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Regulation of cyclin E1 by the breast cancer microenvironment

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REGULATION OF CYCLIN E1 BY THE BREAST CANCER MICROENVIRONMENT

BY

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DISSERTATION

Submitted in Partial Fulfillment of the
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DEDICATION

To JIACONG WU AND XIAOYUE ZHOU
谨以此篇献给我亲爱的父母亲: 吴家聪和周晓悦

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When I first entered the medical university in 1996, I took the Hippocratic Oath. Every step I've taken since then has been as a student of medicine, majoring in both Clinical Medicine and Biomedical Sciences, with my mind set to understanding the basic science underlying disease. Thankfully, the University of New Mexico in the United States provided me a great offer to come across the ocean and let me get to know the real fun of science in a completely new world, thousands of miles away from my hometown. Luckily, in this world, I have never felt alone and received tremendous help from many kind people, especially from my wonderful mentor Dr. Rebecca Hartley and my co-advisor Dr. Helen Hathaway.

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ABSTRACT

The behavior of breast epithelial cells is influenced by their microenvironment, which includes stromal cells and extracellular matrix. During breast cancer progression, the tissue microenvironment fails to control proliferation, resulting in uncontrolled growth and invasion. Upon invasion, the extracellular matrix encountered by breast cancer cells changes from primarily laminin and collagen IV to primarily collagen I. The current study used a three-dimensional (3D) collagen I culture model to address how the microenvironment controls the breast cancer cell cycle after invasion through the basement membrane. We show that culturing breast cancer cells in 3D collagen I inhibits proliferation through direct regulation of cyclin E1, a G_1/S regulator that is overexpressed in breast cancer and implicated in its etiology. When the breast cancer cell line MDA-MB-231 was cultured within 3D collagen I gels, the G_1/S transition was inhibited as compared to cells cultured on conventional 2D collagen or plastic dishes. Cells in 3D collagen downregulated cyclin E1 protein and mRNA, with no

change in cyclin D1 level. Cyclin D1 regulates progression through early G_1, where it is upregulated by signals from the extracellular environment. Cyclin D1 relocalized to the cytoplasm in 3D cultures and phosphorylation of Rb, a nuclear target for both cyclin E1- and cyclin D1-associated kinases, was decreased. Positive regulators of cyclin E1, the transcription factor c-Myc and cold-inducible RNA binding protein (CIRP), were decreased in 3D collagen cultures, while the collagen I receptor β_1 integrin was greatly increased. Inhibition of β_1 integrin function rescued proliferation, cyclin E1 and c-Myc expression, and Rb phosphorylation, but had no effect on cyclin D1 localization. We conclude that cyclin E1 is repressed independent of effects on cyclin D1 in a 3D collagen environment and dependent on β_1 integrin interaction with collagen I, reducing proliferation of invasive breast cancer cells. These results differ from studies of breast epithelial and cancer cells in 2D collagen or 3D Matrigel in which disruption of β_1 integrin function decreased proliferation. We also show that matrix metalloproteinases (MMPs) MMP-2 and MMP-9 increased dramatically in 3D collagen. Their inhibition reversed cyclin E1 reduction, indicating their upregulation may be key to this process. In addition to studying MDA-MB-231 cells, we show that 3D collagen has similar effects on the nontumorigenic breast epithelial cell line MCF10A and the breast cancer cell line HMT-3522 T4-2 cells. Proliferation and cyclin E1 were downregulated in both of these cell lines, with MCF10A cells undergoing global G_1 arrest and subsequent apoptosis. Our data suggest that one way breast cancer cells adapt themselves to a collagen environment is by upregulating a collagen I receptor to decrease proliferation

through cyclin E1 repression. Cyclin E1 therefore appears to link the ECM and cell cycle machinery, arguing the importance of considering the role of ECM in breast cancer etiology, valuing cyclin E1 as a prognostic factor, as well as targeting cyclin E1 overexpression in clinical therapy.

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ABBREVIATIONS

3D: three dimensional

2D: two dimensional

cdks: cyclin-dependent kinases

CERM: cyclin E1 repressor module

CIRP: cold-inducible RNA binding protein

CKIs: cyclin-dependent kinase inhibitors

Cul-3: cullin-3

Ct: threshold cycle

DAPI: 4',6-diamidino-2-phenylindole

BM: basement mambrane

ECM: extracellular microenvironment

EGF: epidermal growth factor

ER: estrogen receptor

ERK: extracellular signal-regulated kinase

FAK: focal adhesion kinase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GSK3β : glycogen synthase kinase-3β

HuR: human antigen R

LMW: low molecular weight

MAPK : Mitogen-activated protein kinase

MCM: mini-chromosome maintenance

ME: mammary epithelial

MMPs: matrix metalloproteinases

PI3K: Phosphoinositide 3-kinases

Rb: retinoblastoma protein

RBPs: RNA binding proteins

RT-PCR: reverse transcription polymerase chain reaction

T4-2: HMT-3522 T4-2

TCNB: Tris, $CaCl_2$, NaCl, Brij35

TEB: terminal end bud

1. INTRODUCTION

1.1. Cyclin E1 as a cell cycle regulator

1.1.1. Cyclins, cyclin-dependent kinases and the mammalian cell cycle

The mammalian cell cycle is controlled by a subfamily of cyclin-dependent kinases (cdks) whose activity is regulated by several activators (cyclins) and cdk inhibitors or CKIs (p16, p21, and p27). It has been over 26 years since cyclins were originally described by Tim Hunt and his colleagues while observing mitotic cleavage of sea urchin embryos (Evans et al., 1983). Cyclins were named based on their dramatic cell cycle periodicity during meiotic and early mitotic divisions. In a "classical" model of the mammalian cell cycle that has been broadly accepted, cyclins, which bind to their catalytic partners, the cdks, are synthesized and destroyed at certain times during the cell cycle thus regulating the kinase activity in a timely manner (Fig.1.1, left). Cdk2, cdk3, cdk4 and cdk6 bind to their specific cyclins to drive each of the main events during interphase; G_1 (Gap phase 1), S (DNA Synthesis), and G_2 (Gap phase 2). Cdk1 binds to its cyclins to drive mitosis. In human cells, 13 loci encoding cdks and 25 loci encoding cyclins have been discovered so far (Malumbres and Barbacid, 2009). Although human genome analysis has found that all the genes that encode cyclins share a conserved stretch of 150 amino acid residues called the "cyclin box", which is formed by 5 helical regions, only 10 cyclins belonging to 4 different classes (the A, B, D, and E-type cyclins) directly drive the cell cycle. Many other cyclins lack known cdk or other kinase partners (Malumbres and Barbacid, 2005). Recently,

genetic evidence from mouse embryos with multiple knockouts has challenged the above model by indicating that cdk1 is sufficient to drive the cell cycle while interphase cdks are only important for specialized cell proliferation (Santamaria et al., 2007). However, these observations do not suggest interphase cdks have no contributions to normal cell cycles even in the cells in which they are dispensable. Thus, in addition to the classical model of the mammalian cell cycle, the “essential cell cycle model’’ and the “specialized cell cycle model” have been proposed (Fig. 1.1) and emphasized how much there is still to learn about the cell cycle (Malumbres and Barbacid, 2009; Murray, 2004). In this dissertation, principles from the classical model are used.

1.1.2. Cyclin E1- one of the G_1 cyclins

Mammalian G_1 cyclins include D-type cyclins (D1, D2 and D3) that associate with and activate cdk4 or cdk6 in early G_1 (Sherr, 1995), and E-type cyclins (E1 and E2) that associate primarily with cdk2, but can also interact with cdk1 and cdk3 (Aleem et al., 2005; Zariwala et al., 1998). Cyclin E2 shares 47% amino-acid homology with cyclin E1 and exhibits similar activities (Hwang and Clurman, 2005). Cyclin E1 is the best studied of the E-cyclins and will be the focus of this dissertation. Cyclin E1 was first discovered by screening human cDNA libraries for genes that could complement G_1 cyclin mutations in *Sacchromyces cerevisiae* (Lew et al., 1991). Its function in promoting the G_1/S transition was further confirmed when ectopic and constitutive expression of cyclin E1, but not of cyclin D1 or cyclin A, led to accelerated G_1 progression (Ohtsubo et al., 1995) and chromosome instability (Spruck et al., 1999). Results from studies using

cyclin E-null mice suggested a function in endoreplication, exit from quiescence, and oncogenic transformation (Geng et al., 2003; Parisi et al., 2003; Zhang, 2007). Cyclin E1 but not cyclin D1 is required for the G_1/S transition in cells lacking functional Rb and cyclin E1 can functionally replace cyclin D1 (Geng et al., 1999). Other functions for cyclin E1 include centrosome duplication (Okuda et al., 2000) and facilitating MCM (Mini-Chromosome Maintenance) complex loading to chromatin for DNA replication (Geng et al., 2007). Besides its role as a G_1 cyclin, cyclin E1 can also function in mitosis and meiosis (Grimison et al., 2006; Keck et al., 2007; Liu et al., 2007). Finally, cyclin E1 has been shown to have an important function in genotoxic stress-induced apoptosis (Mazumder et al., 2007). Originally identified as a regulator of the G_1/S transition, cyclin E1 has been found to have multiple functions and has become an attractive target in cancer research. Understanding the regulation of cyclin E1 will help provide ways to control cell cycle progression in cancer cells and limit their population of distant metastatic sites.

1.2. Regulation of cyclin E1

Cyclin E1/cdk2 activity peaks at the G_1/S transition and is lowest in cells that are arrested in G_0.This tight regulation during the normal cell cycle results from multiple layers of control. These layers include regulation of cyclin E1 transcriptionally, post-transcriptionally, and post-translationally, as discussed below.

1.2.1. Transcriptional regulation

The human cyclin E1 gene is on to chromosome 19q12, where it is regulated by the transcription factor E2F. E2F binding sites are found in both cyclin E1 and cyclin D1 promoters. For cyclin E1, the activity of its promoter is affected by E2F in a cell-cycle dependent manner (Leone et al., 1999; Ohtani et al., 1995). In early G_1, hypophosphorylated Rb binds to E2F1 and inhibits its activity. Cyclin D1 is induced by mitogenic stimulation in early G_1 leading to phosphorylation of Rb by cyclin D1/cdk4/cdk6 and release of E2F1. E2F1 recruits histone acetylases to the promoters of genes required for G_1/S including cyclin E1. Cyclin E1/cdk2 further phosphorylates Rb, reinforcing its own expression (Matsumura et al., 2003). The transient induction of cyclin E1 in late G_1 is also controlled via a cyclin E1 repressor module (CERM), which has an E2F-binding site and an AT-rich sequence that cooperate during G_0/G_1 to delay cyclin E1 expression until late G_1 (Le Cam et al., 1999; Polanowska et al., 2001). In addition, viruses such as adenovirus E1A, human papillomavirus type 16, and human cytomegalovirus have been found to transform cells by transcriptionally activating cyclin E1 (Botz et al., 1996; Bresnahan et al., 1998; Vogt et al., 1999). This subverts the cell cycle restriction point, which occurs before the G_1/S phase transition. The restriction point denotes the point in time when a cell must decide to advance to S phase, to remain in G_1, or to arrest into G_0.

1.2.2. Post-transcriptional regulation

Nine alternatively spliced variants of cyclin E1 mRNA have been identified to date. One, encoding a 45kDa isoform of cyclin E1, was originally considered the full-length protein (Koff et al., 1991; Lew et al., 1991), but a 50kDa isoform is

now known to be predominantly expressed (Ohtsubo et al., 1995). A 43kDa splice variant expressed at 1/10 of the level of full length cyclin E1 is found in several cell lines (Sewing et al., 1994). This isoform lacks the cyclin box necessary for cdk2 activation. Of the other 6 splice variants, 4 can bind to and activate cdk2 *in vitro* (the molecular weights of 3 of these are unknown), while one splice variant and one unspliced variant are not translated into protein. Cyclin E1 protein abundance is also determined via post-transcriptional regulation of its mRNA stability. Two recent studies measured cyclin E1 mRNA stability during the cell cycle using different cell lines and methods. In the first study, human leukemic lymphocytes (MOLT-4) cells were grown on a surface such that when a cell divided, one daughter cell remained attached while the other was released into a collection flask. Newborn cells were collected, grown synchronously, and the half-life of E1 mRNA was determined by real-time RT-PCR. Cyclin E1 mRNA half-life was in the range of 1.5 to 2 h, with little fluctuation in stability during different cell cycle phases (Eward et al., 2004). The second study reported the same half-life using mouse EL4 lymphoma cells and 3T3 fibroblasts synchronized by elutriation or cell sorting (Penelova et al., 2005).

The intracellular half-lives of RNAs are determined by many factors, including their affinity for RNA binding proteins (RBPs). HuR (Human antigen R) is an RBP shown to stabilize mRNAs encoding cyclins A, B, D, and E, among others. HuR is a predominantly nuclear protein but shuttles between the nucleus and the cytoplasm, positively affecting mRNA stability and translation in the cytoplasm (Bevilacqua et al., 2003). It plays an essential role in tumorigenesis by

stabilizing transcripts involved in cell cycle regulation, such as cyclins (Wang et al., 2000); proliferation such as c-Myc (Kim et al., 2009); tumor growth such as vascular endothelial growth factor, cyclooxygenase-2, and β-catenin (Dixon et al., 2001; Levy et al., 1998; Lopez de Silanes et al., 2003); anti-apoptotic effectors such as Bcl-2 and Mcl-2 (Abdelmohsen et al., 2007); and metastasis, possibly through vascular endothelial growth factor-C (Wang et al., 2009b). Cytoplasmic expression of HuR is considered a prognostic factor in breast cancer, where it is overepxressed in about 30% of tumors (Heinonen et al., 2005). Our laboratory has shown that HuR contributes to cyclin E1 overexpression by stabilizing its mRNA in MCF-7 breast cancer cells (Guo and Hartley, 2006). Cold-inducible RBP (CIRP) also stabilizes cyclin E1 mRNA in breast cancer cells. CIRP is overexpressed in several breast cancer cell lines (Guo et al., 2009) as well as in about 30% of primary breast tumors (Artero-Castro et al., 2009). We found that CIRP increased cyclin E1 mRNA stability partially by facilitating the binding of HuR to cyclin E1 mRNA.

The translational efficiency of cyclin E1 mRNA was recently shown to be promoted by the protein kinase Akt in hepatocytes (Mullany et al., 2007). This novel translational mechanism is distinct from the cyclin D1-E2F transcriptional pathway. This study suggested that cells can respond to environmental stimulation (such as mitogens or nutrients) by direct stimulation of cyclin E1 translation by Akt to affect cell cycle progression.

1.2.3. Post-translational regulation

The abundance of cyclin E1 is also regulated by phosphorylation-dependent proteolysis after the cyclin E1/cdk2 complex is activated at the G_1/S boundary. Cyclin E1 turnover is achieved by the ubiquitin-proteasome pathway. Proteins targeted by this pathway require modification by successive actions of ubiquitin-activating (E1) and -conjugating (E2) enzymes, and ubiquitin ligases (E3), which attach polyubiquitin chains to specific lysine residues (Hao et al., 2007). Polyubiquitinated proteins are then targeted and degraded by the 26S proteasome into small peptides. Two pathways for ubiquitin-dependent proteolysis of cyclin E1 have been reported. When cyclin E1 is not associated with cdk2, Cullin-3 (Cul-3) promotes ubiquitination of cyclin E1 (Singer et al., 1999; Wimuttisuk and Singer, 2007). Cul-3 belongs to the Cullin family proteins that comprise the core module of the BTB/Cul3/Rbx1 ubiquitin ligase. When cyclin E1 is bound to cdk2, it is protected from the Cul-3 mediated pathway and multiple phosphorylations are required for its ubiquitination (Welcker et al., 2003). Both cdk2 and glycogen synthase kinase-3β (GSK3β) phosphorylate cyclin E1 on at least 4 different sites. Phosphorylated cyclin E1 is recognized by Fbw7 (hCdc4), the substrate recognition subunit in the Skp1/Cul-1/F-box E3 ligase. There are Cdc4 isoforms required in cyclin E1 ubiquitination (Welcker et al., 2004). The prolyl isomerase Pin1 regulates cyclin E1 turnover, dependent on phosphorylation of cyclin E1 by cdk2 (Yeh et al., 2006). In addition to Cul-1 and Cul-3 as described above, Cul-4B was recently reported to have an essential role in cyclin E1 degradation (Zou et al., 2009).

Another important post-translational modification of cyclin E1 is processing of the full length protein into low molecular weight (LMW) isoforms. These LMW isoforms (ranging from 33-45kDa) have been found in breast, ovarian, gastric, colorectal, and lung cancers, and melanoma (Bales et al., 2005; Bedrosian et al., 2004; Corin et al., 2006; Davidson et al., 2007; Koutsami et al., 2006; Milne et al., 2008; Porter et al., 2001). They have an increased affinity for cdk2, are resistant to cdk inhibitors, and their presence strongly correlates with increasing stage and grade of breast cancer (Wingate et al., 2005a). Transgenic mice expressing LMW cyclin E1 isoforms are susceptible to metastatic mammary carcinoma while full-length cyclin E1 overexpressing mice are not, showing their oncogenic potential. The origin of LMW cyclin E1 isoforms is still under debate. Both alternative splicing (Porter and Keyomarsi, 2000b) and intracellular proteolytic processing (Libertini et al., 2005; Porter et al., 2001; Wang et al., 2003) have been implicated in their production. The LMW cyclin E1 isoforms are thought to be cancer specific (Wingate et al., 2005b), but some studies show that the ability to detect LMW cyclin E1 isoforms correlates only with the level of full length cyclin E1 and they can be detected in both breast cancer and normal mammary epithelial (ME) cells if full length cyclin E1 levels are normalized (Spruck et al., 2006). A better understanding of how LMW isoforms are produced is needed.

1.3. Deregulation of cyclin E1 in cancer

As one of the regulators involved in passing through the restriction point and entry into S phase, cyclin E1 has been extensively studied in human cancers. A

number of cancers overexpress cyclin E1 protein or mRNA, including carcinomas (e.g. of the breast, cervix, endometrium, gastrointestinal tract, and lung), lymphomas, adrenocortical tumors, sarcomas and leukemias (Erlanson and Landberg, 2001; Iida et al., 1997; Molendini et al., 1998; Muller-Tidow et al., 2001; Schraml et al., 2003; Tissier et al., 2004; Yasui et al., 1996). Overexpression of cyclin E1 results in chromosome instability, implicating it in cancer etiology (Spruck et al., 1999).

Several mechanisms contribute to cyclin E1 deregulation in cancer including transcriptional upregulation, disruption of its degradation, downregulation of cdk inhibitors, mRNA stabilization, and processing into LMW isoforms, as described in the previous section (Akli and Keyomarsi, 2004; Guo and Hartley, 2006; Guo et al., 2009; Sutherland and Musgrove, 2004). Rb is inactivated in many human cancers, resulting in increased cyclin E1 abundance transcriptionally through increased E2F activity (Burkhart and Sage, 2008). In addition, transcriptional regulator LIM-only protein 4 has also been shown to transcriptionally upregulate cyclin E1 in breast cancer (Montanez-Wiscovich et al., 2009).

Disruption of cyclin E1 degradation also contributes to cancer etiology. It has been shown that mutation of hCdc4 is sufficient to deregulate cyclin E1 in the cancer cell cycle (Ekholm-Reed et al., 2004) and hCdc4 has been evaluated as a general tumor suppressor that is inactivated by mutation in various human cancers with an overall mutation frequency of 6% (Akhoondi et al., 2007). Ras activity has been shown to regulate hCdc4-mediated cyclin E proteolysis, and impaired cyclin E degradation as a mechanism through which Ras mutations

promote tumorigenesis (Minella et al., 2005). Interestingly, overexpression of E2F stabilizes cyclin E1 protein by reducing its ubiquitination thereby reducing its degradation. This E2F action on cyclin E1 requires E2F transcriptional activity and is specific for cyclin E1, indicating another possible mechanism for increasing cyclin E1 accumulation in proliferating tumor cells (Pajalunga and Crescenzi, 2004).

Downregulation of cdk inhibitors is often seen in cancer. In primary human fibroblasts, deregulated cyclin E1 initiates a p53-dependent response that induces cdk inhibitor p21 expression, preventing excess cyclin E1/cdk2 activity. The response to restrain cyclin E1/cdk2 deregulation by both p53 and p21 is proposed to be a general protective barrier against neoplastic transformation (Minella et al., 2002). p27 is another cdk inhibitor that prevents premature activation of cyclin E/cdk2 in G_1. Although the p27 gene is rarely mutated in human cancers, accelerated p27 proteolysis and its mislocalization from the nucleus to the cytoplasm are often observed in tumor cells. P27 mislocalization was reported in 40% of primary breast cancers (Blain and Massague, 2002). Cytoplasmic localization of p27 prevents it from binding and inhibiting nuclear targets such as cyclin E1/cdk2.

As discussed in section 1.2.2, cyclin E1 mRNA is regulated by the RBPs HuR and CIRP, both of which are overexpressed in many human tumors. In addition to RBPs, a class of small noncoding RNAs, termed microRNAs, deregulate cyclin E1 in cancer cells. MicroRNAs inhibit gene expression by binding to recognition elements, mainly in the 3' untranslated region of mRNA,

resulting in mRNA destablization or translational inhibition (Iorio and Croce, 2009). miR-16 downregulates cyclin E1 by destabilizing its mRNA (Kaddar et al., 2009). miR-16 is downregulated in several cancers (Bottoni et al., 2005; Calin et al., 2002) and has been shown to induce G_0/G_1 cell cycle arrest partially through downregulation of cyclin E1 in human lung and breast cancer cells (Wang et al., 2009a).

1.4. Regulation of cyclin E1 by the cellular microenvironment

1.4.1. Definition, components and function of the cellular microenvironment

The structure and function of normal tissues is largely determined by the interactions between cells within tissue and their microenvironment (Radisky et al., 2002). This communication is so important that loss of tissue architecture becomes a prerequisite for, and one of the critical characteristics of, most cancers. On the other hand, a normal microenvironment that maintains organ structure can act as a strong tumor suppressor, preventing or reversing malignant phenotypes even in cells bearing genomic abnormalities. The cellular microenvironment is defined as local and systemic constituents surrounding a cell, including extracellular matrix (ECM), other cells, and soluble factors released locally or transmitted from other organs, such as hormones, growth factors, and cytokines (Nelson and Bissell, 2006). The ECM, to which the soluble factors can bind, is a three-dimensional (3D) insoluble protein network. There are two major categories of ECM including the basement membrane (BM) and the interstitial matrix. The BM is primarily composed of type IV collagen, laminins,

entactin/nidogen and heparin sulfate proteoglycans. Epithelial and endothelial cells are directly surrounded by the BM, and that surrounding the BM is the interstitial matrix. The interstitial matrix consists of many types of collagens, with type I collagen being the most abundant component, along with elastic fibers, fibronectin, tenascin and proteoglycans. This matrix provides the mechanical strength, hydration, integrity and binding of soluble factors. Originally considered only as a support system, the ECM is now recognized as a central regulator of cell behavior including adhesion, migration, proliferation, differentiation, and survival, by serving as a structural scaffold and an instructive environment (Erler and Weaver, 2009).

1.4.2. Communication between the microenvironment and the cell cycle

Microenvironmental control of the cell cycle has been studied for more than 40 years and led to the concept of anchorage-dependent cell growth (Stoker et al., 1968). It is thought that integrin-mediated cell anchorage to the ECM and growth factors cooperate to regulate cell cycle progression (Fig. 1.2). Expressed by virtually all animal cells, integrins are the most abundant class of ECM protein receptors. Integrins bind to ECM proteins and in some cases, to membrane proteins expressed on the surface of other cells. They are signalling receptors that control both cell binding to ECM and intracellular responses following adhesion (Hehlgans et al., 2007).

Anchorage-dependent regulation controls two different periods of the cell cycle. The first and also the primary control is during the G_1 phase, when the extracellular environment regulates cell cycle progression primarily via regulation

of cyclin D1. Mitogenic growth factors and cell anchorage converge on the signalling pathways that result in cyclin D1/cdk4/6 activity, promoting G_1 phase progression (Bohmer et al., 1996). Adhesion to substratum regulates both cyclin D1 transcription and translation. Nonadherent cells fail to phosphorylate Rb, while enforced expression of cyclin D1 rescues Rb phosphorylation and entry into S phase in nonadherent G_1 cells (Zhu et al., 1996). Cyclin E1 expression is also responsive to extracellular signals. Anchorage-dependent control occurs in Rb/p107 double-null mouse embryonic fibroblasts independent of cyclin D1 but dependent on cyclin E1-associated kinase activity (Gad et al., 2004). Consistent with this study, cyclin E1 and cyclin E1/cdk2 activity were up-regulated independently of cyclin D1 in ME cells adhered to type I collagen (Klekotka et al., 2001b).

Anchorage-dependent control also functions during cytokinesis. Multinucleation and inhibition of cytokinesis were observed in suspended cells, and attachment to proper substratum such as collagen I gel or a thin layer of gelatin, was required in Xenopus embryos for cytokinesis, indicating that cell anchorage may be important in this process (Ben-Ze'ev and Raz, 1981; Winklbauer, 1986). Although non-adhered cells fail to complete cytokinesis, anchorage-independent cytokinesis can be achieved through oncogenic signalling (Thullberg et al., 2007). The role of cyclins has not been addressed except that cyclin B1 localization in G_2 and early M phase was studied but was not changed in suspended as compared to attached cells. From these studies, it

is clear that microenvironmental controls are necessary for the normal cell cycle and that these controls are often disrupted in cancer.

1.4.3. Microenvironmental influences in the normal mammary gland

The mammary gland is the best-studied example of an organ whose structure and function are influenced by reciprocal signalling and communication between cells and their microenvironment. The influences are not only from local constituent tissues, but also from distant organs (Fig. 1.3). The basic mammary structure is a highly organized branched ductal network where milk is produced by a continuous layer of luminal epithelial cells and secreted into a central lumen. The luminal epithelial cells are surrounded by a layer of myoepithelial cells and the double-layered structure is enclosed by the BM. The epithelial ductal tree is embedded in a complex interstitial matrix including non-epithelial cells such as fibroblasts, adipocytes, and capillaries. Although the mammary epithelium (anlage) is specified in the embryo, the mammary gland is one of the few organs that develops and matures postnatally during puberty. At puberty, the breast initiates expansion at the ends of the ducts known as terminal end buds (TEBs). The mammary gland reaches its fully functional differentiation during pregnancy and lactation resulting in the formation of luminal structures that collect milk produced by alveoli. The entire gland undergoes involution after weaning, during which ME cells apoptose and are replaced by mesenchymal and adipose tissue, to return the gland to its dormant state (Bissell et al., 2002).

Hormones, growth factors, cytokines, morphogens, ECM molecules, proteases, physical stress and strain all play important roles in the development

and remodeling of the mammary gland. Estrogen from the ovary and growth hormone from the pituitary regulate the development of the mammary gland at puberty. Progesterone and prolactin are also necessary for the ductal side branching and alveoli development during pregnancy and lactation. Although these systemic hormones are powerful regulators, their effects are not due to direct action but rather through synergistic actions with multiple secondary paracrine effectors that alter expression of transcription factors. Locally, mammary gland branching is controlled by signals from both epithelial cells and their surrounding stroma (Sakakura et al., 1976). Several estrogen receptor (ER) knockout studies have shown the importance of the paracrine signalling events that regulate ME cell proliferation and morphogenesis (Cunha et al., 1997; Mallepell et al., 2006; Mueller et al., 2002). ERα is only expressed in a subset of ME cells and ER positive cells release paracrine factors to control the proliferation of adjacent ER-negative cells (Clarke et al., 1997). Growth hormone is also thought to regulate the development of the mammary gland through a stromal insulin-like growth factor 1 (Kleinberg et al., 2009). During the proliferation of the mammary TEB, local influences from matrix metalloproteinases (MMPs), cadherins, type I collagen and other ECM components integrate with systemic hormones to regulate the whole process (Hinck and Silberstein, 2005). Understanding these microenvironmental influences are important for understanding breast cancer etiology as many common mechanisms and signalling pathways have been observed in both

mammary gland development and carcinogenesis (Lanigan et al., 2007; Noel and Foidart, 1998).

1.5. Cellular microenvironment and breast cancer etiology

1.5.1. Using three-dimensional (3D) models to study breast cancer

More than 90% of human breast cancers arise from the ductal epithelium. Numerous models have been developed to study the normal and neoplastic mammary gland, including the cleared fat pad transplantation system (Deome et al., 1959; Evers et al.) and transgenic mouse models, which have provided significant insight into the genes and signalling pathways regulating normal and aberrant cell behaviors (Muller, 1991; Radisky and Hartmann, 2009). These models are relatively intractable for studying biochemical processes involved in tumor formation. For studying the precise biochemical pathways controlling the normal and malignant states in ME cells, cell culture-based studies are much more amenable to manipulation. However, the mammary gland is not a 2-dimensional (2D) structure. 2D monolayer cell culture systems cannot recapitulate the structural organization and functional differentiation of the mammary gland *in vivo*. Therefore, 3D culture systems have emerged as tractable models that allow ME cells to organize and mimic their *in vivo* structure and allow investigation of the cancer cell behaviors in a biologically relevant context.

When culturing a single cell type in 3D, two different methods are typically used. The first is to completely embed cells within a gelled ECM and culture with

media containing growth factors and hormones. The second is to make a thin ECM gel (~ 1mm), seed single cells on top, and overlay with media containing diluted ECM components. Both systems have been successfully used to induce acini formation [Fig. 1.4; (Hebner et al., 2008)]. Matrices such as Matrigel, collagen I, fibrin and polyacrylamide gels have been utilized and their pros and cons have been summarized in a recent review [Fig.1.5; (Hebner et al., 2008)]. Matrigel is a commercially availaboratoryle reconstituted or laminin-rich BM (lrBM), which contains mainly laminin, type IV collagen, entactin and heparin sulfate proteoglycan (Kenny and Bissell, 2003). Matrigel has been used extensively for promoting ME cell differentiation *in vitro*, because ME cells are surrounded by laminin-rich BM *in vivo* (Nelson and Bissell, 2006). However, Matrigel is an ill-defined mixture of extracellular matrix proteins derived from an Engelbeth-Holm-Swarm murine tumor (Kleinman et al., 1986). Matrigel thus has an intrinsic variability and culture media containing serum or other supplements may unpredictably interfere with its components. Our study has utilized the 3D system by embedding an invasive breast cancer cell line into a 3D environment composed of collagen I, similar to the model shown in the left panel of Fig.1.4. We chose type I collagen because it is the major component of ECM that invasive breast cancer cells contact when the BM is compromised, and it is biologically better defined than Matrigel and can be easily manipulated.

An obvious limitation of 2D culture is lack of stroma, which accounts for greater than 90% of the resting breast volume (Kim et al., 2004). This causes a deficiency of organized architecture and limited transport. Normal ME cells grown

in monolayer can be highly plastic and display tumor cell characteristics. The major advantage of 3D over 2D culture is well-defined geometry, making functional studies directly related to the microenvironment (Kim et al., 2004). Besides resembling *in vivo* phenotypes, cell shape and mechanical force from the microenvironment can also be studied, with the recognition that cell behaviors are affected by these factors (Ghosh et al., 2008). Lastly, 3D cultures support co-cultivation of multiple cell types so that ME-stromal cell interaction can be examined (Gudjonsson et al., 2002).

The use of 3D cell culture systems allows better *in vitro* modeling of the mammary gland. For example, when normal ME cells are plated in traditional 2D cultures, they grow as monolayers and do not differentiate. When they are cultured in 3D reconstituted BM, they form spherical acini in which milk is secreted into a hollow lumen (Barcellos-Hoff et al., 1989). The normal ME cells in the middle of the acini undergo apoptosis due to the lack of ECM contact. In contrast, breast cancer cells grow continuously, resulting in disorganized structures similar to those observed in the premalignant breast (Debnath and Brugge, 2005a). In fact, culturing cells in 3D uncovered not only differences in cell morphology, but also differences in growth rates, identity, quantity, and patterns of expressed proteins between normal and breast cancer cells (Nelson and Bissell, 2005). Using a 3D culture system has also been helpful to the development of new therapy. Notably, Herceptin® (i.e. An epidermal growth factor (EGF) receptor blocking antibody) and Gleevec® (i.e. a KIT-selective

tyrosine kinase inhibitor) have been developed using 3D culture systems and used successfully for cancer therapy (Kim et al., 2004).

Although these 3D systems mimic at least the minimum unit of the mammary gland, the real microenvironment contains a variety of cell types and thus more complicated interactions. As a result, the interpretation of data from monotypic systems needs to consider their limitations.

1.5.2. Collagen I in the mammary microenvironment

The studies discussed thus far have shown that normal and cancer ME cell behaviors are reproducibly distinguished in 3D culture models. *In vivo*, the breast cancer microenvironment is dynamic in that tumor cells recruit numerous stromal and immune cells that produce ECM proteins and other factors to create an environment that favors cancer cell survival and metastasis (Hu and Polyak, 2008). Secretion of matrix metalloproteases (MMPs), fibroblast transdifferentiation to myofibroblasts, and immune cell infiltration all facilitate BM and interstitial matrix degradation, angiogenesis and subsequent invasion, survival and growth (Weaver and Gilbert, 2004). One important change in the interstitial matrix in breast cancer is increased breast density due to increased deposition of collagen I and an increased number of fibroblasts. It is thought that increased matrix stiffness due to these changes may promote cancer progression through altering ECM receptors such as integrins and their downstream signalling pathways. However, the precise mechanisms by which stromal density alteration promotes breast cancer remain unknown (Erler and Weaver, 2009). Fully differentiated myoepithelial cells and intact BM are often

lost during breast cancer progression from *in situ* to invasive carcinoma (Gudjonsson et al., 2003).

There are more than 30 collagens and collagen-related proteins, with type I collagen constituting about 90% of all collagens in the body (Velegol and Lanni, 2001). Collagen I provides a scaffold to which other ECM components adhere. The fundamental structure of collagen I is a long and thin rod-like protein about 300 nm long and 1.5 nm in diameter. Fibroblasts synthesize procollagen molecules and collagen fibers begin to assemble in the endoplasmic reticulum and Golgi, after cleavage of N-terminal and C-terminal propeptides from monomeric collagen. The collagen molecules then polymerize to form collagen fibrils. Collagen fibrils consist of 3 coiled subunits composed of two α1 chains and one α2 chain. Each chain has 1050 amino acids wound around each other in a characteristic right-handed triple helix. After a series of chemical modifications, collagen fibrils are bundled, which enhances mechanical stability. Collagen I forms fibrils spontaneously *in vitro* but not *in vivo*. Multiple factors determine the sites and the initiation of collagen I fibrillogenesis *in vivo*, with integrins involved in this process (Kadler et al., 2008). Type I collagen can be obtained by extraction from rat tail tendon, bovine calf skin, and other sources by acid digestion, and can form gels when returned to neutral pH in the concentration range of 0.3-30 mg/ml (Pedersen and Swartz, 2005).

Cell proliferation has been studied in nontumorigenic ME cells in collagen I-based 2D and 3D models. When collagen I was coated as a thin-layer at the bottom of plastic dishes, both human primary ME and mouse ME cells showed

robust cell proliferation regulated by $\alpha_2\beta_1$ integrin-mediated signalling (Benaud and Dickson, 2001c; Klekotka et al., 2001b). Mouse ME cells grown on floating collagen gels, differentiated, forming alveoli and secreting casein in response to hormones (Emerman et al., 1977). When a mouse ME cell line was grown in 3D attached collagen gels, polarized acini formed (Soriano et al., 1995). Morphogenesis as well as cell cycle progression of mouse ME cells, was controlled by $\alpha_2\beta_1$ integrin and growth factor signalling in 3D collagen culture (Zutter et al., 1999). In contrast to mouse ME cells, human primary ME cells cultured in 3D collagen I formed acini with inverse polarity. Addition of human myoepithelial cells corrected the polarity (Gudjonsson et al., 2002). Correct polarization in collagen gel has been reported for a number of other epithelial cells, indicating that this inside-out polarity in collagen may be unique to normal human ME cells (Gudjonsson et al., 2002).

Collagen cultures have also been used to study malignant ME cells. One study observed that T47D and MCF-7 breast cancer cells were flat and polygonal when grown as monolayers on plastic, but were spherical and formed tumor-like aggregates in 3D collagen I (Leung and Shiu, 1982). However, T47D cells differentiated into duct-like tubules when cultured in a floating 3D collagen gel (Wozniak and Keely, 2005b).

Despite the extensive use of collagen in studying various types of cells including malignant ME cells, there are few studies on the effects of 3D collagen culture on proliferation of invasive breast cancer cells.

1.5.3. $\alpha_2\beta_1$ integrin in the mammary microenvironment

Integrins are the main receptors for the ECM, transmitting signals to and from the cytoskeleton as well as activating signalling pathways that control cell behavior. Specifically, cellular interactions with collagen I are mediated by integrins, with $\alpha_2\beta_1$ being the primary heterodimer on epithelial cells (McCall-Culbreath and Zutter, 2008b).

The integrin family consists of 18 α and 8 β subunits forming 24 known heterodimers dependent on cell type and function. Each integrin subunit has a large extracellular domain, a short transmembrane domain and a small intracellular domain. Different integrins interact with different substrates, but one integrin can also interact with many substrates, and vice versa. α and β subunit phosphorylation following ligand binding is thought to be involved in bi-directional signalling. Cell adhesion via integrin engagement with the ECM results in increased tyrosine phosphorylation of downstream proteins (Hehlgans et al., 2007). One of the first proteins phosphorylated is focal adhesion kinase (FAK). Focal adhesions form by recruiting a number of signalling and structural molecules including FAK, Src, Phosphoinositide 3-kinases (PI3K), integrin linked kinase, and Caveolin-1, among others. Activation of downstream signalling pathways leads to the regulation of proliferation, differentiation, apoptosis, migration, survival and angiogenesis (Hynes, 2002) .

The 5 collagen-binding integrins include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$. As a major collagen I receptor, $\alpha_2\beta_1$ is expressed in epithelial cells, endothelial cells, T cells, natural killer cells, and mast cells. $\alpha_2\beta_1$ integrin knockout mice have reduced mammary gland branching, delayed thrombus formation, and defective

innate immunity (Hynes, 2002). Consistent with the knockout mouse model, in a 3D collagen I floating gel system, $\alpha_2\beta_1$ integrin was found to have a role in supporting branch elongation and cell migration for epithelial morphogenesis (Zutter et al., 1999).

As discussed in the previous section, $\alpha_2\beta_1$ integrin plays an important role in cell cycle progression in nontumorigenic ME cells cultured in 2D or 3D collagen. Studies have also addressed $\alpha_2\beta_1$ integrin function in breast cancer cell proliferation, although few of them used collagen cultures. When MCF-7 breast cancer cells were treated with interferon-α, an inhibitor of cell proliferation, $\alpha_2\beta_1$ integrin-mediated cell adhesion was reduced. Interferon-α had no effect upon the cell surface expression of either α_2 or β_1 integrin (Maemura et al., 1999). Overexpression of β_1 integrin in several breast cancer cell lines increased the phosphorylation of Akt and extracellular signal-regulated kinase (ERK), promoting proliferation and bypassing the antiproliferative effects of Herceptin used for EGF receptor positive breast cancer patients (Lesniak et al., 2009). These studies indicate that β_1 integrin participates in cell proliferation in both normal and malignant ME cells by mediating cell adhesion and common signalling pathways downstream of growth factors.

A transgenic mouse model of human breast cancer showed that β_1 integrin plays an essential role in both the initiation and maintenance of breast cancer growth *in vivo* (White et al., 2004). However, $\alpha_2\beta_1$ integrin expression is often diminished in breast cancer, correlating with breast cancer progression (Zutter et al., 1993). Re-expression of this integrin reduced breast cancer

formation *in vivo* (Zutter et al., 1995), implicating its reduction in breast cancer etiology and progression. In contrast to these *in vivo* studies, studies with breast cancer cells in 3D Matrigel showed that inhibition of β_1 integrin function decreased cell proliferation and reverted the malignant phenotype (Park et al., 2006a; Weaver et al., 1997a). A more recent study showed that β_1 integrin inhibition was not sufficient to suppress tumor-matrix induced breast cancer cell invasion (Castello-Cros et al., 2009). A better understanding of β_1 integrin function in breast cancer is required to clarify these discrepancies.

1.6. Hypothesis

Normal ME cells require attachment to the BM for survival. When integrin-ECM contact is disturbed, ME cells undergo a type of apoptosis termed anoikis. Breast cancer cells often subvert anoikis, surviving without BM contact, allowing them to invade the surrounding interstitial matrix once the BM is compromised. Interestingly, tumor cells still depend on integrin-mediated signalling pathways. It has been shown that integrin expression changes significantly during tumorigenesis (Alexandrova, 2008), and this is also the case in breast cancer (Koukoulis et al., 1991). β_1 integrin regulates behavior of both normal and cancer cells when they adhere to collagen, although the mechanisms are still under investigation. We are interested in how a collagen I environment regulates breast cancer cell proliferation, and the role of β_1 integrin in this process. The ECM primarily controls the expression of D-cyclins in early G_1 phase of the cell cycle, but cyclin E1 is also responsive to extracellular signals. In 2D collagen cultures, $\alpha_2\beta_1$ integrin stimulates the expression of cyclin E1 in nontumorigenic ME cells in

a collagen I-dependent and cyclin D1-independent manner (Klekotka et al., 2001a) . Our goal was to use a 3D collagen culture system to better model the environment of invasive breast cancer cells in order to determine if this environment directly regulates cyclin E1. **We hypothesize that the breast cancer microenvironment controls the cell cycle in part through direct regulation of cyclin E1.** Three specific aims test this hypothesis:

1. Evaluate how 3-dimensional collagen culture regulates the cell cycle in malignant and non-tumorigenic ME cells.

2. Evaluate regulation of cyclin E1 by 2-dimensional versus 3-dimensional collagen culture.

3. Define the mechanisms that regulate cyclin E1 within collagen cultures.

1.7. Figures and Figure Legends

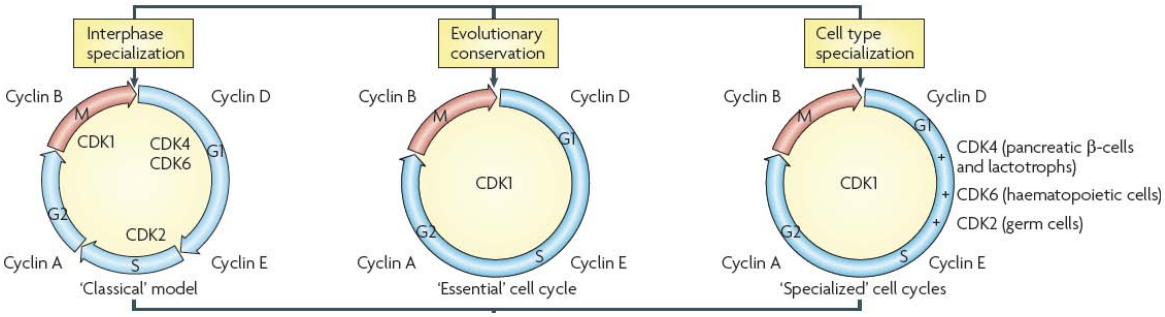

Figure 1.1 Three models of the mammalian cell cycle (Malumbres and Barbacid, 2009).

In the currently accepted classical model, each of the main events during interphase (G_1, S and G_2) is driven by cdks bound to specific cyclins. The essential cell cycle model is based on genetic evidence that cdk1 is sufficient to drive proliferation of all cell types up to midgestation and during adult liver regeneration. The specialized cell cycles are based on the requirements of specialized cell types for specific cdks.

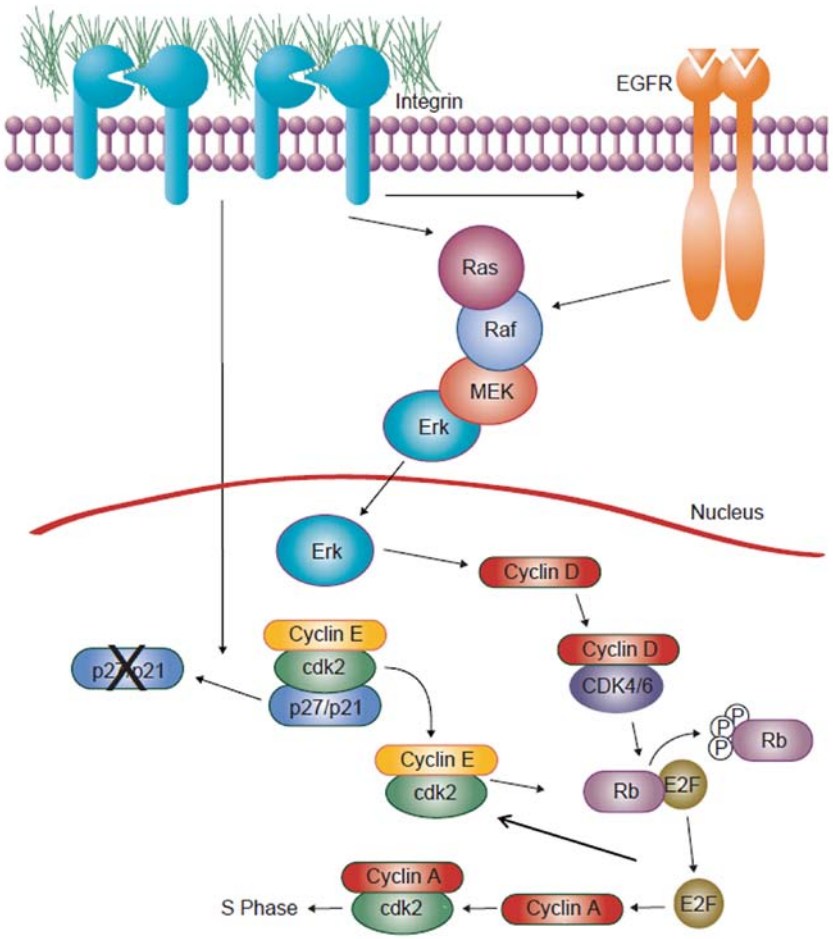

Figure 1.2 Integrin and growth factor cooperation in cell cycle regulation (Miranti and Brugge, 2002).

Both growth factors and cell adhesion are required for transmitting signals to the Ras/Raf/MEK/ERK signalling pathway. Activation of ERK and its nuclear translocation results in an increase in cyclin D transcription. Cyclin D binds to and activates cdk4/6. Integrins also stimulate degradation of the negative cell cycle regulators (cdk inhibitors) p21 and p27, allowing the activation of cyclin E/cdk2 complexes. Activation of both cdk4/6 and cdk2 is required for hyperphosphorylation of retinoblastoma protein (Rb), causing the release of E2F, which is required for increased transcription of cyclins E and A. Cyclin A/cdk2 complexes are required for S phase.

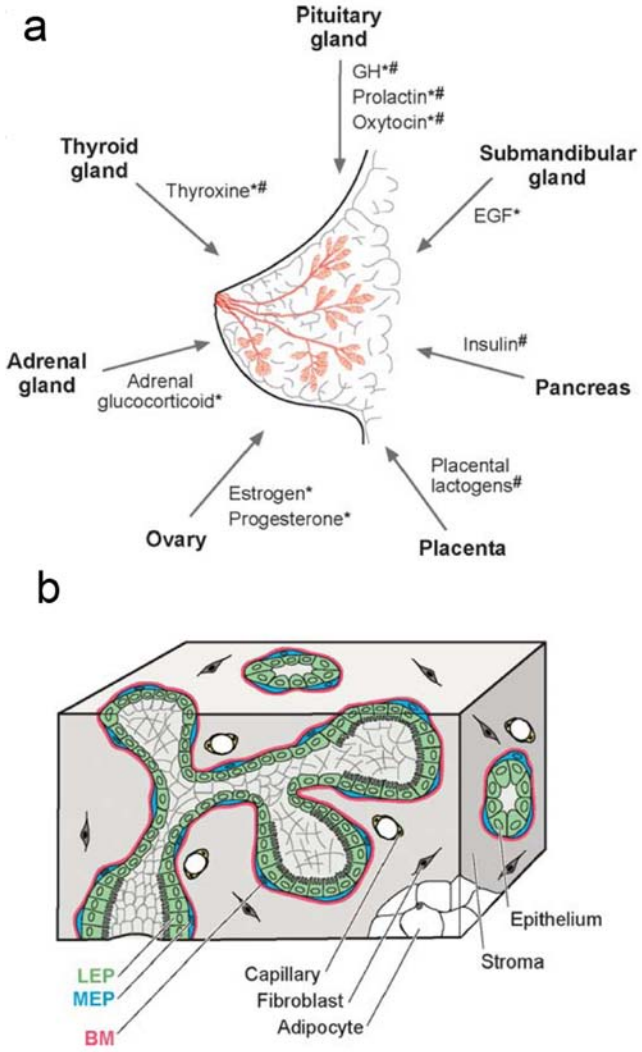

Figure 1.3 The structure and function of the mammary gland are influenced by communication with ECM and distant organs (Nelson and Bissell, 2006).

a. The human breast is a double-layered epithelial ductal tree (pink) embedded in stroma. Distant organs release signals and influence ductal and acinar morphogenesis. b. The epithelium is composed of a layer of luminal epithelial cells (LEP) surrounded by myoepithelial cells (MEP) and enclosed by BM. The epithelium is surrounded by an interstitial matrix and adjacent fatty stroma.

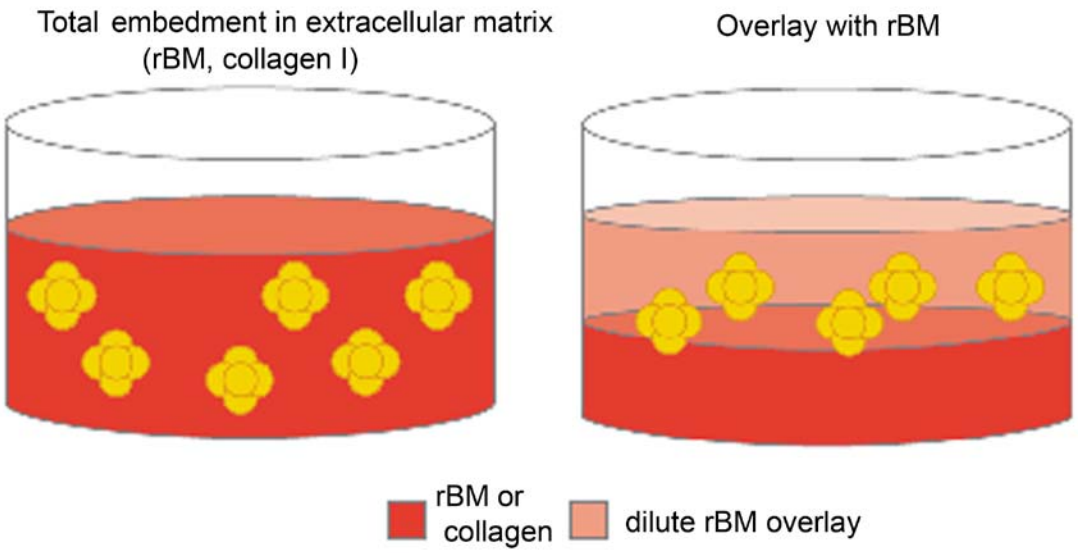

Figure 1.4 3D culture methods (Hebner et al., 2008).

Upper panel: Schematic of commonly used techniques. Left: Complete embedding of ME cells in a matrix such as reconstituted BM (rBM) or type I collagen. We used collagen in 3D culture similar to this method. Right: Mammary epithelial cells seeded on a thin layer of solidified rBM are overlain with a dilute solution of rBM in culture media.

Component	Advantages	Disadvantages
Reconstituted basement membrane	Successfully applied to many 3D systems	Poorly defined content; lot-to-lot variability
Fibrin	Successfully applied to many 3D systems	Easily proteolyzed by cellular proteases
Collagen I	More biologically defined; easy to manipulate	Lot-to-lot variability; limited range of elastic moduli
Polyacrylamide gels	Easy to manipulate; nonreactive; large range of elastic moduli	Acrylamide toxicity; not a true 3D system

Figure 1.5 Advantages and disadvantages of several matrices for 3D culture studies (Hebner et al., 2008).

The comparison between reconstituted BM and collagen I is discussed in detail in section 1.5.1.

2. Chapter 2: Three-dimensional collagen inhibits the cell cycle in breast cancer cells

2.1. Abstract

The behavior of breast epithelial cells is influenced by their microenvironment, which includes stromal cells and extracellular matrix (ECM). During cancer progression, the tissue microenvironment fails to control proliferation and differentiation, resulting in uncontrolled growth and invasion. Upon invasion, the ECM encountered by breast cancer cells changes from primarily laminin and collagen IV to primarily collagen I. We show here that culturing breast cancer cells in 3-dimensional (3D) collagen I inhibits proliferation through direct regulation of cyclin E1, a G_1/S regulator that is overexpressed in breast cancer. When the breast cancer cell line MDA-MB-231 was cultured within 3D collagen I gels, the G_1/S transition was inhibited as compared to cells cultured on conventional 2D collagen or plastic dishes. Cells in 3D collagen downregulated cyclin E1 protein and mRNA with no change in cyclin D1 level. Cyclin D1 relocalized to the cytoplasm in 3D cultures and this was accompanied by decreased phosphorylation of Rb, a nuclear target for both cyclin E1- and cyclin D1-associated kinases. Positive regulators of cyclin E1 expression, the transcription factor c-Myc and cold-inducible RNA binding protein (CIRP), were decreased in 3D collagen cultures, while the collagen I receptor β_1 integrin was greatly increased. Inhibition of β_1 integrin function rescued proliferation and

cyclin E1 expression as well as c-Myc expression and Rb phosphorylation, but had no effect on cyclin D1 localization. We conclude that cyclin E1 is repressed independent of effects on cyclin D1 in a 3-dimensional collagen environment and dependent on β_1 integrin interaction with collagen I, reducing proliferation of invasive breast cancer cells.

2.2. Introduction

Breast cancer is the most common cancer in American women and remains second only to lung cancer as the leading cause of cancer-related deaths among women. The information provided from traditional prognostic factors such as age, lymph node status or tumor grade is often not precise enough to tailor cancer diagnosis and treatment effectively. A better understanding of breast cancer biology and identification of accurate prognostic indicators and predictors of response based on the underlying molecular biology could profoundly decrease metastatic death (Duffy and Crown, 2008; Lu et al., 2009).

One important aspect of cancer biology is uncontrolled cell proliferation. Events affecting checkpoints that govern transit through the first gap phase (G_1 phase) of the cell cycle are observed frequently in human cancer. Cells are responsive to extracellular signals at G_1 phase to decide if cell division continues. Cyclin E1, as regulator of re-entry into G_1 from G_0 (Geng et al., 2003) and the G_1 to S phase (DNA synthesis) transition (Sherr and Roberts, 2004), has been identified as a prognostic factor in breast cancer. Cyclin E1 is overexpressed in

about 32% of breast tumors (Butt et al., 2005) where it is strongly predictive of poor patient outcome and has been implicated directly in breast cancer etiology (Keyomarsi et al., 1994; Potemski et al., 2009; Sieuwerts et al., 2006). Overall, a more detailed understanding of cyclin E1 function in breast cancer biology is needed in order to evaluate its potential as a therapeutic target, prognostic marker and/or predictive marker.

Mammary epithelial (ME) cells respond to signals from the extracellular matrix (ECM) by proliferating and differentiating into highly organized and polarized glands, but in neoplastic progression, the microenvironment is altered such that controls from the ECM are lost. The ECM primarily controls the expression of D-cyclins in early G_1 phase; however, cyclin E1 is also responsive to extracellular signals (Cho et al., 2005; Henriet et al., 2000; Koyama et al., 1996). $\alpha_2\beta_1$ integrin, a receptor for collagen I and laminin, can stimulate expression of cyclin E1 and cdk2 as well as cyclin E1/cdk2 complex formation in ME cells in a collagen I-dependent and cyclin D1-independent manner (Klekotka et al., 2001a). Based on this and the fact that breast cancer cells directly encounter collagen I during invasion once the BM is compromised (Gudjonsson et al., 2003; Ronnov-Jessen and Bissell, 2009), we set out to determine how a 3-dimensional (3D) environment composed of collagen I regulates cyclin E1.

The invasive ME adenocarcinoma cell line MDA-MB-231 was cultured either in conventional 2D monolayer on plastic or collagen I, or within a 3D collagen gel to mimic the environment that invasive breast cancer cells encounter as pericellular proteolysis begins. Our results demonstrate that β_1

integrin can inhibit the breast cancer cell cycle in a 3D collagen environment via negative regulation of cyclin E1, a very different outcome as compared to a 2D collagen environment.

2.3. Materials and Methods

2.3.1. Cell Culture

The human ME adenocarcinoma cell line MDA-MB-231 (HTB-26, <passage 20) and nontumorigenic human ME cell line MCF10A (CRL-10317, <passage 10) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Collagen I was prepared from rat tails using established methods (Elsdale and Bard, 1972) and assessed for polymerization and cell viability. Cells were cultured in 2D monolayer on plastic dishes ($2D^{P}$), 2D monolayer on plastic dishes coated with a thin (< 0.5mm) layer of 2.5% collagen I ($2D^{Cl}$), or 3D culture within intact collagen I ($3D^{Cl}$, 0.5cm). 3D collagen cultures were prepared as described with modifications (Wozniak and Keely, 2005a). Collagen gelation was induced by incubating half of the collagen solution at 37°C for 1h. Cells were suspended with the other half of liquid collagen and spread on top of the gelled layer. After incubation at 37°C for 1h, complete medium was added. Medium was changed every 2 days and cells were collected at 70-80% confluency for experiments.

2.3.2. Cell cycle analysis

For analysis of asynchronous cells, cells were grown for 48h. Cells from 2D cultures were trypsinized and resuspended in complete media followed by brief centrifugation. 3D collagen cultures were treated with 100U/ml highly

purified collagenase (Collagenase VII, Sigma) for 20-30min at 37°C. Cell pellets from the three culture conditions were analyzed by flow cytometry using Becton Dickinson FACScan (San Jose, CA) and CellQuest software. For analysis of synchronized cells, cells grown on $2D^{P}$ and $3D^{Cl}$ to 50%-60% confluence were synchronized in G_1 by serum deprivation for 48h followed by 24h treatment with 5μg/ml aphidicolin. Synchronized cells were stimulated with serum and harvested at the indicated times.

2.3.3. Western analysis

Whole-cell lysates were resolved by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat milk in TBS-0.05% TWEEN20. Primary antibodies against cyclin E1, cyclin D1, HuR, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); CIRP (Proteintech Group, Inc., Chicago, IL); Phospho-Rb(Ser780), total Rb and c-Myc (Cell Signaling Technology, Danvers, MA); and β1-integrin (BD Biosciences, San Jose, CA) were used. Blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Images were captured using Kodak Image Station 4000MM (Kodak Molecular Imaging) and intensities were quantified by KODAK Molecular Imaging Software v4.0.

2.3.4. Real-Time PCR

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Reverse transcription was performed using oligo$(dT)_{12-18}$ or random hexamers (Invitrogen).. cDNAs were amplified by real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Relative quantification

standard real-time PCR was carried out using an AB 7500 Fast Real-Time PCR System with these conditions: 10min at 95°C, 40 cycles of 15sec at 95°C, 1min at 60°C. Primers were: cyclin E1 and GAPDH as previously described (Guo and Hartley, 2006); 18s rRNA, forward 5'-CGAACGTCTGCCCTATCAACTT-3' and reverse 5'-ACCCGTGGTCACCATGGTA-3'. Threshold cycles (Ct) were normalized to GAPDH or 18s rRNA. Data were analyzed by Sequence Detection Software 1.31.22 (Applied Biosystems). For mRNA stability assays, cells were treated with 5μg/ml actinomycin D, total RNA extracted at the indicated time following treatment, and real-time PCR performed to determine mRNA half-life.

2.3.5. Immunofluorescence analysis

Cells grown on glass coverslips in 24-well plates to 70% confluency (Wozniak and Keely, 2005a) were incubated with primary antibodies against cyclin D1, CIRP, HuR, or Ki-67 (BD Biosciences) and Alexa Fluor 488 conjugated secondary antibodies. Coverslips were mounted onto slides with *VECTASHIELD* containing DAPI (Vector laboratoryoratories, Inc, Burlingame, CA). Slides were analyzed using a Zeiss Axiovert 200M or a Zeiss LSM 510 Meta confocal microscope and associated software.

2.3.6. β_1 integrin function blocking assay

MDA-MB-231 cells grown in 3D collagen for 24h were treated with media containing AIIB2, a β_1 integrin function-blocking antibody (Hall et al., 1990), for 2h and then collected for Western or immunofluorescence analysis. AIIB2 (Developmental Studies Hybridoma Bank, Iowa City, IA), or IgG_1 as a control were used at 0.24mg/ml as this concentration efficiently blocked β_1 integrin

function in MDA-MB-231 cells grown in Matrigel (Park et al., 2006b).

2.4. Results

2.4.1. 3D collagen culture inhibits cell cycle progression

To test if the ECM can control ME cell proliferation through direct regulation of cyclin E1, MDA-MB-231 breast cancer cells were cultured in 2D monolayer on plastic or collagen I, or within 3D collagen gels. We chose MDA-MB-231 cells because cyclin E1 is highly expressed in these estrogen-receptor negative, invasive cancer cells as compared to nontumorigenic ME cells, such as MCF10A (Porter and Keyomarsi, 2000a). While MCF10A cells express only full-length cyclin E1, MDA-MB-231 cells express both full length and low molecular weight (LMW) isoforms, consistent with their tumorigenicity [(Harwell et al., 2000), Fig. 2.2a].

Cell cycle phase distribution was obtained by flow cytometry of asynchronized cells as shown in Fig. 2.1a. While 2D monolayer cells had a similar cell cycle profile, in 3D collagen G_1 phase cells increased by 5% while S and G_2/M cells both decreased by 6%. There was also a significant increase in hypodiploid cells in 3D collagen (7.8%) as compared to 2D cultures (1%) indicating increased cell death. This was further confirmed by DAPI staining showing condensed and fragmented nuclei characteristic of apoptosis (data not shown). The cell cycle profile was also analyzed in cells synchronized at the G_1/S border. Cells on 2D plastic were compared to cells in 3D collagen since 2D asynchronized cultures showed similar cell cycle profiles. Synchronized cells on 2D plastic completed an entire cell cycle within 20h after serum stimulation (Fig.

2.1b and c). In 3D collagen, there was a delay entering S phase (Fig. 2.1b and c). While 49% of cells cultured in monolayer had entered S phase by 4h following serum stimulation, only 10% of cells cultured in 3D had done so. By 20h, 47% of 3D cells were still in G_0/G_1 while 41% were in S phase. Surprisingly, up to 72 hours after serum stimulation the majority of 3D cells (45%) were still stagnant in G_1/S and 14% of cells were hypodiploid (data not shown). These data showed that 3D collagen I significantly delayed the G_1/S phase transition in synchronized cells.

Immunofluorescence analysis of Ki-67, a nuclear protein expressed by cells in all phases of the cell cycle but absent in resting (G_0) cells, was used to assess proliferation index in the 3 culture conditions. Fig. 2.1d shows that 68% of cells on 2D plastic, 77% of cells on 2D collagen and only 48% of cells in 3D were Ki-67$^+$. These data suggest that a 3D environment composed of collagen I suppressed cell cycle progression by delaying entry into S phase and increasing the G_0 population.

2.4.2. 3D collagen downregulates cyclin E1 without changing cyclin D1

Since a significant G_1/S delay was observed in cells cultured in 3D collagen and cyclin E1 functions in the G_1/S transition, we first examined potential effects on cyclin E1. The relative level of cyclin E1 (full length and LMW isoforms) was determined by western blotting and quantitated after normalizing to β-actin (Fig. 2.2b). Cyclin E1 was decreased by 56% in 3D collagen compared to 2D plastic ($p<0.01$) and 42% compared to 2D collagen ($p<0.05$), with no significant difference between 2D cultures. Consistent with these results, real

time RT-PCR showed that there was no significant difference in cyclin E1 mRNA level between 2D cultures, while cyclin E1 mRNA was decreased by 71% in 3D compared to 2D plastic ($p<0.01$) and 59% compared to 2D collagen ($p<0.01$) (Fig. 2.2c). Thus inhibition of G_1/S transition was accompanied by cyclin E1 downregulation.

Cyclin D1 regulates entry into and progression through G_1 in response to the extracellular environment (Diehl, 2002) by inactivating Rb, thus freeing E2F for cyclin E1 transcriptional activation. Therefore, we assessed the cyclin D1 protein level. Cyclin D1 ran as a 37-kDa doublet, likely representing different splice variants (Solomon et al., 2003). There was no significant difference in cyclin D1 protein level between culture conditions (Fig. 2.2b), showing that the decrease in cyclin E1 was not secondary to decreased cyclin D1.

2.4.3. Cyclin D1 relocalized from the nucleus to the cytoplasm in 3D collagen culture

Since cyclin D1 is regulated by multiple mechanisms, including changes in subcellular localization (Gladden and Diehl, 2005), its localization was also assessed. Interestingly, cyclin D1 was predominantly nuclear in 2D cultures and relocalized to the cytoplasm in 3D cultures (Fig. 2.3a). Since cyclin D1 exhibits its regulatory function in the nucleus, its presence in the cytoplasm indicated a potential continued activation of Rb and thus transcriptional downregulation of cyclin E1, consistent with the decrease seen in cyclin E1 mRNA. To investigate if cyclin D1 relocalization affected phosphorylation and thus activity of Rb, western blotting was performed using an antibody recognizing Rb phosphorylated on

Ser780, a cyclin D-cdk4/6 phosphorylation site (Lundberg and Weinberg, 1998). Ser780 phosphorylation was decreased in 3D as compared to 2D cultures as was total Rb (Fig. 2.3b). There was also a significant increase of phospho-Rb in 2D collagen compared to 2D plastic, consistent with increased Ki-67 (Fig. 2.1d) in 2D collagen cultures.

2.4.4. Positive regulators of cyclin E1 were decreased in 3D collagen culture

The transcription factor c-Myc is expressed in response to extracellular signals such as mitogens and adhesion and induces expression of cyclin D, cdk4, and E2F, among others. c-Myc was also reported to upregulate cyclin E1 in response to 2D collagen attachment (Benaud and Dickson, 2001a). Therefore, we monitored the c-Myc protein level to determine if it was affected by 3D collagen culture. 3D collagen culture decreased c-Myc levels by 26% compared to 2D cultures (Fig. 2.3c), consistent with the increased G_1 population, decreased Rb phosphorylation, and decreased cyclin E1 mRNA.

In addition to transcriptional control, cyclin E1 mRNA stability is positively regulated in breast cancer cells by the RNA binding proteins HuR and CIRP (Guo and Hartley, 2006; Guo et al., 2009). To ask if regulation of these proteins could also underlie changes in cyclin E1, we assessed their relative protein levels. Western analysis showed no significant difference in HuR while CIRP was decreased by 30% in 3D as compared to 2D cultures (Fig. 2.4a). CIRP and HuR localization was also assessed as they shuttle from nucleus to cytoplasm to stabilize their mRNA targets (Aoki et al., 2003; Brennan and Steitz, 2001). While

HuR was primarily nuclear in all conditions, CIRP was cytoplasmic in 3D cultures (Fig. 2.4c and d). Despite this, cyclin E1 mRNA stability was not significantly altered by 3D culture (Fig. 2.4b) showing that mRNA destabilization did not contribute to the decrease in cyclin E1 mRNA. It is possible that either the decrease in CIRP or its relocalization to the cytoplasm represses cyclin E1 translation (De Leeuw et al., 2007) or affects other CIRP target mRNAs to stall G_1 phase progression (Nishiyama et al., 1997).

2.4.5. β_1 integrin mediated downregulation of cyclin E1

We next asked how 3D collagen could activate signalling pathways that result in downregulation of cyclin E1. Cellular interactions with collagen matrices are mediated by specific receptors, including integrins, with $\alpha_2\beta_1$ integrin being the primary heterodimer on epithelial cells (McCall-Culbreath and Zutter, 2008a). Western blotting for β_1 integrin was performed to see if protein levels differed in the 3 culture conditions. As shown in Fig. 2.5a, there was a marked increase of β_1 integrin in 3D collagen. The nonspecific band below the integrin band was observed in other cell lines (BD Biosciences, San Jose, CA) and did not change between conditions. As $\alpha_2\beta_1$ integrin is a collagen I receptor responsible for delivering ECM signals to the cells (Klekotka et al., 2001a), and the cells were surrounded by collagen I, increased β_1 integrin was likely a functional adaptation to this environment.

To determine if reduced cyclin E1 expression required β_1 integrin interaction with collagen I, a function-blocking antibody (AIIB2) that binds the β1 integrin extracellular domain was used to disrupt β_1 integrin association with

collagen (Hall et al., 1990; Park et al., 2006b). Cells were cultured in 3D collagen for 24h and then treated with AIIB2 or IgG_1 (mock) for 2h. Cyclin E1 levels increased by 34% after blocking β_1 integrin function and cell proliferation increased by 10% compared to the mock group (Fig. 2.5b and d). Despite the increase in cyclin E1, cyclin D1 remained cytoplasmic upon inhibition of β_1 integrin (Fig. 2.5c), suggesting that effects on cyclin E1 were independent of cyclin D1. Notably, there was a dramatic morphological change from spindle to rounded cell shape after treatment, indicating that AIIB2 disrupted cell attachment. Cell rounding was also observed in 2D cultures treated with AIIB2 (Fig. S1b), but cell proliferation was significantly decreased (Fig. S1a). These results indicated that inhibition of β_1 integrin association with collagen rescued cell proliferation in 3D collagen cultures by upregulating cyclin E1 without changing cyclin D1 localization. Blocking β_1 integrin function also restored Rb phosphorylation as well as Rb and c-Myc levels in 3D collagen culture (Fig. 2.6). The increased phospho-Rb and c-Myc were in agreement with increased cyclin E1 and increased cell proliferation upon inhibition of β_1 integrin function.

2.5. Discussion

We have shown that a 3D environment composed of collagen I suppresses proliferation of a highly aggressive human breast cancer cell line. The suppression of proliferation resulted from a delay in S phase entry from G_1 and an increase in G_0 cells. Both cyclin E1 protein and mRNA were decreased, consistent with the inhibition of G_1/S transition, while cyclin D1 protein level was

not affected. There was a dramatic shift in cyclin D1 localization from primarily nuclear to cytoplasmic in 3D collagen, decreased phosphorylation of Rb, a decrease in total Rb and c-Myc proteins, and a dramatic increase in β_1 integrin, a component of the collagen I receptor in epithelial cells. Inhibition of β_1 integrin function in 3D collagen increased cell proliferation and expression of cyclin E1, Rb, phospho-Rb, and c-Myc, while cyclin D1 remained cytoplasmic. These results show that cyclin E1 can be negatively regulated by the ECM downstream of β_1 integrin, bypassing cyclin D1.

β_1 integrin has been implicated in positive regulation of ME cell proliferation both *in vitro* and *in vivo* (Taddei et al., 2003), and is necessary for formation of mammary tumors in murine models (White et al., 2004). Disruption of β_1 integrin function results in decreased ME cell proliferation in a transgenic mouse model (Faraldo et al., 1998) and in both 2D and 3D tissue culture models (Pasqualini and Hemler, 1994; Wang et al., 1998). Proliferation of an ME cell line on 2D collagen was positively regulated via β_1 integrin induction of c-Myc through activation of the Src and MAPK (Mitogen-Activated Protein Kinase) signalling pathways (Benaud and Dickson, 2001b). c-Myc upregulated cyclin E1 expression, downregulated the cyclin-dependent kinase inhibitor (CKI) p27, and increased phosphorylation of Rb by cyclin E/cdk2 complexes (Benaud and Dickson, 2001a). Similarly, in normal primary human ME cells, $\alpha_2\beta_1$ integrin interaction with a 2D collagen matrix stimulated expression of cyclin E1 and cdk2 as well as cyclin E1/cdk2 complex formation (Klekotka et al., 2001a). Consistent with these studies, we noted that 2D collagen culture also increased proliferation

of MDA-MB-231 breast cancer cells, while blocking β_1 integrin reduced proliferation. Likewise, when MDA-MB-231 cells were cultured in 3D Matrigel, a reconstituted BM composed mainly of collagen IV and laminin, the addition of inhibitory β_1-integrin antibody reduced proliferation (Park et al., 2006b) and reverted the malignant phenotype of HMT-3522 T4-2 breast cancer cells (Weaver et al., 1997b). In contrast, nontumorigenic ME cells that form glandular-like structures in Matrigel, were resistant to β_1 integrin inhibition. Neither inhibiting apoptosis nor enhancing proliferation alone blocks lumen formation in these epithelial structures, indicating that nontumorigenic ME cells and breast cancer cells exert different mechanisms to maintain their phenotype in the same environment (Debnath et al., 2002).

In contrast to 3D Matrigel, we show that 3D collagen culture inhibits proliferation of MDA-MB-231 cells and blocking β_1 integrin function rescues this inhibition. Similar to the above studies, the mechanism is at least partially via direct regulation of cyclin E1, as β_1 integrin inhibition restores cyclin E1 expression as well as expression of c-Myc and phosphorylation of Rb without influencing cyclin D1 level or cytoplasmic localization. Thus, our results also implicate c-Myc in transcriptional regulation of cyclin E1 and cyclin E1/cdk2 in phosphorylation of Rb. Results similar to ours were reported for arterial smooth muscle cells, which arrest in G_1 on the surface of polymerized collagen I and proliferate on monomer collagen (Koyama et al., 1996). In this study, the CKI p27 was induced and suppressed cyclin E1/cdk2 without changing cyclin E1 level. Although cyclin E1 was not studied, 3D collagen likewise arrested

glomerular mesangial cells at G_0/G_1 phase (Tsuboi et al., 2000) associated with downregulation of E2F and its targets, including c-Myc, and dephosphorylation of Rb. Our findings add to these by showing that β_1 integrin interaction with collagen I can lead to negative regulation of cyclin E1, c-Myc and Rb. We did not address the role of CKIs such as p27 since cyclin E1 level itself was reduced. p27 is moderately expressed in MDA-MB-231 cells (Sweeney et al., 1998), thus we cannot rule out a possible role in inhibiting cyclin E/cdk2 and/or other cyclin/cdk complexes in 3D collagen, contributing to decreased proliferation.

To our knowledge, this is the first study to show cyclin D1 cytoplasmic relocalization in response to 3D collagen. Cyclin D1 overexpression in cancer is often caused by disruption of its degradation (Pontano and Diehl, 2008), which has been shown to accelerate mammary carcinogenesis. At the G_1/S boundary, cyclin D1 is phosphorylated by GSK3β followed by nuclear export to the cytoplasm, where cyclin D1 is recognized by $SCF^{Fbx4/\alpha B\text{-crystallin}}$ ligase, ubiquitinated, and targeted to the 26S proteasome. Knockdown of either Fbx4 or αB-crystallin inhibits cyclin D1 proteolysis and leads to its nuclear accumulation and cell transformation (Barbash et al., 2008). MDA-MB-231 cells lack αB-crystallin due to a chromosomal deletion (Lin et al., 2008). In light of this, the observation that in 3D collagen, cyclin D1 relocalized from the nucleus to the cytoplasm and remained there after integrin inhibition, was surprising. Our results suggest that cyclin D1 may be phosphorylated by GSK3β in response to a 3D collagen environment, resulting in its inactivation by nuclear export. The ability to directly regulate cyclin E1 in the absence of nuclear cyclin D1 may be

an adaptation of MDA-MB-231 and other cancer cells for survival and growth in a primarily collagen environment after invasion and during metastasis.

We had shown previously that the RNA binding proteins HuR and CIRP stabilize cyclin E1 mRNA, contributing to its overexpression in breast cancer cells. As microenvironment affects mRNA decay (Ross, 1995), we asked if these proteins decreased in 3D collagen cultures. HuR did not change but CIRP decreased and relocalized to the cytoplasm upon 3D collagen culture. Despite this, cyclin E1 mRNA half-life was not changed, suggesting that if the changes in CIRP contribute to cyclin E1 downregulation it is via another mechanism (De Leeuw et al., 2007) or effects on other targets.

In summary, our studies show that a 3D environment composed of collagen I can control the cell cycle in part through negative regulation of cyclin E1. This regulation is through β_1 integrin-activated signalling pathways that decrease c-Myc and ultimately result in downregulation of cyclin E1. The downstream signalling pathways as well as the control of β_1 integrin function in inhibiting proliferation need to be further investigated.

2.6. Figures and Figure Legends

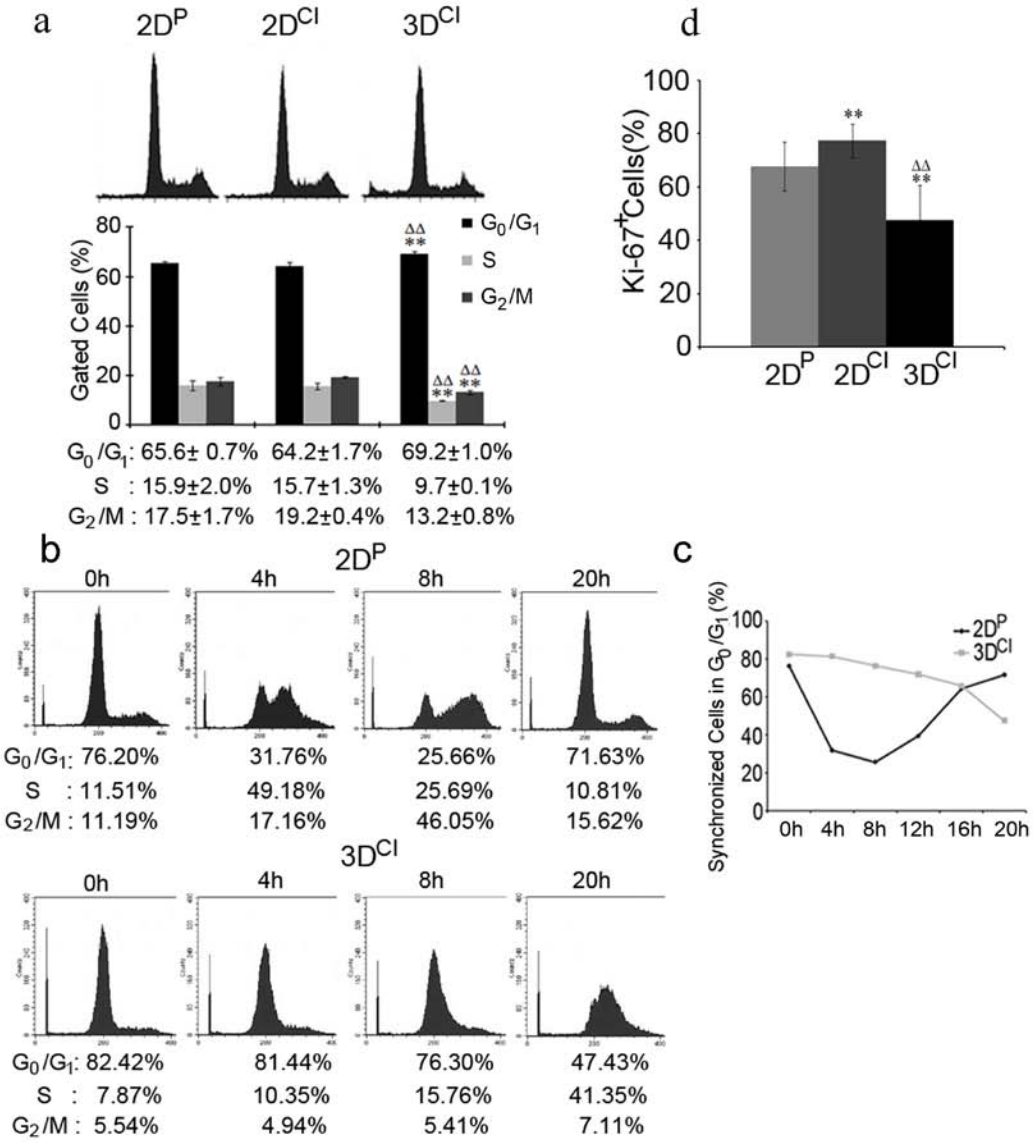

Figure 2.13D collagen culture inhibits cell cycle progression.

a Cell cycle analysis of asynchronous MDA-MB-231 cells in $2D^{P}$, $2D^{Cl}$, or $3D^{Cl}$. Cell cycle phase distribution was obtained and percentage of gated cells in

each phase were expressed as mean ± SD and assessed by Student's *t* test; **p < 0.01 vs. $2D^P$, $^{\Delta\Delta}$p < 0.01 vs. $2D^{CI}$. **b** Cell cycle analysis of synchronized MDA-MB-231 cells on $2D^P$ or in $3D^{CI}$. Data is representative of 2 experiments. **c** Percentage of synchronized cells in G_0/G_1 from B was plotted for the indicated time points. **d** Quantitative analysis of proliferation in asynchronous cells. Percentage of Ki-67^+ cells was calculated by counting at least 10 40X fields (at least 300 cells) per condition. **p < 0.01 vs. $2D^P$, $^{\Delta\Delta}$p < 0.01 vs. $2D^{CI}$

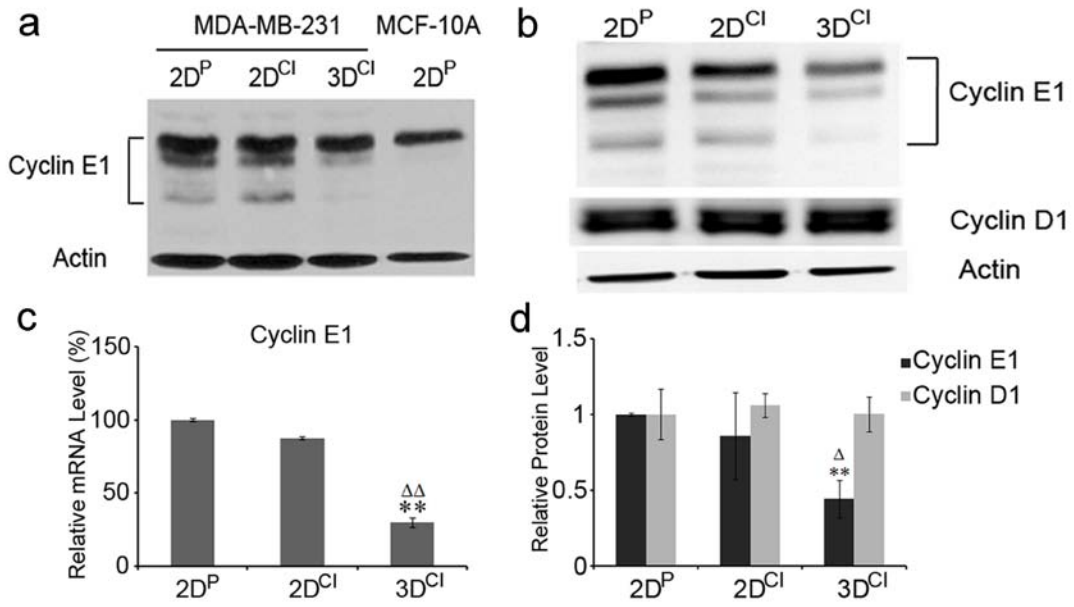

Figure 2.2 3D collagen culture downregulates cyclin E1 without changing cyclin D1.

a Western blot of cyclin E1 in $2D^{P}$, $2D^{CI}$ and $3D^{CI}$ MDA-MB-231 cells and $2D^{P}$ MCF10A cells. β-actin was used as a loading control. Representative of 2 experiments. **b** Western blots of cyclin E1 and cyclin D1 in $2D^{P}$, $2D^{CI}$ and $3D^{CI}$ MDA-MB-231 cells. The relative quantity of cyclin E1 and cyclin D1 was calculated after normalization to actin, expressed as mean ± SD and assessed by Student's *t* test. $^{\Delta}p < 0.05$ vs. $2D^{CI}$, $^{**}p < 0.01$ vs. $2D^{P}$. **c** Cyclin E1 mRNA level was determined by real-time RT-PCR. Threshold cycles (*C*t values) were normalized to GAPDH or 18s rRNA and plotted as relative mRNA levels. Values were expressed as mean ± SD and assessed by Student's *t* test. $^{**}p < 0.01$ vs. $2D^{P}$, $^{\Delta\Delta}p < 0.01$ vs. $2D^{CI}$

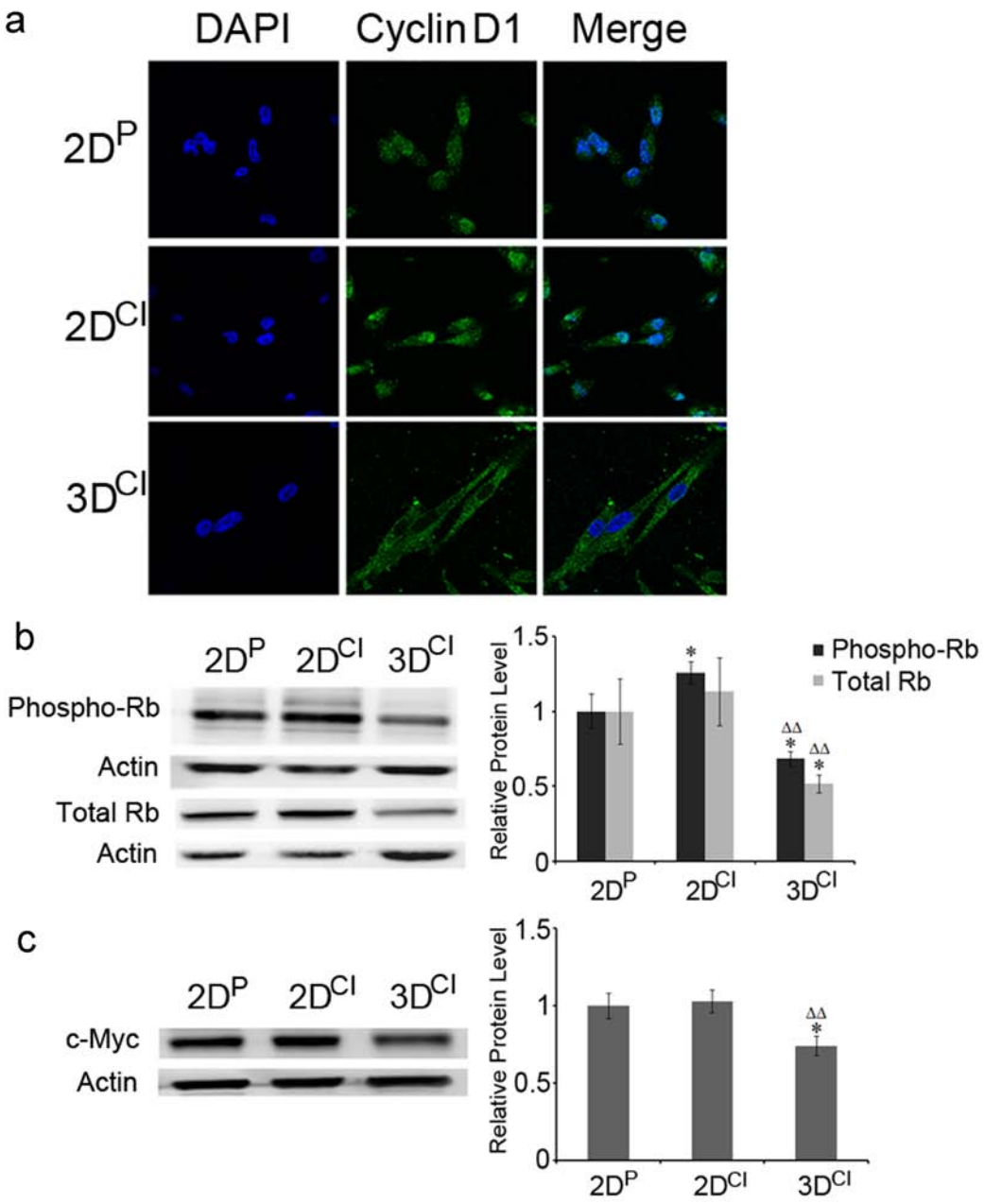

Figure 2.3 Cyclin D1 relocalized from the nucleus to the cytoplasm in 3D collagen culture.

a Confocal images of cyclin D1 in MDA-MB-231 cells. Cyclin D1 staining was visualized with Alexa Fluor 488 conjugated secondary antibody (green).

Nuclei were stained with DAPI (blue). Magnification 63X. **b** Western blots of phospho-Rb (Ser780) and Rb in $2D^{P}$, $2D^{Cl}$ and $3D^{Cl}$ MDA-MB-231 cells. *p < 0.05 vs. $2D^{P}$, $^{\Delta\Delta}$p < 0.01 vs. $2D^{Cl}$. **c** Western blot of c-Myc in $2D^{P}$, $2D^{Cl}$ and $3D^{Cl}$ of MDA-MB-231 cells. *p < 0.05 vs. $2D^{P}$, $^{\Delta\Delta}$p < 0.01 vs. $2D^{Cl}$. The relative quantity of phospho-Rb**,** total Rb or c-Myc protein was calculated as in Fig. 2b

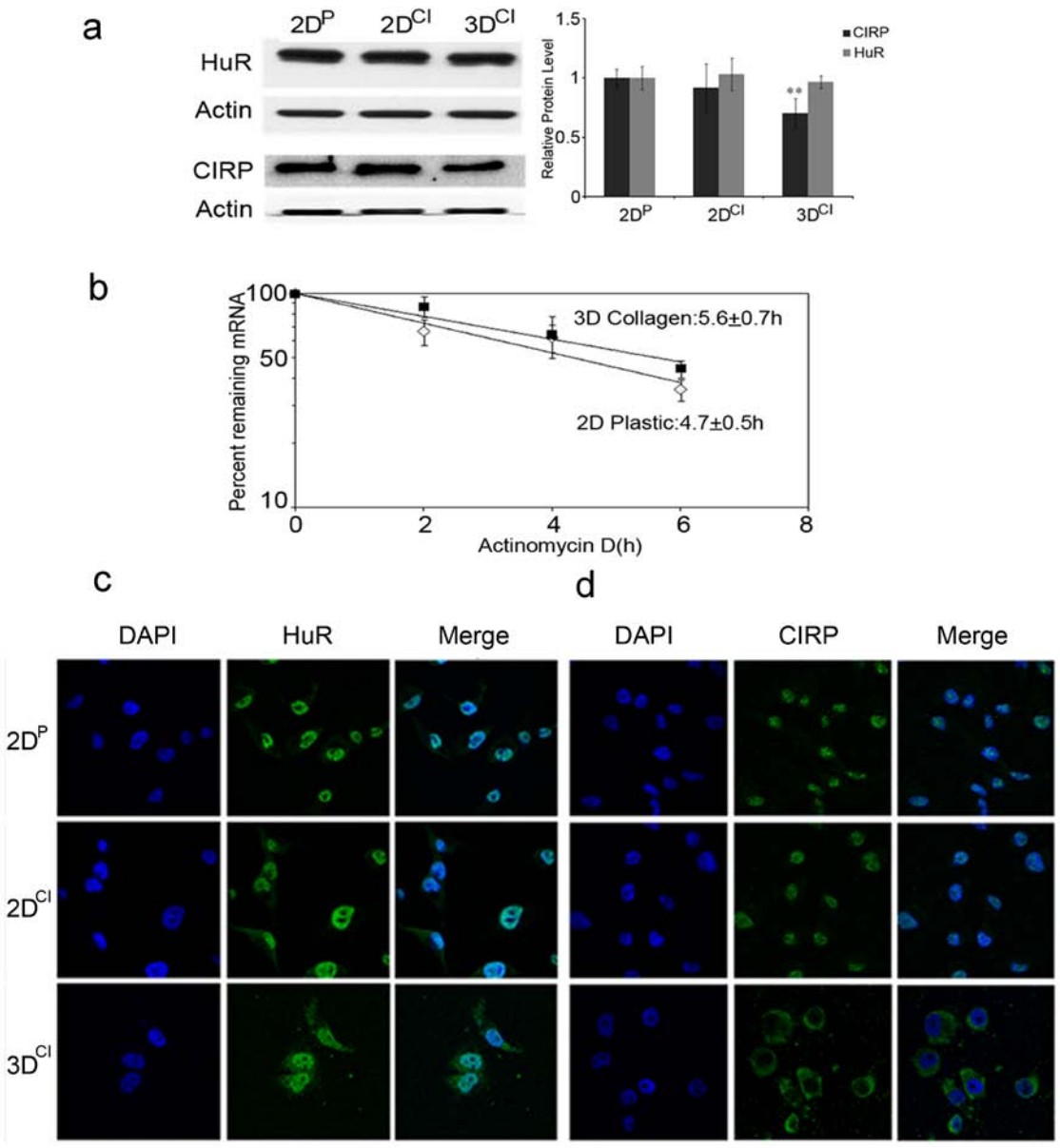

Figure 2.4 CIRP was decreased and relocalized to the cytoplasm in 3D collagen culture.

a Western blots for HuR and CIRP in 2DP, 2DCI and 3DCI MDA-MB-231 cells. ** $p < 0.01$ vs. $2D^P$. The relative quantity of HuR and CIRP proteins was calculated as in Fig. 2b. b Half-life determination for cyclin E1 mRNA. Total RNA

was extracted from cells grown on 2DP or in 3DCI at the indicated time after addition of actinomycin D. Real time RT-PCR was used to analyze cyclin E1 mRNA level. Data were normalized to GAPDH mRNA or 18s rRNA and plotted on a semi-logarithmic scale. c,d Confocal images of HuR and CIRP localization. HuR or CIRP staining was visualized with Alexa Fluor 488 conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Magnification 63X

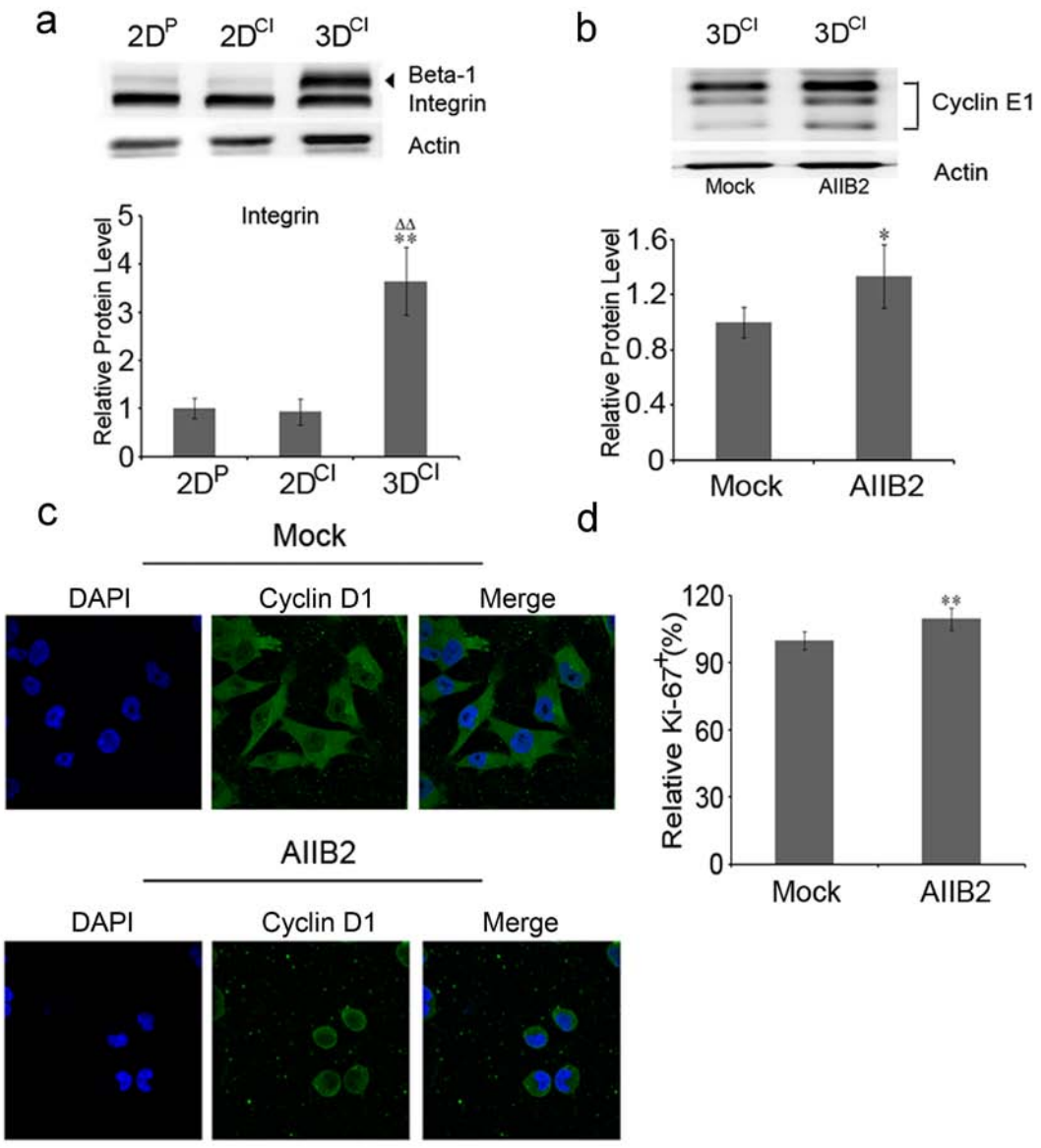

Figure 2.5 β_1 integrin was dramatically increased by 3D collagen culture.

a Western blot for β1 integrin in $2D^P$, $2D^{CI}$ and $3D^{CI}$ MDA-MB-231 cells. **p < 0.01 vs. $2D^P$, $^{\Delta\Delta}$p < 0.01 vs. $2D^{CI}$. The relative quantity of β1 integrin protein was calculated after normalization to actin, expressed as mean ± SD and assessed by Student's *t* test. **b** Western blot for cyclin E1 in $3D^{CI}$ cultures of

MDA-MB-231 cells with (AIIB2) or without AIIB2 (mock) antibody. *p < 0.05. **c** Confocal images of cyclin D1 in $3D^{Cl}$ MDA-MB-231 cells with or without AIIB2 treatment (63X magnification). **d** Quantitation of Ki-67 in $3D^{Cl}$ MDA-MB-231 cells with or without AIIB2 treatment. Percentage of $Ki\text{-}67^{+}$ cells was calculated by counting at least 10 microscope fields at 40X (at least 600 cells) for each condition. **p < 0.01

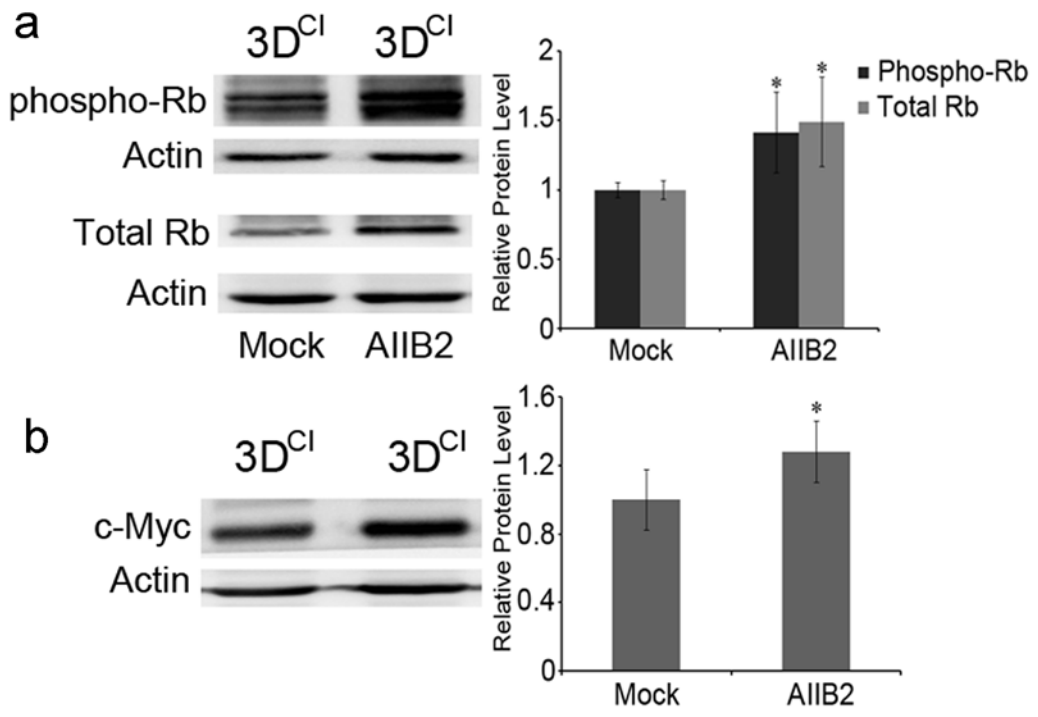

Figure 2.6 β_1 integrin inhibition increased Rb and its phosphorylation as well as c-Myc in 3D collagen.

a Western blots of phospho-Rb and total Rb in $3D^{Cl}$ cultures of MDA-MB-231 cells with (AIIB2) or without (Mock) β_1 integrin antibody. Cells were grown in $3D^{Cl}$ for 24h and then treated with AIIB2 or IgG_1 for 2h. *$p < 0.05$. **b** Western blot of c-Myc in $3D^{Cl}$ cultures of MDA-MB-231 cells with or without AIIB2 antibody. *$p < 0.05$. The relative quantity of phospho-Rb, total Rb or c-Myc was calculated as in Fig. 2b

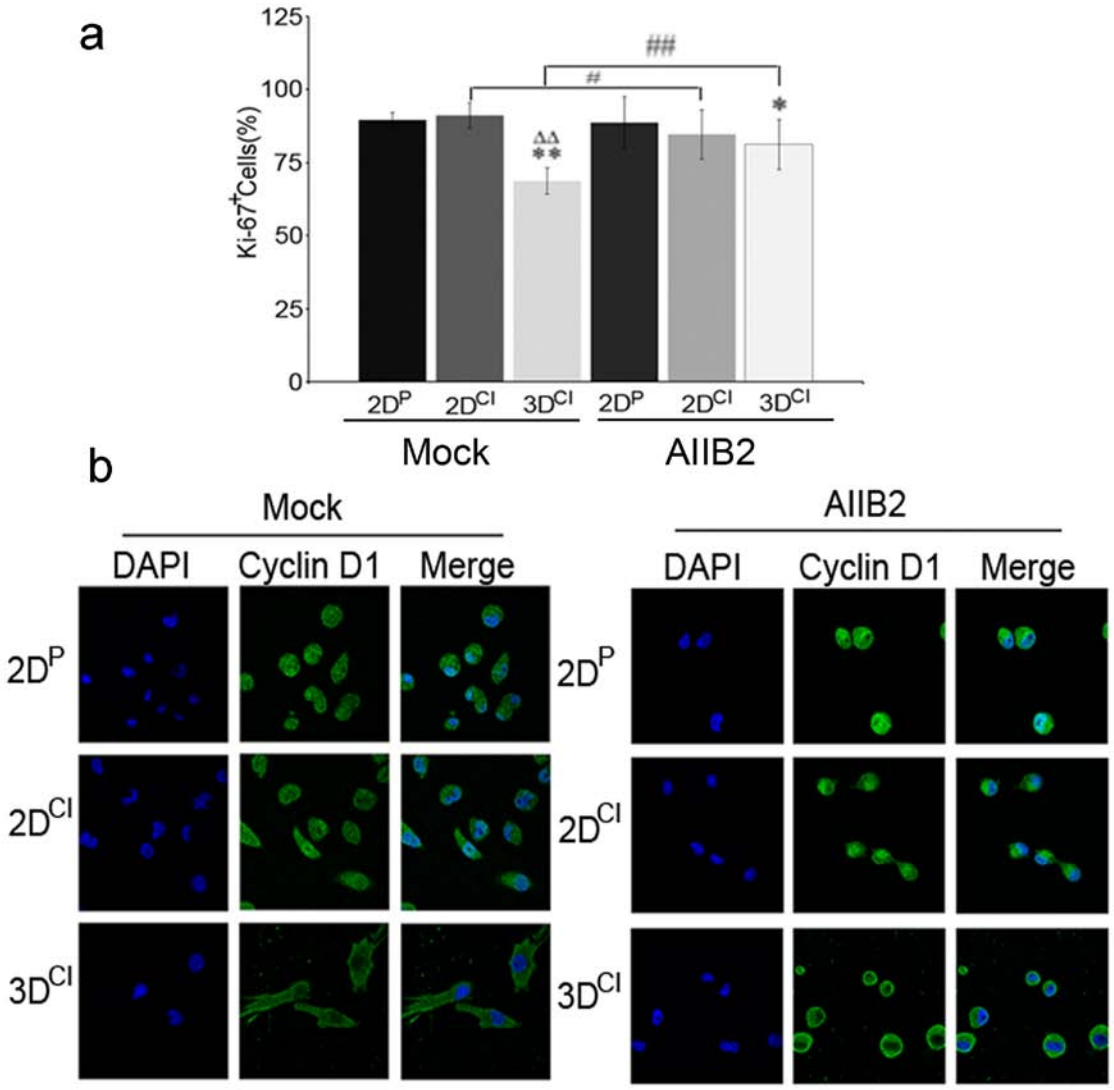

Figure S 1 Supplementary data.

a Quantitation of Ki-67 in $2D^{P}$, $2D^{CI}$ and $3D^{CI}$ MDA-MB-231 cells with or without AIIB2 treatment. Percentage of $Ki\text{-}67^{+}$ cells was calculated by counting at least 10 microscope fields at 40X (at least 500 cells) for each condition and repeated 3 times. $^{**}p < 0.01$ vs. Mock $2D^{P}$, $^{\Delta\Delta}p < 0.01$ vs. Mock $2D^{CI}$, $^{*}p < 0.05$

vs. AIIB2 $2D^{P}$, $^{\#}p < 0.05$ vs. Mock $2D^{Cl}$, $^{\#\#}p < 0.01$ vs. Mock $3D^{Cl}$. **b** Confocal images of cyclin D1 in $2D^{P}$, $2D^{Cl}$ and $3D^{Cl}$ MDA-MB-231 cells with or without AIIB2 treatment (63X magnification)

3. Chapter 3: The regulation of cyclin E1 in 3D collagen I by Matrix Metalloproteinases

3.1. Abstract

Matrix Metalloproteinases (MMPs) have multifaceted roles in breast cancer progression. In addition to promoting tumor metastasis through ECM degradation, MMPs function in all steps of cancer progression through their signalling functions including relieving proliferation suppression. Due to these important roles of MMPs in breast cancer, we set out to determine if MMPs are involved in cyclin E1 regulation within collagen cultures. The effects of exogenous MMPs and MMP inhibitors on regulation of cyclin E1 by 3D collagen were assayed. We found that exogenous addition of MMPs did not affect cyclin E1 but endogenous MMPs were highly induced in 3D collagen. Inhibition of these MMPs may rescue cyclin E1 suppression but did not change cyclin D1 localization in 3D collagen, suggesting negative regulation of cyclin E1 by MMPs induced by 3D collagen culture.

3.2. Introduction

The presentation of an intact ECM is one of several mechanisms that control ME cell proliferation *in vivo* (Jarvelainen et al., 2009). During cancer invasion the ECM is degraded by several proteinases, including MMPs (Brinckerhoff et al., 2000; Rudolph-Owen and Matrisian, 1998). Several MMPs are highly expressed in breast cancer and invasive breast cancer growth is preceded by an increase in MMP activity (Duffy et al., 2000). High levels of

MMP-2 (Talvensaari-Mattila et al., 2003), MMP-9 (Mylona et al., 2007), and MT1-MMP (Jiang et al., 2006; Tetu et al., 2006) all have a poor prognostic significance; while MMP-2 and -9 also have clinical value as diagnostic factors or predictive factors of metastases (Jinga et al., 2006; Somiari et al., 2006a; Somiari et al., 2006b).

In normal breast tissue, MMPs are tightly controlled to remodel the breast microenvironment during mammary gland branching and involution. However, during breast cancer progression, this normal control of MMP expression and activity is lost (Strongin, 2006). MMPs have long been thought to associate with late stages of tumor progression by promoting tumor metastasis through ECM degradation. MMP association with cancer has been dramatically expanded with evidence showing that MMPs function in all steps of cancer progression through their signalling functions (Egeblad and Werb, 2002). These signalling functions include release and/or activation of growth factors sequestered in the matrix or complexed to associated proteins, cleavage of cell adhesion molecules, and activation and /release of angiogenic factors and integrin subunits. MMPs thus regulate tumor cell proliferation/apoptosis; the epithelial to mesenchymal transition; angiogenesis and migration; and invasion and metastasis.

The sources of MMPs and their roles in breast cancer progression are reviewed and summarized in Fig. 3.1(Chabottaux and Noel, 2007). We are interested in how MMPs relieve proliferation suppression. This could be due to removal of physical constraints, loss of ECM epitopes that suppress proliferation, unmasking of epitopes that promote proliferation, changes in the

availaboratoryility of paracrine factors that suppress or promote proliferation, and/or changes in cell shape that activate cross-talk between growth and adhesion signalling networks (Chabottaux and Noel, 2007). In this study, we use a 3D collagen culture system to determine if MMPs relieve proliferation suppression via upregulation of the G_1/S regulator cyclin E1.

Collagen I, a major ECM component, is degraded by several MMPs including MMP-1, MMP-2, MMP-8, MMP-13, MT1-MMP, and MT3-MMP (Wolf et al., 2007). A 3D collagen culture system has been used extensively to study the contribution of MMPs to cancer cell migration and invasion (Barbolina et al., 2007; Sarkar and Yong, 2009; Wolf and Friedl, 2009). However, few studies have addressed the mechanism by which MMPs regulate cell proliferation. This may be due to the fact that MMPs usually function extracellularly and the observations that overexpressing several MMPs (MMP-1, -2, -3, -7, -9, -11, -13, MT1-, MT2-, MT3-MMP) in epithelial cells cultured atop collagen I gel did not alter cell proliferation (Hotary et al., 2000). In contrast, MT1-MMP conferred breast cancer cells with a growth advantage by directly driving proliferative responses within a 3D culture system rich in type I collagen, emphasizing the importance of using well-defined 3D culture systems to study cancer cell behaviors (Hotary et al., 2003).

MT1-MMP is also implicated in MMP-2 processing and its overexpression induces ECM remodeling and tumor formation in the mammary glands of transgenic mice (Ha et al., 2001). Cyclin D3-associated kinase activity increased in MT1-MMP transfected squamous cell carcinoma cell line UM-SCC-1(Hotary et

al., 2003). Similarly, impaired tyrosine phosphorylation of MT1-MMP in human fibrosarcoma cell line HT-1080 induced growth arrest only in 3D collagen. This growth arrest correlated with decreased cyclin D3 and Cdk4, and increased p16, a cdk4/6 inhibitor, without changing cyclin D1 expression (Nyalendo et al., 2008). MMP-9 and MMP-12 were shown to promote vascular smooth muscle cell proliferation via increasing cyclin D1 expression through β-Catenin-mediated signalling pathways (Dwivedi et al., 2009). Interestingly, siRNA against cyclin D1 decreased both MMP-2 mRNA and protein in non-small cell lung cancer along with an inhibition of tumor growth and metastasis (Huang et al., 2009). Taken together, these studies suggest a reciprocal regulation between MMPs and positive cell cycle regulators, such as D-type cyclins.

Based on the observations that cell-ECM interactions may link extracellular MMP function to the intracellular cell cycle machinery, and our results showing that 3D collagen inhibits the breast cancer cell cycle by decreasing cyclin E1 and bypassing cyclin D1 (Chapter 2), we sought to investigate if a 3D collagen environment decreases cyclin E1 via downregulation of MMPs. To address this question, the effects of exogenous MMPs and MMP inhibitors on regulation of cyclin E1 by 3D collagen were assayed. We also stably expressed MT1-MMP in the MDA-MB-231 cell line to see if cyclin E1 downregulation in 3D collagen was rescued (Appendix, Fig.1). As cyclin D1 was previously shown to be regulated by MMPs, we also assayed effects on cyclin D1.

3.3. Materials and Methods

3.3.1. MMP treatment

MMP-1, MMP-2 and MMP-9 were purchased from R&D Systems (Minneapolis, MN) and activated according to the product instructions. 2D plastic, 2D collagen and 3D collagen culture conditions are described in Chapter 2, section 2.3.1. MDA-MB-231 cells were cultured on 2D plastic with addition of soluble activated MMP-1, MMP-2 and MMP-9 as a pilot experiment before adding MMPs into 3D collagen cultures. Cells cultured within 3D collagen were incubated with no addition, TCNB (Tris, $CaCl_2$, NaCl, Brij35) buffer, or increasing amounts of MMPs in TCNB buffer in serum-free media at 37°C for 4h before lysis.

3.3.2. Western and Immunofluorescence Analyses

Western blotting for cyclin E1 was performed as described in Chapter 2, section 2.3.3. Cyclin D1 immunofluorescence assay was performed as in Chapter 2, section 2.3.5.

3.3.3. MMP Zymography

Conditioned media were collected from cells cultured on 2D plastic, 2D collagen, and within 3D collagen for 24h in serum-free medium. Endogenous MMP-2 and MMP-9 activities were assayed by substrate zymography with conditioned media. Briefly, SDS-polyacrylamide gels were co-polymerized with 1mg/ml gelatin. Samples were normalized by an equal cell number and loaded without reductant. Gels were washed with 2.5% Triton X-100 for 5min at room temperature and rinsed with water. Gels were then incubated for 24-48h in a buffer containing 50mM Tris-HCl, pH 7.6, 200mM NaCl, 5mM $CaCl_2$, 0.02% Brij-

35 and 0.02% NaN_3 (TCNB), stained with Coomassie Blue and destained with 10% acetic acid.

3.3.4. MMP activity inhibition

To inhibit endogenous MMP activity, cells were incubated with serum free medium containing 30µg/ml BB94 (a gift from Dr. Paul McGuire, UNM-HSC) for 24h and then collected for western blotting or immunofluorescence assays. We used 30µg/ml BB94 for 24h because this treatment condition reduced MMP activity in the conditioned media to basal levels in a pilot time course enzyme activity assay, in which cleavage of a fluorogenic peptide substrate ES010 by MMPs in the conditioned media with or without serial concentrations of BB94 for different periods of time was measured and compared (data not shown).

3.4. Results

3.4.1. Exogenous MMPs did not alter cyclin E1 protein level

MDA-MB-231 cells overexpress cyclin E1 and its tumor-associated low molecular weight (LMW) isoforms. We showed previously that cyclin E1 expression was reduced when MDA-MB-231 cells were grown within 3D collagen, as compared to cells grown on 2D plastic or collagen-coated dishes. Reduction of cyclin E1 by 3D collagen required interaction of β_1-integrin with the collagen matrix. As discussed above, a reciprocal regulation between MMPs and positive cell cycle regulators has been observed. Collagen I can be degraded by MMPs and MMPs can also regulate cell motility through activating integrin subunits such as β_3(Deryugina et al., 2002). Whether MMPs can regulate cyclin

E1 through integrin-mediated pathway in collagen is unknown. In order to determine if we could rescue cyclin E1 expression in 3D collagen by adding MMPs, we first needed to confirm that cyclin E1 levels in 2D cultures would not be affected by MMP addition as they already highly express MMPs. MDA-MB-231 cells grown on 2D plastic dishes were incubated with exogenous MMP-1, MMP-2 and MMP-9 for 24h before collection. Cells without any addition (control) or with buffer (TCNB) alone were collected as controls. Indeed, there was no change in cyclin E1 protein level upon treatment with increasing amounts of MMP-1, MMP-2, or MMP-9 (Fig. 3.2a). However, when cells cultured in 3D collagen I were treated with MMP-1, no change was observed in cyclin E1 level either (Fig. 3.2b), although cyclin E1 level appeared higher than 2D plastic due to blot overexposure. Therefore, exogenously adding soluble active MMPs did not affect cyclin E1 level in 2D plastic or in 3D collagen MDA-MB-231 cells.

3.4.2. Endogenous MMP-2 and MMP-9 were increased in 3D collagen cultures

Although exogenous MMPs did not appear to affect cyclin E1 in collagen cultures, breast cancer cell lines including MDA-MB-231, were found to activate serum-derived MMP-2 independent of MMP-2 production when they were plated on collagen I gels (Azzam et al., 1993). Therefore, we investigated whether these cells secret and activate MMPs when cultured in 3D collagen I. We looked at endogenous MMP expression and activity by zymography assays. 3D collagen not only induced pro-MMP-2 and pro-MMP-9 expression but also significantly increased active MMP-2 and MMP-9 levels (Fig. 3.3). Interestingly, 2D collagen

also increased pro-MMP-9 and MMP-9 expression as compared to 2D plastic. Although pro-MMP-2 was not increased in 2D collagen cultures compared to 2D plastic, active MMP-2 was significantly higher in 2D collagen cultures. These results show that a collagen environment significantly induced endogenous MMP-2 and MMP-9 expression and activity, with 3D collagen having the greatest effect.

3.4.3. Endogenous MMPs may participate in altering cyclin E1 level

To determine if the upregulation of MMPs was necessary for downregulation of cyclin E1 in 3D collagen cultures, we inhibited endogenous MMP activity by adding a broad-spectrum MMP inhibitor (BB94, Batimastat) to the culture media. While the cyclin E1 level in 2D cultures was similar with or without BB94, the decrease seen in cyclin E1 upon 3D culture (**$p<0.01$ vs. $2D^P$ in serum free media) was partially rescued upon BB94 treatment. This rescue trended towards significance, with a p value of 0.05 (Fig. 3.4). The variability in cyclin E1 level in 3D BB94 treated cultures could result from the variable penetration of BB94 through the 3D collagen between independent experiments, as MMP could be trapped in the collagen gel (Azzam et al., 1993).

3.4.4. Inhibition of MMP activity did not alter cyclin D1 localization in 3D collagen

In addition to reducing cyclin E1 expression, culturing cells in 3D collagen also changed the localization of cyclin D1 from the nucleus to the cytoplasm (Fig. 2.3), which would results in inactivation of its associated kinase. This relocalization was not dependent on β_1 integrin interaction with the matrix and did

not appear to be causal for reducing cyclin E1. Since MMPs are known to upregulate cyclin D1 and its associated kinase, we asked if inhibition of MMP activity in 3D collagen would reverse cyclin D1 relocalization. As shown by immunofluorescence analysis (Fig. 3.5), Cyclin D1 remained cytoplasmic upon BB94 treatment of cells in 3D collagen. This experiment indicated that cyclin D1 re-localization in 3D collagen was independent of endogenous MMP activity.

3.5. Discussion

Our results in Chapter 2 showed that 3D collagen I reduced cyclin E1 expression as compared to 2D cultures and this reduction required cell-collagen interaction through β_1 integrin. 3D collagen I also re-localized cyclin D1 and this did not appear to be dependent on β_1 integrin or the cause for cyclin E1 suppression. MMPs are highly expressed in breast cancer and have multiple functions in tumor etiology and a poor prognostic significance, mainly because of their enzymatic degradation of ECM, including type I collagen (Chabottaux and Noel, 2007). MMPs have also been suggested to positively regulate the cell cycle regulators, as well as to activate integrins to facilitate tumor cell behaviors. We set out to determine if MMPs are involved in the regulation of cyclin E1 by 3D collagen. Specifically, we asked if 3D collagen environment decreases cyclin E1 via downregulation of MMPs. We found that exogenous addition of MMPs did not affect cyclin E1 but endogenous MMPs were highly induced in 3D collagen. Inhibition of these MMPs may rescue the cyclin E1 suppression but did not change cyclin D1 localization in 3D collagen, suggesting a negative regulation between MMPs and cyclin E1 without changing cyclin D1.

The finding that adding soluble MMPs into 3D collagen cultures did not change cyclin E1 level was not surprising because as a highly invasive breast cancer cell line, MDA-MB-231 cells were recently reported to express 26 MMPs. These MMPs include the 3 used in our study, which enhance invasiveness (Hegedüs, L, 2008). As a result, exogenous MMPs may not have any effect due to constitutively high expression of MMPs by MDA-MB-231 cells.

We did observe a robust increase of MMP-2 expression and activity in 3D collagen compared to both 2D plastic and 2D collagen cultures. This is intriguing since it was previously reported that MMP-2 mRNA was not detectable in MDA-MB-231 cells cultured on 2D plastic (Morini et al., 2000). However, MMP-2 mRNA was increased when MDA-MB-231 cells were grown in Matrigel compared to 2D plastic cultures (Figueira et al., 2009). Consistently, culturing ovarian cancer cells on 3D collagen stimulated activation of pro-MMP-2 (Ellerbroek et al., 1999). Both of these cell types bind to collagen via β_1 integrins and integrin clustering stimulates MT1-MMP expression and activation, which ultimately promotes MMP-2 processing (Ellerbroek et al., 2001). In our study, β_1 integrin was significantly increased in 3D collagen (Fig. 2.5), suggesting that MDA-MB-231 cells possibly activate MMP-2 through β_1 integrin-mediated interaction with collagen I. MT1-MMP is a membrane-type MMP and is present and active on the cell surface (Barbolina and Stack, 2008). Therefore, the role of MT1-MMP in suppression of cyclin E1 and upreguation of MMP-2 in 3D collagen needs further study. We have successfully cloned MT1-MMP from nontumorigenic MCF10A ME cells (Appendix, Fig. 6.1). We will first monitor MT1-MMP expression and

activity in 3D collagen. If MT1-MMP expression and activity is similarly high as MMP-2 and MMP-9 in 3D MDA-MB-231 cells, MT1-MMP catalytic inactive mutants based on this clone can be used to see if downregulation of MT1-MMP will rescue cyclin E1 reduction.

We also showed that MMP-9 expression and activity was induced by both 2D and 3D collagen. MMP-9 has been found to be highly expressed and directly involved in MDA-MB-231 cell invasion in 2D cultures (Morini et al., 2000). Function blocking $\alpha_3\beta_1$ integrin antibody inhibited MDA-MB-231 cells migration and invasion, correlating with a reduction of MMP-9 activity. However, how blocking integrin function led to reduced MMP-9 was not addressed. Our results show that MMP-9 is induced to an even higher level by 3D as compared to 2D collagen culture in MDA-MB-231 cells, accompanied by increased expression of β_1 integrin. These results suggest that β_1 integrin-mediated regulation of MMP-9 may be different between 2D and 3D culture systems. They also suggest that upregulation of β_1 integrin may result in increased MMP-9 expression and activity, when breast cancer cells are embedded in 3D collagen. It has been shown that 3D collagen increases MMP-9 expression and activation in hepatic stellate cells via activation of the ERK signalling pathway (Takahara et al., 2003). The effect on MMP-9 was inhibited by neutralizing antibody against $\alpha_2\beta_1$ integrin, tyrosine kinase inhibitors, or a MEK inhibitor. Whether MMP-9 was induced through similar signalling pathways and which α integrin subunit (α_2 or α_3) was functioning in our model needs to be further investigated.

Increased MMP-2 and MMP-9 expression and activity in 3D collagen cultures of MDA-MB-231 cells also correlated with suppressed cell proliferation and a decrease in cyclin E1. The decrease seen in cyclin E1 upon 3D culture was rescued upon MMP inhibition, with a trend towards significance ($p = 0.05$). This was independent of effects on cyclin D1 since it remained cytoplasmic upon BB94 treatment (Fig.3.5).

Among its many functions, integrin is also involved in tumor invasion (Gui et al., 1995; Jia et al., 2004; Strizzi et al., 2004) and metastasis (Brakebusch et al., 1999; White and Muller, 2007). Since tumor cells do not appear to undergo invasion and proliferation simultaneously (Jung et al., 2001; Koochekpour et al., 1995; Palmqvist et al., 2000; Svensson et al., 2003), the increase seen in β_1 integrin and MMPs coupled to decreased cell proliferation in 3D collagen cultures might indicate a cell behavior transition from growth to invasion. In a recent study, 8 determinants of cancer cell invasiveness that are located upstream of MMPs in cell signaling pathways were transiently overexpressed in MDA-MB-231 cells, and alterations of 9 different MMPs (including MMP-2, MMP-9, and MT1-MMP) mRNAs were seen (Delassus et al., 2008). Each of these determinants regulated MMP expression through different sets of signaling pathways, resulting in identification of 20 new pathway links including 5 in breast cancer. Notably, many components of the cell signalling pathways linking these upstream determinants and MMPs are also involved in integrin-mediated signalling pathways, such as the Ras/Raf/MEK/MAPK/ERK shown in Fig.1.2. Future experiments will determine whether MDA-MB-231 cells become more invasive in

3D collagen and which integrin-linked signalling pathways regulate both MMP activity and cyclin E1.

3.6. Figures and Figure Legends

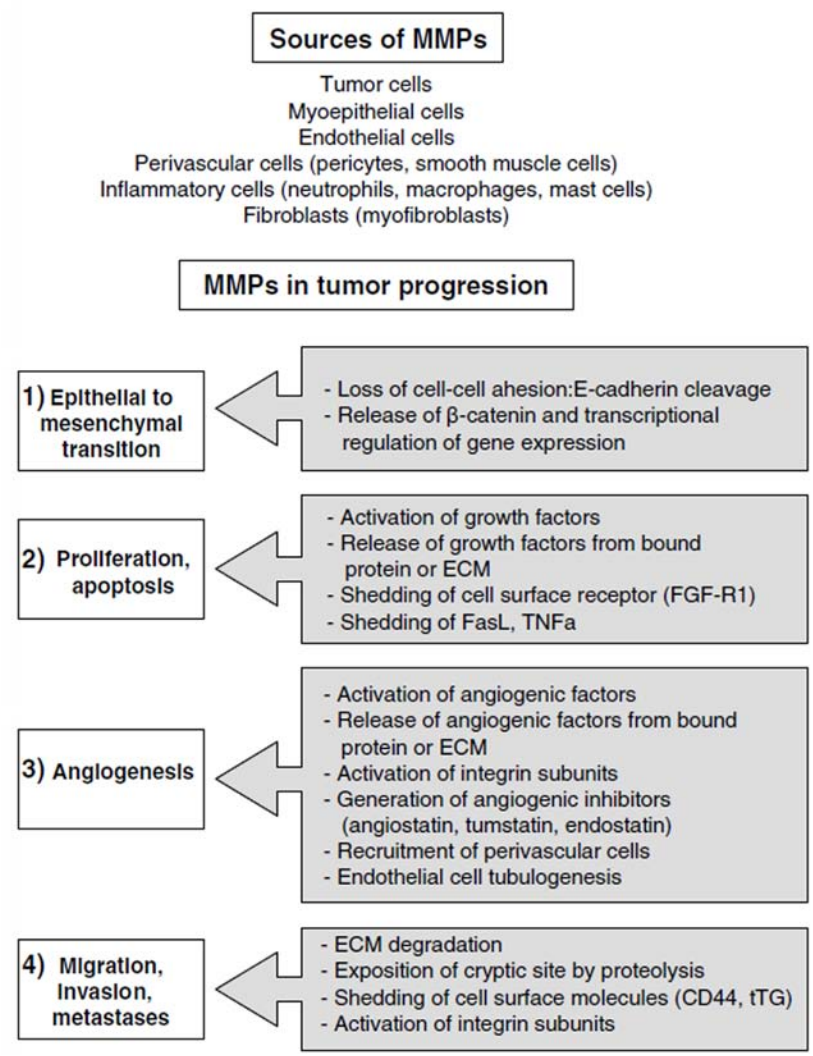

Figure 3.1 The sources and implication of MMPs in breast cancer progression. Modified from Fig. 2 in (Chabottaux and Noel, 2007).

Top panel indicates the sources of MMPs within the breast cancer microenvironment. 1)-4) refer to breast cancer cell behaviors during breast cancer progression. Boxes with arrows show the contributions of MMPs to each behavior and the possible mechanisms.

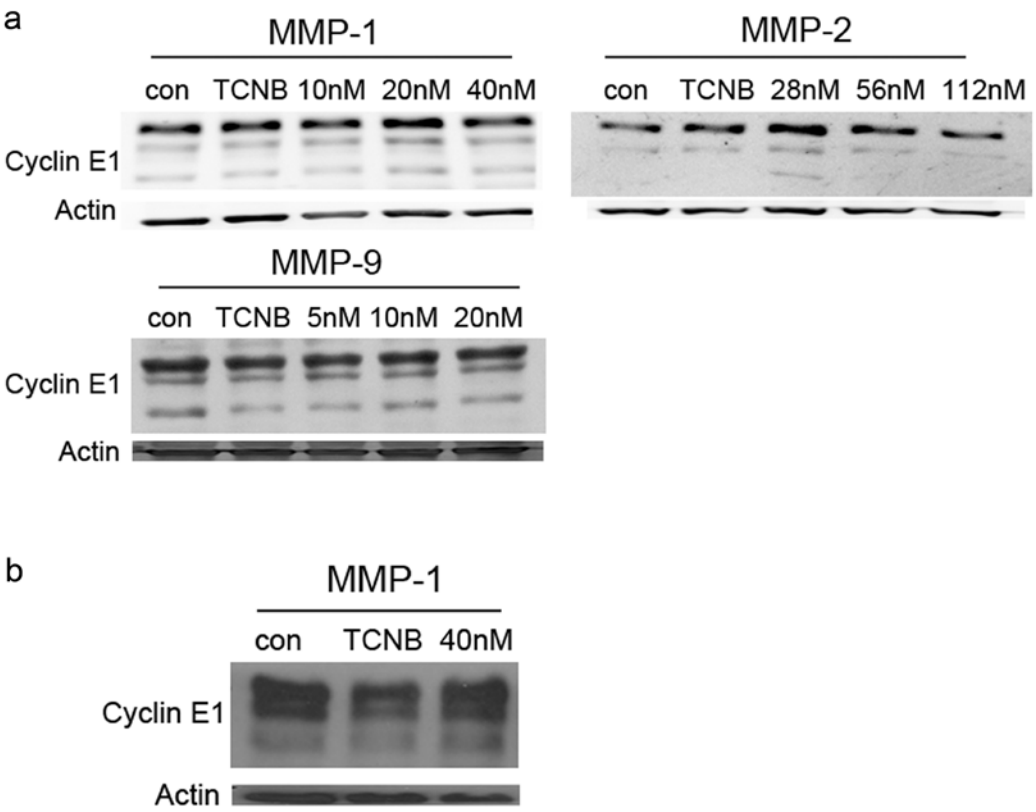

Figure 3.2 Exogenous MMPs did not change cyclin E1 protein level.

a. Western blotting for cyclin E1 in MDA-MB-231 cells cultured on 2D plastic and treated with the indicated MMPs in increasing concentration in serum-free media at 37°C for 4h before lysis. Controls were no addition (con) or buffer alone (TCNB). Blots are representative of 2 experiments. b. Western blotting for cyclin E1 in MDA-MB-231 cells cultured in 3D collagen and treated with 40 nM MMP-1 before lysis. Blot is representative of 2 experiments

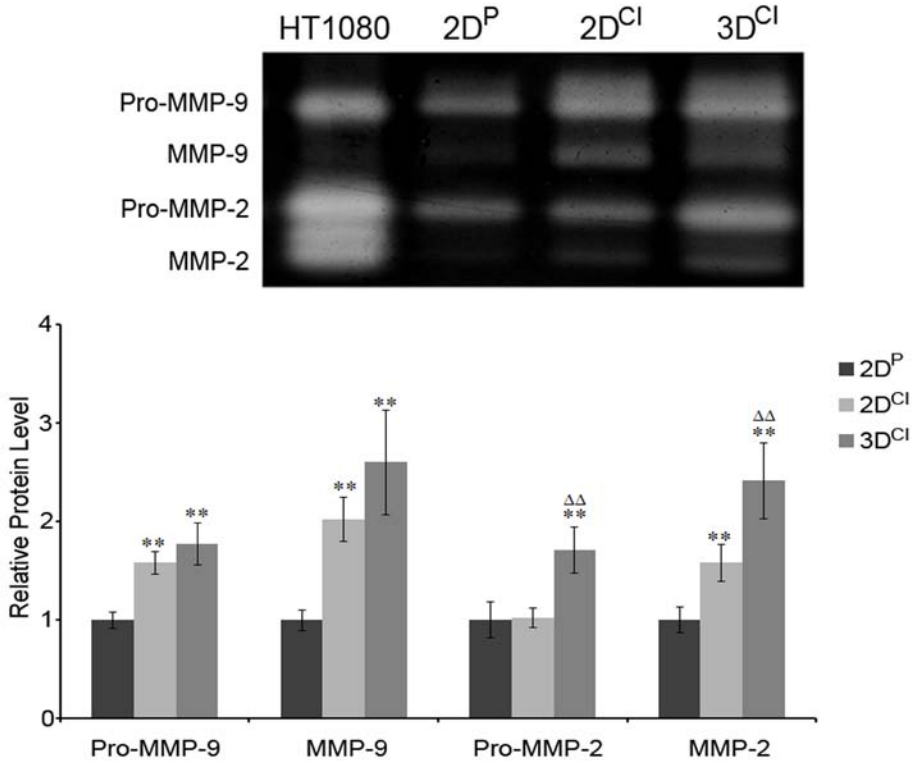

Figure 3.3 Endogenous MMP-2 and MMP-9 expression and activation by 2D and 3D collagen culture.

Conditioned media were collected from MDA-MB-231 cells cultured on 2D plastic ($2D^{P}$), 2D collagen ($2D^{CI}$), or within 3D collagen ($3D^{CI}$) for 24h in serum-free medium. Endogenous MMP-2 and MMP-9 activities were assayed by 1mg/ml gelatin substrate zymography with conditioned media. The volumes of conditioned media loaded was normalized to an equal cell number in each condition. HT1080 cell conditioned medium was used as a positive control. The relative quantity of pro-MMPs and MMPs were measured by intensity analysis on a Kodak imager system, expressed as mean ± SD, and assessed by Student's *t* test. $^{\Delta\Delta}p < 0.01$ vs. $2D^{CI}$, $^{**}p < 0.01$ vs. $2D^{P}$. Results are representative of 5 experiments

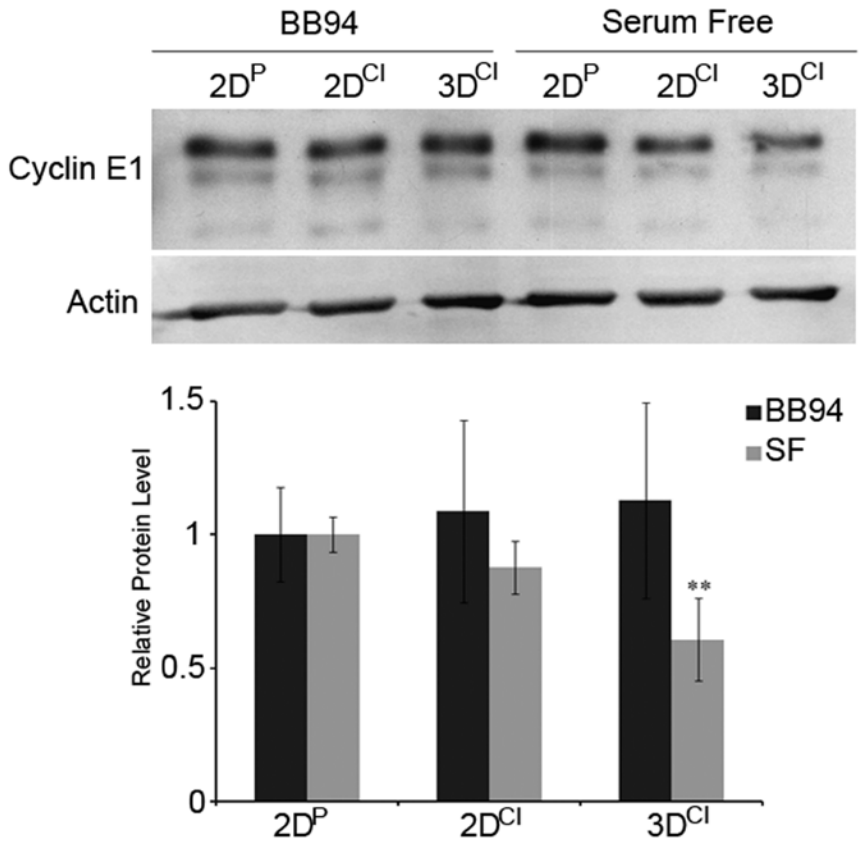

Figure 3.4 Endogenous MMP activity may regulate cyclin E1 level.

MDA-MB-231 cells cultured on 2D plastic ($2D^{P}$), 2D collagen ($2D^{CI}$), or within 3D collagen ($3D^{CI}$) were incubated with serum free medium alone or containing 30µg/ml BB94 for 24h and then collected for cyclin E1 western blotting. The relative quantity of cyclin E1 was calculated after normalization to actin, expressed as mean ± SD and assessed by Student's *t* test. **p < 0.01 vs. $2D^{P}$. Results are representative of 4 experiments

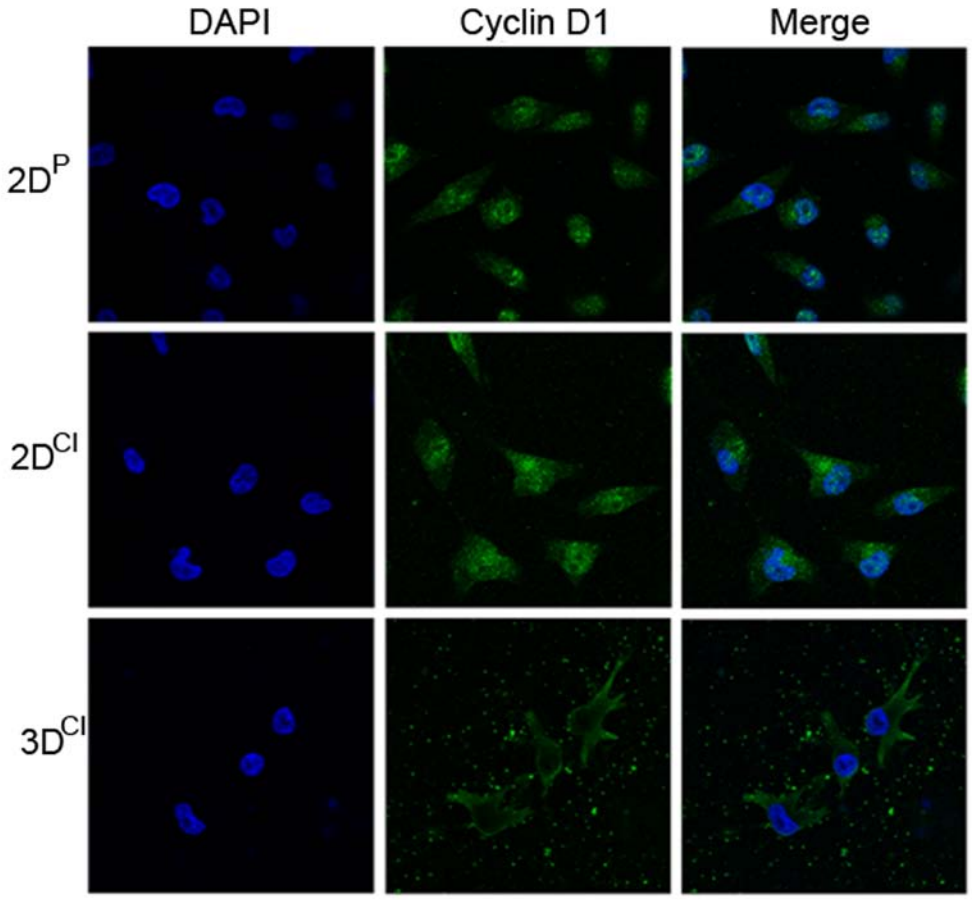

Figure 3.5 Inhibition of MMPs did not alter cyclin D1 cytoplasmic localization in 3D collagen cultures.

Confocal images of cyclin D1 localization after BB94 treatment of 3D collagen cultures of MDA-MB-231 cells. Cells were incubated with serum free medium containing 30µg/ml BB94 for 24h and then fixed. Immunofluorescence assay for cyclin D1 was performed as described in Chapter 2, section 2.3.5. Cyclin D1 staining was visualized with Alexa Fluor 488 conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Magnification 63X

4. Chapter 4: Regulation of cyclin E1 by 3D collagen in nontumorigenic MCF10A and tumorigenic HMT-3522 T4-2 mammary epithelial cells

4.1. Abstract

To address the hypothesis that cyclin E1 inhibition by a 3D collagen environment occurs in both nontumorigenic and tumorigenic ME cells other than MDA-MB-231 cells, we assayed effects of 3D collagen culture on the cell cycle and cyclin E1 level of nontumorigenic MCF10A and tumorigenic HMT-3522 T4-2 ME cells. Results showed that an ECM composed of collagen I negatively regulates the cell cycle in part through downregulation of cyclin E1 in these cell lines, similar to what we have shown for MDA-MB-231 cells. In contrast to the tumorigenic cell lines, MCF10A cells underwent a global G_1 arrest followed by apoptosis. These results suggest that tumorigenic ME cells are better adapted for survival in a 3D collagen environment.

4.2. Introduction

All of the data shown thus far are based on one invasive breast cancer cell line, MDA-MB-231, thus limiting the impact of our results. To begin to determine if the general model that an ECM composed of collagen I negatively regulates the ME cell cycle in part through downregulation of cyclin E1, we used the nontumorigenic ME cell line MCF10A and the malignant ME cell line HMT-3522 T4-2 (T4-2).

In this study, we addressed the hypothesis that cyclin E1 inhibition by a 3D collagen environment occurs in both tumorigenic and nontumorigenic ME cells. We proposed that the tumorigenic cells are able to partially overcome this inhibition by upregulating β_1-integrin, a collagen receptor, thus adapting themselves to this environment. We assayed effects of 3D collagen culture on the MCF10A cell cycle and on cyclin E1 levels in MCF10A and T4-2 cells. Results suggested that our model held true for the ME cells studied.

4.3. Materials and Methods

4.3.1. Cell Lines

The T4-2 cell line was a generous gift from Dr. Mina Bissell (Lawrence Berkeley National Laboratoryoratory) and was maintained on 2D collagen as recommended (Blaschke et al., 1994). MCF10A and MDA-MB-231 cells were cultured as described in Chapter 2, section 2.3.1.

4.3.2. Cell Cycle Analysis

MCF10A cells grown in 3D collagen were synchronized at G_1/S boundary by serum deprivation for 48h followed by 24h treatment with 5μg/ml aphidicolin in order to obtain cell cycle profile by FACS. The FACS profile of synchronous MCF10A cells on 2D plastic was provided courtesy of Dr. Xun Guo (UNM-HSC) and published (Guo and Hartley, 2006). Cells grown on 2D plastic were synchronized and collected by the same method.

T4-2 cell proliferation was assessed by immunofluorescence analysis with an antibody recognizing phosphorylation of serine 10 of histone H3. Phosphorylation of histone H3 begins at the end of S phase, is maximal in M

phase and is rapidly lost as cells enter G_1. Phospho-Histone-H3 (Ser 10) antibody was from Upstate Biotech (Millipore Corporate,Billerica, MA). Cells were grown in 2D collagen or 3D collagen with glass coverslips in 24-well plates to 70% confluency (Wozniak and Keely, 2005a), then incubated with phospho-Histone-H3 (Ser 10) antibody and Alexa Fluor 488 conjugated secondary antibodies. Coverslips were mounted onto slides with *VECTASHIELD* containing DAPI (Vector laboratoryoratories, Inc, Burlingame, CA). Slides were analyzed using a Zeiss Axiovert 200M microscope and associated software. Percentage of phospho-H3 positive cells was calculated by counting at least twenty 40X fields (at least 500 cells) per condition. For assays of apoptosis, cells were fixed and nuclei were stained with DAPI in order to visualize morphology. Percentage of apoptotic cells was calculated by counting condensed and fragmented nuclei characteristic of apoptosis, at least 20 40X fields (at least 500 cells) per condition.

4.3.3. Cyclin E1 mRNA analysis

The Cyclin E1 mRNA level was studied by quantitative real-time PCR and protein level by western analysis as described in Chapter 2, sections 2.3.3 and 2.3.4.

4.4. Results

4.4.1. 3D collagen arrested MCF10A cells in G_1

When tumorigenic MDA-MB-231 cells were cultured in 3D collagen, the G_1 population increased, consistent with the decrease seen in cyclin E1. To determine if 3D collagen culture had similar effects on nontumorigenic ME cells,

MCF10A cells were cultured in 3D collagen I, synchronized at the G_1/S boundary, and the cell cycle profile assessed by FACS (Fig. 4.1b). We had previously shown that synchronous MCF10A cells cultured on 2D plastic have a rapid cell cycle of 10-12 h [Fig. 4.1a, (Guo and Hartley, 2006)]. In contrast, when cultured within 3D collagen, MCF10A cells arrested in G_1 and hypodiploid cells increased in a time-dependent manner, indicating continuous cell death. For example, when the majority of the 2D MCF10A cells were in S phase at 4h after serum addition (79%), only 6% of 3D cells were in S phase and 37% were hypodiploid. By 10h, when 2D cells had almost completed the cell cycle, 37% of the 3D cells were still in G_0/G_1 phase and 50% of the cells were hypodiploid. Although these results are preliminary (the experiment was performed once), there appears to be a major difference in response to 3D collagen culture between nontumorigenic MCF10A and tumorigenic MDA-MB-231 cells, with a 5-10% increase in the G_1 population in tumor cells and at most 10% hypodiploid cells, while the majority of nontumorigenic cells underwent G_1 arrest and subsequently died (Fig. 4.3a-b and Fig. 2.1).

4.4.2. Collagen culture decreased cyclin E1 mRNA level in MCF10A cells

We next asked if a decrease in cyclin E1 accompanied the nearly global G_1 arrest. Real time RT-PCR showed that there was a significant decrease in cyclin E1 mRNA levels in MCF10A cells cultured on both 2D collagen (35%) and 3D collagen (48%) compared to 2D plastic. These results suggest that a type I collagen environment might inhibit cyclin E1 transcription (Fig. 4.2). We have not

yet assessed cyclin E1 protein level, but based on results with MDA-MB-231 cells in which the decrease of cyclin E1 mRNA was always accompanied by decreased protein (Fig. 2.2), and on the fact that cyclin E1 is a very unstable protein, we expect that cyclin E1 protein level will also be decreased. This will be investigated in future experiments.

4.4.3. 3D collagen effects on tumorigenic T4-2 cells.

To determine if 3D collagen would have similar effects on a different tumorigenic ME cell line, we studied the malignant breast cancer cell line HMT-3522 T4-2. We chose this cell line because it has a genetically matched nontumorigenic ME cell line HMT-3522 S1 (S1) with the same origin (Debnath and Brugge, 2005b). Due to technical difficulties in growing the cells, data from these nontumorigenic S1 cells have not yet been obtained. T4-2 cell proliferation was assessed by immunofluorescence analysis for phosphorylated serine 10 of histone H3, a mitotic cell marker. As T4-2 cells are routinely maintained on 2D collagen, 2D collagen data was compared to that of cells embedded in 3D collagen. As shown in Fig. 4.3b, 6.5% of cells were undergoing mitosis on 2D collagen but only 1.5% in 3D collagen. We also assessed apoptosis by DAPI staining followed by counting condensed and fragmented nuclei. Only 0.3% of cells showed characteristics of apoptosis on 2D collagen, while about 20% appeared apoptotic in 3D collagen. Although this experiment needs to be repeated, it is consistent with MDA-MB-231 data in which mitosis decreased and apoptosis increased in 3D as compared to collagen (Fig. 4.3a).

4.4.4. 3D collagen decreased LMW cyclin E1 in T4-2 cells

As preliminary data indicates that proliferation of T4-2 cells is decreased in 3D collagen, we next investigated cyclin E1 protein level by western analysis. Although full length cyclin E1 level was not significantly affected, the 40kDa LMW isoform of cyclin E1 was decreased by 70% in 3D collagen compared to 2D collagen culture (Fig. 4.4). The decrease of this LMW isoform was also seen in MDA-MB-231 cells, where 3D collagen significantly decreased by 77% (data not shown), although it was accompanied by a decrease of full-length cyclin E1. This result indicated that 3D collagen generally suppressed breast cancer cell proliferation at least partly through downregulation of cyclin E1.

4.5. Discussion

We showed previously that a 3D collagen environment suppresses synchronous breast cancer MDA-MB-231 cell proliferation by significantly delaying the G_1/S phase transition. We have now shown that in the same 3D collagen environment, synchronous nontumorigenic MCF10A cells arrest in G_0/G_1 phase and subsequently die. This cell cycle arrest was accompanied by decreased cyclin E1 mRNA, indicating that the mechanism for cell cycle arrest may be similar in these cells. MDA-MB-231 cells underwent limited apoptosis in both asynchronous (about 10%, Fig. 4.3a) and synchronous 3D collagen cultures (about 5%, Fig. 4.3b and Fig. 2.1). The increase in cell death in MCF10A cells suggests that nontumorigenic ME cells are either more sensitive to a collagen environment or that tumorigenic cells are more adaptable, as suggested by their ability to upregulate β_1 integrin (Fig. 2.5). When deprived of an appropriate ECM, i.e. BM *in vivo*, ME cells undergo detachment-induced apoptosis, a process

termed anoikis (Debnath and Brugge, 2005b). In contrast, breast cancer cells survive independent of attachment (Simpson et al., 2008). Our results are consistent with this, as nontumorigenic MCF10A arrested and died while the majority of MDA-MB-231 breast cancer cells survived in 3D collagen I, probably via upregulation of β_1 integrin. Although consistent with cell cycle arrest, the decrease of cyclin E1 mRNA in MCF10A cells could also be a result of cell death. It has been shown that under genotoxic stress, caspase-mediated cleavage of cyclin E1 generated a p18-cyclin E fragment in hematopoietic tumor cells. This fragment is unable to interact with cdk2 but can sensitize cells to apoptotic stimuli (breast ME cells were not studied), indicating a role for cyclin E1 in regulation of apoptosis (Mazumder et al., 2007). We have not seen this cyclin E1 fragment in MDA-MB-231 cells but we cannot exclude its possible role in promoting MCF10A cell apoptosis under the stress of a 3D collagen environment. Western blotting for cyclin E1 will be the first step to examine the existence of this cyclin E1 fragment.

Since MCF10A cells have a different genetic background from MDA-MB-231 cells, it is possible that the difference in response to a 3D collagen environment is due partially or completely to the differences in genetics, rather than a difference in tumorigenicity. However when ME cells having a matched genetic background (T4-2 and S1) were grown in 3D Matrigel, they had completely different responses (Debnath and Brugge, 2005b). While the nontumorigenic S1cells formed acini and the cells in the middle underwent apoptosis, tumorigenic T4-2 cells continuously grew and showed disorganized

structures. MCF10A cells express much lower cyclin E1 protein and do not express LMW cyclin E1 isoforms compared to MDA-MB-231 cells (Fig. 2.2), leading us to believe that the differences seen upon 3D collagen culture between these cells are at least partially due to the tumorigenic potential of MDA-MB-231 cells. In order to compare cells with a similar genetic background, we have begun to revert MDA-MB-231 cells to a non-invasive phenotype. MDA-MB-231 cells lack E-Cadherin expression and have been successfully reverted to form morphologically normal structures in 3D Matrigel by combining E-Cadherin expression with a β1-integrin/PI3K/MAPK inhibitor (Wang et al., 2002). We have successfully overexpressed E-Cadherin in MDA-MB-231 cells (Appendix, Fig. 6.2), as the first step towards this goal.

The preliminary results from the breast cancer cell line T4-2 were consistent with those from MDA-MB-231 cells. This cell line evolved from a line of immortalized normal human ME cells and is tumorigenic, exhibiting highly disordered growth in 3D Matrigel culture (Debnath and Brugge, 2005b). Growth on collagen coated dishes is required for them to maintain their tumorigenic phenotype. T4-2 breast cancer cells showed decreased proliferation in 3D collagen when compared to 2D collagen, along with increased apoptotic cells, similar to MDA-MB-231 cells, although this experiment needs to be repeated. Western analysis showed that only LMW cyclin E1 decreased in 3D collagen, not full length cyclin E1. The decrease of this LMW isoform in 3D collagen was also seen in MDA-MB-231 cells (data not shown), although it was along with a decrease of full-length cyclin E1. The ratio of each cyclin E1 LMW isoform as

compared to the full length protein has not been reported, except for a 43-kDa splice variant that was reported to be expressed at 1/10 the level of full length cyclin E1 (Sewing et al., 1994). The preferential decrease in LMW cyclin E1 may be due to a 3D collagen environment decreasing full length isform and thus the LMW isoforms, if they result from proteolytical processing of overexpressed full length protein (Porter et al., 2001); or by affecting alternative splicing of the cyclin E1 mRNA (Porter and Keyomarsi, 2000a). Considering that LMW cyclin E1 isoforms have higher affinity for cdk2 and are resistant to cdk inhibitors, as well as strongly correlate with decreased survival in breast cancer, their reduction by a 3D collagen environment in both T4-2 and MDA-MD-231 cells warrants further investigation. In addition, we transfected MDA-MB-231 cells with one of the LMW isoforms (Appendix, Fig. 6.3), providing the first step to re-rexpressing cyclin E1 to determine if proliferation reduction in 3D collagen will be rescued.

In summary, we used the nontumorigenic ME cell line MCF10A and the malignant ME cell line HMT-3522 T4-2 to show that an ECM composed of collagen I negatively regulates the cell cycle in part through downregulation of cyclin E1, similar to what we have shown for MDA-MB-231 cells. Although both cell lines showed suppressed cell proliferation in 3D collagen, differences from MDA-MB-231 cells were also obvious, as synchronous MCF10A cells subsequently underwent apoptosis and T4-2 cells decreased only a LMW isoform of cyclin E1. Whether cyclin E1 contributed to apoptosis and whether the cyclin E1 LMW isoform is differentially regulated compared to full-length cyclin E1 in T4-2 cells embedded in 3D collagen, need further investigation.

4.6. Figures and Figure Legends

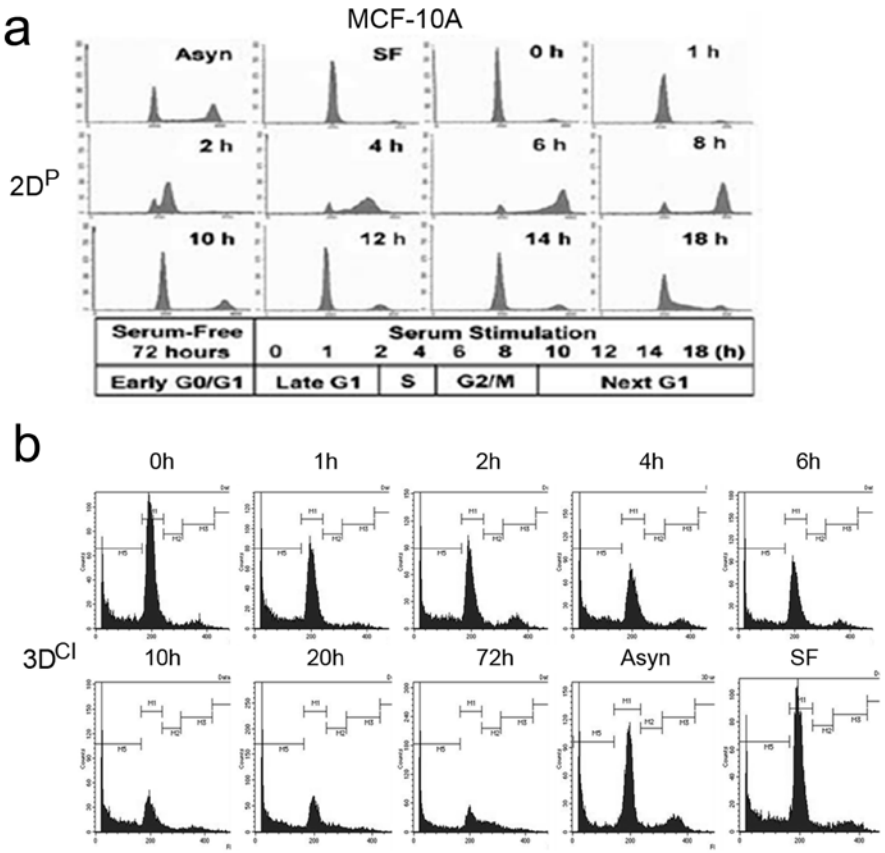

Figure 4.1 MCF10A cells undergo G_1 cell cycle arrest in 3D collagen.

Cells grown on $2D^P$ and $3D^{CI}$ to 50%-60% confluence were synchronized in G_1/S boundary by serum deprivation for 48h followed by 24h treatment with 5μg/ml aphidicolin. Cells were then stimulated with serum and harvested at the indicated times for FACS as described in Chapter 2, section 2.3.2. a. Cell cycle analysis of synchronous MCF10A cells on $2D^P$, courtesy of Dr. Xun Guo (Guo and Hartley, 2006). b. Cell cycle analysis of synchronous MCF10A cells in $3D^{CI}$. Experiment was performed once.

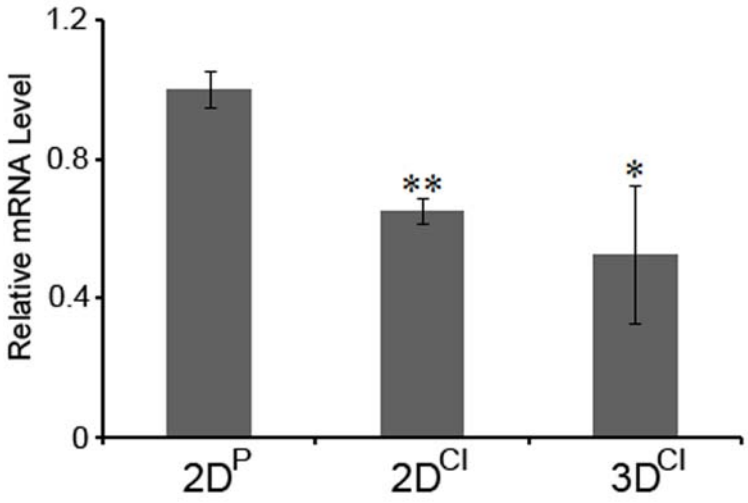

Figure 4.2 Collagen culture decreased cyclin E1 mRNA in MCF10A cells.

Cyclin E1 mRNA level was determined by real-time RT-PCR. Threshold cycles (*C*t values) were normalized to GAPDH and plotted as relative mRNA levels. Data is average of 3 experiments. Values were expressed as mean ± SD and assessed by Student's *t* test. *$p < 0.05$ vs. $2D^P$, **$p < 0.01$ vs. $2D^P$

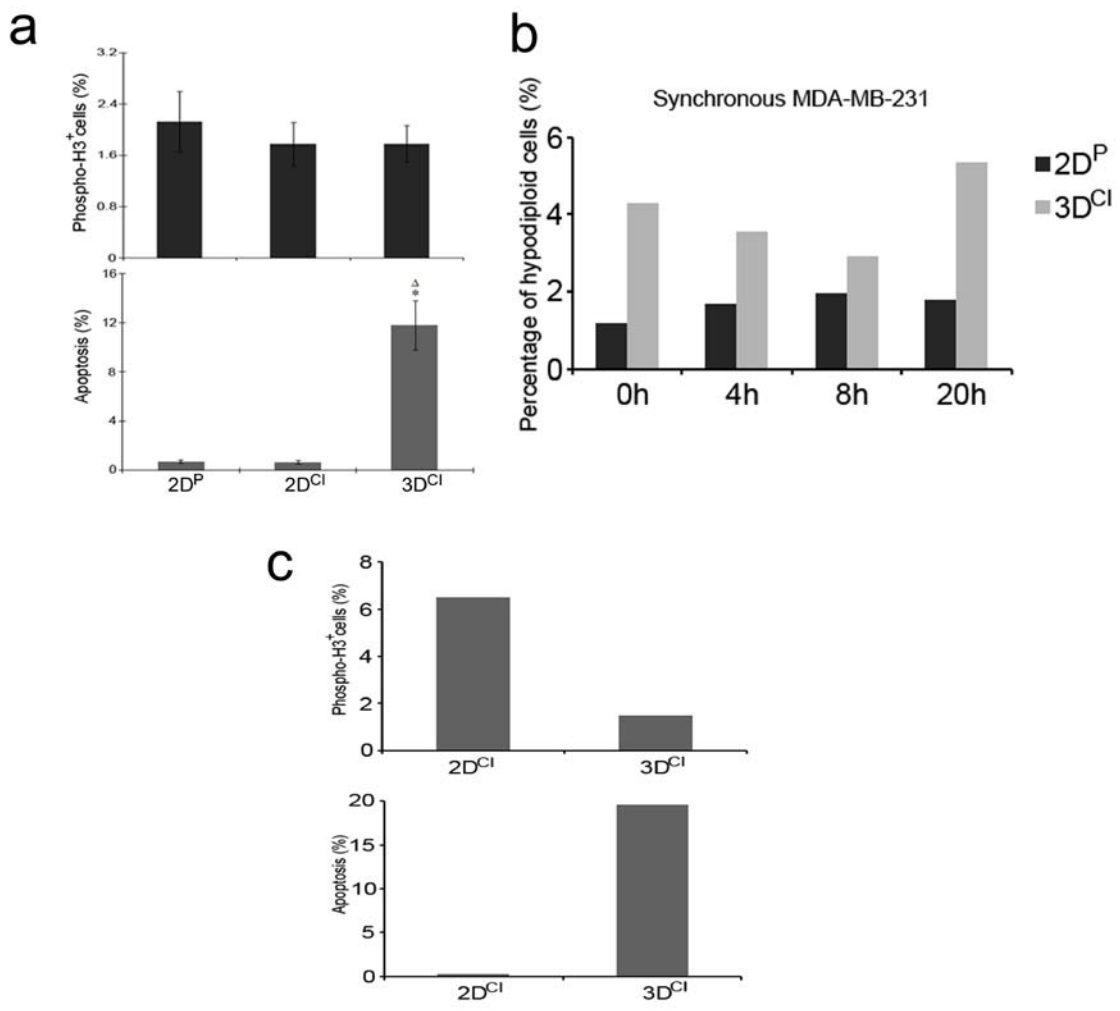

Figure 4.3 Quantitative analysis of mitosis and apoptosis in MDA-MB-231 and T4-2 cells.

a. Quantitative analysis of mitosis and apoptosis in asynchronous MDA-MB-231 cells. Immunofluorescence assay using Phospho-Histone H3 (Ser 10) antibody was performed to monitor cell proliferation in MDA-MB-231 cells cultured in $2D^{P}$, $2D^{CI}$ and $3D^{CI}$. Upper panel: Percentage of phospho-$H3^{+}$ cells was calculated by counting at least 20 40X fields (at least 500 cells) per

condition. Bottom panel: Percentage of apoptotic cells was calculated by counting condensed and fragmented nuclei stained with DAPI, at least 20 40X fields (at least 500 cells) per condition. Data is the average from 3 experiments. Values were expressed as mean ± SD and assessed by Student's *t* test. *p < 0.05 vs. $2D^{P}$, $^{\Delta}p < 0.05$ vs. $2D^{Cl}$ b. Percentage of hypodiploid cells in synchronous MDA-MB-231 cells was plotted for the indicated time points of Fig. 2.2. Data is representative of 2 experiments. c. 3D collagen decreased proliferation and increased apoptosis in HMT-3522 T4-2 cells. Immunofluorescence assay monitoring Phospho-Histone H3 (Ser 10) staining was done as described above in T4-2 cells cultured in $2D^{Cl}$ and $3D^{Cl}$. Upper panel: Percentage of phospho-$H3^{+}$ cells. Bottom panel: Percentage of apoptotic cells. Values were expressed as a mean of 2 experiments.

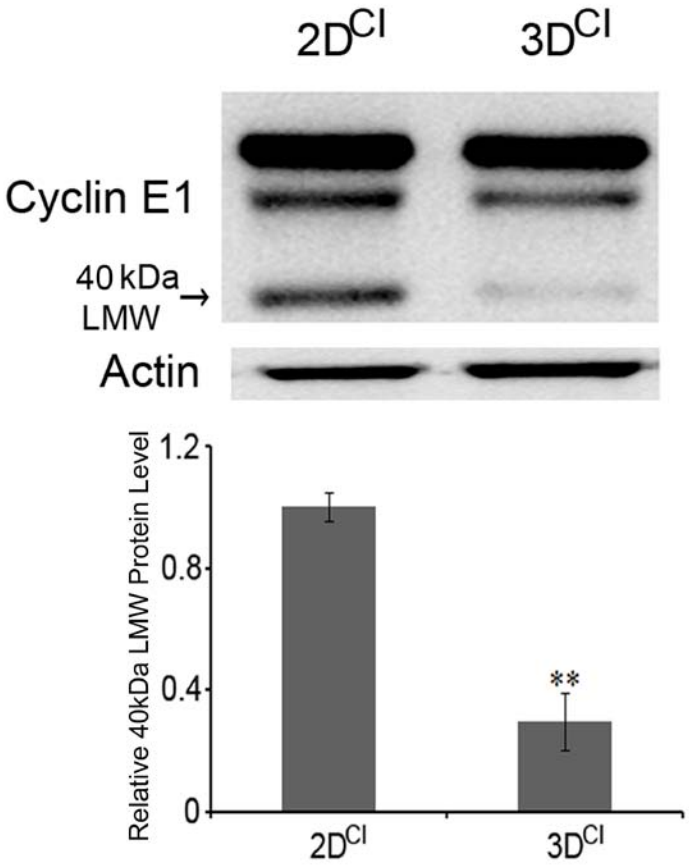

Figure 4.4 3D collagen decreased the 40kDa LMW isoform of cyclin E1 in T4-2 cells.

Western blots of cyclin E1 in T4-2 cells cultured on 2DCI and 3DCI. Data is representative of 3 experiments. The relative quantity of cyclin E1 and was calculated after normalization to actin, expressed as mean ± SD and assessed by Student's t test. **$p < 0.01$ vs. $2D^{CI}$.

5. Chapter 5: Final Conclusion/Discussion

In this project, we set out to determine if the breast cancer microenvironment controls the cell cycle in part through direct regulation of cyclin E1. We conclude that a 3D environment composed of collagen I, which mimics the environment when breast cancer cells first invade through the basement membrane into the interstitial stroma, can control the cell cycle in part through negative regulation of cyclin E1. Support for this conclusion has been obtained in different mammary epithelial cell lines including nontumorigenic MCF-10A cells, and tumorigenic MDA-MB-231 and HMT-3522 T4-2 cells, although most of our data are based on the highly invasive MDA-MB-231 adenocarcinoma cells. Importantly, this negative regulation is specific to 3D collagen as compared to 2D plastic and 2D collagen, providing strong evidence for the essential differences between 2D and 3D culture systems, and emphasizing the importance of considering the role of ECM when studying human cancers.

A model based on our data is presented in Fig. 5.1, to summarize the possible mechanisms by which 3D collagen controls the breast cancer cell cycle in comparison to 2D collagen. As shown in the left panel, when ME cells adhere to 2D collagen, mitogenic growth factors and cell anchorage through β_1 integrin converge on signalling pathways that result in activating cyclin D1/cdk4/6 activity (Bohmer et al., 1996). In the nucleus, cyclin D1 inhibits Rb by phosphorylation and this releases transcription factor E2F, promoting cyclin E1 transcription. Collagen adhesion also induces the transcription factor c-Myc through activation of the Src and MAPK signalling pathways (Benaud and Dickson, 2001b). This

can be independent of cyclin D1 and directly upregulates cyclin E1 expression (Benaud and Dickson, 2001a). Increased cyclin E1 promotes the G_1/S transition. In contrast, when ME cells are embedded in 3D collagen I (Fig. 5.1, right panel), cell proliferation is suppressed. Contact with 3D collagen induces a dramatic increase in β_1 integrin, followed by a decrease in c-Myc, cyclin D1 relocalization to the cytoplasm, and dephosphorylation of Rb. Thus E2F remains inactive and cyclin E1 transcription decreases. CIRP decreases in 3D collagen as well as relocalizes to the cytoplasm, without changing cyclin E1 mRNA stability.

In this model, several unresolved questions are worth further investigation. First, although β_1 integrin-mediated signalling seems to be involved in both 2D and 3D collagen regulation of cell cycle, the detailed signalling pathways in the 3D culture system have not been identified. β_1 integrin has been shown to positively regulate c-Myc expression through activation of Src and MAPK in 2D collagen. However, increased expression of β_1 integrin is associated with a decrease of c-Myc in 3D collagen. Whether Src or MAPK activation is decreased in 3D collagen and is involved in c-Myc regulation, or whether other signalling pathways are involved, needs future study.

Second, the mechanism of Rb downregulation and its phosphorylation in 3D collagen needs further study. Although decreased cyclin E1 can result in hypophosphorylation of Rb and further decrease cyclin E1 itself (Donnellan and Chetty, 1999), cyclin D1 as another direct Rb inhibitor, does not change its expression. Since cyclin D1 phosphorylates Rb in the nucleus, its relocalization to the cytoplasm could also contribute to Rb hypophosphorylation. Arguing

against this, its relocalization is not affected by integrin inhibition while cyclin E1 expression and Rb phosphorylation are restored. This indicates that if cyclin D1 regulates Rb phosphorylation in 3D collagen, it is independent of integrin-mediated pathways. Whether there are other signalling pathways downstream of β_1 integrin that directly mediate Rb phosphorylation independent of effects on cyclin D1 and cyclin E1 in 3D collagen needs further study. We are not aware of any studies describing the direct regulation of Rb downstream of integrin bypassing cyclin-cdk regulation.

Third, MDA-MB-231 cells lack αB-crystallin, a component of the E3 ligase that mediates cyclin D1 degradation due to a chromosomal deletion (Lin et al., 2008). Knockdown of αB-crystallin inhibits cyclin D1 proteolysis and leads to its nuclear accumulation and cell transformation (Barbash et al., 2008), explaining the predominantly nuclear cyclin D1 in MDA-MB-231 cells cultured in 2D. The observation that in 3D collagen, cyclin D1 relocalizes from the nucleus to the cytoplasm and remains there after integrin inhibition is surprising. GSK3β phosphorylates cyclin D1 and triggers its nuclear export. Therefore, cyclin D1 may be phosphorylated by GSK3β in response to a 3D collagen environment, resulting in cyclin D1 nuclear export. GSK3β also phosphorylates cyclin E1 and promotes cyclin E1 degradation. Whether GSK3β plays a role in regulating cyclin E1 degradation and cyclin D1 localization in 3D collagen should be assessed in future experiments (Cohen and Goedert, 2004).

Lastly, as a relatively novel RBP, the regulation of CIRP in 3D collagen is also worth further study. CIRP is overexpressed in about 30% of primary breast

cancers (Artero-Castro et al., 2009). We have shown that CIRP stabilizes cyclin E1 mRNA in breast cancer cells and localizes both in the nucleus and in discrete cytoplasmic foci identified as stress granules (Guo et al., 2009). In a 3D collagen environment, CIRP decreases and relocalizes to the cytoplasm without changing cyclin E1 mRNA half-life. CIRP has been reported to act as a translational repressor (De Leeuw et al., 2007). Cytoplasmic CIRP contributing to cyclin E1 downregulation via repressing its translation remains a possibility.

The ability to directly regulate cyclin E1 in the absence of nuclear cyclin D1 may be an adaptation of MDA-MB-231 and other cancer cells for survival and growth in a primarily collagen environment after invasion and during metastasis. This adaptation may result from upregulation of integrin and integrin mediated signalling pathways that promote cell survival. β integrins deliver prosurvival messages by activating downstream signalling pathways such as FAK/JNK (c-Jun N-terminal kinase) or NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) in ME cells (Hehlgans et al., 2007). Whether these signalling pathways are activated in 3D collagen needs future investigation.

MMPs are highly expressed in breast cancer and invasive breast cancer growth is preceded by an increase in MMP activity (Duffy et al., 2000). Few studies have addressed the mechanism by which MMPs regulate cell proliferation. This may be due to the fact that MMPs usually function extracellularly. Although MMPs have been reported to function within nuclei and promote apoptosis via their catalytic activities (Si-Tayeb et al., 2006; Yang et al., 2009) and cyclin E1 decreases in 3D collagen and also might be involved in

apoptosis, we have not found MMPs in the nucleus of 3D collagen culture by immunofluorescence assays (courtesy of Dr. Paul McGuire, UNM-HSC, data not shown), excluding the possibility that MMPs cleave cyclin E1 in the nucleus. We have shown that inhibition of MMPs might rescue cyclin E1 ($p=0.05$) without changing cyclin D1 relocalization. In order to determine the mechanisms responsible for MMP regulation of cyclin E1, other factors involved in cyclin E1 regulation, such as c-Myc, Rb, CIRP or signalling pathways, can be examined upon MMP inhibition. Our results suggest that MDA-MB-231 cells possibly activate MMP-2 and MMP-9 through β_1 integrin-mediated interaction with collagen I (as discussed in Chapter 3, section 3.4). The increase seen in β_1 integrin and MMPs coupled to decreased cell proliferation in 3D collagen might indicate a cell behavior transition from growth to invasion. Determining whether MDA-MB-231 cells become more invasive in 3D collagen and whether β_1 integrin is involved in regulating cell invasion would provide evidence for this. Further elucidation of the mechanisms by which a 3D collagen ECM controls ME cell proliferation, invasion and survival via a balance adjustment between extracellular proteases, cell surface receptors and cell cycle regulators is needed.

Monotypic 3D culture models are only one of several models that have been successfully utilized to study microenvironmental contributions to the normal and malignant mammary gland. Heterotypic 3D models in which different cell types are co-cultured, better mimic the *in vivo* environment and are better suited to investigate more complicated cellular interactions (Gudjonsson et al.,

2002; Krause et al., 2008). The “humanized” mammary gland model has also allowed the study of mammary gland development and carcinogenesis *in vivo* (Kuperwasser et al., 2004). This model was generated by clearing ME cells from the mammary gland of immunocompromised mice and sequentially injecting irradiated human fibroblasts and then normal human mammary tissue mixed with human primary fibroblasts and collagen/Matrigel into the cleared mammary fat pad. Human ME cells survived and integrated within the mouse mammary stroma and development of human breast ducts were observed. However, these models are limited in that the tumor microenvironment differs from normal tissue stroma; mutations that cause embryonic lethality or impede mammary gland development are difficult if not impossible to study in these models. The development of innovative 3D culture systems will be invaluable in modeling breast cancer progression and identifying new strategies for its therapy.

Although using a relatively simple monotypic 3D culture system, our study directly answered an important question about how the major component of the early stage breast cancer microenvironment, collagen I, exerts its control on cancer cell proliferation. Our studies focused on regulation of cyclin E1, a G_1/S regulator often perturbed in breast cancer and involved in its etiology. Our results show a network of regulation involving other cell cycle regulators, transcriptional and post-transcriptional regulatory factors as well as cell surface receptors and extracellular proteases. A number of promising future directions and possible signalling pathways merit continued study and exploration.

Figure and Figure Legend

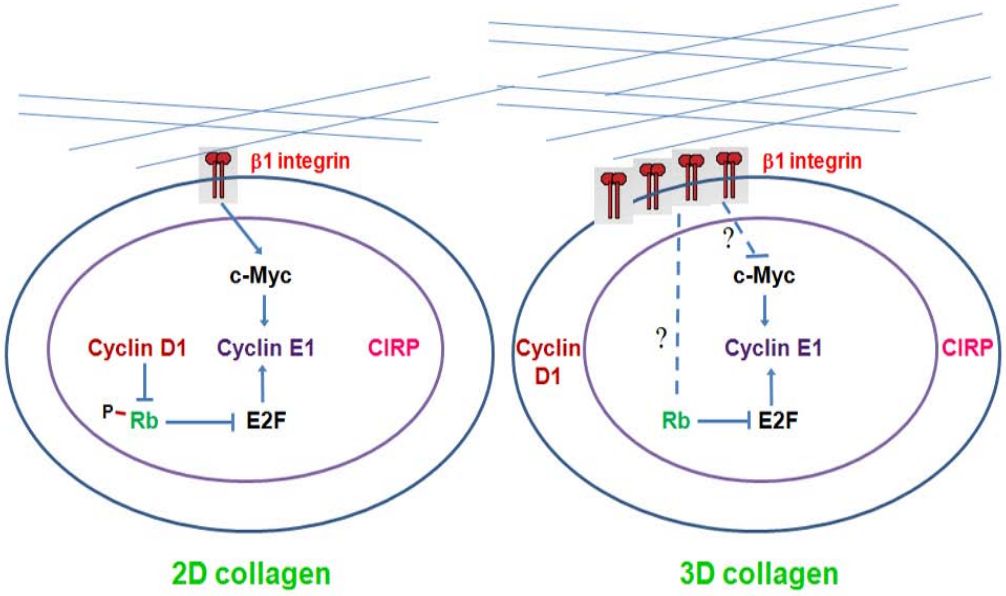

Figure 5.1 Model.

A model to summarize the possible mechanisms identified in our project by which 3D collagen controls the cell cycle in part through cyclin E1, compared with regulation by 2D collagen. Crossed lines on top indicate ECM composed of collagen I. Outer circle indicates cell surface and inner circle indicates nucleus. Mechanisms are discussed in the text. Left panel: β_1 integrin mediated signalling pathways when ME cells adhere to 2D collagen. Right panel: β_1 integrin mediated signalling pathways when ME cells are embedded in 3D collagen. Solid line arrows: positive regulation shown previously or in the current study. Solid lines with perpendicular bar: negative regulation shown previously or in the current study. Dashed line: regulation proposed based on published data.

6. Appendix-Pilot Studies

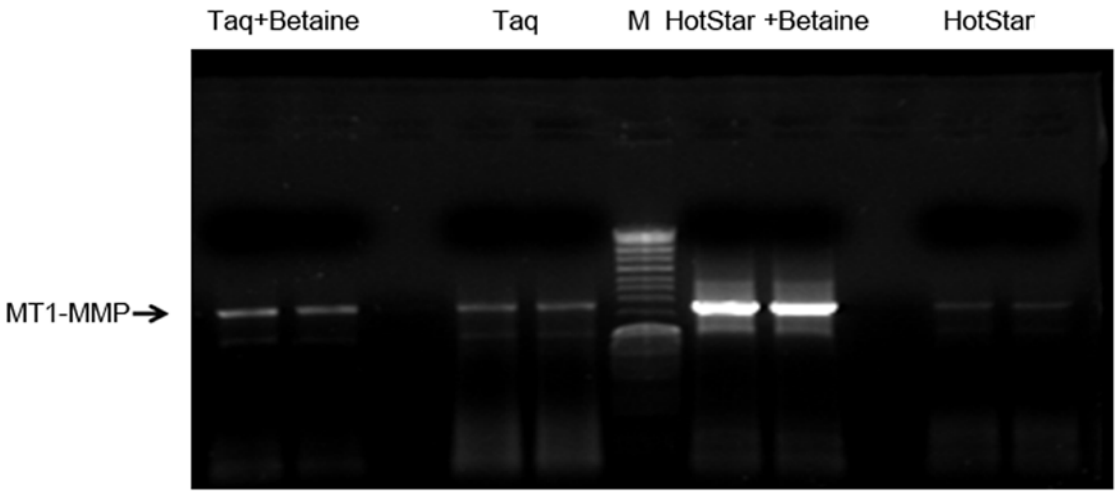

Figure 6.1 MT1-MMP cloning.

MT1-MMP was cloned from MCF10A cDNA. Agarose gel showing PCR cloning of the full length MT1-MMP coding sequence (~1.7kb). PCR was performed under 4 different conditions: with Taq polymerase (Taq), Taq polymerase plus Betaine (Taq+Betaine), HotStart Taq polymerase (HotStar), or HotStar Taq polymerase plus Betaine (HotStar+Betaine). M: DNA HyperLadder I (Bioline, Taunton, MA). Total RNA from MCF10A cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcription was performed using Oligo $(dT)_{12\text{-}18}$ as described in Chapter 2, section 2.3.4. MT1-MMP was cloned from the resulting cDNA with the following primer pairs by PCR: forward: 5'-GAA AGATCT ACC ATG TCT CCC GCC CC-3' and reverse: 5'-CCG GAATTC TCA GAC CTT GTC CAG-3'. The reaction was performed using HotStar Taq PCR kit (QIAGEN, Valencia, CA) with 1M Betaine (Sigma, St. Louis,

MO) to improve the yield by reducing the formation of secondary structure in GC-rich regions. PCR was performed as: 95°C 15min followed by 35 cycles of 94°C 1min, 60°C 30sec, 72°C 2.5min, and 72°C 10min.

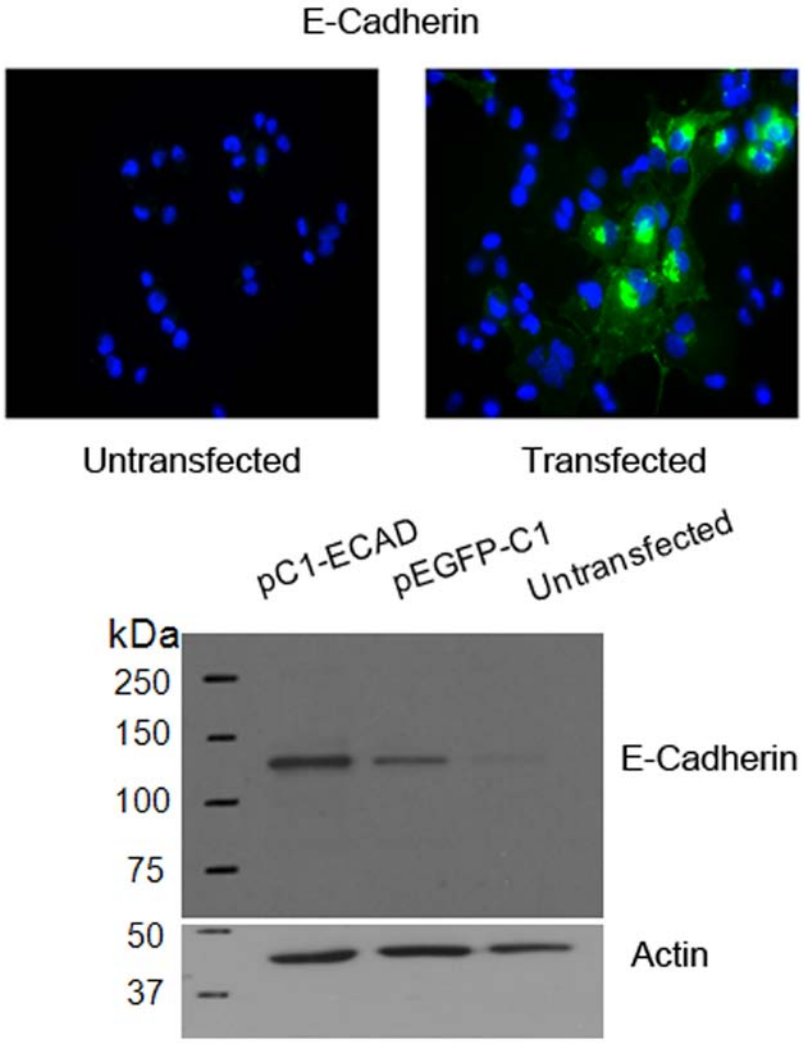

Figure 6.2 E-Cadherin was overexpressed in MDA-MB-231 cells.

Upper panel: E-Cadherin was visualized with Alexa Fluor 488 conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). Transfection efficiency was about 10%. Magnification 63X. Bottom Panel: E-Cadherin expression was monitored by western blotting of cell lysates from untransfected MDA-MB-231 cells or cells stably transfected with empty vector (pEGFP-C1) or untagged E-Cadherin (pC1-ECAD). Actin was used as a loading control. A pBluescript II phagemid bearing the coding sequence of E-Cadherin was

provided courtesy of Dr. Helen Hathaway (UNM-HSC). E-Cadherin was subcloned into pEGFP-C1 with or without the EGFP tag. Primers used for cloning E-Cadherin downstream of EGFP were: forward 5'5'CCG CTCGAG CGG ATG GGC CCT TGG AGC CGC AGC 3'; reverse 5'TCC CCGCGG GGA CTA GTC GTC CTC GCC GCC TCC GTA 3'. Primers used for replacing EGFP sequence with the E-Cadherin coding sequence were: forward 5'GTT GCTAGC ATG GGC CCT TGG AGC CGC AGC 3'; reverse 5'TTA CTCGAG TCC CCT AGT CGT CCT CGC CGC CTC CG 3'. Both constructs were sequenced to confirm that E-Cadherin was correctly cloned. Lipofectamine2000 (Invitrogen, Carlsbad, CA) was used to transfect pEGFP-E-Cadherin, or pC1-E-Cadherin (no EGFP) or empty vector into MDA-MB-231 cells. Transfection efficiency was monitored by visualizing EGFP fluorescence or western blotting for E-Cadherin in cell lysates. Immunofluorescence assays using untagged E-Cadherin antibody (BD Biosciences, San Jose, California) and nonspecific mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) also performed to visualize E-Cadherin.

6.1.1.

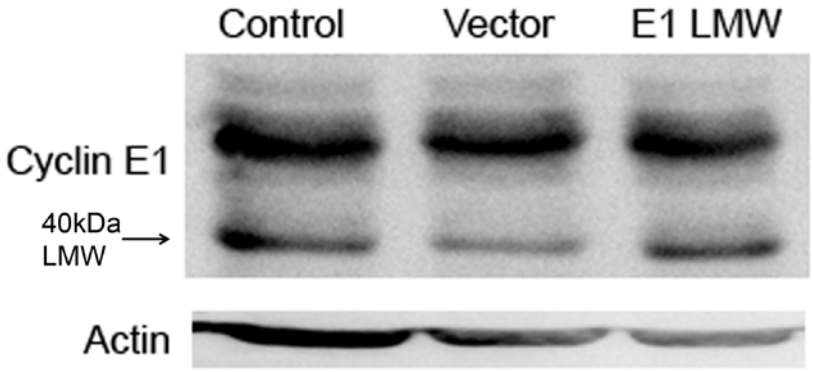

Figure 6.3 40kDa LMW cyclin E1 was transfected in MDA-MB-231 cells.

Expression of a 40kDa LMW isoform of cyclin E1 in MDA-MB-231 cells. Expression was monitored by western blotting for cyclin E1 using cell lysates from untransfected (control), empty vector transfected (vector) and 40kDa isoform (E1 LMW) stably transfected MDA-MB-231 cells. Actin was used as a loading control. pTracer-CMV2-E1Trunk2 vector containing a LMW cyclin E1 isoform of 40kDa was provided by Dr. Xun Guo (UNM-HSC). Fugene 6 was used to transfect LMW cyclin E1 vector or empty vector into MDA-MB-231 cells (Roche, Basel, CN) according to the manufacturer's directions. Western Blotting for Cyclin E1 verified expression of the 40kDa cyclin E1 isoform.

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