeugen.

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Curriculum Vitae

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