A NOVEL ROLE OF GELSOLIN IN CANCER CELL SURVIVAL THROUGH REGULATION OF FIBRONECTIN

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Dinh Thuy Duong
28 September 2014

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SUMMARY

The interaction between cancer cells and the extracellular matrix (ECM) holds a special position in tumor progression. While the ECM modulates cancer initiation and progression, cancer cells are able to remodel the ECM to facilitate their survival, migration and invasion. During metastasis, cancer cells develop a special ability called anoikis resistance or extracellular matrix (ECM)-independent survival to thrive in the condition without ECM attachment. Actin cytoskeleton plays an important role in anoikis resistance of cancer cells, with unknown modes of action. In this study, we investigated the effects of gelsolin, a well-known regulator of actin cytoskeleton, on cancer cell survival in an anoikis condition and the underlying mechanism of those effects.

We showed that gelsolin protected colorectal cancer HCT 116 cells from apoptosis and promoted HCT 116 cell survival in an anoikis model. Our data suggested that gelsolin exerted the effects by upregulating fibronectin expression and secretion in HCT 116 cells. Conditioned media obtained from gelsolin over-expressing cells protected HCT 116 cells against apoptosis in the anoikis condition, suggesting that gelsolin-overexpressing cells secreted factors that promoted cancer survival. On the other hand, fibronectin expression and secretion, which were regulated by gelsolin, protected cancer cells against apoptosis and promoted cancer cell survival through Akt signaling. Fibronectin also rescued the apoptotic effects of gelsolin knock-down in cells cultured in the anoikis condition.

In addition, our results suggested that gelsolin upregulated fibronectin expression in HCT 116 cells through activation of the transcription factor nuclear factor kappa B (NFκB). NFκB activity was significantly enhanced in gelsolin-overexpressing cells and attenuated in gelsolin-knockdown cells. Activation of NFκB by tumor necrosis factor alpha (TNFα) led to increased fibronectin expression in HCT 116 cells, whilst inhibition of NFκB attenuated fibronectin expression. Additionally, upregulation of fibronectin by gelsolin could be attenuated by NFκB inhibition. Fibronectin expression was also upregulated by the early growth response proteins 1 (EGR-1). However, gelsolin did not affect either EGR-1 expression or EGR-1 activity. These findings suggest that gelsolin upregulated fibronectin expression via NFκB but not EGR-1 pathway.

In summary, our findings reveal a novel role of gelsolin in promoting ECM-independent cancer cell survival by upregulating fibronectin expression and secretion via NFκB pathway.

LIST OF FIGURES

ABBREVIATIONS

GSN	Gelsolin
PIP2	Phosphatidylinositol 3,4 or 4,5-bisphosphate
SDF-1	Stromal derived factor 1
PARP	Poly-adenosine diphosphate ribose polymerase
DNase	Deoxyribonuclease
HIF1α	Hypoxia-inducible factor-1alpha
VDAC	Voltage-dependent anion channel
PLD	Phospholipase D
PLC	Phospholipase C
AR	Androgen receptor
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment Receptors
Syn4	Syntaxin 4
VAMP2	Vesicle-associated membrane protein 2
AP-1	Activator protein-1
NFκB	Nuclear factor kappa B
ATF-1	Activating transcription factor-1
ECM	Extracellular matrix
MMP	Matrix metalloproteinase
ADAMTS	A disintegrin and metalloproteinase with Thrombospondin motifs
LOX	Lysyl oxidase
EIIIA or EDA	Type III extra domains A
EIIIB or EDB	Type III extra domains B
TNFα	Tumor necrosis factor alpha
bFGF	Basic fibroblast growth factor
PKCα	Protein kinase C alpha
HBV	Hepatitis B virus
HBVxAg	HBVx antigen
EGR-1	Early growth response 1

HGF	Hepatocyte growth factor
MAPK	Mitogen-activated protein kinase
NAB2	NGFI-A binding protein
UTR	Untranslated region
LC3	Light chain 3 of microtubule-associated proteins 1A/1B
ER	Endoplasmic reticulum
RGD	Arg-Gly-Asp
FAK	Focal adhesion kinase
PI3K	Phosphoinositol 3 kinase
HPCs	Hematopoietic progenitor cells
Bcl-2	B-cell lymphoma 2
p-Akt	Phospho-Akt
IκB	Inhibitor of kappa B

-CHAPTER 1-

INTRODUCTION

1.1. GELSOLIN

1.1.1. Gelsolin structure

Gelsolin (GSN) is a member of the gelsolin superfamily of actin-binding proteins, with a molecular weight of about 80-84kDa. Three isoforms of gelsolin have been identified including plasma gelsolin, cytoplasmic gelsolin and gelsolin-3. The gelsolin isoforms are encoded by one single gene. Transcription of the gelsolin gene is controlled by promoters at different sites, resulting in alternative splicing variants. Compared to cytoplasmic gelsolin, the plasma isoform has an additional signal peptide of 25-amino acid and plasma extension. Gelsolin-3 has extra 11-amino acids at the N-terminus compared to cytoplasmic gelsolin [1, 2]. While cytoplasmic gelsolin is expressed in all tissues, gelsolin-3 is only detected in brain, lung and testis [3].

Gelsolin consists of 6 domains (G) with binding sites for actin and Ca^{2+}. Domains G1 and G4 have binding sites for actin monomers (G-actin), while domain G2 contains binding sites for actin filaments (F-actin) [2]. In the inactive form, the helix tail of G6 interacts with G2 to form a latch, masking actin-binding sites on the gelsolin molecule. Upon activation by Ca^{2+}, the latch between G2 and G6 opens, allowing conformational rotation of G3 and G6 compared to G1 and G4, hence exposing actin-binding sites for capping and severing [2, 4].

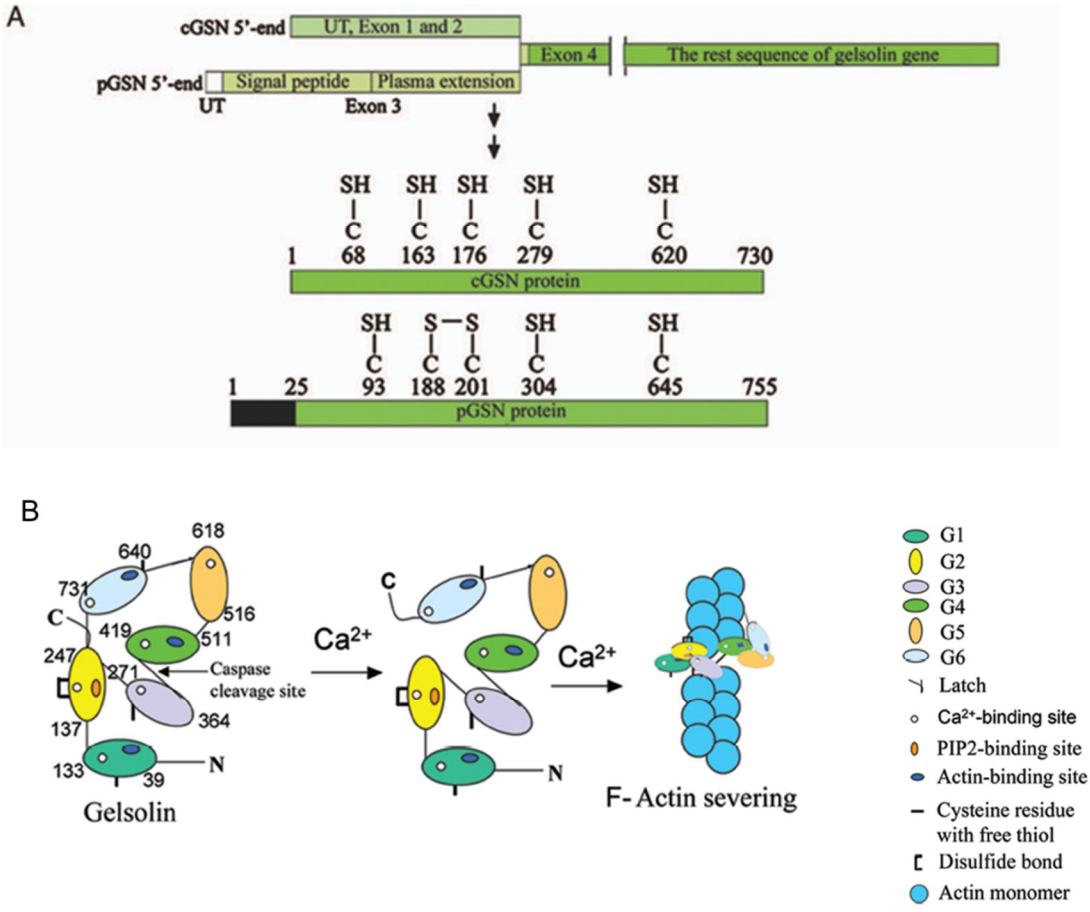


Figure 1.1. Gelsolin structure. (A) The gelsolin gene encoding plasma and cytoplasmic gelsolin isoforms. (B) Conformational changes of gelsolin for capping and severing actin filaments. *Figure is from a paper by Li G.H. et al, Med Res Rev, 2012. 32(5): p. 999-1025* [2].

1.1.2. Functions of gelsolin in cell biology

The three isoforms of gelsolin, including plasma gelsolin, gelsolin-3 and cytoplasmic gelsolin, serve different functions in cell biology. The main role of plasma gelsolin is to scavenge actin filaments generated from necrotic cells, which are toxic to living cells. Plasma gelsolin was also shown to transport inflammatory factors to their receptors, and hence plays a role in immune system. Indeed, plasma gelsolin level decreases in patients with sepsis, trauma or liver injury. Therefore, plasma gelsolin was proposed to be a prognostic biomarker for the above-mentioned diseases. Gelsolin-3 has limited expression in brain, lung and testis with unknown cellular and physiological functions. In this study we focused on the most common form of gelsolin, the cytoplasmic isoform, and its roles in cell biology will be discussed in the following part.

1.1.2.1. Roles of gelsolin in normal cell biology

Regulation of actin dynamics:

Gelsolin is well-known as an actin regulator, playing important roles in regulating actin dynamics. Upon binding to an actin filament, gelsolin severs the filament into two and remains to cap the newly severed end (or barbed end), and hence inhibits polymerization of the filament. In the presence of phosphatidylinositol 3,4 or 4,5-bisphosphate (PIP2), which competitively binds to barbed ends, gelsolin is released from its binding with actin filaments. The length of actin filaments is maintained, in stable conditions, by continuous addition of actin monomers to the barbed ends and deletion of monomers from the pointed ends. By capping and inhibiting the addition of monomers at the barbed ends, gelsolin directly depolymerizes actin filaments, creating

shortened filaments and more newly-severed ends. The newly-generated barbed ends, when uncapped by PIP2, serve as acting sites for further polymerization and/or branch formation [5, 6], which induce membrane protrusion for locomotion. On the other hand, by regulating the actin network near the plasma membrane, gelsolin modulates stress fibers that affect the shape of a cell. As a result, gelsolin promotes cell motility and defines cell shape. Indeed, overexpression of gelsolin in fibroblasts stimulates cell migration. Hematopoietic stem cells with low gelsolin expression migrate slower in response to the chemokine stromal-derived factor 1 (SDF-1) than those with high gelsolin expression [7-10]. In gelsolin knock-out mice, osteoclasts were unable to form podosomes for cell adhesion, leading to decreased osteoclastic motility in both basal and osteopontin-induced conditions [11, 12].

Apoptosis:

Gelsolin shows contradictory effects on apoptosis, depending on stimuli and cell types. Upon stimulation by apoptotic stimuli, gelsolin is cleaved by caspases-3, -7 or -9 at the 252-253 residues to generate a 39 kDa N-terminal half and a 41 kDa C-terminal half. Gelsolin cleavage was observed concomitantly with poly-adenosine diphosphate ribose polymerase (PARP) disruption, which is associated with enhanced apoptosis *in vivo* [2]. The N-terminal half binds to actin filaments independent of Ca^{2+}, resulting in uncontrolled apoptosis. The N-terminal half of gelsolin also stimulates apoptosis by competing with deoxyribonuclease (DNase) I for binding to actin. DNase I is thus dissociated from its binding to actin and induces apoptotic

degradation. In addition, gelsolin was suggested to activate DNase I for apoptosis via its association with both DNases I and hypoxia-inducible factor-1alpha (HIF1α), which is a possible regulator of DNases I [13].

On the other hand, full-length gelsolin and the C-terminal half of gelsolin have been observed to exert anti-apoptotic effects. Gelsolin was shown to inhibit mitochondria potential loss, resulting in blockage of voltage-dependent anion channel (VDAC), which is, in normal conditions, responsible for the release of cytochrome c from mitochondria to cytosol for caspase activation, and ultimately apoptosis [14, 15].

Signal transduction:

Besides its functions on actin remodeling, gelsolin also regulates different aspects of cell biology by its involvement in different signaling pathways. Gelsolin physically interacts with phospholipase D (PLD) to activate both PLD downstream signals and actin remodeling [16]. Gelsolin also modulates PLC signaling by competing with phospholipase C (PLC) beta and gamma in binding to PIP2. Inhibition of PLC's association with its substrate, PIP2, leads to suppression of downstream signals of PLC [7]. In addition, a study by Azuma *et al* [17] suggested that gelsolin is a downstream effector of EGF-induced Rac activation. Experiments on Gsn-/- fibroblasts demonstrated that Rac was overexpressed when gelsolin was knocked out. Rac overexpression in this condition was postulated to compensate for the decreased motility induced by gelsolin knock-out. However the compensatory overexpression of Rac was unable to completely reverse the effects of gelsolin

depletion on cell ruffling and motility, indicating that the gelsolin plays an essential role in Rac signaling.

Transcriptional co-activation:

Mounting evidence has shown the involvement of gelsolin in co-regulating several transcription factors or transcription co-activators. Gelsolin interacts with androgen receptor (AR) at DNA-binding and ligand-binding sites of AR molecule in the nucleus of human prostate cancer cells, resulting in enhanced AR activity. Interestingly, *in vitro* and *in vivo* studies showed that gelsolin expression was highly detected after treatment with androgen deprivation or AR antagonists. This suggests that inhibition of AR activity may be compensated by high gelsolin expression, which aids to magnify AR activity, hence to contribute to prostate cancer resistance. Targeting gelsolin, therefore, is perhaps a possible strategy to treat prostate cancer recurrence [18]. In thyroid cancer mouse model, the C-terminus of gelsolin was shown to interact with thyroid hormone receptor-β1 at the DNA binding sequence of thyroid hormone receptor. The interaction with gelsolin inhibited the effects of thyroid hormone receptor on tumor progression [19].

Recently, gelsolin has been shown to serve as an activity checkpoint of the transcription co-activators YAP and TAZ, which are important regulators of cell survival and proliferation [20]. In normal condition, YAP and TAZ localize in cytoplasm in binding with gelsolin. When cells are stimulated by mechanical stresses or stiffness changes of ECM, gelsolin releases YAP and TAZ for their translocation to the nucleus, where they bind TEAD

transcription factors for transcription of target genes. Therefore, gelsolin indirectly affects transcription of various genes downstream of YAP and TAZ.

Gelsolin also inhibits tumor suppressor protein p53, which acts as a transcription factor to regulate cell differentiation and apoptosis. By interacting with p53 and retaining it in cytoplasm, gelsolin suppresses nuclear translocation of p53 in hepatocarcinoma cells, resulting in attenuation of apoptosis [21].

Regulation of vesicle secretion:

Cytoskeleton plays an important part in exocytosis of secretory vesicles. At the proximity of plasma membrane, actin filaments form a meshwork that blocks the transport of secretory vesicles to plasma membrane for fusion and secretion [22]. By regulating actin dynamics, gelsolin indirectly promotes movement of secretory vesicles to exocytosis sites on plasma membrane. Gelsolin also plays an important role in regulated secretion, a process by which secretory vesicles bind to soluble N-ethylmaleimide-sensitive factor attachment receptors (SNARE) upon stimulation before being secreted outside. In basal conditions, gelsolin interacts with Sytaxin 4 (Syn4), a SNARE protein, to hinder binding of insulin vesicles to Syn4. When cells are stimulated by glucose or potassium chloride, gelsolin is disconnected from the interaction with Syn4. Thus, insulin vesicles are able to bind to Syn4, and then fuse with plasma membrane for exocytosis [23]. A summary of these findings are summarized in Figure 1.2.

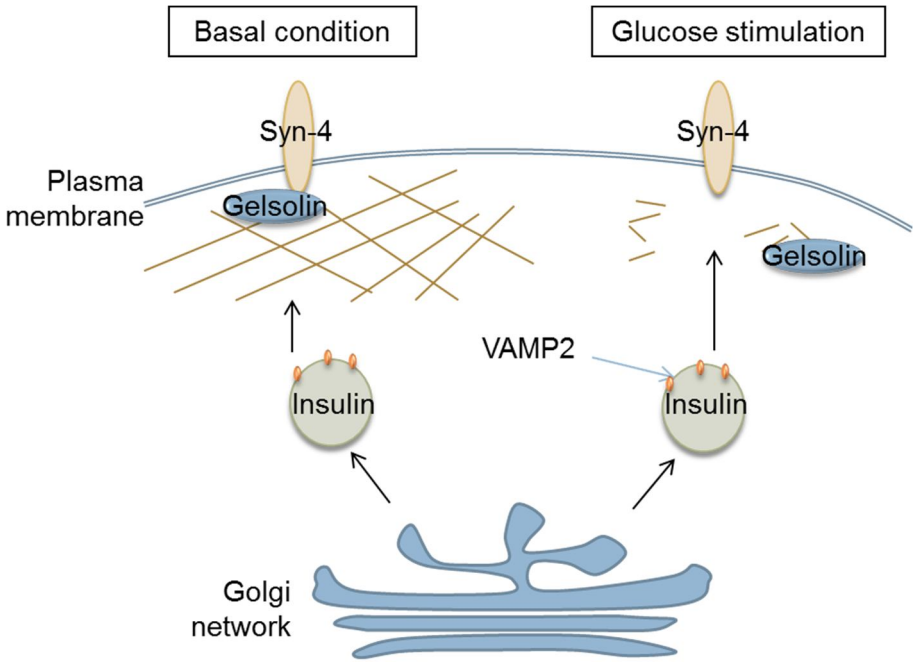


Figure 1.2. Gelsolin promotes the regulated-secretion of insulin in mouse pancreatic β-cells. In basal condition, gelsolin interacts with Syn4, a SNARE protein, on plasma membrane to hinder the fusion of insulin vesicles to plasma membrane. When cells are stimulated by glucose, gelsolin is dissociated from the interaction with Syn4. The vesicle-associated membrane protein 2 (VAMP2), a SNARE protein on insulin secretory vesicles, binds to Syn4 for fusion of insulin secretory vesicles with plasma membrane and insulin vesicles are secreted outside.

1.1.2.2. Roles of gelsolin in tumor biology

Numerous studies have shown that gelsolin is involved in cancer development. However, the exact roles of gelsolin in tumor biology are still under debate. Decreased expression of gelsolin was observed in some cancers such as colon, gastric, breast and lung cancers [24-27], suggesting the possible roles of gelsolin as a tumor suppressor. The findings were supported by

several studies in which overexpression of gelsolin reduced proliferation of bladder cancer cells as well as attenuated carcinogenesis in a mouse model [28]. In addition, gelsolin siRNA knock-down induced epithelial-mesenchymal transition in human mammary cells, resulting in enhanced cancer progression [29].

However mounting evidence suggested an opposite role of gelsolin in cancer progression, based on the fact that gelsolin is highly expressed in other cancers. Recent studies have provided more information to explain the contradictory results regarding gelsolin expression in cancers. Although gelsolin expression is decreased in some tumors, its higher levels correlate with more advanced stages of the diseases. A study with non-small lung cell carcinoma tissues demonstrated that high gelsolin expression correlated with high recurrence risk of cancers in lung [30, 31]. Similar correlation was also found in cancers in kidney [32] and urinary track [33]. Therefore, gelsolin was recommended to be used as a biomarker for progression and recurrence of these cancers. Interestingly, gelsolin was highly detected at the invasive front of liver metastatic tissues of colorectal cancers. Gelsolin stimulated cancer invasion and metastasis by up-regulating invasion-promoting factors such as urokinase plasminogen activator and its receptor, and/or by repressing E-cadherin expression [24, 34]. These results highlight an important role of gelsolin in cancer invasion and metastasis. The findings also suggest that, gelsolin serves as a tumor suppressor at primary sites, but when re-emerges, it confers tumor-promoting roles by stimulating invasion and metastasis.

1.1.3. Regulation of gelsolin expression

Gelsolin has been found with decreased expression in various cancers. However, no mutant regulatory sites or regions in the gelsolin gene have been identified, suggesting that downregulation of the gesolin gene is not by mutation but other mechanisms.

Mielnicki *et al.* [35] was the first group to report the downregulation of gelsolin by epigenetic modification via histone deacetylation. Normally, histone deacetylation is catalyzed by histone deacetylases, resulting in increased positive charge of histone tails, strengthening histone-DNA binding and stimulating post-translational modification by histone methyl transferases. Thus, histone deacetylation blocks accessibility of transcriptional complexes to DNA, leading to gene silencing [36]. Gelsolin expression in breast cancer was shown to be upregulated by inhibition of histone deacetylases [35]. Subsequent studies on cervical cancer cells [37], urinary cancer cells [38] and gastric cancer tissues [39] confirmed the regulatory effect of histone deacetylation on gelsolin depression in those cancers. On the other hand, interaction of transcriptional complexes to DNA can also be interfered by DNA methylation, a process characterized by addition of methyl groups to cytosine in CpG islands. Hyper-methylation in intron 1 of the gelsolin gene was observed, suggesting that gelsolin is probably downregulated by DNA methylation [35].

Besides the epigenetic modification mentioned above, some transcription factors are also involved in regulation of the gelsolin gene. Sequence analysis of the gelsolin gene revealed interacting sites for the TATA-binding protein (TBP), specificity protein 1 (Sp1), activator protein-1

(AP-1), and nuclear factor kappa B (NFκB). Indeed, a study in HeLa cells confirmed that Sp1 bound to gelsolin promoter to upregulate the gelsolin gene [40]. Additionally, gelsolin was downregulated in breast cancer by interacting with the activating transcription factor-1 (ATF-1) [41].

At protein level, a study in pancreatic cancer showed that gelsolin was regulated by ubiquitin-dependent proteosomal degradation [42]. The conclusion was supported by the evidence demonstrating that gelsolin expression was suppressed in the early stages of pancreatic cancer at protein level but not mRNA level, suggesting post-translational down-regulation of gelsolin. The finding was further confirmed by data showing that poly-ubiquitinated gelsolin was detected in pancreatic cancer cells and inhibition of 26s proteasome significantly increased gelsolin expression.

1.2. EXTRACELLULAR MATRIX

1.2.1. Extracellular matrix composition and structure

Extracellular matrix (ECM) is a complex structure present in all tissues (Figure 1.3). Basically ECM consists of 2 main structural components, which are fibers and interstitial matrix. ECM fibers are made of glycoproteins, such as collagens and elastin, forming a flexible fibrous scaffold or basement membrane. Interstitial matrix or ground substance is made of amorphous proteoglycans (e.g. aggrecan, syndecan), glycosaminoglycans (e.g. dermatan sulfate, keratan sulfate, hyaluronan) and adhesive glycoproteins (e.g. fibronectin, laminin). Interstitial matrix interacts with both basement membrane and cells in the matrix vicinity. The proportion of fibers and interstitial matrix of the ECM varies between different tissues, contributing to tissue-specific characteristics such as strength, elasticity and stickiness. [43]

Although ECM proteins are particularly diverse, they are similar in a way that they all consist of multi-domains with high molecular weight. Most of ECM proteins are normally decorated with glycosylation, which makes ECM components greatly bigger and more complex. Multi-domains of ECM proteins are repeated homologous structures, which share similar functions and may work independently or together to exert certain functions. Multi-domains of different proteins can also interact with each to form fiber networks [44].

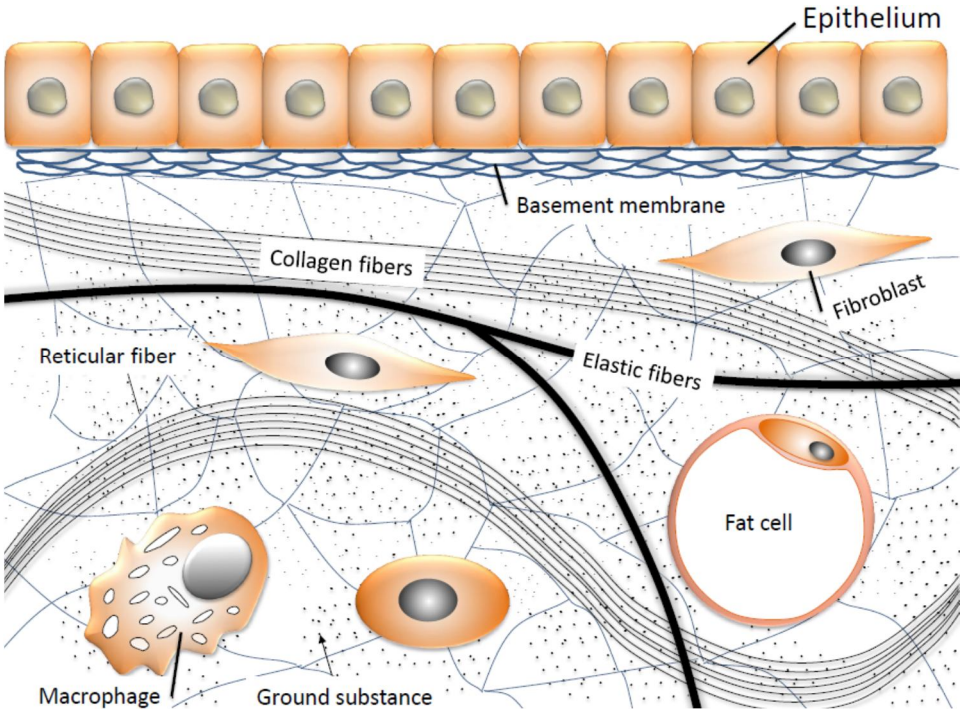


Figure 1.3. A schematic illustration of extracellular matrix structure in connective tissues

Adapted figure from a chapter by Engel J. et al. (The Extracellular Matrix: an Overview, R.P. Mecham, Editor. 2011, Springer Berlin Heidelberg. p. 1-39.)[44]

1.2.2. Extracellular matrix remodeling

ECM is not a static structure as usually being thought. In fact, it is extremely dynamic at microscopic level due to the constant remodeling of ECM components. ECM remodeling consists of formation steps, which are secretion and deposition, and deformation processes, which are modification and degradation. A balance of secretion, deposition, modification and degradation is always maintained in normal condition. [44]

1.2.2.1. Secretion and deposition of ECM components

ECM proteins are secreted locally by resident cells in the matrix, which are mostly fibroblasts and probably some epithelial cells. Except for collagen, secretion of other ECM proteins is poorly understood. Thus, information about synthesis, secretion and deposition of collagen will help to understand possible productional mechanisms of other ECM proteins.

Large precursors of collagen peptides are synthesized on rough-ribosomes and transported to endoplasmic reticulum due to signal peptides in N-termini. Then the precursors are hydroxylated at selected prolines and lysines. Some are also glycosylated at specific hydroxylysines. The precursors interact via hydrogen bonds to form higher structures such as pro-collagen, a stable triple-stranded helix. After secretion, pro-collagen molecules are converted to collagen by enzyme-cleavage of pro-peptides. Collagen molecules, which are about 1000-fold more insoluble than pro-collagen, self-assemble to form large collagen fibrils. Fibrils are formed extracellularly at the promixity of plasma membrane where secretory vesicles fuse with cell surface

in exocytosis. Covalent cross-links between ECM fibers at lysine residues further fortify fibril structures. Then, ECM-secreting cells such as fibroblasts interact with the secreted molecules mechanically and chemically to compact the collagen fibrils into sheet-like or packed structures and push other secreted proteins into that meshwork. In turn, the meshwork modulates fibroblast distribution as it occupies most of the area in the proximity. Different fibrils such as inelastic collagen and elastic fibrils can interweave with each other to further enrich the complexity and plasticity of the ECM.

1.2.2.2. Modification and degradation of ECM components

The ECM is degraded by a wide range of matrix-degrading enzymes, including:

- Metalloproteinase family such as matrix metalloproteinase (MMP), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families and their inhibitors;
- Other proteases such as heparanase, cathepsins, hyaluronidases, serine and threonine proteases. [45]

Most of matrix-degrading enzymes have their specific targets, for example proteoglycans, fibronectin and laminin are targeted by MMP-3 and -10 and serine protease plasmin, whilst collagen III is degraded by MMP-1, collagen I and II is degraded by MMP-8 and -13 [46]. Matrix-degrading enzymes cause cleavage and alteration of ECM structure. The main function of these processes is to ensure proper disposal of unwanted proteins during development. Any changes in the homeostasis of the enzymes will affect ECM

structure and ultimately tissue functions. For example, lysyl oxidase (LOX) and lysyl hydroxylases modify formation of collagen and elastin cross-linking, resulting in altered physical properties of tissues such as enhanced tensile strength and stiffness [47].

1.2.3. Roles of extracellular matrix in cell biology

1.2.3.1. Roles of ECM in normal cell biology

The ECM plays intricate and crucial roles in cell biology (Figure 1.4). In normal conditions, ECM serves as a platform for cell anchorage, division, proliferation and migration. Cells anchor to the ECM when it comes to asymmetry division (step 1). Depending on the conditions, the ECM may be a barrier against cell migration, or may act as a track for cell movement (step 2 and 3). The ECM is also a reservoir of growth factors, which are secreted and deposited into the ECM (step 4). The growth factors trigger cells directly or are presented to cells via ECM proteins (step 5 and 6). Some ECM proteins in the proximity of cells, such as fibronectin, or some ECM fragments are able to directly influence cell survival, proliferation or migration (step 7). The ECM also exerts biomechanical forces via actin cytoskeleton to affect changes in cell behaviors (step 8) [48].

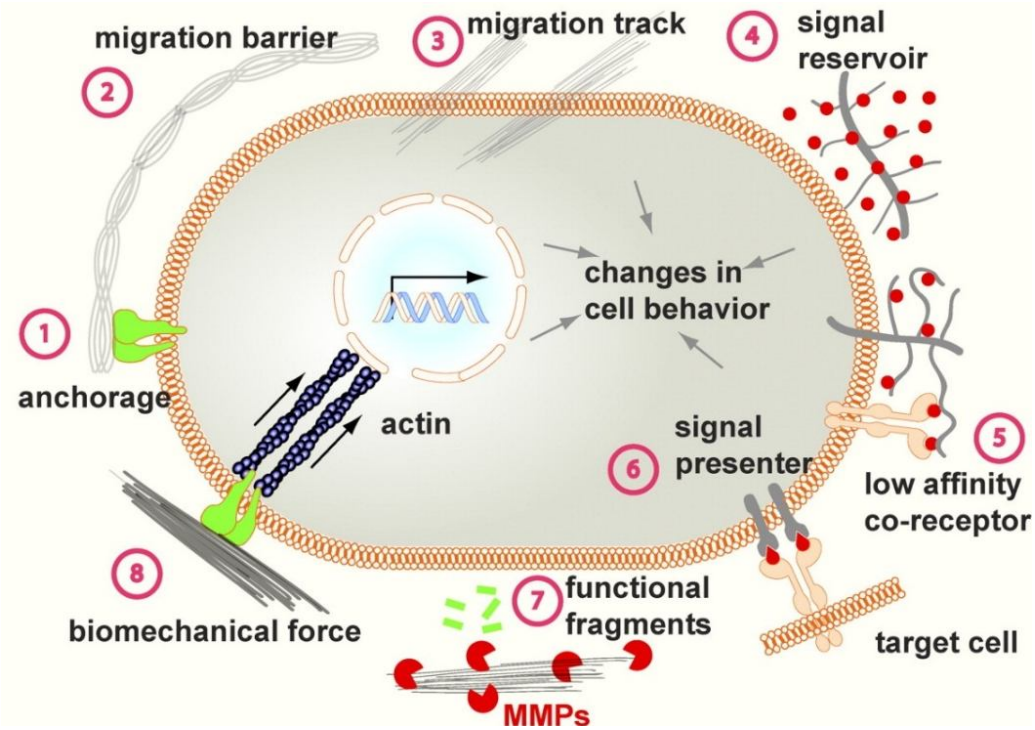


Figure 1.4. Roles of extracellular matrix in cell biology.

A figure selected from a paper by Lu P. et al. (J Cell Biol, 2012. 196(4): p. 395-406) [48]

1.2.3.2. Roles of ECM in tumor biology

During normal cellular development, the homeostasis of the ECM is maintained by a regulatory system that ensures the balance of formation and deformation processes. Disruption of that regulatory system is often observed in some abnormal conditions such as cancer. The ECM plays important roles in the initiation, progression and recurrence of different cancers.

Cancer initiation:

Extracellular components influence stem cell polarity by maintaining mitotic spindles for asymmetric division, therefore ensure the self-renewal capacity of stem cells [49]. The expansion and differentiation of stem cells are

strictly regulated by ECM factors. Disruption of this regulating system results in uncontrolled expansion, loss of polarity and differentiation and ultimately tumor development [50]. The ECM derived from bone marrow stromal cells or bone marrow mesenchymal cells was shown to stimulate mesenchymal stem cell expansion and prevent stem cell differentiation [51, 52]. The ECM expands stem cell populations by activating Erk1/2 and cyclin D1, which are responsible for cell-cycle progression and cells' commitment to S phase. The effects of the ECM on survival signaling were postulated to be via integrin activation or inhibition of reactive oxygen species (ROS) levels. As stem cell expansion has been proved to drive tumor development and recurrence [53], the ECM may contribute to tumor development via its effects on stem cell populations. Besides regulating stem cell expansion, secreted molecules in the ECM and intercellular junctions are required for stem cell differentiation [54]. Stem cells grown in ECM conditions similar to brain or bone express neuronal or musculoskeletal markers, suggesting that the ECM provides driving forces for the differentiation of some stem cell lineages [55].

Cancer progress and metastasis:

The ECM also plays crucial roles in cancer progress. Degradation of the basement membrane, a physical barrier against cell migration, by matrix-degrading enzymes leads to migration and invasion of cancer cells to distant sites [46]. In fact, changes in ECM structure, such as thickened and linearized collagen, are usually observed in tissues with active invading cells or angiogenesis [56]. The ECM influences angiogenesis or tumor vasculature due to its stimulatory roles in blood vessel formation. In addition, ECM anomalies

trigger recruitment of various factors, such as fibroblasts and immune cells, to create a tumor microenvironment that favors tumor progression [56].

Cancer recurrence:

The ECM drives cancer recurrence by activating tumor cells that are in dormant state. Quiescent cancer cells present at distant sites are converted to malignant forms when there is a favorable environment. Laminin-5 and fibronectin have been shown to trigger that transition process resulting in cancer metastasis or recurrence [57]. Details of the important roles of fibronectin in tumor recurrence will be reviewed in the following part.

1.3. FIBRONECTIN

1.3.1. Fibronectin structure

Fibronectin is a crucial and ubiquitous component of the ECM. Fibronectin exists in the fibril structure of the ECM and basement membrane to function as ECM scaffold, together with other fibers from collagen or elastin. Fibronectin is also present in the interstitial matrix, which interacts with both cells and basement membrane [58]. Fibronectin consists of two identical glycoprotein molecules connecting via disulfide bonds at the C-termini, with molecular weight of about 220-250 kDa each. The fibronectin monomer contains 3 types of repeating domains, which are types I, II and III, and a variable (V) region. There are numerous binding sites in a fibronectin molecule for binding with other ECM proteins, such as collagen, laminin, and elastin, and for binding with cellular surface via integrin receptors. Fibronectin are further modified by proteolysis or incorporation of type III extra domains A or B (EDA or EIIIA and EDB or EIIIB respectively) to expose cryptic positions for additional binding. [59]

All human fibronectin isoforms are encoded by one pre-mRNA generated from one single gene. The pre-mRNA is alternatively spliced at extra domains EDA, EDB or the variable (V) region to generate different fibronectin variants [60]. As a result of alterative splicing, there are more than 20 isoforms generated from the human fibronectin gene [59].

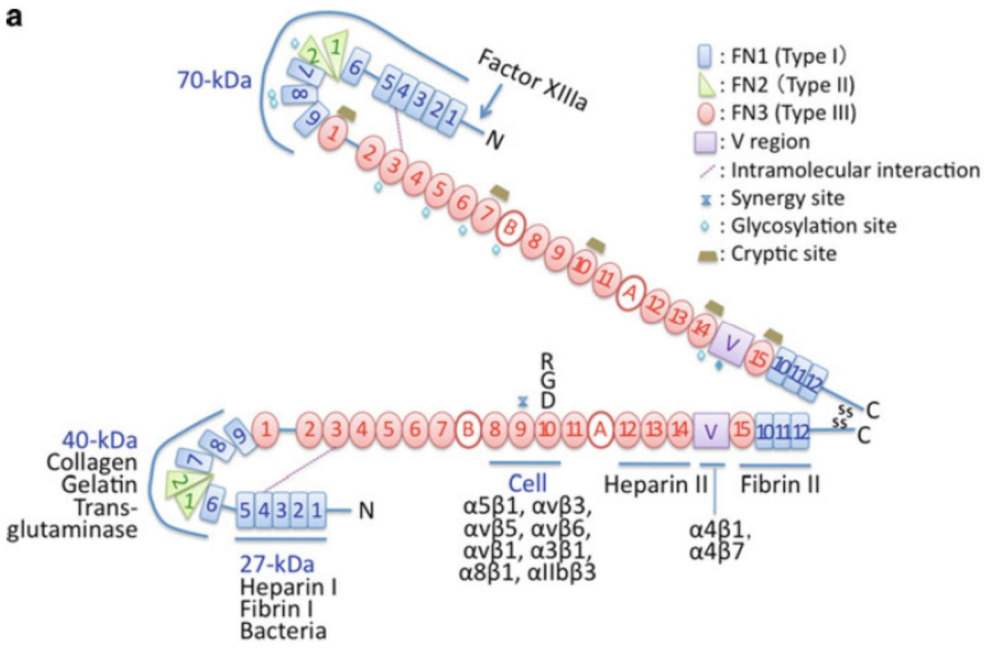


Figure 1.5. Fibronectin structure. Fibronectin consists of 2 monomer glycoproteins connecting via disulfide bonds. Each monomer contains repeated types I, II and III domains, a variable (V) region and alternatively splicing domains (A and B). *A figure from a chapter by Jielin Xu D.M. et al. (The Extracellular Matrix: an Overview 2011, Springer Berlin Heidelberg. p. 41-75) [61]*

1.3.2. Regulation of fibronectin expression

Fibronectin production in cells is regulated by different factors. Tumor necrosis factor alpha (TNF-α) stimulates fibronectin expression in coronary artery smooth muscle cells by a post-transcriptional mechanism dependent on nitric oxide [62, 63]. Basic fibroblast growth factor (bFGF) activates phosphatidylinositol phospholipase gamma 2 (PLCγ2), protein kinase C alpha (PKCα), c-Src and NFκB in osteoblasts, resulting in fibronectin synthesis [64]. In rat hepatocytes, the transcription of fibronectin is also regulated by NFκB by the binding of p65/p65 homodimer to fibronectin promoter [65]. High fibronectin expression was observed in the livers of Hepatitis B virus (HBV) carriers and co-localized with HBVx antigen (HBVxAg). HBVx Ag was shown to control fibronectin expression by transactivating fibronectin promoter via NFκB and repressing tumor suppressor p53 [66].

In some cancer cells, fibronectin gene is regulated by the transcription factor early growth response 1 (EGR-1), which plays important roles in regulating cell proliferation and differentiation. Overexpression of EGR-1 in human glioblastoma cells resulted in high fibronectin expression. EGR-1 exerted its effect by binding to 2 binding sites between -75 to -52 and -4 to +14 of the fibronectin promoter [67]. In melanoma cell lines, EGR-1 was also shown to bind to fibronectin promoter upon stimulation by hepatocyte growth factor (HGF) via activation of mitogen-activated protein kinase (MAPK). Consistenly, overexpression of NGFI-A binding protein (NAB2), the co-supressor of EGR-1, in melanoma cells reversed the effect of HGF on fibronectin expression [68].

Fibronectin expression is also regulated by post-transcriptional mechanisms including modification of mRNA stability and translation efficiency [69]. The 3'-untranslated region (UTR) in fibronectin mRNA consists of putative regulatory elements that play an important role in translation of fibronectin. Activation of the regulatory elements resulted in enhanced fibronectin mRNA turnover as well as fibronectin translation. Zhou *et al* [69, 70] demonstrated that light chain 3 of microtubule-associated proteins 1A/1B (LC3) bound to AU-rich region in UTR of fibronectin mRNA and facilitated attachment of ribosomes on fibronectin mRNA in endoplasmic reticulum (ER). As a result, LC3 directly augmented translation fibronectin mRNA. Taken the association of LC3 with microtubules into account, Zhou *et al.* [70] additionally showed that microtubules work in tandem with LC3 to increase fibronectin translation.

1.3.3. Regulation of fibronectin secretion

Two main forms of fibronectin exist, including plasma fibronectin in blood and cellular fibronectin in cytoplasm of cells. The two forms are different in structure and secreted by different origins. Plasma fibronectin is secreted by hepatocytes in a soluble form, without the presence of alternative splicing domains EDA and EDB. Fibroblasts, epithelial cells and endothelial cells are the main sources of cellular fibronectin, which is secreted in soluble forms and then converted to insoluble forms by incorporating into the fibril structure of the ECM. Unlike plasma fibronectin, cellular fibronectin contains alternative splicing EDA and/or EDB domains [59].

As mentioned above, fibroblasts have long been thought to be the main source of fibronectin secretion into ECM. However, mounting evidence has shown that some cancer cells such as melanoma [71] and hepatoma cells [72] also secrete fibronectin for purposes not-fully-understood.

Information about the secretion process of fibronectin is not abundant in literature. Like other secretory vesicles, fibronectin is synthesized in ER, post-translationally modified while it traffics from ER to Golgi network and finally secreted outside. Post-translational modification of fibronectin include glycosylation, phosphorylation and sulfation [73] to stabilize fibronectin from proteolytic degradation as well as to promote its binding capacity to other molecules [74, 75]. As a result, post-translational modification greatly contributes to the heterogeneity of fibronectin isoforms.

1.3.4. Functions of fibronectin in cell biology

1.3.4.1. Functions of fibronectin in normal cell biology

Fibronectin mediates the interactions between the ECM and cells via its binding to integrin receptors on the cell surface due to numerous binding domains in fibronectin molecule. The fibronectin-regulated interactions between the ECM and cells provide downstream signals of integrin pathways to promote cellular proliferation, survival and migration (Figure 1.6) [76].

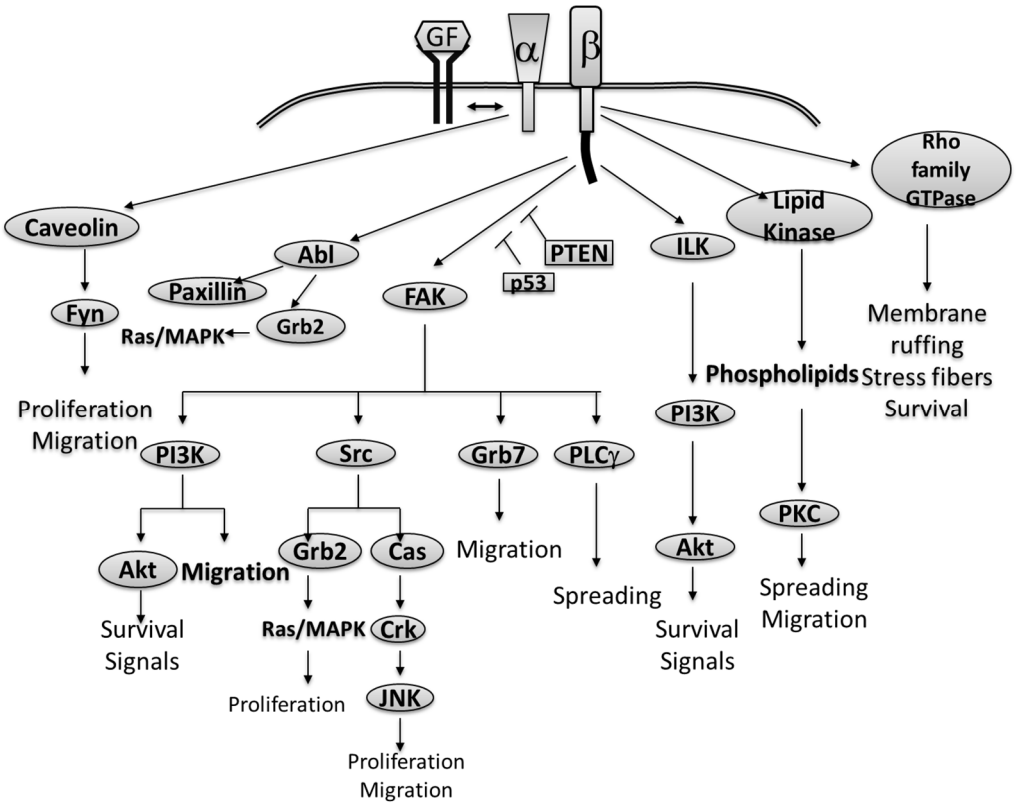


Figure 1.6. Integrin signaling pathways. *An adapted figure from a chapter by Reiske H.R. (Encyclopedia of Cancer (Second Edition), J.R. Bertino, Editor. 2002, Academic Press: New York. p. 493-499) [76]*

Fibronectin interacts with numerous transmembrane integrin receptors. It specifically binds to integrin α5β1, αIIbβ1, α3β1, αvβ3 via the Arg-Gly-Asp (RGD)-binding sites. RGR-binding sites are synergized by PHSRN peptide sequence at the 10^{th} and 9^{th} type III domains. Fibronectin also binds to integrin α4β1 via EDGIHEL sequence on EDA domain, and binds to α4β7 via LDV sequence on the variable (V) domain [59]. Upon binding to fibronectin, the heterodimers of integrin reorganize to expose binding sites for other intracellular molecules to bind to and be activated, such as focal adhesion kinase (FAK) and phosphoinositol 3 kinase (PI3K), c-Src, protein kinase C (PKC) and RhoGTPase family [76].

Not only does fibronectin affect cellular behavior, it also influences several physiological processes such as wound healing and thrombosis. Plasma fibronectin exists in circulation in an inactive form inside platelets. Once activated by the coagulation cascade following wound response, plasma fibronectin is secreted from platelets and deposited into ECM, where it attracts and stimulates adherent platelets and fibroblasts. Recruited fibroblasts secrete more cellular fibronectin, whilst platelets produce more plasma fibronectin. Cellular fibronectin, in turn, attracts more fibroblasts as well as other ECM proteins for thrombosis and wound healing processes [77, 78].

1.3.4.2. Functions of fibronectin in tumor biology

Cancer survival:

Several isoforms of fibronectin have been detected at high expression levels in some malignancies such as lung and colorectal cancers. By binding to integrin receptors, fibronectin stimulates numerous signaling pathways that are responsible for cancer tumorigenesis, anti-apoptosis and proliferation. The signaling pathways regulated by fibronectin-integrin include mitogen-activated protein kinases (MAPK), GTPase, PI3K/Akt/m-TOR [79-82], which have been established as pro-survival signaling pathways. Indeed, inhibition of fibronectin-intergrin binding by anti-α5β1 integrin or inhibition of PI3K abrogated the effects of fibronectin on cancer survival and anti-apoptosis [83]. In suspending culture condition, aggregates of human oral squamous carcinoma cells expressed higher fibronectin and integrin α5 receptors, and hence survived better than single cells [84].

Cancer invasion and metastasis:

It has been shown in ovarian cancer cells that fibronectin binding to integrin α5β1 triggered activation of c-Met in a mechanism independent of HGF, followed by stimulation of Src and FAK. The activation of this signaling resulted in increased cancer invasion and metastasis both in vitro and in vivo. In addition, inhibition of fibronectin-integrin binding by injection of anti-integrin α5 reversed the effects of fibronectin on invasion and metastasis in a mice model [85]. Interestingly, Kaplan *et al.* [86] have shown in a xenograft model that when a primary tumor was established, fibronectin was secreted by

stromal cells at the sites where metastasis often appears, followed by recruitment of VEGFR1-expressing hematopoietic progenitor cells (HPCs) from bone marrow. HPCs traversed through endothelium to the distal sites and created a pro-metastatic tumor microenvironment, even before tumor cells metastasized to those sites. Barkan *et al.* [57] observed that when fibronectin was produced by dormant mammary cancer cells, the cells left the quiescent state and proliferated. Moreover, exogenous fibronectin drove cancer cells form dormancy to metastasis. Taken together, these findings emphasize the important involvement of fibronectin in cancer invasion and metastasis.

Anoikis resistance:

Normal epithelial cells, when detached from ECM anchorage, undergo a type of anchorage-dependent apoptosis named anoikis. However, cancer cells survive anoikis by developing various measures to become anchorage-independent. Hamster ovary cells survived anoikis condition in serum-withdrawn culture medium due to fibronectin. Fibronectin exerted the anti-apoptotic effects by upregulating expression of B-cell lymphoma 2 (Bcl-2) independent of FAK [87]. On the other hand, fibronectin was shown to induce anoikis resistance in human squamous carcinoma (HSC-3) cells in a FAK-dependent mechanism [84]. Interaction of fibronectin and intergrin $\alpha 5$ resulted in phosphorylation of FAK at tyrosine 397 and suppression of p53, and hence protected the cells from anoikis. Anoikis resistance in HSC-3 in the suspension condition could be reversed by inhibition of FAK phosphorylation. The findings suggest that HSC-3 used fibronectin in ECM or produced by other cells to acquire anoikis resistant capacity. Interestingly, fibronectin

structure is of crucial importance in regulating cancer cells' survival in anoikis condition. Instead of inducing anoikis resistance, aberrant fibronectin structure with non-functional high-affinity heparin-binding domain promotes apoptosis of squamous carcinoma cells in a suspension condition. By down-regulating the expression of integrin α5 receptor, the mutated fibronectin suppresses FAK phosphorylation and ERK phosphorylation resulting in enhanced anoikis. [88]

Drug resistance:

Fibronectin stimulated downstream signaling of integrin β1 and changed the sensitivity of cancer cells to chemotherapy-induced apoptosis [89], suggesting that fibronectin promotes resistance of cancer cells to apoptotic reagents. Indeed, several studies have shown that fibronectin protects cancer cells from chemo-reagents. Xenograft-derived endothelial cells, when cultured on fibronectin, became resistant to DNA-breakage induced by etoposide [90]. Fibroblast-produced fibronectin protected colon cancer cells form apoptosis induced by etoposide and camptothecin [91]. Consistently, when fibronectin-integrin connection was inhibited by α5β1 antibodies, the cisplastin-resistant ovarian cancer cells became more sensitive to apoptotic-induced conditions such as anchorage-independence or serum derivation [92].

1.4. INTERACTION BETWEEN ACTIN CYTOSKELETON AND EXTRACELLULAR MATRIX REMODELING

1.4.1. The role of actin cytoskeleton in ECM-dependent apoptosis

Abnormal cytoskeletal remodeling and anoikis resistance are two common features of cancer cells. Normal epithelial cells require attachment to the ECM for survival and proliferation. When normal cells are detached from the ECM, they undergo a process called anoikis, which is an ECM-anchorage-dependent cell death. Anchorage-independent survival and anoikis-resistance are special abilities that cancer cells develop to allow them to survive without ECM attachment to colonize at distant metastatic sites. [93]

Actin cytoskeleton is one of the important factors that regulate anoikis-resistance in cancer cells. The stress fiber-stimulating factor tropomyosin isoform-1 (TM1), which stabilizes actin filaments, was shown to stimulate anoikis in breast cancer cells MDA-MB-231. Consistently, disruption of actin filaments by latrunculin A rescued breast cancer cells from anoikis induced by TM1 [94]. Although the role of actin cytoskeleton in regulating anoikis in cancer cells is established, the mechanisms underlying this effect still need to be elucidated.

1.4.2. Direct interaction between actin cytoskeleton and the ECM

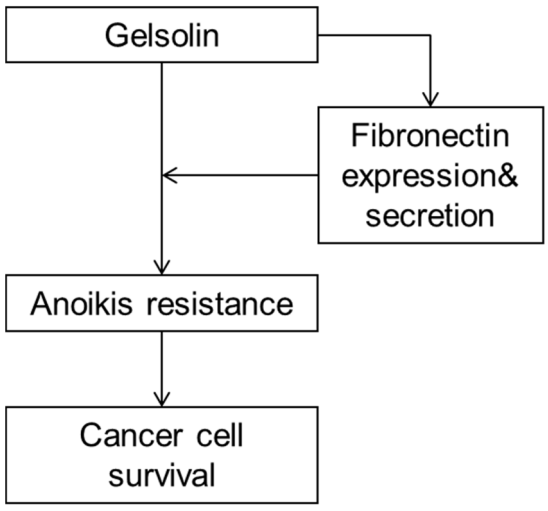
Actin cytoskeletal reorganization plays important roles in a number of cell activities including maintaining cell shape, facilitating cell motility and involving in cellular reactions to external environment [95]. Fibrils of fibronectin are formed in the infolding area of cell surfaces, which is formed

by fusion of secretory vesicles and plasma membrane. Actin filaments mechanically link with fibronectin and other ECM proteins via specific surface receptors of the integrin family. This connection allows the transmission of signals and mechanical forces from the ECM to the actin cytoskeleton and vice versa [96]. Indeed, actin and myosin are able to pull on extracellular fibronectin via integrin to expose binding sites for fibronectin or other ECM proteins. Thus, the cytoskeleton indirectly stimulates the assembly of fibronectin and other ECM proteins into fibrils. In addition, the cytoskeleton regulates the orientation of these fibrils. When organization of actin filaments is disturbed, the fibrils are disconnected from the surface, releasing cells from interactions with the ECM. This process often happens when cells undergo mitosis in normal conditions [43] or in some malignant processes such as anchorage-independent survival and dissemination of cancer cells. Therefore, by regulating actin reorganization, actin-binding proteins such as gelsolin pose importance roles in the interactions between the ECM and cellular behavior.

1.5. RATIONALE AND OBJECTIVES OF THE STUDY

The interaction between cancer cells and the ECM has a special position in tumor progression. The ECM serves as a platform for cancer cell anchorage, proliferation, survival and migration. In return, cancer cells are able to remodel the ECM to facilitate their survival, migration and invasion. Cancer cells exert these effects by degrading some ECM proteins and/or secreting some other components of the ECM. ECM-independent survival or anoikis resistance is a special way that cancer cells develop to escape apoptosis and cell death in ECM detachment conditions, which normally happen during cancer metastasis. Actin cytoskeleton remodeling is an important factor that regulates anoikis resistance in cancer cells. As disruption of actin cytoskeleton has been shown to induce enhanced anoikis resistance in cancer cells, it is possible that actin-severing proteins, such as gelsolin, may exert similar effects on cancer survival in anoikis conditions. In addition, as mentioned previously, the actin cytoskeleton and gelsolin may affect the secretion of ECM proteins. Moreover, gelsolin has been shown to regulate transcription of various genes by modulating their transcription factors or transcription co-activators. Interestingly, fibronectin has binding sites for plasma gelsolin [97, 98] suggesting a direct connection between the two proteins for as yet unknown cellular functions. Taken together, actin-binding proteins in general and gelsolin in particular probably play a role in regulating fibronectin secretion and expression. Given that fibronectin is a known promoter of anoikis resistance in cancer cells, it is possible that gelsolin contribute to anoikis resistance via its regulation of fibronectin secretion and expression and ECM reorganization.

In this study, we postulate that gelsolin affects anoikis resistance and cancer cell survival, and gelsolin exerts the effects by regulating fibronectin expression and secretion. In order to elucidate the hypothesis, we examine whether gelsolin modulates cancer cell survival in an anoikis condition by regulating fibronectin secretion. We also investigate the influence of gelsolin in the expression and secretion of fibronectin in colorectal cancer cell lines. We further explore the mechanisms by which gelsolin regulates fibronectin expression and secretion in colorectal cancer cells.



OBJECTIVES OF THE STUDY

The main objectives of this study are:

1. To investigate whether gelsolin influences colon cancer survival by regulating fibronectin expression and secretion

2. To explore the mechanisms by which gelsolin regulates fibronectin expression and secretion in colon cancer.

-CHAPTER 2-

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Chemicals and reagents

1st Base

siRNA against gelsolin, biotechnology grade water (Buf1180); Tris-HCl (B10-1500), 10% SDS (Buf 2051)

Abcam

Mouse monoclonal anti-gelsolin (ab11081) (1:1000 dilution); Rabbit polyclonal anti-fibronectin antibody (ab2413) (1:400 dilution).

Ambion

Silencer FN1 siRNA (4392421)

Applied Biosystems

Taqman PCR primers for Gelsolin (Hs00609275_m1); FN1 (Hs01549976_m1); EGR-1 (Hs 00152928_m1) and GAPDH (Hs99999905_m1).

Bio-Rad

Protein assay dye reagent (500-0006); Blotting-grade blocker (170-6404).

Cell Signaling Technology

Phospho-Akt (Ser473) Antibody #9271 (1:1000 dilution); Akt1 (C73H10) Rabbit mAb #2938 (1:1000 dilution).

Gibco

Geneticin G418 (10131-027); Opti-MEM (31985); Penecillin-Streptomycin (10,000 U/mL) (15140-122).

Fisher

Glycine (G/0800/60)

Hyclone

Fetal bovine serum (SV30160.03)

Invitrogen

Lipofectamine™ RNAiMAX transfection reagent (13778150); Lipofectamine™ 2000 (11668-019); Stealth RNAi™ siRNA Negative Control Med GC Duplex (12935-112).

PAA Laboratories

Trypsin EDTA (L11-003)

Promega

Dual-Luciferase® Reporter Assay System (E1910); ImProm-II Reverse Transcription System (A3800).

Qiagen

Cignal EGR1 Reporter (luc) Kit (CCS-8021L); RNeasy Mini Kit (250) (74106).

QREC

Potassium chloride (KCl) (7447407)

Roche

Cell proliferation ELISA, BrdU kit (colorimetric) (11647229001); Complete Protease inhibitor cocktail tablets (05-872-988-001);

Phosphatase inhibitor cocktail (04-906-837-001); Annexin-V-FLUOS Staining Kit (11 858 777 001).

Santa Cruz Biotechnologies

Mouse monoclonal anti-GAPDH (sc-32233); Goat anti-rabbit IgG conjugated with HRP (sc-2004); Goat anti-mouse IgG conjugated with HRP (sc-2005).

Sigma Aldrich

Dimethylsulfoxide (DMSO) (D2650); Triton X-100 (T8787); McCoy's 5A modified medium (M4892); RPMI 1640 (R4130); Sodium deoxycholate (D6750); Sodium bicarbonate (S6297); Bovine serum albumin (A9418); Trypan blue dye (72-57-1); Ammonium per sulfate (A3678); Hepatocyte growth factor (H9661-5UG); Propidium iodide (P4170); BAY 11-7085 (B5681); Tumor necrosis factor-α human (T0157).

Thermo Scientific

Super signal, West Dura, extended duration substrate (34076); Stripping buffer (21059); LDS Sample Buffer; Non-Reducing (4X) (84788); TEMED (Tetramethylethylenediamine) (17919); RNase A, DNA and protease-free (EN0531).

Vivantis

Tris-Base (PR0612); 10X PBS (PB0344).

2.1.2. Cell lines

HCT116, RKO and DLD-1 are human colon cancer cell lines obtained either from the laboratories of Prof. Shing Chuan Hooi or Dr Richie Soong. E1 cells, the *in vivo*-derived metastatic HCT116 cells, were obtained from Prof. Shing Chuan Hooi.

2.2. METHODS

2.2.1. Cell culture

HCT116 and E1 cells were cultured in McCoy's 5A modified medium (Sigma-Aldrich); DLD-1 and RKO were cultured in RPMI 1640 (Sigma-Aldrich). Serum-free media were media without serum but supplemented with 1% Penicillin-Streptomycin (Gibco). Complete media contained 10% fetal bovine serum (FBS) (Hyclone) and 1% Penicillin-Streptomycin. Stable transfected HCT 116 cell lines (gelsolin-overexpressing cell clones C1 and C8 and vector control cells) were cultured in McCoy's 5A medium supplemented with 500μg/mL Geneticin G418 (Gibco). All cell lines were grown at 37^{o}C in a humidified incubator supplemented with 5% CO_2.

2.2.2. DNA stable transfection

Stable overexpression of gelsolin in HCT 116 cells was described previously [34]. Gelsolin-encoding plasmids were generated by insertion of human cytoplasmic gelsolin cDNA into pIRES2-EGFP vectors (BD Biosciences Clontech). HCT 116 cells were transfected with gelsolin-encoding plasmids using FuGENE 6 (Promega) and selected using 500μg/ml G418 (Gibco). Vector control cells were generated by transfection of empty pIRES2-EGFP vector into HCT 116 cells. Transfected cells were selected by green fluorescence using Fluorescence Activated Cell Sorting (BD FACSCalibur) including clones C1 and C8 as gelsolin-overexpressing cells and V4 as vector control cells.

2.2.3. siRNA transfection

The following siRNA duplex oligoribonucleotide sequences (Invitrogen) were used for silencing expression of the gelsolin gene:

Oligo 1: UCUUGAAGAACUGCUUGAACAGUGG

Oligo 3: AAACGUCCAAUCUUGUUGGAGCAGG

Fibronectin siRNA for knocking down fibronectin was a pre-designed siRNA sequence against the FN1 gene obtained from Ambion.

For siRNA transfection, cells were cultured in 6-well plates at the density of $2.5x10^6$ cells/well in complete McCoy's 5A medium. The complexes of siRNA and Lipofectamine RNAiMAX transfection reagent (Invitrogen) were prepared by mixing a desired amount of 10nM or 20nM siRNA with Lipofectamine RNAiMAX in Opti-MEM reduced serum media (Gibco) and incubated at room temperature for 15 minutes. The complexes were then added to the cell culture in a drop-wise manner and incubated for four hours at 37ºC in an incubator. After that transfection media were replaced with complete media. Non-targeting siRNA control with medium GC content (Invitrogen) was used as a negative control for siRNA transfection.

2.2.4. Protein assay

Protein concentration was determined using the Bio-Rad protein dye reagent (Bio-Rad) with different concentrations of bovine serum albumin (BSA) (Sigma-Aldrich) as standards. One part of the dye reagent was diluted with four parts of water and filtered. 10µL of protein samples were added into 96-well plate with 200µL of 1X Bio-Rad protein dye reagents at room

temperature for 5 minutes. Absorbance was measured at 595nm using a microplate reader.

2.2.5. Western blot

Cell lysate preparation

Cells were harvested by trypsinisation for 5 minutes and then washed with 1X PBS and centrifuged. Cell pellets were lysed by radioimmunoprecipitation assay (RIPA) buffer (Sigma Aldrich) supplemented with protease inhibitors (Roche), vortexed, put on ice for 15 minutes and microcentrifuged at 10,000g for 10 minuties at 4ºC. The supernatants were collected in pre-chilled tubes. Protein concentration was determined using Bradford Protein Assay described above. In general, 25-40µg of extracted proteins and 20-30µl of culture supernatants were loaded on gels to measure the expression and secretion of protetins respectively. The exact amounts of proteins were stated in specific experiments.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The resolving gels used in the study (8 and 10%) were prepared with 30% bis – acrylamide, 1.5M Tris HCl pH 8.8, 10% SDS, ammonium persulphate (APS) and N, N, N', N'- tetramethyl – ethylenediamine (TEMED). 5% stacking gel was prepared with 30% bis - acrylamide, 1.0M Tris – HCl pH 6.8, 10% SDS (w/v), and polymerised with ammonium per sulfate (Sigma-Aldrich) and TEMED (Thermo Scientific). Protein samples were diluted with LDS sample loading buffer (Thermo) supplemented with 2-mercaptoethanol (Bio-Rad), and were heated at 95ºC for 5 min before loading into the gels. Equal

total protein amount of samples were loaded into each well for Western blot analysis. Housekeeping protein, GAPDH were used to as loading control. Samples were run at 80mV for 10 minutes and 150mV for 60-90 minutes depending on sizes of the proteins of interest. After that, SDS – PAGE resolved proteins were transferred onto activated polyvinylidene difluoride (PVDF) membrane, which had been activated by dipping in 100% methanol for 15 seconds, water for 2 minutes and then transfer buffer. Electroblotting was conducted at 350mA for 1 hour at 4ºC.

Immunodetection

After transfer, PVDF membrane was blocked with 5% w/v non-fat milk (Bio-Rad) in 1X TBST (20mM Tris – HCl, pH 7.6, 137mM NaCl, 0.1% Tween 20) for 1 hour at room temperature. The membrane was then incubated with primary antibodies (diluted in 5% milk w/v in 1X TBST or 5% BSA w/v in 1X TBST) and incubated overnight at 4ºC with gentle shaking. Unbound primary antibodies were washed off with 1X TBST (repeated 3 times, 10 minutes each with vigorous shaking). After that, the membrane was incubated with the respective horse radish peroxidase- conjugated secondary antibodies in 5% milk with gentle shaking for 1 hour at room temperature. Unbound secondary antibodies were again washed off with 1x TBST (repeated 3 times, 10 minutes each with shaking). The resulting immunocomplexes formed were detected using chemiluminescence substrate (Thermo Scientific, West Dura, Super signal extended duration substrate). For re-probing of the same membrane for different proteins, the membrane was incubated with stripping buffer (Thermo Scientific) for 15 minutes at room temperature. Membranes were rinsed once with 1X TBST for 15 minutes with agitation at room

temperature. Stripped membranes were then re-probed with the respective primary and secondary antibodies.

2.2.6. Real-time PCR

RNA Extraction

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. Samples were lysed and homogenised by passing through a 21-gauge needle ten times. Ethanol was added in a 1:1 ratio to provide ideal binding conditions before the lysates were loaded onto the silica membrane columns. The samples in columns were washed a few times using washing buffer to remove contaminants. RNA was then eluted in 30-50µl of RNase-free water. RNA concentration and quality was determined using NanoDrop ND-1000 spectrophotometer. All RNA samples were stored at -80^{o}C before cDNA synthesis.

cDNA sysnthesis

6µg of total RNA was converted to cDNA per 20µL reaction using ImProm-II Reverse Transcription System reagents (Promega). Oligo(dT) primer (2µg RNA in 1µL Oligo (dt)) was added to RNA in nuclease-free water. The reaction mix was heated at 70^{o}C for 5 minutes for target RNA and primer combination. This reaction mix was immediately chilled on ice for 5 minutes before adding reverse transcription reaction mix (ImProm-II5X Reaction Buffer, 3mM MgCl2, 0.5mM dNTP Mix, 20 units of Recombinant RNasein Ribonuclease Inhibitor and ImProm-II Reverse Transcriptase). The reaction mix was then incubated at 25^{o}C for 5 minutes, 42^{o}C for 50 minutes for

annealing and extension step respectively. A no-template (RNA) control was included. The cDNAs were stored at -20^{o}C until real-time PCR.

Real-Time PCR

Real-time PCR was carried out on the ABI 7500 Fast Real-time PCR system. cDNA was added to a custom Taqman Gene Expression Assay comprising a primer and Taqman probe, and combined with Taqman Universal PCR Master Mix (Applied Biosystems) according to manufacturer's instructions. PCR cylcles include one cycle at 95^{o}C for 10 minutes, followed by 40 cycles of denaturation at 95^{o}C for 15 seconds and annealing extension at 60^{o}C for 1 minute. GAPDH primer was included as an internal control.

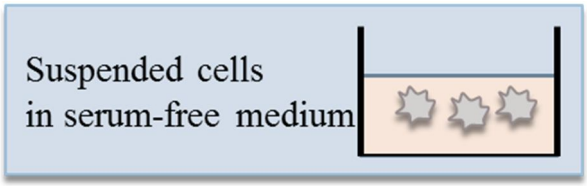
2.2.7. Immunofluorescence microscopy

Cells were plated onto a cover slip at a density of 2.5x10^{4} in a 24-well plate in complete media for 24h. After that cells were fixed with 4% paraformaldehyde in 1X PBS for 15 minutes at room temperature and washed with 1X PBS. Cells were permeabilised with 0.1% Triton X-100 in 1X PBS for 10min, and then blocked with 20% goat serum in PBS for 1h at room temperature. Cells were then incubated with fibronectin antibodies (Abcam) (1:100 dilution in 1% BSA in PBS) at 4^{0}C overnight in a humidifier chamber. Cells were washed three times with 1X PBS and incubated with secondary antibodies (1:400 dilution in 1% BSA in PBS) for 1 hour at room temperature followed by washing three times. The coverslips were mounted on a glass slide with VECTASHIELD® mounting medium containing nuclear counter

stain DAPI (Vector Laboratories). Samples were imaged with 100X oil immersion using Olympus Fluoview FV1000. The images were processed using Olympus Fluoview Viewer (Ver 2.0).

2.2.8. Anoikis model for studies of apoptosis and cell death

Cells were cultured in attachment plates in complete media before being trypsinized and collected. Cells were then counted and put into non-attachment plates (Corning Cat#3471) in serum-free media for 24 h for apoptosis study or cell death study.



2.2.9. Cell death analysis by flow cytometry

Cultured cells were collected for centrifugation at 405g in 5 min. Pellets were collected and re-suspended in 1X PBS. Cell pellets were then fixed with 70% ethanol while vortexing to avoid clumping. Suspensions were then kept at 4^{o}C overnight. After that cell pellets were centrifuged, washed with 1X PBS and re-suspended in propidium iodide (Sigma Aldrich) (200µg/mL DNase-free Rnase A, 20µg/mL propidium iodide in 0.1% (w/v) Triton X-100 in PBS) for 30 min at room temperature. Cells were filtered and subjected to flow cytometry using Becton Dickinson FACScalibur with excitation wavelength 288 nm and emission wavelength 610 nm. Data was analyzed using CellQuest Pro or WinMDI 2.9 software.

2.2.10. Apoptotic analysis by flow cytometry

Cultured cells were collected for centrifugation at 405g in 5 min. Pellets were collected and washed twice with 1X PBS. After that cell pellets were centrifuged and incubated in Annexin-V FLUOS labeling solution (Roche) for 15 min at room temperature. Cells were then diluted with incubation buffer, filtered and analyzed Becton Dickinson FACScalibur using 488 nm excitation and a 515 nm bandpass filter for fluorescein detection. Data was analyzed using CellQuest Pro or WinMDI 2.9 software.

2.2.11. Luciferase reporter assay

Transcriptional activity of EGR-1 and NFκB was measured by dual-luciferase reporter assay as per manufacturer's instructions (Qiagen). For testing the effects of gelsolin overexpression, transcription factor (EGR-1 or NFκB)-responsive reporter, negative control, and positive control constructs were mixed with the transfection reagent Lipofectamine 2000 (Invitrogen) and transfected to HCT 116 cells, vector control cells and gelsolin-overexpressing cells. For testing the effects of gelsolin knock-down, EGR-1 or NFκB-responsive reporter, negative control, and positive control constructs were diluted and mixed with diluted gelsolin siRNA or control siRNA (Invitrogen). The diluted nucleic acids were then mixed with Lipofectamine 2000 (Invitrogen) and transfected to cells. After 24h, culture media was replaced with serum-free media and incubated for another 24h. Following transfection, cells were harvested into cell lysis buffer (Promega) and luciferase activity was measured using the Dual-Luciferase Assay System (Promega) using a

Varioskan Flash Multimode Reader (Thermo Scientific). Relative transcriptional activity was calculated by the following fomula:

$$\text{Transcriptional activity (luciferase unit)} = \frac{\text{Ratio Firefly/Renilla of experiment samples}}{\text{Ratio Firefly/Renilla of negative controls}}$$

At least 3 independent transfections were performed in duplicate for each of the conditions tested with each reporter assay.

2.2.12. Statistical analysis

All statistical analyses were performed using Mann-Whitney U test. Differences between sample means were considered statistically significant with $p < 0.05$.

-CHAPTER 3-

GELSOLIN PROMOTES CANCER CELL SURVIVAL BY UPREGULATING FIBRONECTIN EXPRESSION AND SECRETION

3.1. BACKGROUND

Gelsolin has been associated with increased cancer cell survival, migration and invasion. Several investigations have shown that overexpression of gelsolin enhanced cell survival, migration and invasion, whilst loss of gelsolin resulted in decreased motility and invasion capacity. Gelsolin was considered as a tumor suppressor in some studies on primary tumors. However it was found highly expressed in invasive and metastatic cancers such as urothelial carcinoma, EGFR-positive breast cancer, and non-small cell lung cancer [26, 99-104]. These findings suggest that gelsolin may have different roles in different stages of cancer.

We have recently shown that overexpression of gelsolin enhanced invasion of colon cancer cells by increasing the expression and secretion of urokinase plasminogen activator (uPA), a potent matrix-degrading enzyme. We also observed increased gelsolin expression at the invasive border of metastatic colorectal tumors [105]. These data show an important contribution of gelsolin in remodeling the tumor ECM to promote tumor progression.

Among ECM proteins, fibronectin stands a special position in tumor biology as it has pivotal roles in cancer survival, proliferation, migration and invasion. High fibronectin expression was reported in several cancers. Fibronectin was identified to be one of the top 10 genes that were expressed at high levels in human colorectal cancer tissues compared to normal lesions [106]. High fibronectin expression was also observed in breast cancer tissues at advanced stages compared to earlier stages [107]. EDA isoform of fibronectin was found to be highly expressed in metastatic melanoma tissues compared to benign lesions [108]. Although fibronectin has long been thought

to be secreted by stromal cells like fibroblasts, mounting evidence has shown that cancer cells such as melanoma and hepatoma cells are also important sources of fibronectin [68, 109]. Interestingly, the presence of fibronectin was detected along migratory trails of cancer cells, suggesting that fibronectin was secreted when cancer cells migrate [110]. Fibronectin bound to integrin receptors on cell surface and triggered various signaling pathways such as MAPK, GTPase, PI3K/Akt/m-TOR to favor cell survival [79-82]. Inhibition of the interaction between fibronectin and integrin receptors using anti-integrin α5 antibodies reversed the anti-apoptosis and pro-survival effects of fibronectin [83]. Collectively, these findings emphasize the stimulatory involvement of fibronectin in cancer progression.

However, despite the important roles of fibronectin in cell biology in general and in tumor biology in particular, information about the secretion of fibronectin by cancer cells is still poorly understood. Moreover, it needs to be elucidated how the expression and secretion of fibronectin is regulated in cancer cells. In this chapter, we investigated the roles of gelsolin in the regulation of fibronectin expression and secretion in colorectal cancer cells. We explored the roles of gelsolin in cancer cell survival and found that gelsolin promoted survival of cancer cells under suspension conditions. Our findings indicated that gelsolin promoted cancer survival by enhancing fibronectin expression and secretion in colorectal cancer cell lines.

3.2. OBJECTIVES

1) To establish the roles of gelsolin in regulating fibronectin expression and secretion in colorectal cancer

2) To examine whether gelsolin regulates fibronectin expression and secretion to promote cancer cell survival.

Study model:

To investigate the roles of gelsolin in regulating fibronectin expression and secretion, we modulated gelsolin levels using gain-of-function and loss-of-function models in HCT 116, as described previously by our research group [34]. The gain-of-function model of gelsolin was achieved by stably overexpressing a plasmid encoding the cytoplasmic gelsolin sequence in HCT 116 colorectal cancer cells. The loss-of-function model was established by siRNA knock-down of gelsolin in HCT 116, RKO, DLD-1 colorectal cancer cells. We also used these models to investigate the effects of gelsolin on apoptosis and cell death in HCT 116 cells. The apoptosis and cell death studies were performed using an anoikis model, which was adapted from a study by Lee *et al.* [111]. Briefly, HCT 116 cells were cultured in suspension conditions in non-attachment plates with serum-free media. The anchorage-inhibition due to the suspension culture as well as the absence of serum in media induced apoptosis and cell death in HCT 116 cells.

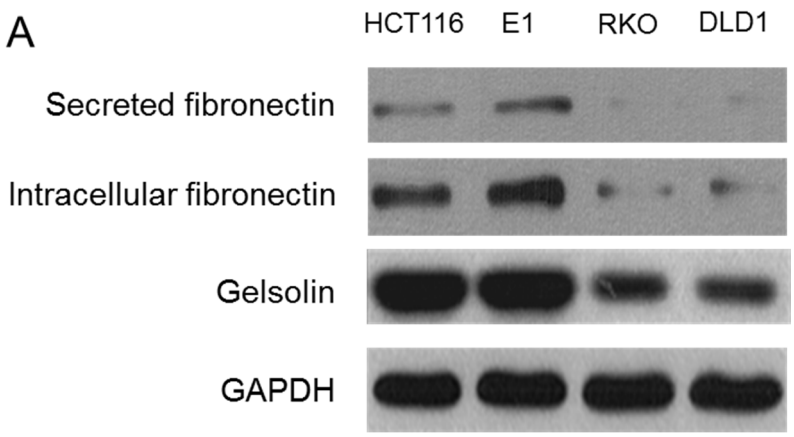
3.3. RESULTS

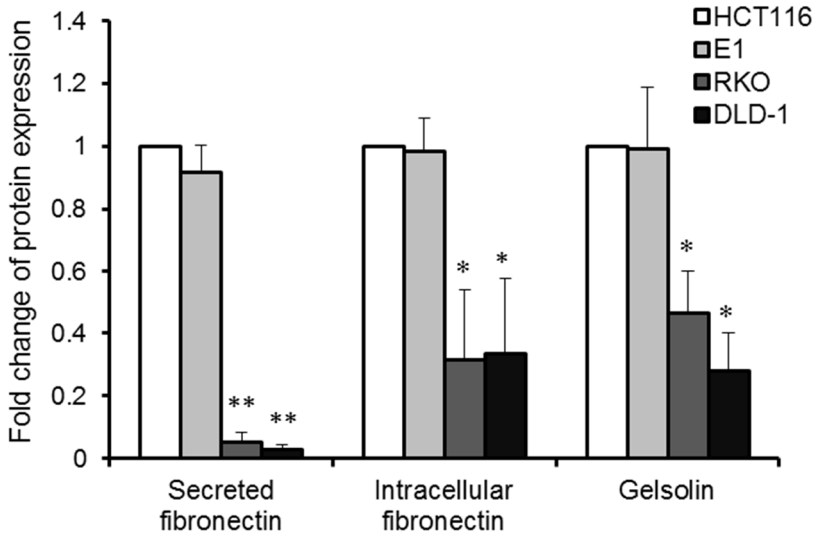
3.3.1. Expression and secretion of fibronectin correlate with gelsolin expression in colorectal cancer cells

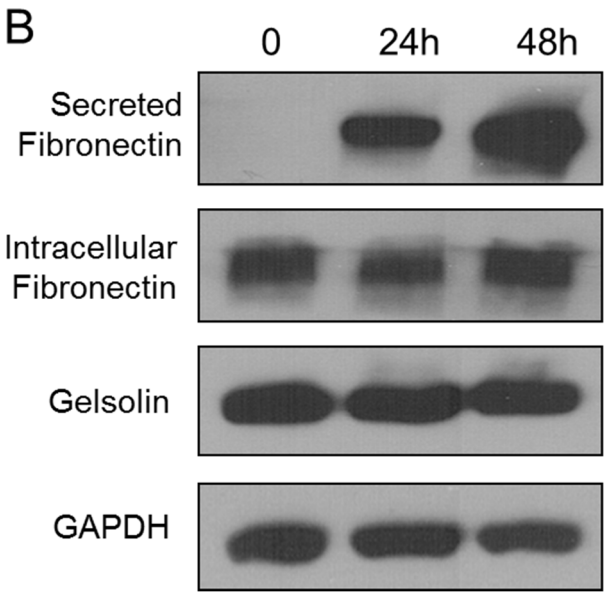
Previous studies have shown that fibronectin expression and secretion were detected in melanoma and hepatocellular carcinoma cells [71, 72]. We investigated whether fibronectin was also expressed and secreted in colorectal cancer cell lines HCT 116, E1, DLD-1 and RKO. As described previously, E1 was the *in vivo* derivation of HCT 116 cells in liver metastasis by repeated injection of HCT 116 cells into the spleen of nude mice [34]. Colorectal cancer cells were cultured in serum-free media for 48h. Supernatants and cells were collected for analysis of gelsolin and fibronectin. Western blot results showed that fibronectin was secreted and expressed in colorectal cancer cell lines HCT 116, E1, RKO and DLD-1. Fibronectin expression and secretion were higher in HCT 116 and E1 cells than that in RKO and DLD-1 cells. Moreover, fibronectin expression and secretion correlated with gelsolin levels in the cell lines. (Figure 3.1A)

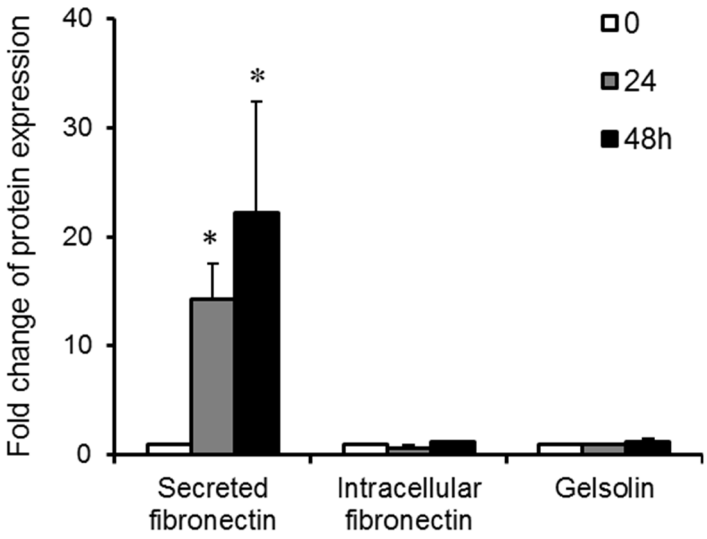
We choose HCT 116 cells, which exhibited relatively high fibronectin expression and secretion, for further investigation. HCT 116 cells were cultured in serum-free media. Supernatants and cultured cells were collected for Western blot analysis after 24h or 48h. Figure 3.1B shows that fibronectin was detected intracellularly and increasing amounts of fibronectin were detected in the supernatants after 24h and 48h of culture. To confirm that fibronectin detected in the supernatants of HCT 116 cell culture was not resulted from disruption of cell membrane integrity, but it was secreted by HCT 116 cells, we treated the cells with brefeldin A, an inhibitor of secretory

pathways. Brefeldin A was reported to induce collapse of Golgi stacks resulting in inhibition of secretion of all secretory vesicles [112]. Figure 3.1C shows that fibronectin in the culture media of HCT 116 cells was detected at lower levels when the cells were treated with brefeldin A, coinciding with higher expression of intracellular fibronectin. These findings confirmed that fibronectin detected in the supernatants of HCT 116 cell culture was secreted by HCT 116 cells. This secretion was attenuated by treatment with brefeldin A, resulting in intracellular fibronectin trapped inside the cells.


A
HCT116
E1
RKO
DLD1
Secreted fibronectin
Intracellular fibronectin
Gelsolin
GAPDH


Fold change of protein expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
HCT116
E1
RKO
DLD-1
**
**
*
*
*
*
Secreted fibronectin
Intracellular fibronectin
Gelsolin


B
0
24h
48h
Secreted Fibronectin
Intracellular Fibronectin
Gelsolin
GAPDH


Fold change of protein expression
40
30
20
10
0
*
*
0
24
48h
Secreted fibronectin
Intracellular fibronectin
Gelsolin

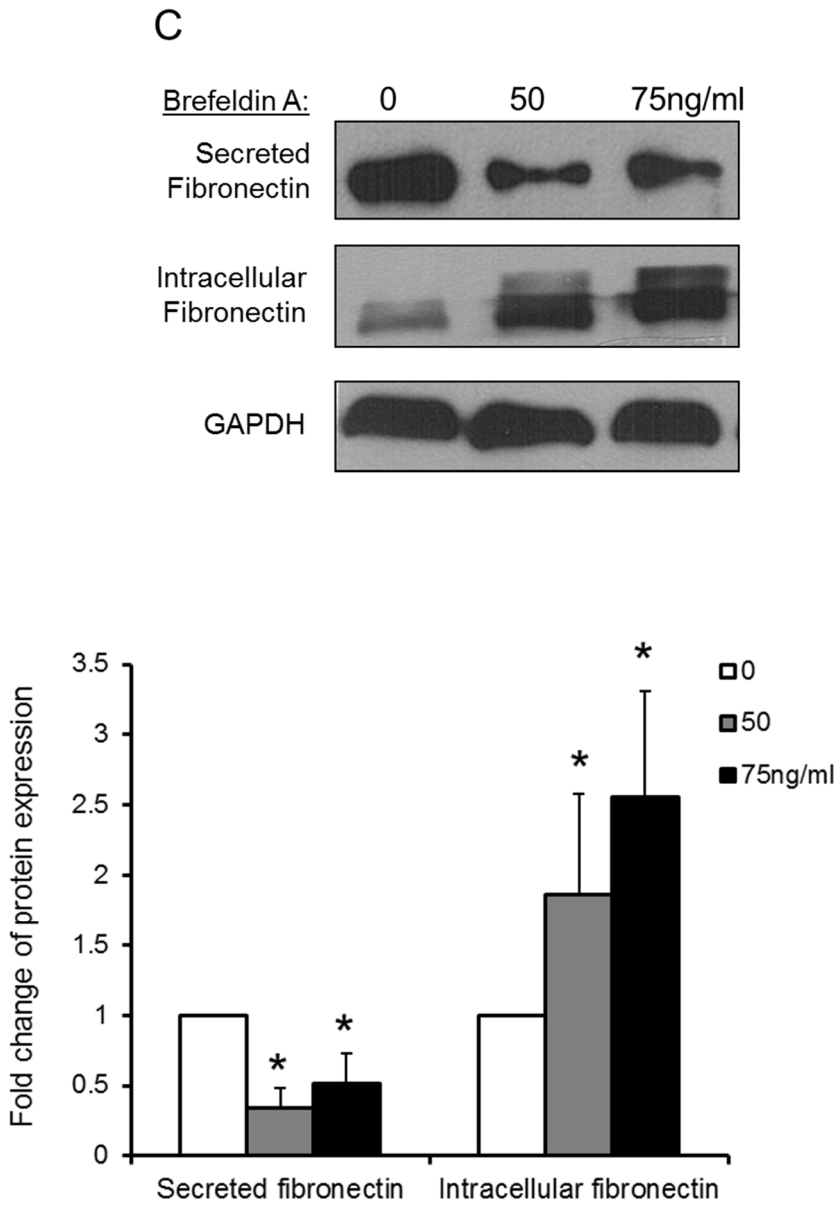


Figure 3.1. Fibronectin expression and secretion in colorectal cancer cells. (A) Colorectal cancer cell lines HCT 116, E1, DLD-1, RKO were cultured in serum-free media for 48h. After that, 15µl of supernatants and 25µg of extracted proteins were analyzed by Western blot to measure secreted fibronectin and intracellular proteins respectively. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; p-value<0.01, *p-value<0.05 (Mann-Whitney U test) compared to HCT 116 in the same group. (B) HCT 116 cells were cultured in serum-free media for 24 and 48h. 20µl of supernatants and 25µg of extracted proteins were analyzed by Western blot

to measure secreted fibronectin and intracellular proteins respectively. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments. *p-value<0.05 (Mann-Whitney U test) compared to 0h in the same group. (C) HCT 116 cells were cultured in serum-free media in the presence of 0, 50, 75ng/ml brefeldin A for 12h. 30μl of supernatants and 25μg of extracted proteins were analyzed by Western blot to measure secreted fibronectin and intracellular fibronectin respectively. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test) compared to no treatment in the same group (0ng/ml).

3.3.2. Models of gelsolin overexpression and siRNA knock-down used to investigate the effects of gelsolin on fibronectin expression and secretion

Previous data showed the correlation of gelsolin expression and the expression and secretion of fibronectin. In order to investigate the effects of gelsolin on fibronectin, we modulated gelsolin levels by gain-of-function and loss-of-function models. We overexpressed gelsolin in HCT 116 cells by stable transfection with the pIRES2-EGFP plasmid encoding human cytoplasmic gelsolin cDNA as reported previously (Appendix I) [34]. Control cells were generated by stable transfection with empty pIRES2-EGFP plasmid. Gelsolin in HCT 116 cells was knocked down by transfection with either of two siRNA sequences, oligo 1 or oligo 3. Control cells were transfected with non-targeting siRNA control. Our results showed that gelsolin-overexpressing cells (C1 and C8) expressed higher levels of gelsolin compared to vector control cells and wild-type HCT 116 cells (Figure 3.2A), whilst cells with knock down using siRNA (oligo 1 or oligo 3) against gelsolin expressed lower gelsolin compared to control cells (Figure 3.2B).

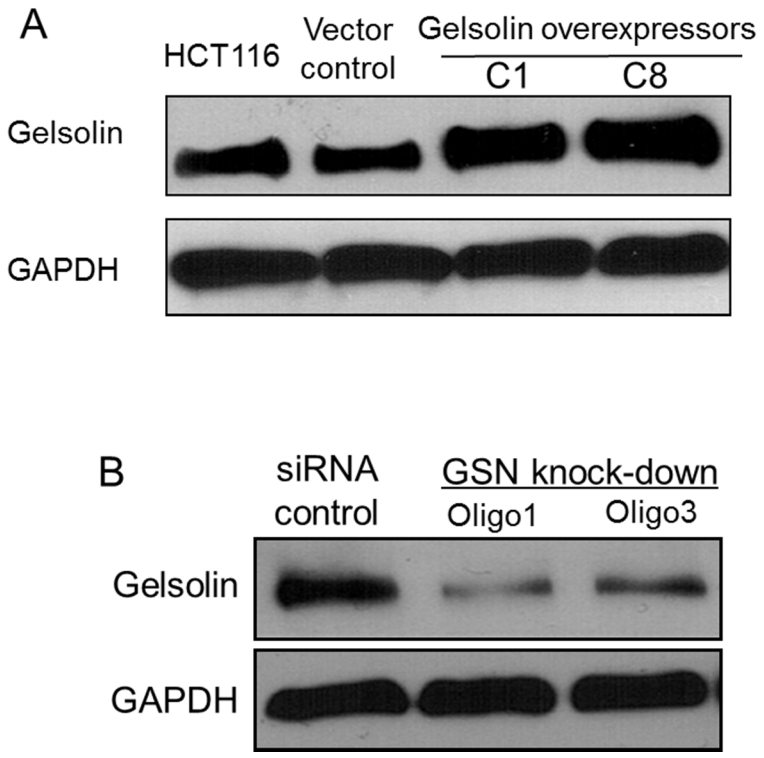


Figure 3.2. Modulation of gelsolin expression in HCT 116 cells. (A) Western blots of geloslin overexpression. HCT116 cells were stably transfected with pIRES2-EGFP plasmid encoding human cytoplasmic gelsolin cDNA. Two clones C1 and C8 were chosen for investigation. Vector controls cells were transfected with empty pIRES2-EGFP plasmid. 25μg of extracted proteins were analyzed by Western blot. (B) Western blots of gelsolin knock-down at 72h after transfection. HCT 116 cells were transfected with 10nM either of two siRNA sequences against gelsolin, oligo 1 or oligo 3, whilst siRNA control cells were transfected with non-targeting negative control siRNA for 72h. 25μg of extracted proteins were analyzed by Western blot to measure intracellular gelsolin expression.

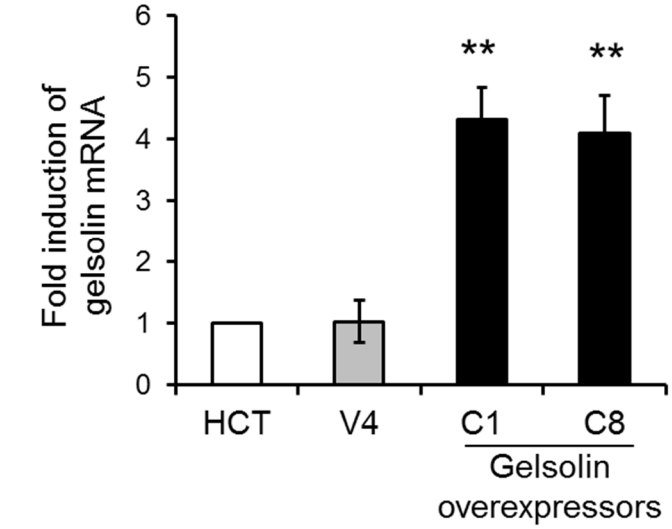
3.3.3. Gelsolin upregulates fibronectin expression and secretion

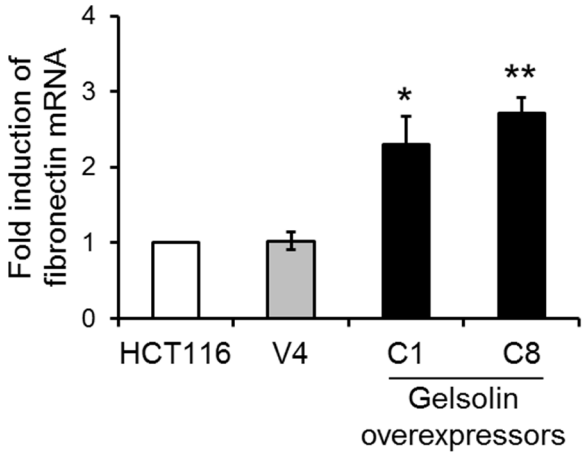
Our previous results demonstrated that fibronectin was secreted by HCT 116 cells. In this part of the study, we modulated levels of gelsolin to examine the effects of gelsolin on fibronectin expression and secretion.

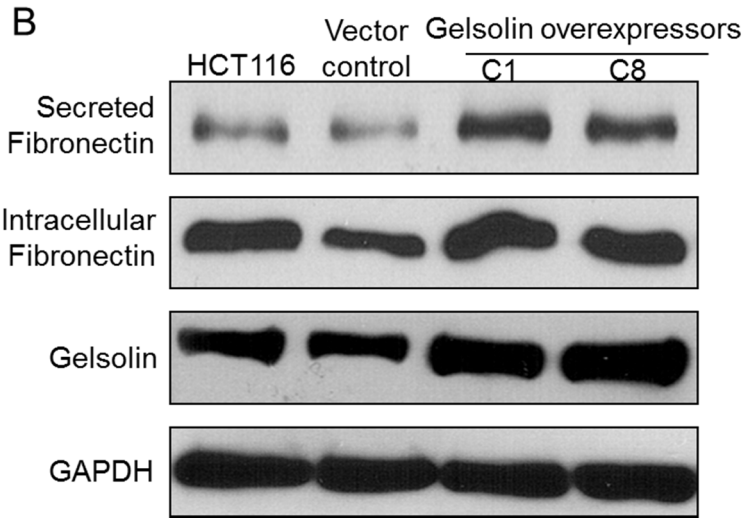
Wild-type HCT 116 cells, gelsolin-overexpressing cells (C1 and C8) and vector control cells (V4) were cultured in serum-free media. Supernatants and cultured cells were collected for real-time PCR, Western blot and immunofluorescence microscopy.

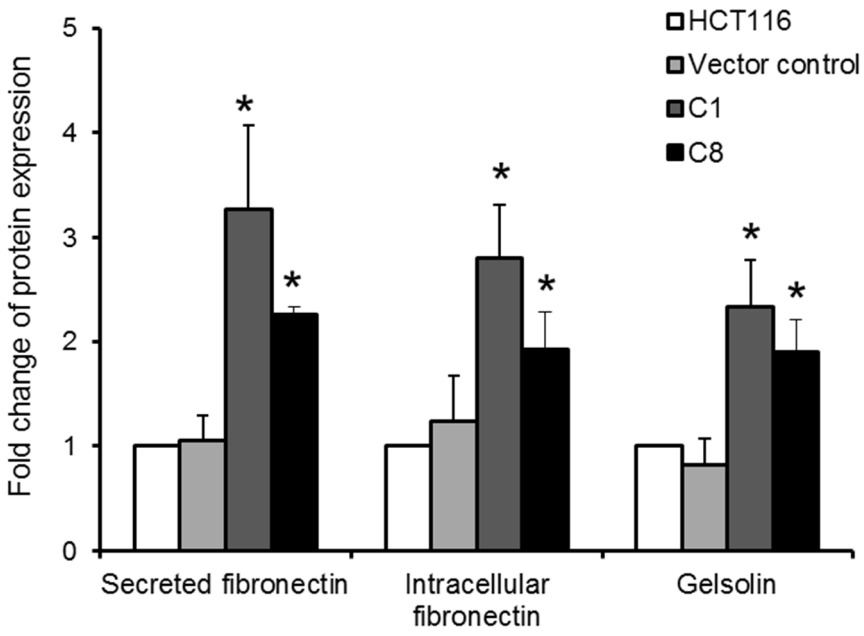
As shown in Figure 3.3, overexpression of gelsolin induced an increase in fibronectin expression in both mRNA (Figure 3.3A) and proteins levels (Figure 3.3B&C). Moreover, fibronectin secretion was also enhanced by gelsolin overexpression compared to control cells (Figure 3.3B).

A


Fold induction of gelsolin mRNA
6
5
4
3
2
1
0
**
**
HCT
V4
C1
C8
Gelsolin overexpressors


Fold induction of fibronectin mRNA
4
3
2
1
0
*
**
HCT116
V4
C1
C8
Gelsolin overexpressors


B
HCT116
Vector control
Gelsolin overexpressors
C1
C8
Secreted Fibronectin
Intracellular Fibronectin
Gelsolin
GAPDH


HCT116
Vector control
C1
C8
Fold change of protein expression
0
1
2
3
4
5
*
Secreted fibronectin
Intracellular fibronectin
Gelsolin

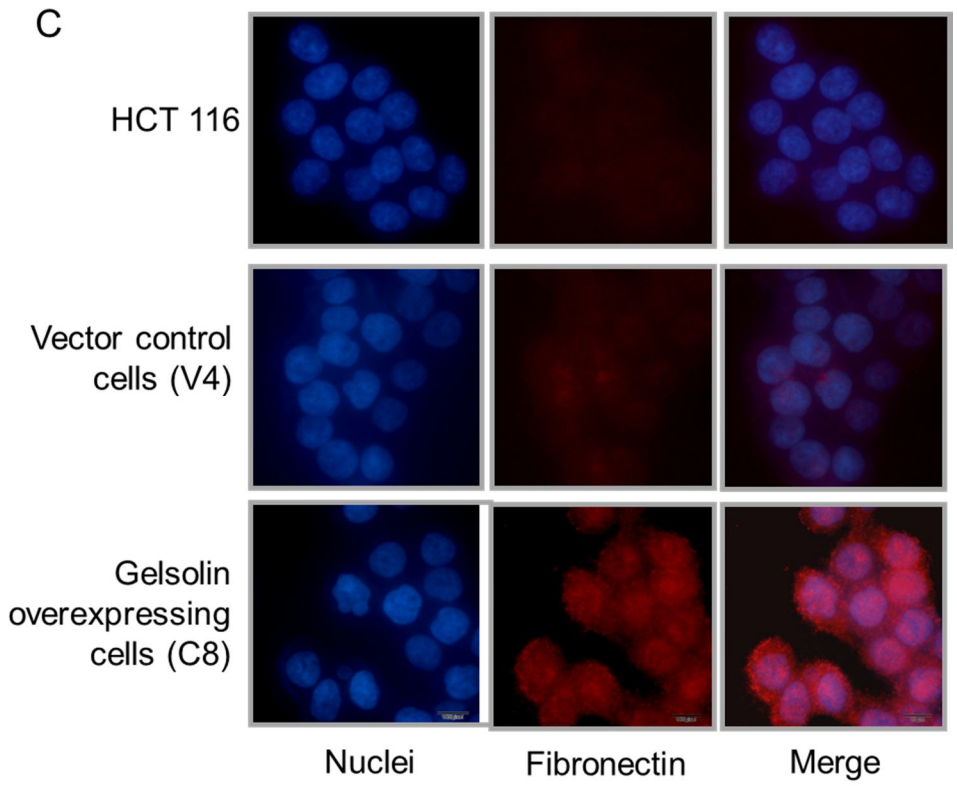


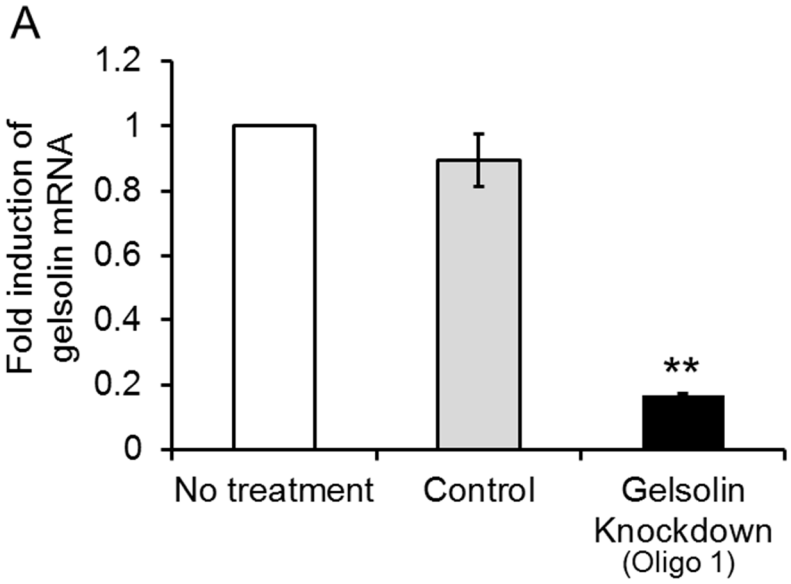
Figure 3.3. Gelsolin overexpression upregulates fibronectin expression and secretion by HCT 116 cells.

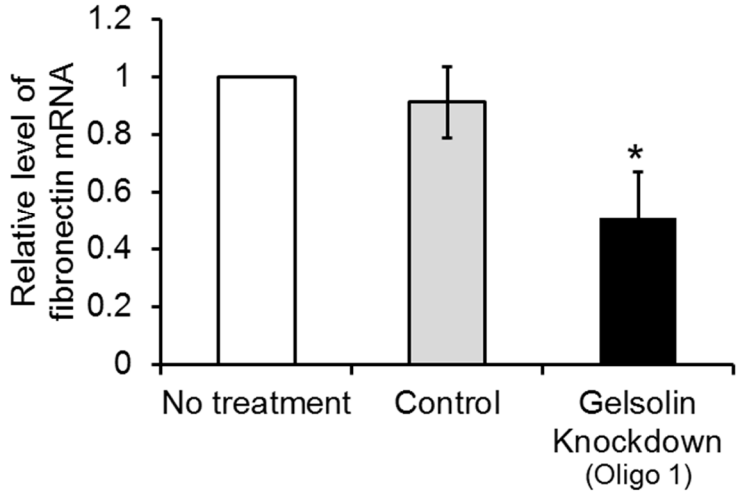
(A) HCT 116 cells, gelsolin-overexpressing cells (C1 and C8) and control cells (V4) were cultured in serum-free media for 24h. Cultured cells were collected for real-time PCR analysis. Data shown are mean± SD of at least three independent experiments. *p-value <0.05; **p-value <0.01 (Mann-Whitney U test).

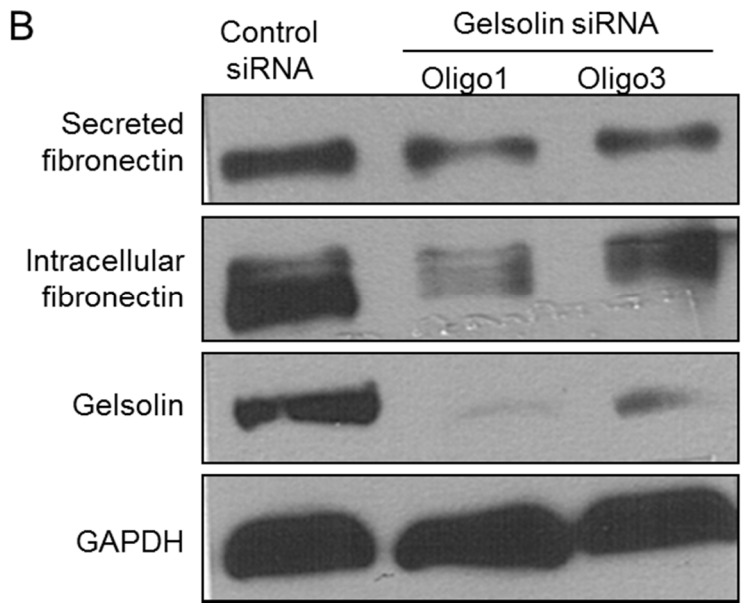
(B & C) HCT 116 cells, gelsolin-overexpressing cells (C1 and C8) and vector control cells (V4) were cultured in serum-free media for 48h. (B) 20µl of supernatants and 25µg of extracted proteins were analyzed by Western blot to measure secreted fibronectin and intracellular proteins respectively. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to HCT 116 in the same group. (C) Cells were collected, fixed, permeabilized and incubated with fibronectin antibodies and then Alexa Fluor 568-conjugated secondary antibodies (red). Nuclei were counter-stained with DAPI (blue).

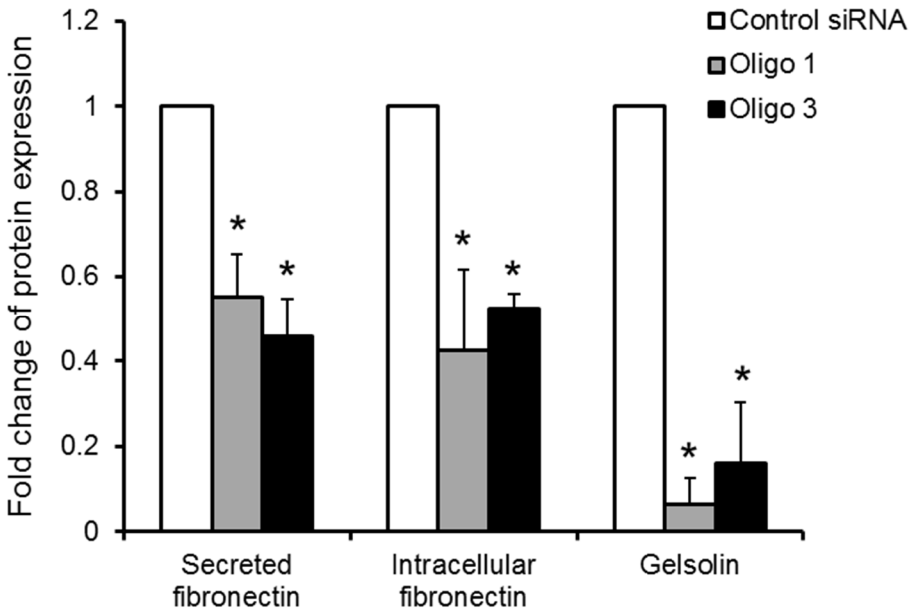
In order to investigate the effects of gelsolin knock-down on fibronectin, HCT 116 cells were transfected with gelsolin siRNA (oligo 1 or oligo 3) or control siRNA for 72h, and re-cultured in serum-free media. Cultured cells and supernatants were collected for real-time PCR, Western blot and immunofluorescence microscopy.

As shown in figure 3.4, knock-down of gelsolin induced a decrease in fibronectin expression in both mRNA (Figure 3.4A) and proteins levels (Figure 3.4B&C). Moreover, fibronectin secretion was also attenuated by gelsolin knock-down compared to control cells (Figure 3.4B).


A
Fold induction of gelsolin mRNA
1.2
1
0.8
0.6
0.4
0.2
0
**
No treatment
Control
Gelsolin Knockdown (Oligo 1)


Relative level of fibronectin mRNA
1.2
1
0.8
0.6
0.4
0.2
0
*
No treatment
Control
Gelsolin Knockdown (Oligo 1)


B
Control siRNA
Gelsolin siRNA
Oligo1
Oligo3
Secreted fibronectin
Intracellular fibronectin
Gelsolin
GAPDH


Fold change of protein expression
1.2
1
0.8
0.6
0.4
0.2
0
Control siRNA
Oligo 1
Oligo 3
*
Secreted fibronectin
Intracellular fibronectin
Gelsolin

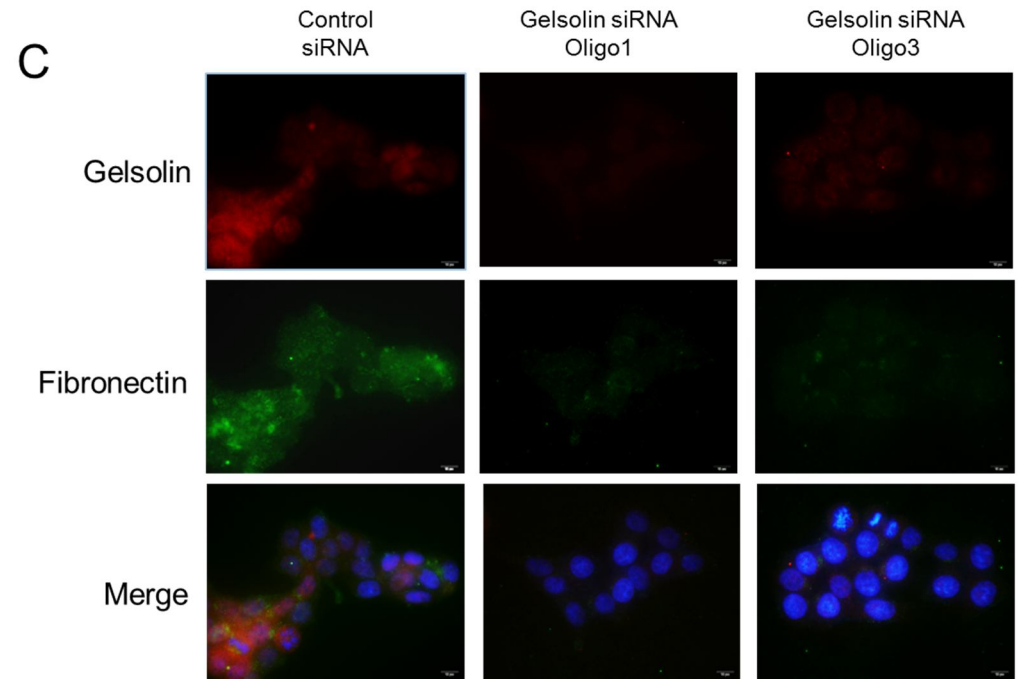


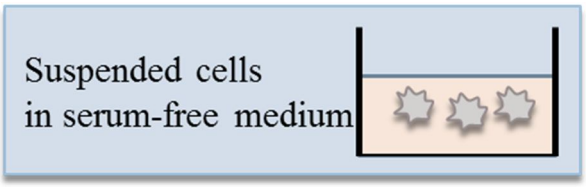
Figure 3.4. Gelsolin knock-down attenuates fibronectin expression and secretion in HCT 116 cells. HCT 116 cells were transfected with gelsolin siRNA (oligo 1 or oligo 3) or control siRNA for 72h and then re-plated in serum-free media.

(A) Cells were cultured in serum-free media for 24h, and then cell pellets were collected for real-time PCR analysis. Data shown are mean± SD of at least three independent experiments. *p-value <0.05; **p-value <0.01 (Mann-Whitney U test).

(B & C) Cells were cultured in serum-free media for 48h. (B) After that 20µl of supernatants and 25µg of extracted proteins were analyzed by Western blot to measure secreted fibronectin and intracellular proteins respectively. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to control siRNA in the same group. (C) Cultured cells were collected, fixed, permeabilized and incubated with fibronectin antibodies and then with Alexa Fluor 488-conjugated secondary antibodies (green). Cells were then stained with gelsolin antibodies and Alexa Fluor 568-conjugated secondary antibodies (red). Nuclei were counter-stained with DAPI (blue).

3.3.4. Gelsolin protects cancer cells from cell death in anoikis model

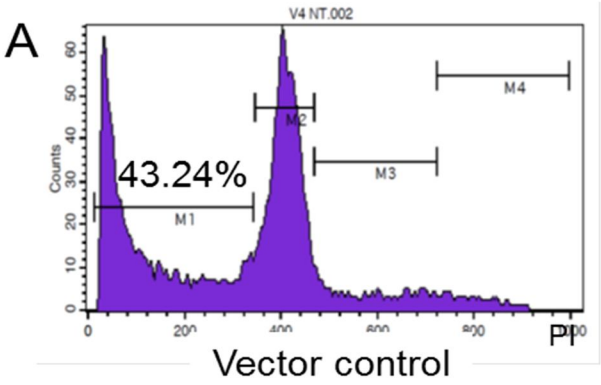
We investigated the protective effects of gelsolin on cancer cell survival using a model of anoikis, in which HCT 116 cells were cultured in non-attachment plates in serum-free media as illustrated below.

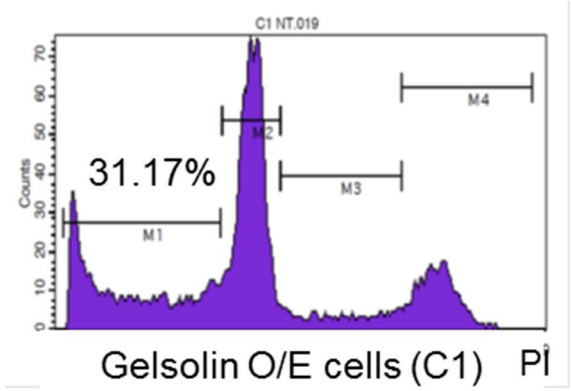


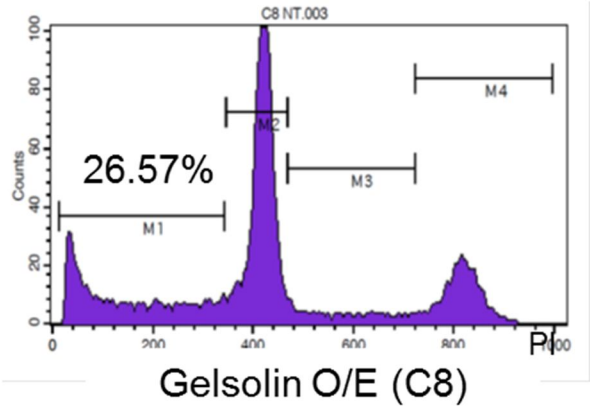
Anoikis model of apoptosis and cell death

After 24h of culture, cells were then collected for fixing with ethanol and staining with propidium iodide and subjected to flow cytometry for cell cycle analysis. Cell death was determined by measuring percentages of subG1 populations. Our results showed that when cultured in the anoikis condition, gelsolin-overexpressing cells (C1 and C8) exhibited less cell death compared to vector control cells (Figure 3.5). When HCT 116 cells were treated with gelsolin siRNA to knock down gelsolin, cell death was significantly increased compared to cells treated with control siRNA (Figure 3.6). These data suggest that gelsolin protects cancer cells against anchorage-dependent cell death.

A


V4 NT.002
Counts
43.24%
M1
M2
M3
M4
PI
Vector control


C1 NT.019
Counts
31.17%
M1
M2
M3
M4
Gelsolin O/E cells (C1)
PI


C8 NT.003
Counts
26.57%
M1
M2
M3
M4
PI
Gelsolin O/E (C8)

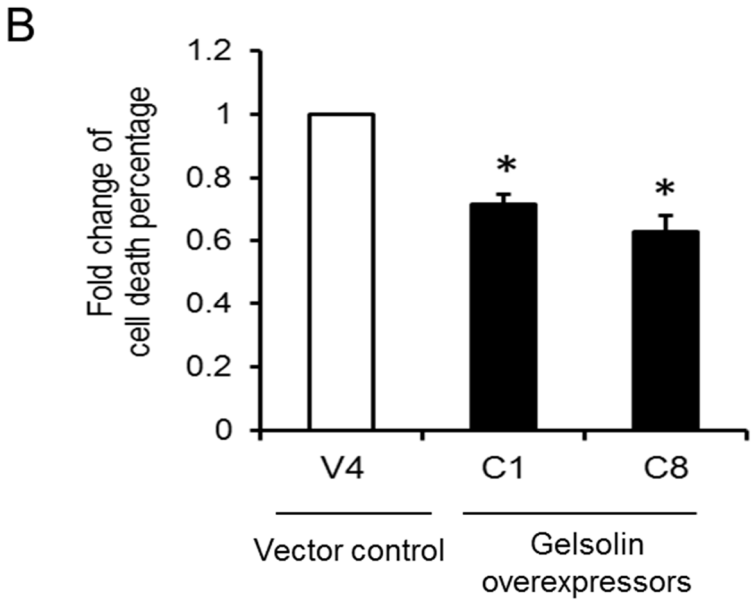
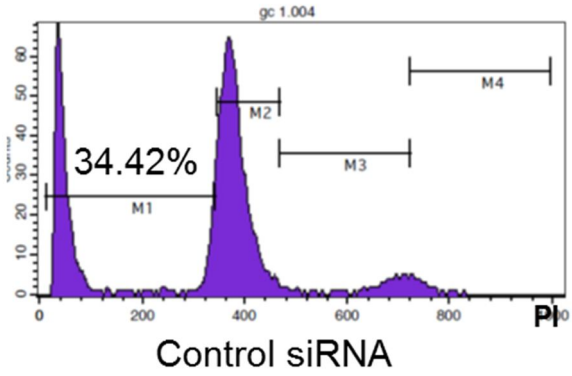
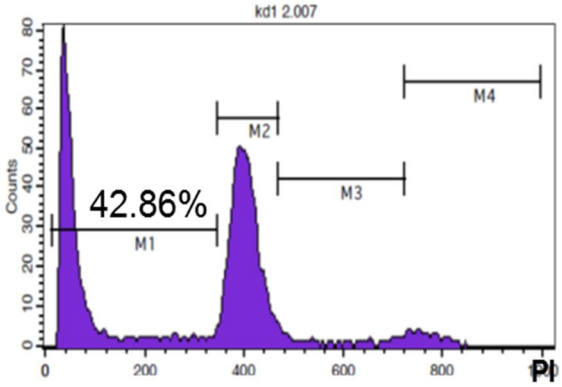


Figure 3.5. Gelsolin overexpression protects HCT 116 cells from cell death in anoikis model (flow cytometric analysis of propidium iodide staining). HCT 116, vector control (V4) and gelsolin-overexpressing cells (C1 and C8) were cultured in non-attachment plates at density of $2x10^6$ cells/24-well in serum-free media for 48h. After that, cells were collected, fixed with ethanol, stained with propidium iodide and then subjected to cell cycle analysis by flow cytometry. (A) Representative results of cell cycle analysis of HCT116, vector control cells and gelsolin-overexpressing cells (labeled as Gelsolin O/E cells (C1) and Gelsolin O/E cells (C8) in the flow histograms). Percentages of subG1 populations are indicated. (B) Cell death in HCT 116, vector control cells and gelsolin-overexpressing cells (C1 and C8) measured by percentages of subG1 population in cell cycle analysis. Data shown are mean± SD of three independent experiments. **p-value <0.01 (Mann-Whitney U test).

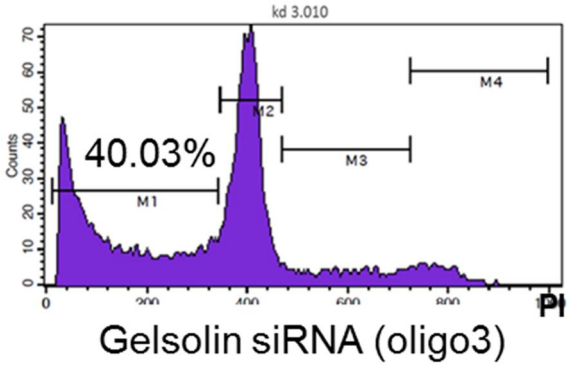
A



Control siRNA



Gelsolin siRNA (oligo1)



Gelsolin siRNA (oligo3)

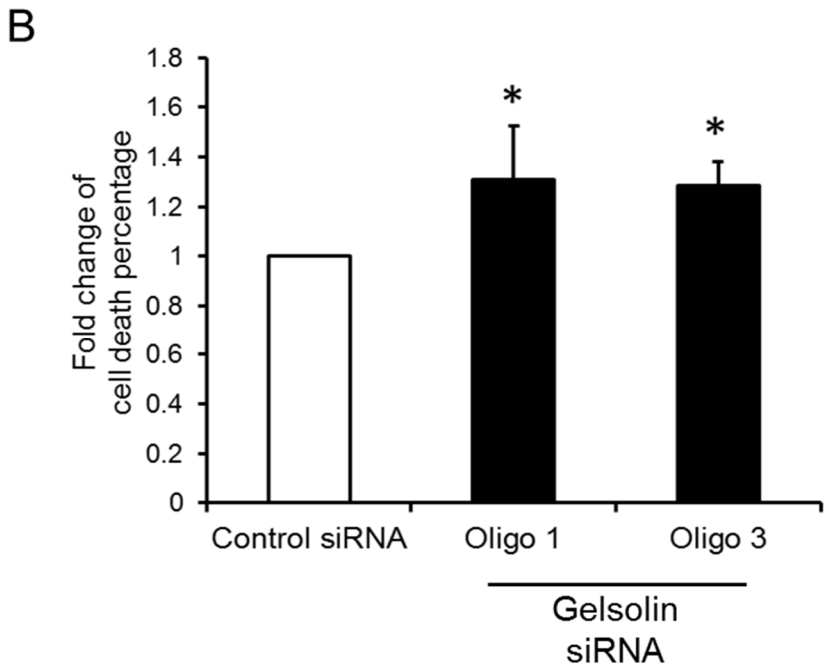


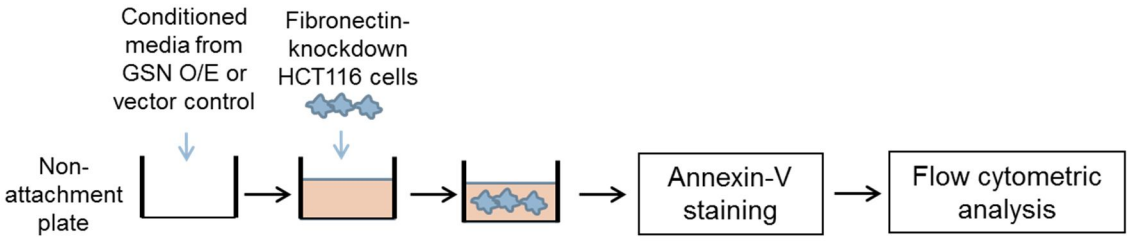
Figure 3.6. Gelsolin knock-down stimulates cell death of HCT 116 cells in anoikis model (flow cytometric analysis of propidium iodide staining). HCT 116 cells were treated with either gelsolin siRNA or control siRNA for 72h, and collected for re-culture at density of $3x10^6$ cells/24-well in non-attachment plates in serum-free media for 48h. After that, cells were collected, fixed with ethanol, stained with propidium iodide and then subjected to cell cycle analysis by flow cytometry. (A) Representative results of cell cycle analysis of cells transfected with control siRNA, gelsolin siRNA (oligo 1) or gelsolin siRNA (oligo 3). Percentages of subG1 populations are indicated. (B) Cell death in control cells, gelsolin siRNA knock-down cells (oligo 1 and oligo 3) was measured by percentages of subG1 population in cell cycle analysis. Data shown are mean± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test) compare to control siRNA.

3.3.5. Conditioned media of gelsolin-overexpressing cells protect HCT 116 cells from apoptosis in anoikis model

Literature suggests that gelsolin plays an important role in secretory pathway. Our results in this study showed that gelsolin promotes fibronectin secretion in HCT 116 cells and gelsolin protected HCT 116 cells from anoikis. We wanted to know if secreted molecules from gelsolin-overexpressing cells were the factors that protect HCT 1116 cells from apoptosis in the anoikis condition. In order to investigate this postulation, we used conditioned media from culture of gelsolin-overexpressing cells to culture HCT 116 cells in non-attachment plates. To focus on the effects of fibronectin secretion from gelsolin-overexpressing cells, and rule out the effects of endogenous fibronectin in HCT 116 cells, HCT 116 cells were treated with fibronectin siRNA to knock down their endogenous fibronectin before being cultured in conditioned media from gelsolin-overexpressing cels.

The effects on apoptosis were measured by annexin-V staining with flow cytometry. The basis for this assay is that when a cell undergoes apoptosis, phosphatidylserine, which is normally located on the inner side of plasma membrane, translocates to the outer side and is exposed for binding with annexin-V. FITC-conjugated annexin-V can be detected by flow cytometry and the intensity of annexin-V staining, therefore, represents apoptotic levels of the cell.

The set-up of this experiment is illustrated in the following diagram:

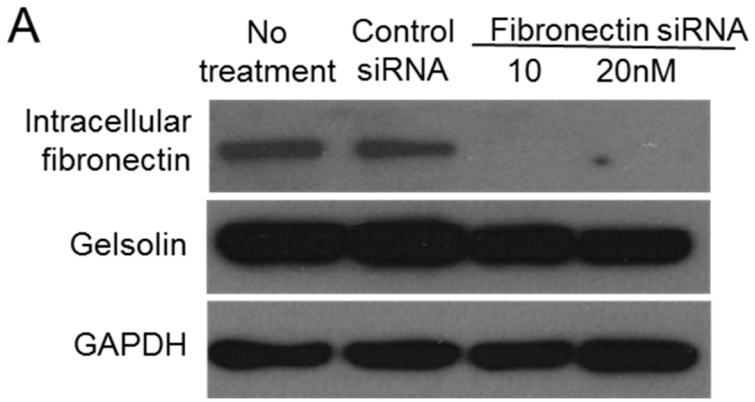


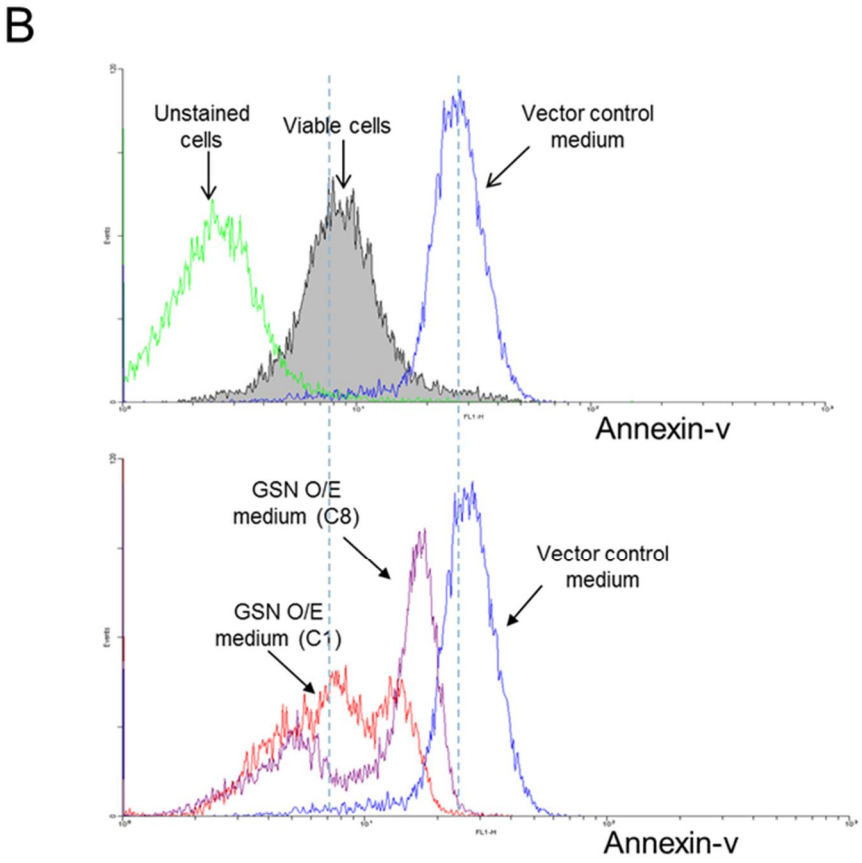
Experimental set-up for investigation of the effects of conditioned media from gelsolin-overexpressing cells on HCT 116 cell apoptosis

Briefly, gelsolin-overexpressing cells and vector control cells were cultured in serum-free media in attachment plates for 24h. Conditioned media of the cell culture were collected and put into non-attachment plates. In another setting, HCT 116 cells were transfected with fibronectin siRNA for 48h and then re-cultured in the non-attachment plates with conditioned media from gelsolin-overexpressing cells or vector control cells described above. After 24h, fibronectin-knockdown HCT 116 cells were collected, stained with annexin-V and subjected to flow cytometry. As a negative control for apoptosis, wild-type HCT 116 cells were cultured in a standard culture condition, which is in attachment plates with complete media.

Fibronectin-knockdown HCT 116 cells cultured in conditioned media from vector control cells experienced apoptosis in the anoikis model as shown by higher annexin-V staining compared to viable cells. When cultured in condition media from gelsolin-overexpressing cells (GSN O/E media from C1 or C8), fibronectin-knockdown HCT 116 cells exhibited lower annexin-V staining than those cultured in media from vector control cells (Vector control

medium) (Figure 3.7), suggesting that condition media from gelsolin-overexpressing cells contain anti-apoptotic factor(s).


A
No treatment
Control siRNA
Fibronectin siRNA
10
20nM
Intracellular fibronectin
Gelsolin
GAPDH


B
Unstained cells
Viable cells
Vector control medium
Annexin-v
GSN O/E medium (C8)
GSN O/E medium (C1)
Vector control medium
Annexin-v

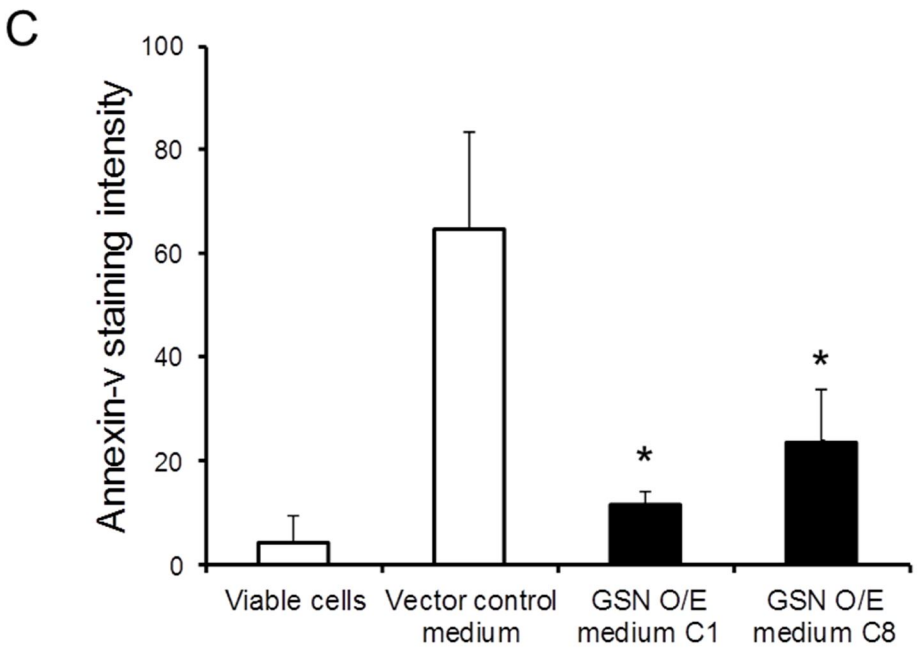


Figure 3.7. Conditioned media from gelsolin-overexpressing cells protect cancer cells from anoikis-induced apoptosis. HCT 116 cells and gelsolin-overexpressing cells were cultured in serum-free media in attachment plates in 24h. Supernatants of the gelsolin-overexpressing cells and of the vector control cells were collected to put into non-attachment plates. Wild-type HCT 116 cells were transfected with fibronectin siRNA for 48h, collected and re-cultured in the non-attachment plates with conditioned media (collected from culture of gelsolin-overexpressing cells or vector control cells). After 24h, cells were collected for staining with annexin-V and flow cytometric analysis.

(A) HCT 116 cells were transfected with either fibronectin siRNA or control siRNA for 48h. Western blot results showed that fibronectin was knocked down after transfection with fibronectin siRNA compared to control siRNA.

(B) Histograms obtained from one representative experiment. The following are details of the experimental groups in the flow histograms:

- Viable cells: as a negative control for apoptosis, HCT 116 cells were cultured in a standard culture condition (i.e. in attachment plates with complete media) for 24h, and stained with annexin-V before flow cytometry analysis.

- Unstained cells: as a negative control for annexin-V staining, HCT 116 cells were cultured in a standard culture condition (i.e. in attachment plates with complete media) for 24h, and NOT stained with annexin-V.
- Vector control medium: HCT 116 cells transfected with fibronectin siRNA and cultured in conditioned media from vector control cells.
- GSN O/E medium (C1): HCT 116 cells transfected with fibronectin siRNA and cultured in conditioned media from gelsolin-overexpressing cells C1.
- GSN O/E medium (C8): HCT 116 cells transfected with fibronectin siRNA and cultured in conditioned media from gelsolin-overexpressing cells C8.

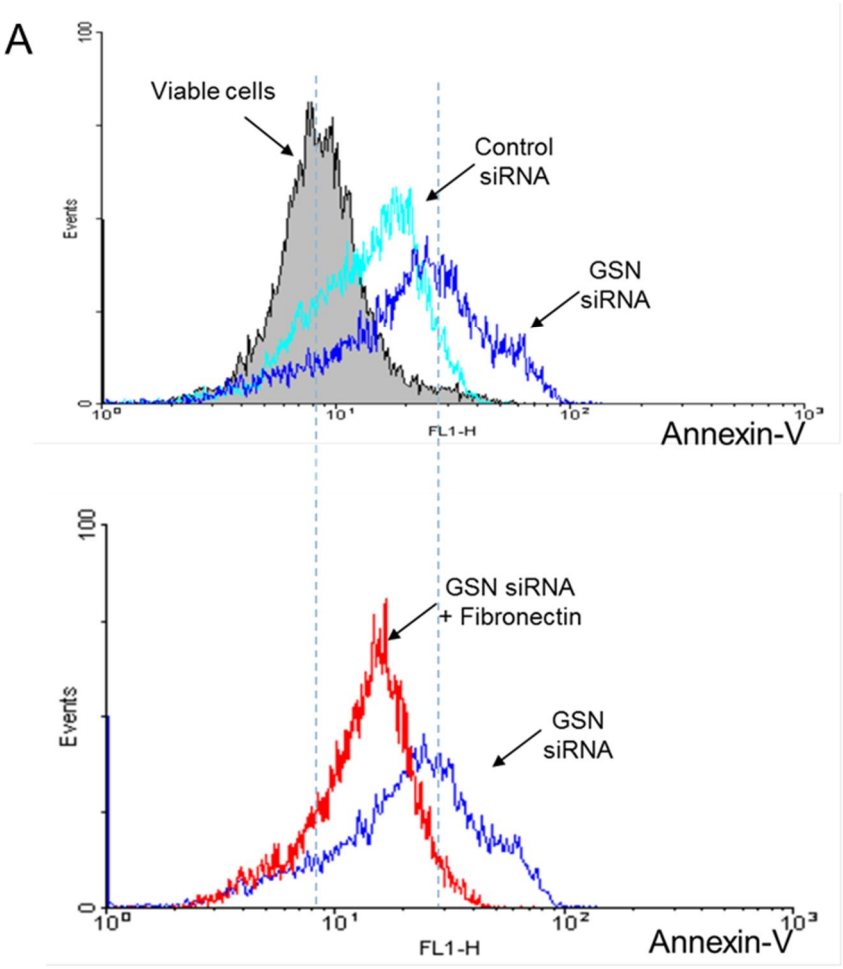
(C) Quantitative analysis of median of annexin-V staining. Data shown are median ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test).

3.3.6. Fibronectin rescues the effects of gelsolin knock-down on apoptosis of HCT 116 cells in anoikis model

We have shown that gelsolin promotes survival of HCT 116 cells in the anoikis condition and gelsolin knock-down induces HCT 116 cell death. Our data also highlighted that gelsolin-overexpressing cells secret soluble factors that protect HCT 116 cells from apoptosis. As far as the important role of fibronectin in cell survival is concerned, it is possible that secreted fibronectin from gelsolin-overexpressing cells the factor that protected HCT 116 cells against apoptosis, and hence promoted HCT 116 cell survival. In this part of the study, we investigated the effects of gelsolin knock-down on apoptosis in the anoikis condition and examined whether exogenous fibronectin could reverse the effects of gelsolin knock-down on apoptosis.

HCT 116 cells transfected with either gelsolin siRNA or control siRNA were re-cultured in non-attachment plates in serum-free media for 24h. After that, the cells were collected, washed and stained with annexin-V for 15min and subjected to flow cytometry. As a negative control for apoptosis, untransfected HCT 116 cells were cultured in a standard culture condition (i.e. in attachment plates with complete medium) for 24h, and then stained with annexin-V. In order to assess the effects of fibronectin on apoptosis, exogenous fibronectin 50μg/ml was added to gelsolin-knockdown cells, which were cultured in the anoikis condition. Based on published investigations on the effects of fibronectin on cell survival, in which fibronectin was used to coat culture plate at concentration from 0.5-20μg/ml [81, 82], the concentration of fibronectin was optimized and chosen at 50μg/ml for this experiment.

As shown in figure 3.8, when gelsolin was knocked down using siRNA, more apoptosis was induced in the cells, indicated by higher annexin-V staining compared to cells treated with control siRNA. When cells were treated with exogenous fibronectin, gelsolin knock-down cells exhibited lower intensity of annexin-V staining, in a level comparable to control siRNA cells. These data indicate that fibronectin rescued the effects of gelsolin knock-down on apoptosis in HCT 116 cells.


A
Viable cells
Control siRNA
GSN siRNA
Events
100
0
10^0
10^1
10^2
10^3
FL1-H
Annexin-V
GSN siRNA + Fibronectin
GSN siRNA
Events
100
0
10^0
10^1
10^2
10^3
FL1-H
Annexin-V

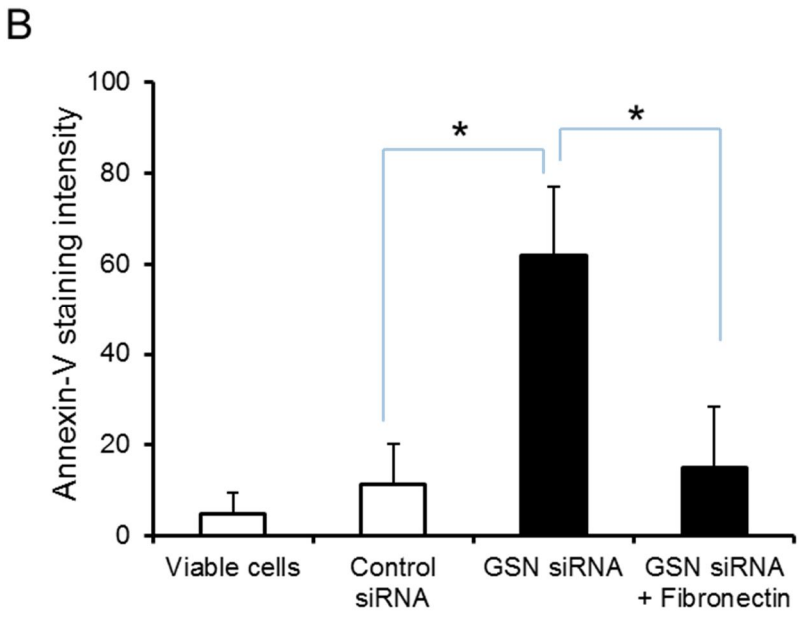
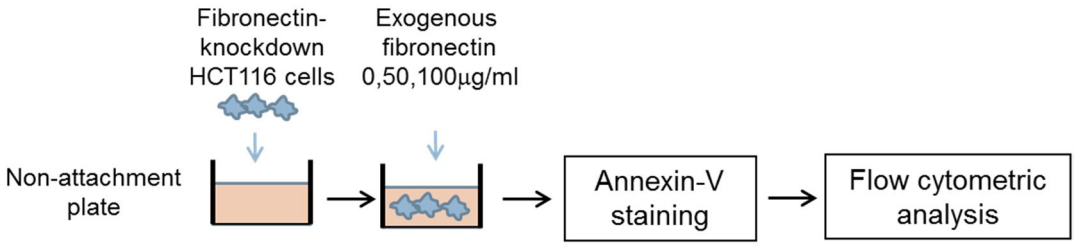

B
Annexin-V staining intensity
100
80
60
40
20
0
*
*
Viable cells
Control siRNA
GSN siRNA
GSN siRNA + Fibronectin

Figure 3.8. Fibronectin rescued the effects of gelsolin knock-down on apoptosis in HCT 116 cells (flow cytometric analysis of annexin-V staining). HCT 116 cells were transfected with either geloslin siRNA or control siRNA for 48h. The cells were then re-cultured in non-attachment plates in serum-free media for 24h. After that, the cells were collected and stained with annexin-V for 15min and subjected to flow cytometry (this group is labeled as 'GSN siRNA' and 'Control siRNA' in the representative flow histogram). As a negative control for apoptosis, untransfected HCT 116 cells were cultured in a standard culture condition (i.e. in attachment plates with complete media) for 24h, and then stained with annexin-V (this group is labeled as 'Viable cells'). Exogenous fibronectin 50μg/ml was added to gelsolin-knockdown cells, which were cultured in the anoikis condition (this group is labeled as 'GSN siRNA + Fibronectin' in the histogram). (A) Histograms from one representative experiment. (B) Quantitative analysis of median of annexin-V staining. Data shown are median ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test).

3.3.7. Fibronectin protects HCT 116 cells from apoptosis in anoikis model

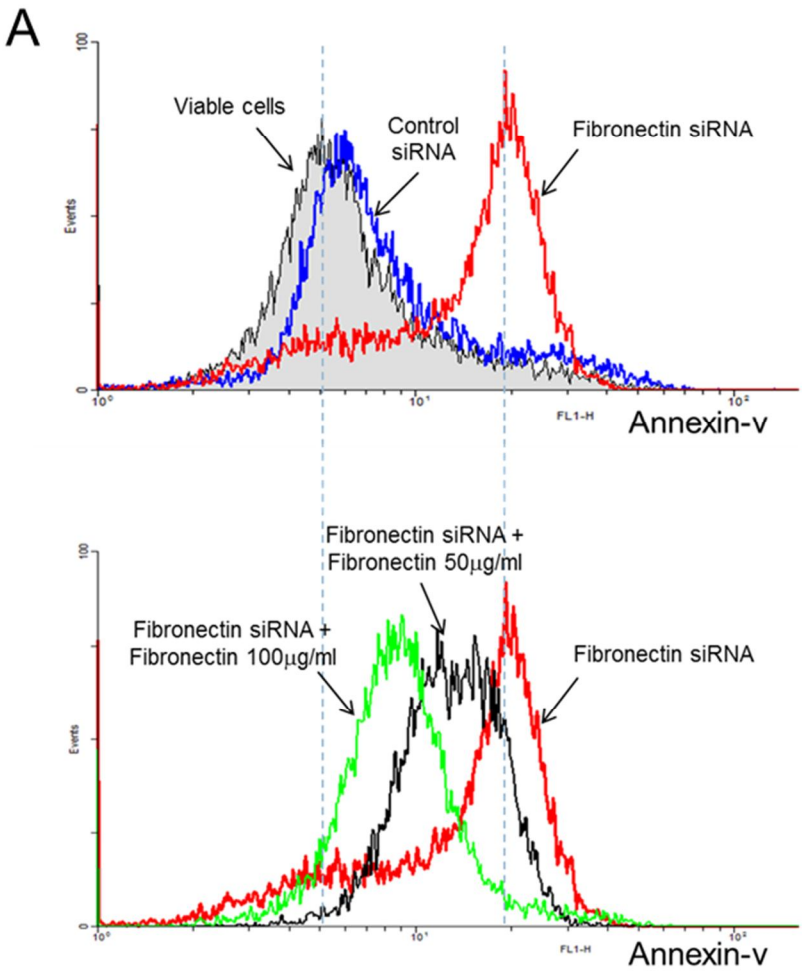
In the previous part, we have shown that fibronectin rescues the effects of gelsolin knock-down on apoptosis of HCT 116 cells in anoikis model. In this part of the study, we wanted to determine whether fibronectin expression and secretion in HCT 116 cells exerted the pro-survival effects.

To investigate the effects of endogenous fibronectin on apoptosis, HCT 116 cells were transfected with fibronectin siRNA or control siRNA before being re-cultured in non-attachment plates in serum-free media. To investigate the effects of exogenous fibronectin on cell survival, exogenous fibronectin was added with different concentrations 0-100μg/ml to the culture media of fibronectin-deficient HCT116 cells (ie. fibronectin-knockdown cells) in non-attachment plates. After 24h, cells were collected for staining with annexin-V and then flow cytometric analysis. The experimental set-up is illustrated below:



Experimental set-up for investigating the effects of exogenous fibronectin on HCT 116 cell apoptosis.

Western blot analysis of transfected HCT 116 cells showed that fibronectin was knocked down after 48h transfection with fibronectin siRNA (Figure 3.9A). The flow cytometric results displayed that when fibronectin was knocked down, there was increased annexin-V staining intensity compared to transfection with control siRNA (Figure 3.9B), indicating that endogenous fibronectin protected HCT 116 cells from apoptosis in the anoikis condition. In the presence of exogenous fibronectin, fibronectin-knockdown HCT 116 cells remained detached from the non-attachment plates and were collected without trypsinization. Figure 3.9C shows that exogenous fibronectin treatment resulted in lower annexin-V staining intensity in a dose-dependent manner. Taken together, these data indicated that both endogenous and exogenous fibronectin protected HCT 116 cells from apoptosis in the anoikis condition.


A
Viable cells
Control siRNA
Fibronectin siRNA
Events
FL1-H
Annexin-v
Fibronectin siRNA + Fibronectin 50μg/ml
Fibronectin siRNA + Fibronectin 100μg/ml
Fibronectin siRNA
Events
FL1-H
Annexin-v

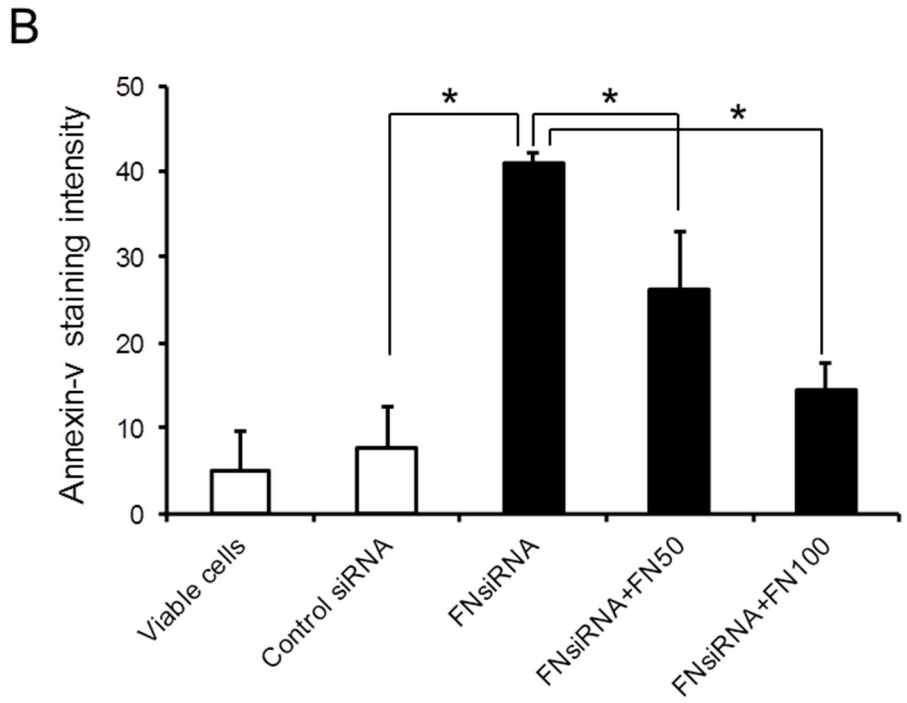


Figure 3.9. Fibronectin expression and secretion protects HCT 116 cells from apoptosis in anoikis model.

HCT 116 cells were transfected with either fibronectin siRNA or control siRNA for 48h. After transfection, cells were re-cultured in non-attachment plates in serum-free media for 24h, and then collected, washed and stained with annexin-V for 15min before being subjected to flow cytometry.

(A) Histograms obtained from one representative experiment. As a negative control for apoptosis, untransfected HCT 116 cells were cultured in attachment plates in complete medium and stained with annexin-V (labeled as 'Viable cells' in the flow histogram). Cells transfected with fibronectin siRNA and control siRNA are respectively labelled as 'Fibronectin siRNA' and 'Control siRNA' in the flow histogram (upper). Fibronectin-knockdown cells in the anoikis condition were treated with exogenous fibronectin at the concentration of 0, 50 or 100μg/ml for 24h before flow cytometric analysis (lower).

(B) Quantitative analysis of median of annexin-V staining. (FNsiRNA: Fibronectin siRNA; FNsiRNA+FN50: Fibronectin siRNA+Fibronectin 50μg/ml; FNsiRNA+FN100: Fibronectin siRNA+Fibronectin 100μg/ml). Data

shown are median ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test). Higher annexin-V staining intensity was detected in cells with fibronectin knock-down. In addition, exogenous fibronectin reduced annexin-V staining intensity in fibronectin-knockdown HCT 116 cells in a dose-dependent manner.

3.3.8. Fibronectin stimulates activation of Akt signaling pathway in HCT 116 cells

To further assess the significance of exogenous fibronectin in HCT 116 cell survival, we investigated if exogenous fibronectin could stimulate activation of survival signaling in our anoikis model. HCT 116 cells were cultured in serum-free media in non-attachment plates and stimulated with exogenous fibronectin at different concentrations. The cells were collected at different time-points for Western blot analysis. Figure 3.10 shows that Akt signaling was activated in HCT 116 cells upon treatment with fibronectin as shown by increased levels of phospho-Akt. The effects of exogenous fibronectin on activating Akt signaling were dependent on treatment duration as well as fibronectin concentration.

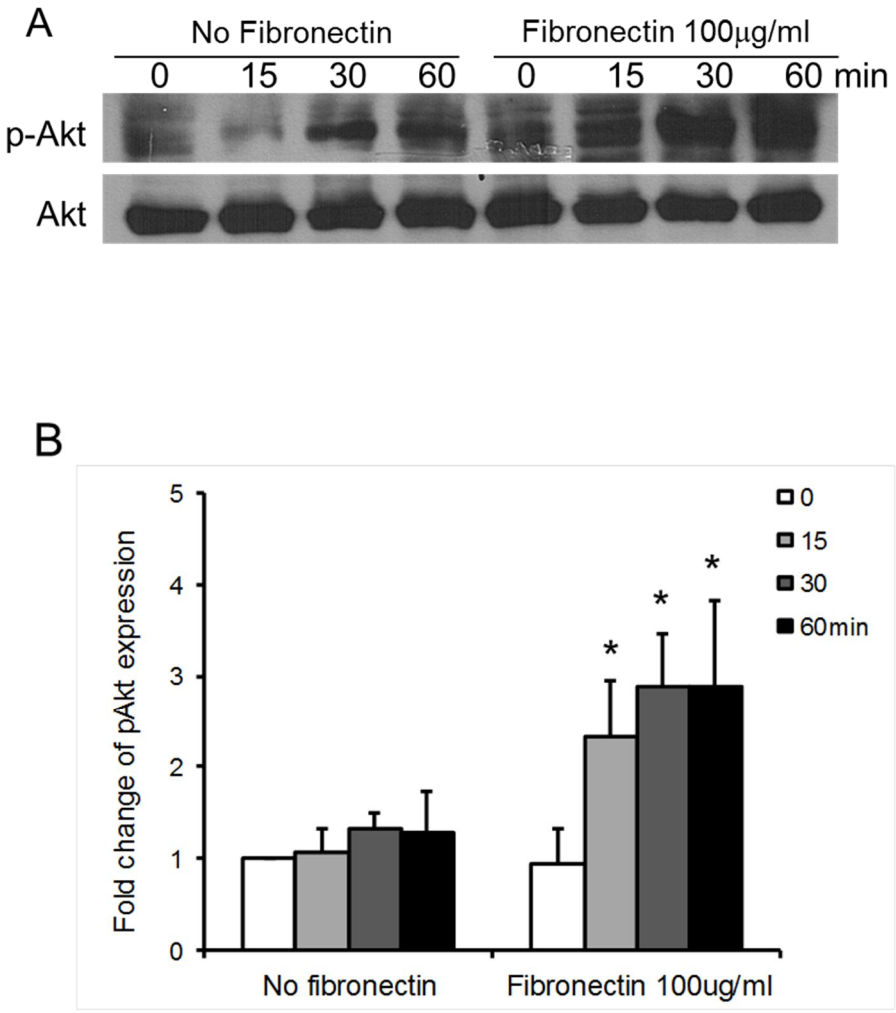


Figure 3.10. Fibronectin promotes Akt signaling in HCT 116 cells. HCT 116 cells were cultured in non-attachment plates in serum-free media. (A) Fibronectin was added into the HCT 116 cell culture at a concentration of 100µg/ml. Cells were collected at different time points 0, 15, 30 and 60min and 25µg of extracted proteins were analyzed by Western blot to measure phospho-Akt (p-Akt) and total Akt expression. (A) A representative western blot and (B) quantitative analysis of western blots; data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test).

3.4. DISCUSSION

3.4.1. Gelsolin regulates fibronectin expression and secretion in colorectal cancer cells

Secretion of fibronectin, an important survival stimulator, has been shown in some cancers such as melanoma or hepatocellular carcinoma [71, 72]. Our data showed that colorectal cancer cells, including HCT116, its metastatic derivative E1, DLD-1 and RKO, also expressed and secreted fibronectin.

Colorectal cancer is heterogeneous with various mutations. The most common mutations in colorectal cancers include KRAS, BRAF and APC [113, 114]. The three cell lines in this study, HCT 116, RKO and DLD-1 respectively have mutations in KRAS, BRAF and APC genes [115]. Therefore, the chosen cell lines can represent, to some extent, the most common genetic changes in colorectal cancers. However, there is no connection between the documented mutations in the cell lines and fibronectin expression and secretion. Among the above-mentioned mutated genes, BRAF is potentially related to fibronectin expression. BRAF is a proto-oncogene downstream of RAS and upstream of MAPK. Mutations in BRAF are reported to result in enhanced malignant proliferation. On the other hand, MAPK has been reported to regulate HGF-induced fibronectin expression in melanoma [71]. Therefore, it is possible that cells with activated BRAF may induce fibronectin upregulation. However, RKO cells with hyper-activating BRAF [116] have low fibronectin expression, and HCT 116 cells with wild-type BRAF express high level of fibronectin. Therefore, documented mutations cannot help to explain the difference in fibronectin expression in HCT 116, RKO and DLD-1.

Our data also show that the expression and secretion of fibronectin detected in colorectal cancer cell lines HCT 116, E1, RKO and DLD-1 correlate with intracellular gelsolin level. This finding suggests the involvement of gelsolin in regulating fibronectin expression and/or secretion. In addition, literature has suggested the participation of gelsolin in ECM remodeling, in regulating gene regulation and vesicle-mediated secretion. The information prompted us to investigate if gelsolin regulated fibronectin expression and secretion. In order to do that, we modulated gelsolin levels by the gain-of-function and loss-of-function models. Our results demonstrated that when gelsolin was overexpressed in HCT 116 cells, there was upregulation of fibronectin (at both protein and mRNA levels) and increased fibronectin secretion. Consistently, when gelsolin was knocked down, fibronectin expression (protein and mRNA) and secretion were suppressed accordingly. These data indicate that gelsolin upregulates fibronectin expression as well as secretion of fibronectin.

Our results not only reveal gelsolin as a novel regulator of fibronectin, an important part of the ECM, but also suggest that gelsolin regulates the fibronectin gene and possibly the secretory pathways that involved in fibronectin secretion. These findings are in accordance with the previous studies that suggested the role of gelsolin in vesicle-mediated secretion. Gelsolin regulates actin remodeling, which is important in the regulation of secretion pathways [22]. Gelsolin has also been shown to be involved in regulated secretion of insulin by a mechanism independent of actin regulation. Kalwat M.A. *et al.* [23] demonstrated that gelsolin interacted with the SNARE protein Syn4, which was important for the fusion of insulin vesicles to plasma

membrane for exocytosis. When cells were stimulated with glucose, gelsolin was released from the binding with Syn4, allowing insulin vesicles to fuse with Syn4 and then were secreted outside. On the other hand, literature has shown the roles of gelsolin as a transcription co-activator of several transcription factors or co-activators. Gelsolin promoted nuclear translocation of androgen receptor and thyroid hormone receptor-β1 resulting in increased androgen receptor activity and thyroid hormone receptor-β1 activity respectively [18, 19]. In addition, gelsolin was shown to regulate the transcription factors YAP/TAZ and p53. Gelsolin localized YAP/TAZ and p53 in cytoplasm, leading to suppression of YAP/TAZ and p53 signaling. When gelsolin was knocked down, YAP/TAZ and p53 translocated to the nucleus and exerted their effects on transcription of various downstream genes [20, 21]. Our findings about the possible roles of gelsolin in the regulation of the fibronectin gene and fibronectin secretion process contribute to elucidate the diverse and potent roles of gelsolin in cell biology, besides its famous role as a regulator of actin dynamics.

3.4.2. Gelsolin protects cancer cells from apoptosis and cell death in anoikis condition

Gelsolin has been shown to exert anti-apoptotic effects in several studies *in vitro* [14, 15]. In addition, higher gelsolin expression was associated with poorer prognosis in clinical research [30, 31]. Our findings in this study are in agreement with the anti-apoptotic and pro-survival effects of gelsolin described above. Our results showed that when cultured in the anoikis condition, gelsolin-overexpressing cells exhibited less cell death compared to

control cells. Consistently, when gelsolin was knocked down with siRNA, there was more cell death in comparison to control groups.

The anoikis model used in this research was adapted from previous studies relating to anoikis and cancer development [111, 117]. In our study, HCT116 cells were cultured in serum-free media to avoid the effects of exogenous fibronectin present in serum. In addition, to rule out the effects of massive signals triggered by binding of cancer cells to attachment plates [118], we cultured cells in non-attachment plates, which had been coated with hydrogel to prevent adhesion-induced signaling.

Anoikis is a programmed cell death due to loss of anchorage to the ECM. While normal cells undergo anoikis in non-attachment conditions, cancer cells can acquire anchorage-independence or anoikis-resistance [119] to survive in stress conditions or during dissemination process. The findings from our study suggest a potential mechanism by which cancer cells can survive anoikis condition. Taking the anti-apoptosis and pro-survival effects of gelsolin into account, it is possible that cancer cells that express high levels of gelsolin are able to promote their own survival, and hence do not need to rely on anchorage-related survival signaling during invasion and metastasis. This postulation also helps to partially explain the increased gelsolin expression in cells with high invasive or metastatic capacity as well as high gelsolin expression in cancer invasion and metastasis.

3.4.3. Gelsolin promotes HCT 116 survival by upregulating fibronectin expression and secretion

Our results showed that gelsolin upregulated fibronectin expression and secretion in colorectal cancer cells. In addition, gelsolin promoted HCT 116 cell survival. Taking the reported pro-survival effects of fibronectin into account, we postulated that gelsolin-regulated fibronectin expression and secretion possibly played a part in the pro-survival effects of gelsolin on HCT 116 cells in the anoikis condition. Indeed, both endogenous and exogenous fibronectin protected HCT 116 cells from apoptosis in the anoikis condition. In addition, conditioned media from gelsolin-overexpressing cells, which had been shown to contain high levels of secreted fibronectin, also exerted pro-survival effects on HCT 116 cells. The results suggest that secreted fibronectin by gelsolin-overexpressing cells was possibly the factor that protected HCT 116 cells from apoptosis. This postulation was supported by our findings showing that exogenous fibronectin inhibited apoptosis and also stimulated the pro-survival Akt signaling pathway. Moreover, exogenous fibronectin could rescue the apoptotic effects induced by gelsolin knock-down.

These findings are in accordance with numerous studies emphasizing the roles of fibronectin in the survival of both normal and tumor cells [76, 81, 84]. The stimulation of Akt signaling by fibronectin is also supported by previous studies [81, 82]. Akt signaling is widely known for its protective effects against apoptosis induced by serum deprivation, inflammatory cytokines or UV-irradiation in different cell types [120-122]. Akt confers anti-apoptotic effects by inhibiting pro-apoptotic factors such as p53 or the Bcl-2-associated death promoter (BAD) or stimulating anti-apoptotic molecules such as NFκB

or Bcl-x_L [123]. In agreement with our findings, it has been shown in literature that pre-coated fibronectin in attachment plates activated Akt signaling [81, 82]. However, of note is that in fibronectin-coated plates, cell survival is possibly stimulated by not only fibronectin but also mechanical forces dependent on matrix physical properties such as stiffness and topography [20, 124]. Our anchorange-independent model used in this study rules out the effect of the mechanical factors, and hence confirms that secreted fibronectin promotes cancer cell survival by activation of Akt. In addition, the finding that increased Akt phosphorylation in HCT 116 cells not treated with exogenous fibronectin at 30min and 60min suggests Akt could be activated by other factors rather than the exogenous fibronectin. Of note is that HCT 116 cells have significant endogenous level of fibronectin and can secrete fibronectin. Therefore, it is possible that fibronectin was secreted by HCT 116 cells and activated Akt signaling in the cells in a paracrine mechanism.

Based on the findings on pro-survival effects of both endogenous and exogenous fibronectin, our findings suggest that gelsolin protects cancer cell from apoptosis by upregulating fibronectin expression and secretion. Gelsolin has been reported to be involved in promoting cancer survival. Higher expression of gelsolin correlated with poorer prognosis in patients at later stages or recurrent lung cancer [30, 31]. As shown in our previous study, high gelsolin expression was detected at the invasive front of metastatic tumor in liver [34]. Moreover, cell-based studies have indicated that the association of gelsolin with cancer survival was not only a correlative but also causative relation. Full-length gelsolin and C-terminal half of gelsolin were demonstrated to exhibit pro-survival effects by inhibiting apoptosis [14, 15].

The anti-apoptotic effects of gelsolin was demonstrated due to its ability to bind and close voltage-dependent anion channel (VDAC), which inhibited cytochrome c release from mitochondria resulting in attenuated caspases 3, 8, 9 [14, 15]. In this study, we propose a novel mechanism by which gelsolin protects cancer cells from apoptosis and cell death. By upregulating the expression and secretion of fibronectin, gelsolin indirectly exhibits anti-apoptotic capacity and promotes cancer cell survival.

SUMMARY

In this chapter, we demonstrated that gelsolin upregulated fibronectin expression and secretion in colorectal cancer HCT 116 cells. In addition, gelsolin exhibited pro-survival and anti-apoptotic effects in HCT 116 cells in an anoikis condition. Conditioned media obtained from gelsolin over-expressing cells protected HCT 116 cells against apoptosis, gelsolin-overexpressing cells secreted unknown factors that promoted HCT 116 cells survival. Our results suggested that one of the secreted factors that promoted HCT 116 cell survival was fibronectin. Fibronectin protected HCT 116 cells from apoptosis and stimulated Akt signaling. Moreover, fibronectin rescued the apoptotic effect of gelsolin knock-down on HCT 116 cells. Taken together, these findings elucidated an important role of gelsolin in cancer cell survival in the anoikis condition by promoting fibronectin expression and secretion.

-CHAPTER 4-

THE MECHANISMS BY WHICH GELSOLIN UPREGULATES FIBRONECTIN EXPRESSION

4.1. BACKGROUND

Regulation of fibronectin expression and secretion is not yet fully understood. Studies in different cells show diverse signaling pathways involved in fibronectin expression. Normal cells such as coronary artery smooth muscle cells or osteoblasts express fibronectin upon stimulation of growth factors such as TNF-α and bFGF [62-64]. TNF-α induces nitric oxide-dependent post-transcriptional modification of the fibronectin gene, whereas bFGF activates NFκB via PLCγ2/PKCα/c-Src signaling. However, in glioblastoma or melanoma cells, the fibronectin gene has been shown to be regulated by the transcription factor EGR-1 upon stimulation of hepatocyte growth factor (HGF) [67, 68]. The limited information about fibronectin regulation necessitates further research on the factors that control fibronectin expression.

Besides its famous role as an actin regulator, gelsolin has been shown to modulate various genes by either promoting or suppressing their transcription factors or transcription co-activators. When in cytoplasm, fibronectin binds to transcription co-activators YAP/TAZ or p53, inhibiting their translocation to the nucleus. The downstream genes of YAP/TAZ and p53 are therefore down-regulated by gelsolin overexpression [20, 21]. On the other hand, gelsolin in the nucleus binds to androgen receptor at its DNA-binding and ligand binding sites to promote androgen receptor activity in human prostate cancer cells [18]. Collectively, these findings have highlighted the involvement of gelsolin in gene transcription.

In the previous chapter, we have demonstrated that gelsolin promoted the secretion of fibronecin by colorectal cancer cells HCT 116. In addition, gelsolin upregulated fibronectin expression in both protein and mRNA levels. In this part of the study, we investigated whether gelsolin exerted the regulation effects on fibronectin expression and secretion by affecting the reported modulators of the fibronectin gene, the transcription factors EGR-1 and NFκB.

4.2. OBJECTIVES

1) To investigate the involvement of the transcription factor EGR-1 in gelsolin-regulated expression of fibronectin

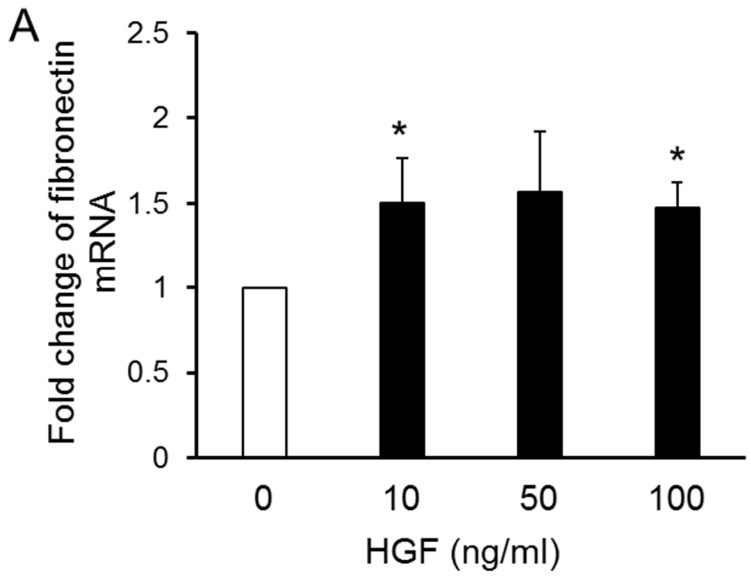
2) To investigate the involvement of the transcription factor NFκB in gelsolin-regulated expression of fibronectin

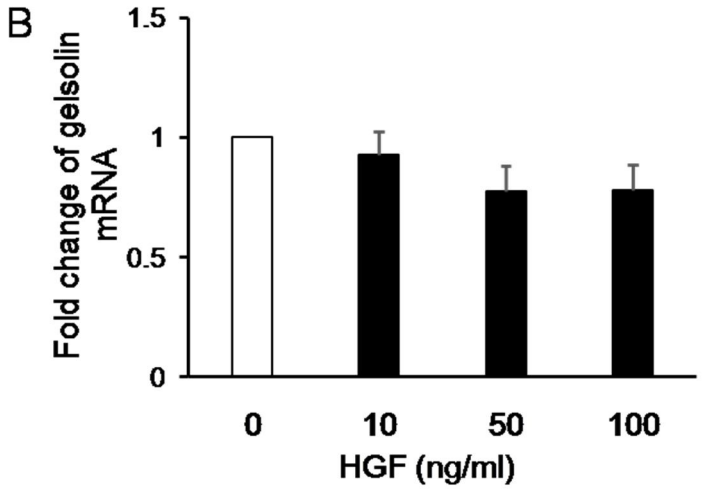
4.3. RESULTS

4.3.1. Fibronectin expression in HCT 116 cells is stimulated by hepatocyte growth factor

Hepatocyte growth factor (HGF) was shown to stimulate fibronectin expression in melanoma cells [71]. We investigated if HGF upregulated fibronectin in colorectal cancer HCT 116 cells. Cells were cultured in serum-free media with HGF at different concentrations. After 24h, cells were collected for Western blot and real-time PCR.

As shown in Figure 4.1, while gelsolin expression was not affected by HGF, fibronectin mRNA was upregulated by HGF stimulation at concentrations from 10ng/ml. Consistently, stimulation of HCT 116 cells with HGF induced increased fibronectin expression at protein level, whilst HGF did not affect gelsolin expression.


A
Fold change of fibronectin mRNA
2.5
2
1.5
1
0.5
0
*
*
0
10
50
100
HGF (ng/ml)


B
Fold change of gelsolin mRNA
1.5
1
0.5
0
0
10
50
100
HGF (ng/ml)

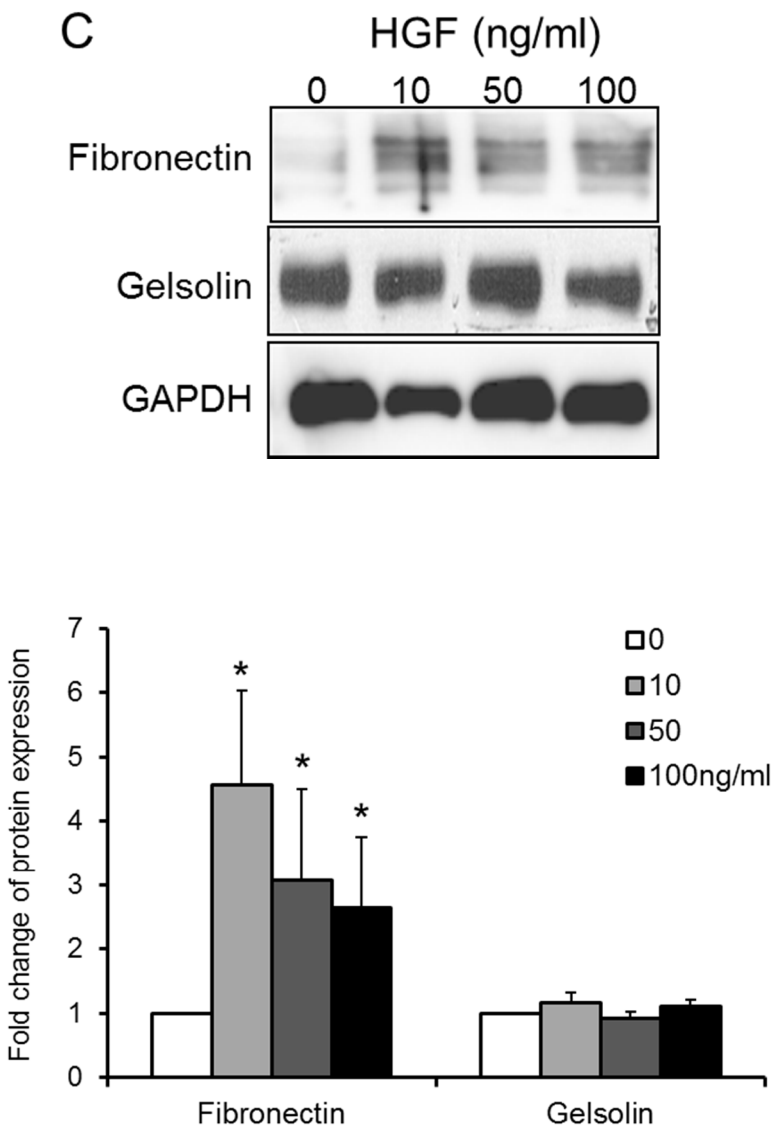


Figure 4.1. HGF stimulates fibronectin expression in HCT 116 cells. HCT 116 cells were cultured in serum-free media. HGF with concentrations of 0, 10, 50, 100 ng/ml was added into culture media of HCT 116 cells.

(A&B) After 24h, cells were collected for real-time PCR to measure (A) fibronectin and (B) gelsolin mRNA levels. Data shown are mean± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test).

(C) After 24h of stimulation with HGF, 25μg of extracted proteins were analyzed by Western blot. A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to no treatment (0ng/ml) in the same group.

4.3.2. Hepatocyte growth factor stimulates EGR-1 in HCT 116 cells

HGF was reported to activate EGR-1 expression, which is a known regulator of the fibronectin gene in melanoma cells and hepatocellular carcinoma cells [71, 125]. We examined whether HGF enhanced EGR-1 expression in HCT 116 cells. HCT 116 cells were cultured in serum-free media and stimulated with HGF 10ng/ml for different periods of time. After that the cells were collected for real-time PCR or Western blot.

As demonstrated in Figure 4.2A, EGR-1 mRNA was observed to be upregulated at 60-120 minutes after HGF treatment, with highest levels observed at 60 minutes. In agreement with that, upon stimulation with HGF, EGR-1 protein expression in HCT 116 cells was also enhanced at 60 and 120 minutes after HGF treatment (Figure 4.2B).

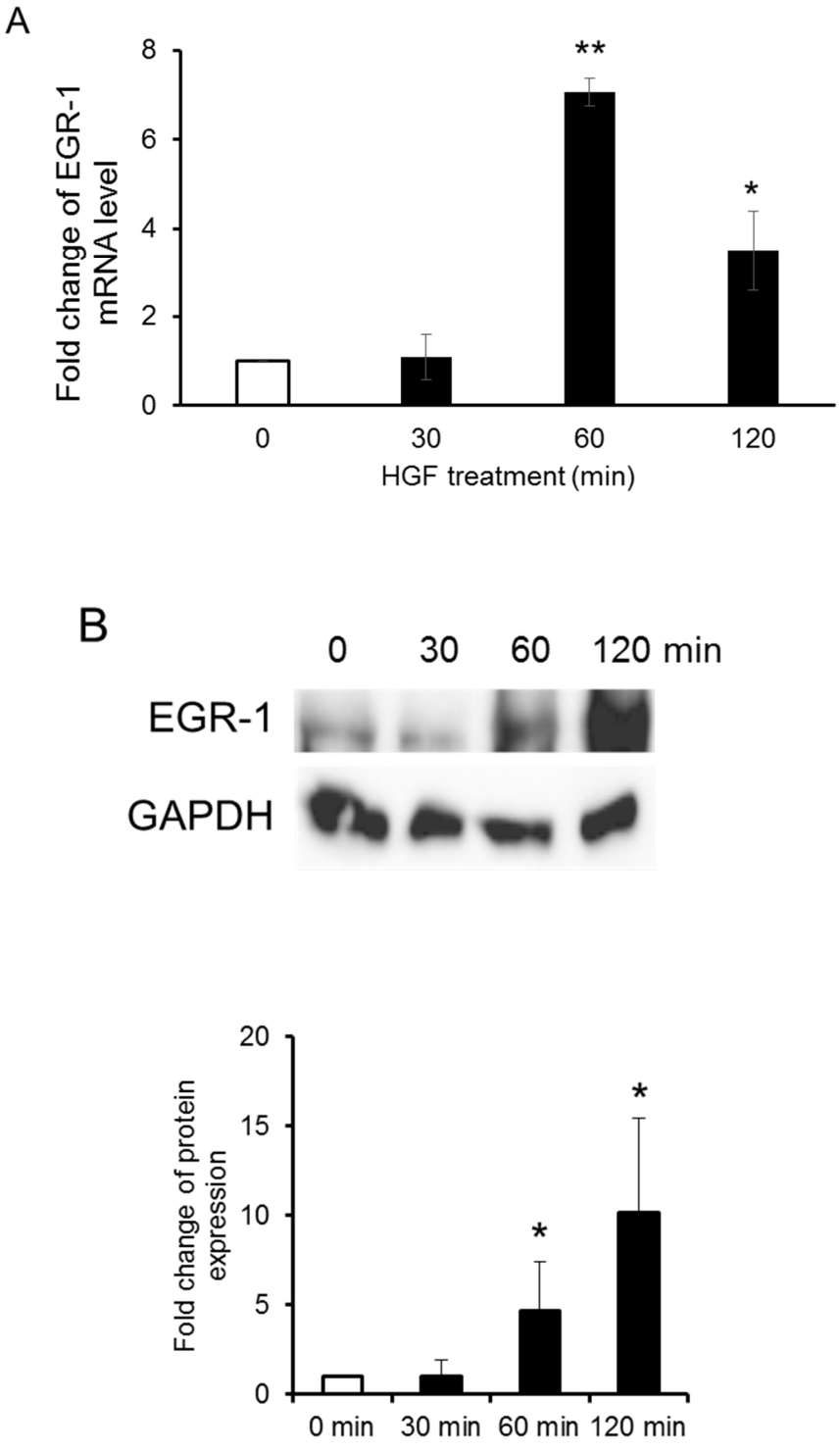


Figure 4.2. HGF stimulates EGR-1 expression in HCT 116 cells. HCT 116 cells were cultured in serum-free media. 10 ng/ml HGF was added into culture

media of HCT 116 cells. After 0, 30, 60 and 120 minutes, cells were collected for real-time PCR (A) or Western blot (25μg of extracted proteins were loaded) (B); data shown in (A) are mean± SD of three independent experiments. *p-value <0.05, **p-value <0.01 (Mann-Whitney U test). (B) A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to 0 min in the same group.

4.3.3. Gelsolin does not affect EGR-1 expression and activity in HCT 116 cells

We have demonstrated that HGF upregulated fibronectin as well as the known regulator of the fibronectin gene, EGR-1 [71]. In addition, in the previous chapter, our data indicated that gelsolin upregulated fibronectin expression. In order to determine whether gelsolin upregulated fibronectin gene via HGF-EGR-1 signaling, we investigated the roles of gelsolin on EGR-1 expression and activity.

We modulated gelsolin levels by overexpression or siRNA knock-down and measured EGR-1 expression by Western blot. EGR-1 activity was measured by dual-luciferase reporter assay. HCT 116, vector control cells and gelsolin-overexpressing cells were transfected with EGR-1-responsive luciferase reporter, negative control, or positive control luciferase constructs. To test the effects of gelsolin knock-down on EGR-1 activity, HCT 116 cells were co-transfected with individual luciferase constructs as well as either gelsolin siRNA or control siRNA. Following transfection, cells were harvested for cell lysis and measurement of luciferase activity.

Relative transcriptional activity was calculated by the following formula:

$$\text{EGR-1 transcriptional activity } \textit{(luciferase unit)} = \frac{\text{Ratio of Firefly/Renilla of experimental samples}}{\text{Ratio of Firefly/Renilla of negative controls}}$$

As shown in Figure 4.3, gelsolin overexpression (in C1 and C8 cells) did not alter either EGR-1 expression nor EGR-1 transcriptional activity compared to HCT 116 and vector control cells. Consistently, Figure 4.4 shows that neither EGR-1 expression nor transcriptional activity was affected by gelsolin knock-down. These data indicate that gelsolin does not regulate EGR-1 expression nor activity, and thus fibronectin regulation by gelsolin is unlikely via gelsolin's influence on EGR-1.

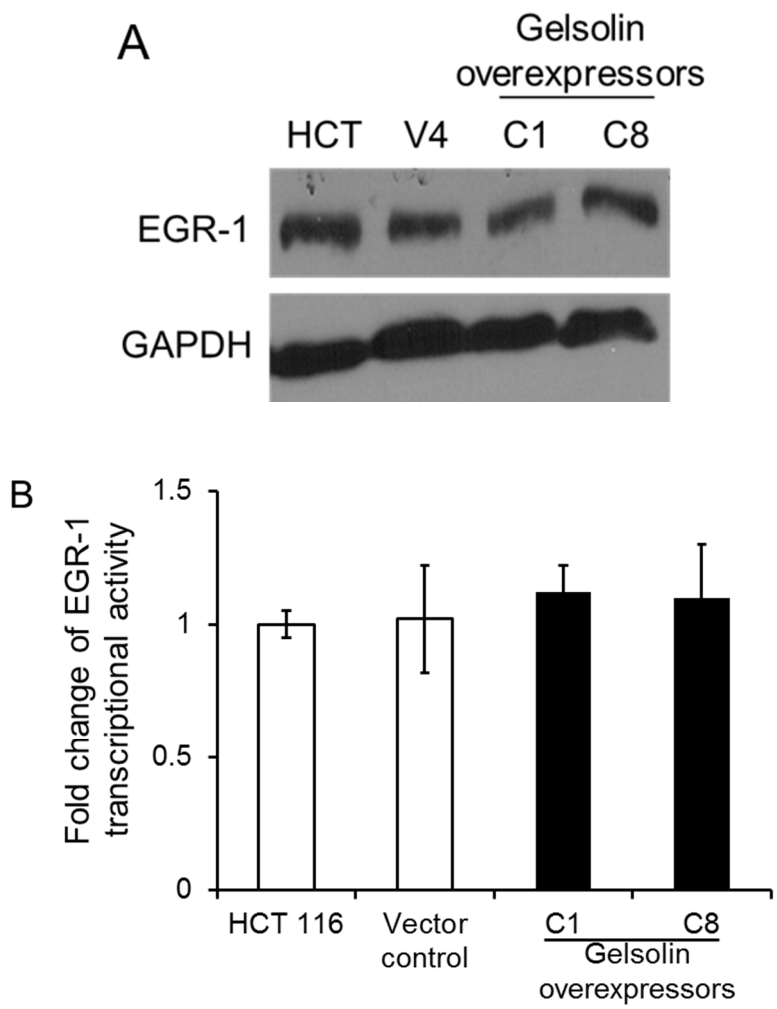


Figure 4.3. Gelsolin overexpression does not affect EGR-1 expression and transcriptional activity. (A) Western blot of EGR-1 expression in HCT 116, vector control cells and gelsolin-overexpressing cells (C1 and C8) (25μg of extracted proteins were analyzed). (B) EGR-1-responsive reporter, negative control, and positive control constructs were mixed with the transfection reagent Lipofectamine 2000 and delivered to HCT 116, vector control and gelsolin-overexpressing cells (C1, C8). After 24h, culture media was replaced with serum-free media and incubated for another 24h. Following transfection, cells were harvested with cell lysis buffer and luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Transcriptional activity was calculated from the ratio between firefly/renilla from experimental samples and firefly/renilla from negative control transfection (refer to main text for the formula). Data shown are mean± SD of three independent experiments.

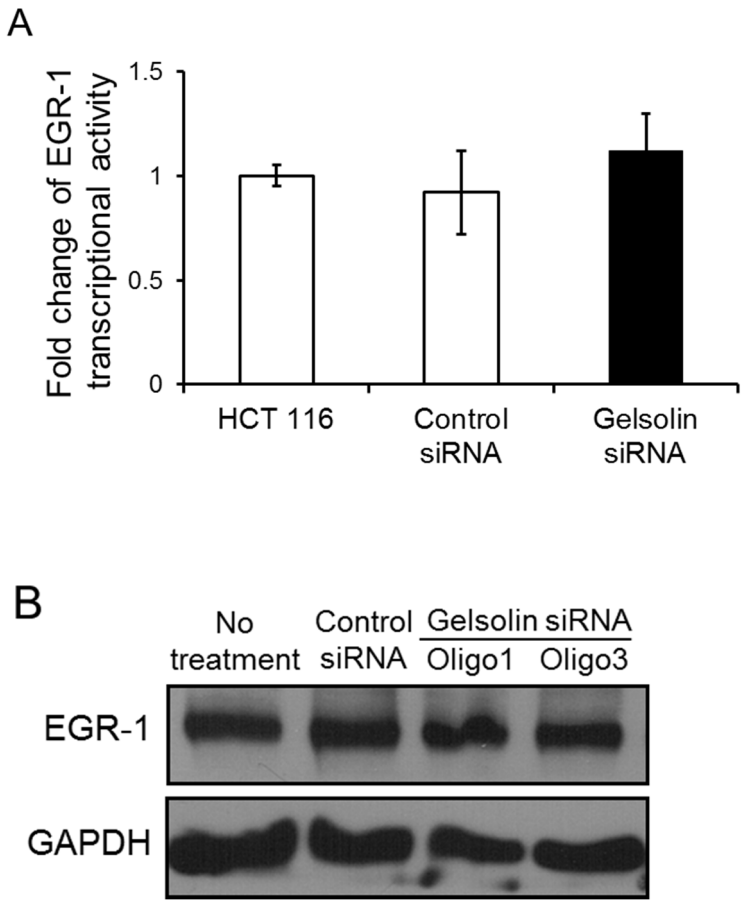


Figure 4.4. Gelsolin knock-down does not affect EGR-1 expression and transcriptional activity. (A) Western blot of EGR-1 expression in HCT 116 cells transfected with either of control siRNA or gelsolin siRNA (25μg of extracted proteins were analyzed). (B) EGR-1-responsive reporter, negative control, and positive control constructs were mixed with gelsolin siRNA or control siRNA. The nucleic acids were then mixed with Lipofectamine 2000 and transfected to HCT 116 cells. After 24h, culture media was replaced with serum-free media and incubated for another 24h. Following co-transfection, cells were lysed and luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Transcriptional activity was calculated from the ratio between firefly/renilla from experimental samples and firefly/renilla from negative control transfection (refer to main text for the formula). Data shown are mean± SD of three independent experiments.

4.3.4. Fibronectin expression in HCT 116 cells is regulated by NFκB

NFκB has been shown to upregulate fibronectin expression in normal cells such as osteoblasts or hepatocytes. In order to investigate the involvement of NFκB in fibronectin expression in HCT 116 cells, cells were treated with NFκB inhibitor, BAY 11-7085 at different concentration 0, 10 or 20 ng/ml, for 12 h, and then collected for Western blot. At these concentrations, BAY 11-7085 was shown not to induce apoptosis nor cell death in HCT 116 cells [126]. As shown in Figure 4.5, NFκB inhibitor reduced fibronectin expression in HCT 116 cells. This result suggests that the fibronectin level in HCT 116 cells is regulated by NFκB.

We next investigated if NFκB activation promoted fibronectin expression in HCT 116 cells. HCT 116 cells cultured in serum-free media were stimulated with different concentrations of TNFα, 0, 10, 20 or 40ng/ml. NFκB was shown to be activated by TNFα at these concentrations [127]. After 12h, cells were collected for real-time PCR or Western blot. Figure 4.6 shows that TNFα increased fibronectin mRNA to about 2-3 times higher than in the absence of TNFα, whereas TNFα did not affect gelsolin mRNA levels in HCT 116 cells. These findings were substantiated with Western blot results, which showed that TNFα stimulates the expression of fibronectin but not that of gelsolin.

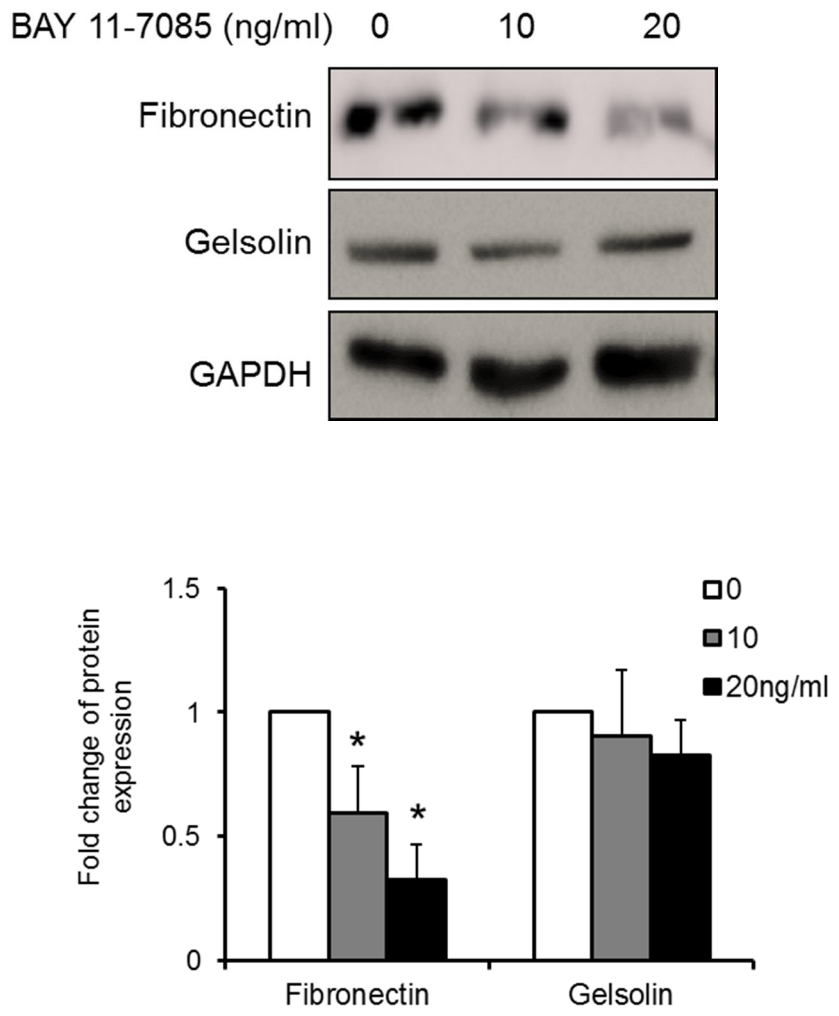
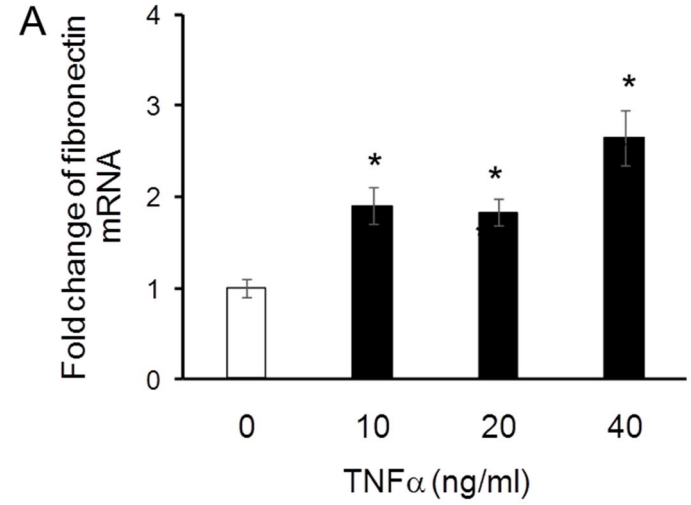
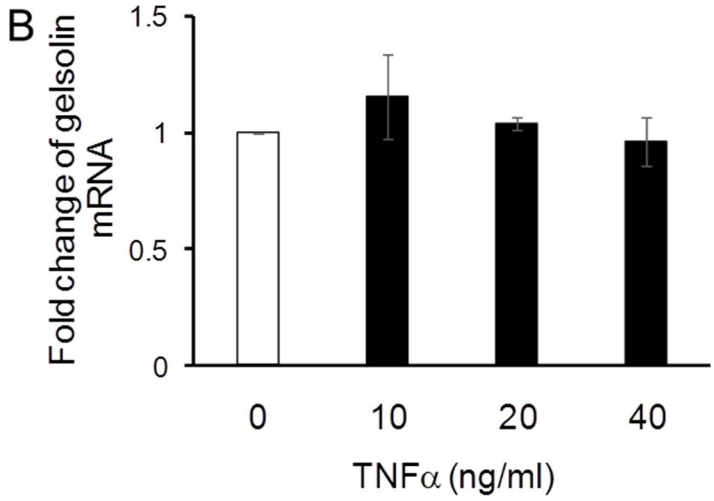


Figure 4.5. NFκB inhibitor BAY 11-7085 attenuates fibronectin expression in HCT116 cells. HCT 116 cells were cultured in serum-free media. BAY 11-7085 at the concentrations of 0, 10, 20 ng/ml was added into cultured cells. After 12h, cells were collected for Western blot analysis (25μg of extracted proteins were analyzed). A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to no treatment (0ng/ml) in the same group.


A
Fold change of fibronectin mRNA
4
3
2
1
0
*
*
*
0
10
20
40
TNFα (ng/ml)


B
Fold change of gelsolin mRNA
1.5
1
0.5
0
0
10
20
40
TNFα (ng/ml)

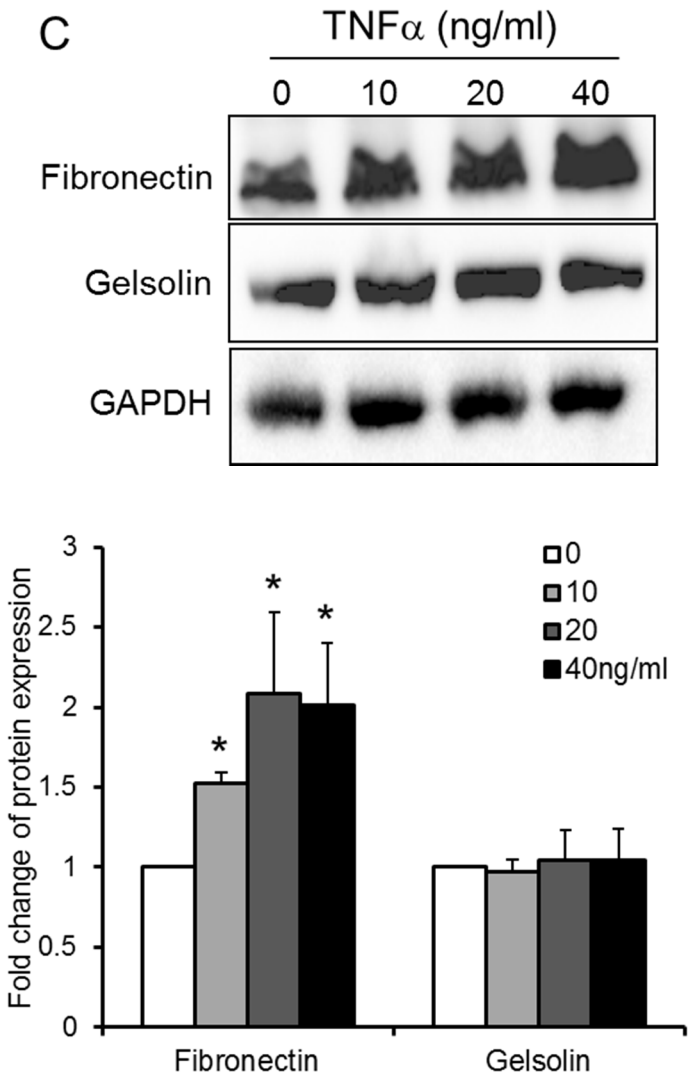


Figure 4.6. TNFα stimulates fibronectin expression in HCT 116 cells. HCT 116 cells were cultured in serum-free media. TNFα with concentrations of 0, 10, 20, 40 ng/ml was added into culture media of HCT 116 cells. After 12h, cells were collected for real-time PCR to measure fibronectin mRNA (A) and gelsolin mRNA (B) and Western blot (25μg of extracted proteins were analyzed) (C). Data shown in (A) and (B) are mean± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test). (C) A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test) compared to no treatment (0ng/ml) in the same group.

4.3.5. Gelsolin stimulates NFκB activity in HCT 116 cells

We have demonstrated that TNFα, a known stimulator of NFκB, upregulated fibronectin expression in HCT 116 cells but did not affect gelsolin levels. We also showed in the previous chapter that gelsolin upregulated fibronectin expression. In order to ascertain whether gelsolin upregulated the fibronectin gene via NFκB signaling, we investigated the roles of gelsolin on NFκB expression and activity.

We modulated gelsolin levels by overexpression or siRNA knock-down and measured NFκB expression by Western blot. NFκB activity was measured by dual-luciferase reporter assay. HCT 116, vector control cells and gelsolin-overexpressing cells were transfected with NFκB-responsive luciferase reporter, negative control, or positive control luciferase constructs. To test the effects of gelsolin knock-down on NFκB activity, HCT 116 cells were co-transfected with individual luciferase constructs as well as with either gelsolin siRNA or control siRNA. Following transfection, cells were harvested for cell lysis and measurement of luciferase activity.

Relative transcriptional activity of NFκB was calculated by the following formula:

$$\text{NFκB transcriptional activity } \textit{(luciferase unit)} = \frac{\text{Ratio of Firefly/Renilla of experimental samples}}{\text{Ratio of Firefly/Renilla of negative controls}}$$

Our data showed that although NFκB expression was not affected by gelsolin overexpression, NFκB activity was significantly enhanced in gelsolin-overexpressing cells (C1 and C8 cells) compared to wild-type HCT 116 cells and vector control cells (Figure 4.7). Consistent with this, gelsolin knock-down in HCT 116 cells did not alter NFκB expression but attenuated NFκB activity compared to control siRNA-transfected cells. These findings were further confirmed by knock-down of gelsolin in gelsolin-overexpressing cells and other colorectal cancer cells DLD-1 and RKO (Figure 4.8). Collectively, our data suggest that gelsolin stimulates NFκB activity but not NFκB expression in colorectal cancer cells.

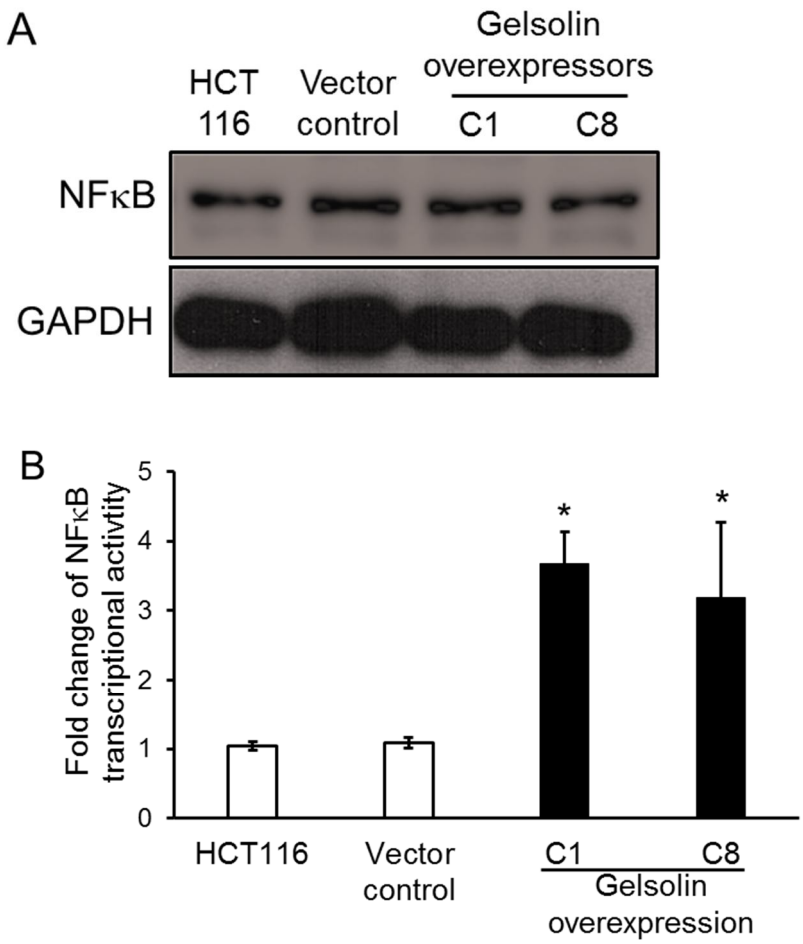


Figure 4.7. Gelsolin overexpression promotes NFκB activity but not NFκB expression. (A) Western blot of NFκB expression in HCT 116, vector control cells and gelsolin-overexpressing cells (C1 and C8) (25μg of extracted proteins were analyzed). (B) Transcriptional activity of NFκB in gelsolin-overexpressing cells and control cells. NFκB-responsive reporter, negative control, and positive control constructs were transfected to HCT 116, vector control cells and gelsolin-overexpressing cells (C1, C8). After 24h, culture media was replaced with serum-free media and incubated for another 24h. Following transfection, cells were harvested with cell lysis buffer and luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Transcriptional activity of NFκB was calculated from the ratio between firefly/renilla from experimental samples and firefly/renilla from negative control transfections (refer to main text for the formula). Data shown are mean± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test).

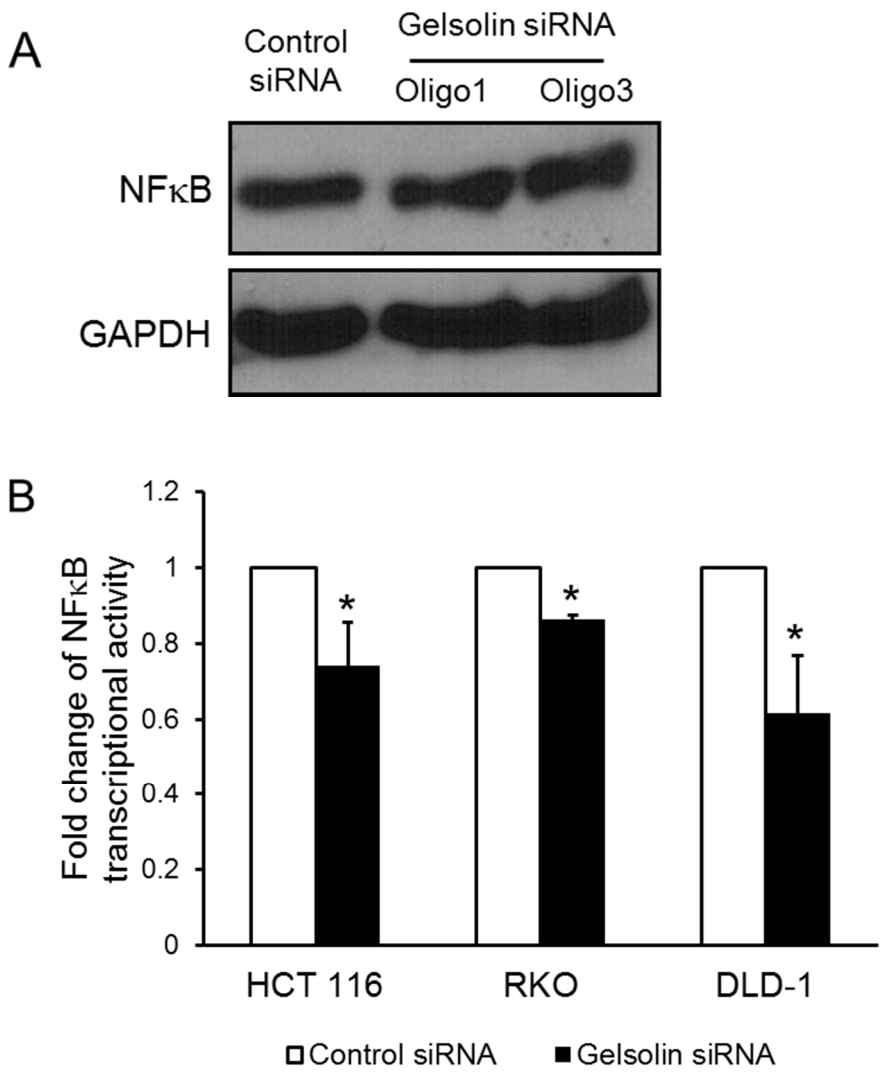


Figure 4.8. Gelsolin knock-down attenuates NFκB activity but not NFκB expression. (A) Western blot of NFκB expression in HCT 116 cells transfected with either of control siRNA or gelsolin siRNA (oligo 1 or oligo 3) (25μg of extracted proteins were analyzed). (B) Transcriptional activity of NFκB in gelsolin-knockdown cells and control cells. NFκB-responsive luciferase reporter, negative control, or positive control constructs was mixed with gelsolin siRNA or control siRNA. The combination of constructs were then transfected into HCT 116 cells, RKO or DLD-1. After 24h, culture media was replaced with serum-free media and incubated for another 24h. Following

co-transfection, cells were lysed and luciferase activity was measured using the Dual-Luciferase Assay System (Promega). Transcriptional activity of NFκB was calculated from the ratio between firefly/renilla from experimental samples and firefly/renilla from negative control transfections (refer to maintext for the formula). NFκB transcriptional activity was normalised within each cell line against its relevant control siRNA-treated sample. Data shown in are mean± SD of three independent experiments. *p-value <0.05 (Mann-Whitney U test).

4.3.6. Gelsolin promotes fibronectin expression by activating NFκB activity

In the previous parts of this chapter, we have shown that NFκB upregulated fibronectin expression and gelsolin activated NFκB activity. We explored whether gelsolin upregulated fibronectin expression by activating NFκB activity.

Gelsolin-overexpressing cells and vector control cells were cultured in serum-free media with or without NFκB inhibitor, BAY 11-7085. After 12 h, cells were collected for Western blot. Figure 4.9 shows that gelsolin-overexpressing cells exhibited higher fibronectin expression compared to vector control cells. However, when cells were treated with NFκB inhibitor, fibronectin expression in gelsolin-overexpressing cells was reduced to similar levels as untreated vector control cells. This shows that the upregulation of fibronectin by gelsolin could be attenuated by NFκB inhibition. Taken together with our previous findings that gelsolin stimulates NFκB activity, these results suggest gelsolin upregulates fibronectin expression through the NFκB pathway.

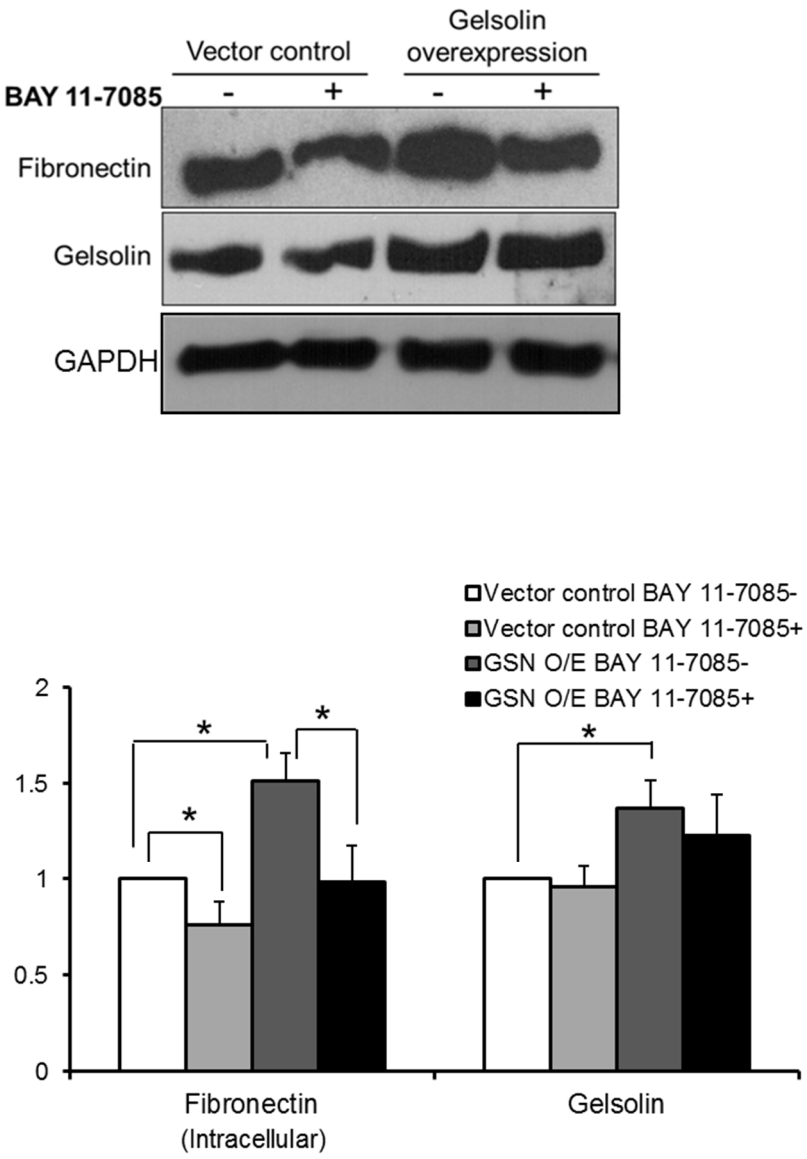


Figure 4.9. Gelsolin-upregulated fibronectin expression is attenuated by NFκB inhibition. Gelsolin-overexpressing cells and vector control cells were cultured in serum-free media. BAY 11-7085, NFκB inhibitor, at the concentration of 20ng/ml was added into cultured cells. After 12 h, the cells were collected for Western blot analysis (40μg of extracted proteins were analyzed). A representative Western blot (upper) and quantitative analysis of Western blots (lower); data shown are mean ± SD of three independent experiments; *p-value<0.05 (Mann-Whitney U test).

4.4. DISCUSSION

4.4.1. Fibronectin expression is regulated by the transcription factors EGR-1 and NFκB in HCT 116 cells

In this chapter, we have shown that fibronectin expression in HCT 116 cells was upregulated by HGF. Furthermore, HGF stimulated EGR-1 expression. Fibronectin upregulation was also induced by TNF-α treatment, a known stimulator of NFκB pathway.

The two transcription factors EGR-1 and NFκB were mentioned in several studies to be involved in regulating the fibronectin gene. It was reported that, in normal cells such as hepatocytes and osteoblasts, fibronectin expression is upregulated by NFκB upon different stimulations. TNF-α, a known regulator of NFκB, stimulated fibronectin expression in coronary artery smooth muscle cells by a posttranscriptional mechanism dependent on nitric oxide [62, 63]. In osteoblasts, NFκB was activated by basic fibroblast growth factor (bFGF) via PLCγ2/PKCα/c-Src signaling, resulting in enhanced fibronectin synthesis [64]. In rat hepatocytes, the transcription of fibronectin was also regulated by NFκB by the binding of p65/p65 homodimer to the fibronectin promoter [65].

The fibronectin gene has also been shown to be regulated in cancer cells by EGR-1, which plays important roles in regulating cell proliferation and differentiation. Overexpression of EGR-1 in human glioblastoma cells was observed to result in high fibronectin expression [67]. EGR-1 exerted its effect by binding to 2 binding sites between -75 to -52 and -4 to +14 of the fibronectin promoter. In melanoma cell lines, EGR-1 also bound to fibronectin

promoter upon stimulation by HGF via activation of mitogen-activated protein kinase MAPK. Overexpression of NGFI-A binding protein NAB2, the co-suppressor of EGR-1 in melanoma cells, reversed the effect of HGF on fibronectin expression [68].

Our findings are in accordance with previous studies showing that both TNFα and HGF induce upregulation of the fibronectin gene. In HCT 116 cells, TNFα upregulated fibronectin expression at both mRNA and protein levels in a dose-dependent manner. As TNFα did not affect gelsolin expression, TNFα may regulate fibronectin a gelsolin-independent mechanism. However, it is also possible that gelsolin was involved as a downstream effector of the TNFα signaling. Further studies should be conducted to clarify these possibilities. The effects of HGF on fibronectin expression are different from what we observed with TNFα. Our data shows that the effects of HGF on fibronectin mRNA saturated at HGF 10ng/ml, and there were no significant differences when concentration of HGF was increased to 50 or 100ng/ml. However, Western blot results showed that there was a slight decrease of fibronectin expression at protein level. This phenomenon can be explained by possible effects of HGF on secretory pathways. HGF was previously shown to promote secretion of prostaglandin E2 by lung cancer cell line H23 [128] and exocytosis of lysosomal enzymes by neutrophils [129]. Therefore, it is possible that, in our study, increased level of HGF promoted fibronectin secretion in HCT 116 cells, and enhanced fibronectin secretion resulted in slightly lower intracellular fibronectin. In order to clarify the postulation, fibronectin secretion should be measured and effects of HGF at 50ng/ml and 100ng/ml on secretory pathways should be studied.

While different studies showed the effects either of EGR-1 or NFκB, our results suggest that both transcription factors EGR-1 and NFκB were involved in the regulation of the fibronectin gene in HCT 116 cells. As a crosstalk between EGR-1 and NFκB has been reported [130], our findings do not exclude a possibility that the 2 transcription factors may work in tandem to induce transcription of fibronectin.

4.4.2. Gelsolin enhances NFκB activity but not EGR-1 to upregulate fibronectin expression

We have shown that fibronectin expression in HCT 116 cells was stimulated by both EGR-1 and NFκB transcription factors. In addition, our data suggested that gelsolin upregulated fibronectin expression by promoting NFκB activity but not EGR-1.

Our findings showing fibronectin gene regulation by gelsolin are in agreement with reported functions of gelsolin in modulating transcription co-activators or transcription factors. Gelsolin was shown to regulate gene transcription via two main mechanisms:

(1) Cytoplasmic gelsolin directly binds to transcription co-activators/factors, inhibiting their translocation to the nucleus. For example, it was demonstrated that binding of gelsolin to YAP/TAZ transcription factors restricted their localization to the cytoplasm, leading to down-regulation of transcriptional activity. Similar effects of gelsolin on p53 and its transcriptional activity were also reported. [20, 21].

(2) Nuclear gelsolin interacts with transcription co-activators/factors to promote their activity. Gelsolin was shown to interact with androgen receptor at its DNA-binding and ligand-binding sites to promote androgen receptor activity in human prostate cancer cells [18]. In a thyroid cancer mouse model, the C-terminus of gelsolin was shown to interact with the DNA binding sequence of thyroid hormone receptor-β1, resulting in reduced effects of thyroid hormone receptor on tumor progression [19].

Our results in this chapter indicated that gelsolin overexpression or knock-down did not affect either EGR-1 nor NFκB expression. However NFκB activity was significantly stimulated by gelsolin overexpression and inhibited by gelsolin knock-down. Nevertheless, how gelsolin activates NFκB activity remains to be elucidated in future studies.

In general, changes of NFκB activity can be explained by the following possibilities [131-133]:

(1) Enhanced NFκB expression, which leads to increased nuclear transclocation of NFκB, and hence enhanced NFκB activity;

(2) Reduced level of NFκB inhibitors: In normal conditions NFκB exists in binding with the inhibitory kappa B proteins, IκB, which mask the nuclear signal of NFκB. When cells are stimulated, IκB proteins are phosphorylated and degraded by ubiquitination, allowing NFκB to translocate into the nucleus for transcription of various NFκB target genes. Therefore, reduction of IκB proteins usually results in increased nuclear translocation of NFκB as well as enhanced NFκB activity;

(3) Increased NFκB nuclear translocation, which results in an increase of NFκB activity;

(4) Increased NFκB activity by direct stimulation: NFκB activity was reported to be directly modulated by post-translational modification. Various studies have shown that phosphorylation or acetylation of NFκB at different sites results in changes in NFκB activity. Phosphorylation of NFκB at Ser-529 and Ser-536 stimulated NFκB transactivation, whereas acetylation of NFκB at Lys-218, -221 and -310 enhanced DNA binding of NFκB [133]. Thus, factors that stimulate phosphorylation of acetylation of NFκB at these sites may confer stimulation of NFκB activity.

If gelsolin activates NFκB by either of the four above-menioned mechanisms, it would all result in enhanced NFκB activity, which can be measured by dual-luciferase reporter assay. Therefore, dual-luciferase reporter assay was used to assess the effects on NFκB instead of other indirect assays such as electrophoretic mobility shift assay (EMSA) or enzyme-linked immunosorbent assay (ELISA).

As no changes in NFκB expression were observed when gelsolin was overexpressed, or knocked down, it is possible to rule out the possibility (1). If gelsolin activates NFκB via either mechanism (2) or (3), increased NFκB nuclear translocation will be detected when gelsolin expression is enhanced. If gelsolin exerts its effect by directly modulating NFκB post-translational modification (4), an increase of NFκB activity but not NFκB nuclear translocation will be observed. As increased gelsolin translocation to nucleus was detected in gelsolin-overexpressing cells (data not shown), it is possible

that gelsolin activates NFκB by enhancing nuclear translocation of NFκB or by directly stimulating NFκB activity in the nucleus. The possible influence of gelsolin on nuclear translocation of NFκB can be determined by cytoplasmic and nuclear fractionation followed by Western blot. To investigate whether gelsolin directly stimulates NFκB activity, further studies on the effects of gelsolin modulation on phosphorylation and acetylation of NFκB are suggested. If gelsolin induces phosphorylation or acetylation at the positive regulatory sites of NFκB, it suggests that gelsolin may stimulate NFκB activity via direct post-translational modification of NFκB, which induces enhanced DNA binding capacity or transactivation of NFκB.

SUMMARY

In this chapter, we showed that fibronectin expression in HCT 116 cells was stimulated by HGF. HGF in turn activated EGR-1 expression, which is a known regulator of fibronectin. Gelsolin upregulated fibronectin expression but not did not regulate EGR-1 expression nor activity. Therefore, fibronectin regulation by gelsolin is unlikely via gelsolin's influence on EGR-1.

On the other hand, TNFα, a known stimulator of NFκB, did not affect gelsolin levels but increased fibronectin expression in HCT 116 cells. Athough NFκB expression was not affected by gelsolin overexpression, NFκB activity was significantly enhanced in gelsolin-overexpressing cells and attenuated in several gelsolin knock-down cells. Moreover, upregulation of fibronectin by gelsolin could be attenuated by NFκB inhibition. Taken together, these results suggest that gelsolin upregulates fibronectin expression through NFκB pathway.

-CHAPTER 5-

GENERAL CONCLUSIONS AND FUTURE WORK

5.1. GENERAL CONCLUSIONS

In this study, we have identified a novel role of gelsolin in regulating fibronectin, an important ECM component, as well as one of the underlying mechanisms of that regulation.

Firstly, we showed that gelsolin protected colorectal cancer HCT 116 cells from apoptosis and promoted HCT 116 cell survival in an anoikis model. Gelsolin exerted the effects by upregulating fibronectin expression and secretion in HCT 116 cells. Conditioned media obtained from gelsolin over-expressing cells protected HCT 116 cells against apoptosis, indicating that gelsolin-overexpressing cells secreted factors that promoted cancer survival. On the other hand, fibronectin expression and secretion, which were regulated by gelsolin, protected cancer cells against apoptosis and promoted the survival pathway through Akt signaling in the anoikis condition. Fibronectin also rescued the apoptotic effects of gelsolin knock-down in HCT 116 cells cultured in anoikis conditions. These findings elucidated the important roles of gelsolin in cancer cell survival by upregulating fibronectin.

Secondly, our results suggested that gelsolin upregulated fibronectin expression in HCT 116 cells through activation of the transcription factor nuclear factor kappa B (NFκB) pathway. Gelsolin stimulated NFκB activity, although did not affect NFκB expression. NFκB activity was significantly enhanced in gelsolin-overexpressing cells and attenuated in gelsolin-knockdown cells. Activation of NFκB by tumor necrosis factor alpha (TNFα) leaded to increased fibronectin expression in HCT 116 cells, whilst inhibition of NFκB attenuated fibronectin level. Additionally, upregulation of

fibronectin by gelsolin could be attenuated by NFκB inhibition. Although the transcription factor early grow response protein 1 (EGR-1) was also involved in fibronectin regulation in HCT 116 cells, fibronectin upregulation by gelsolin was unlikely via EGR-1 pathway. Activation of EGR-1 by the hepatocyte growth factor (HGF) resulted in upregulation of fibronectin expression. However, gelsolin did not affect either EGR-1 expression or EGR-1 activity. These findings suggest that gelsolin upregulates fibronectin expression via NFκB but not EGR-1 pathway.

In conclusion, our results demonstrated a role of gelsolin in upregulating the expression and secretion of fibronectin to promote tumor cell survival. Based on the above findings, we propose a model for the novel role of gelsolin in cancer cell survival as illustrated in Figure 5.1. Results from this study provide further insights into the mechanisms behind the important roles of actin cytoskeleton as well as gelsolin in tumor biology. The findings would contribute to understanding of how cancer cells modulate the microenvironment to favor tumor progression, and this understanding may contribute to the development of novel strategies for cancer therapy.

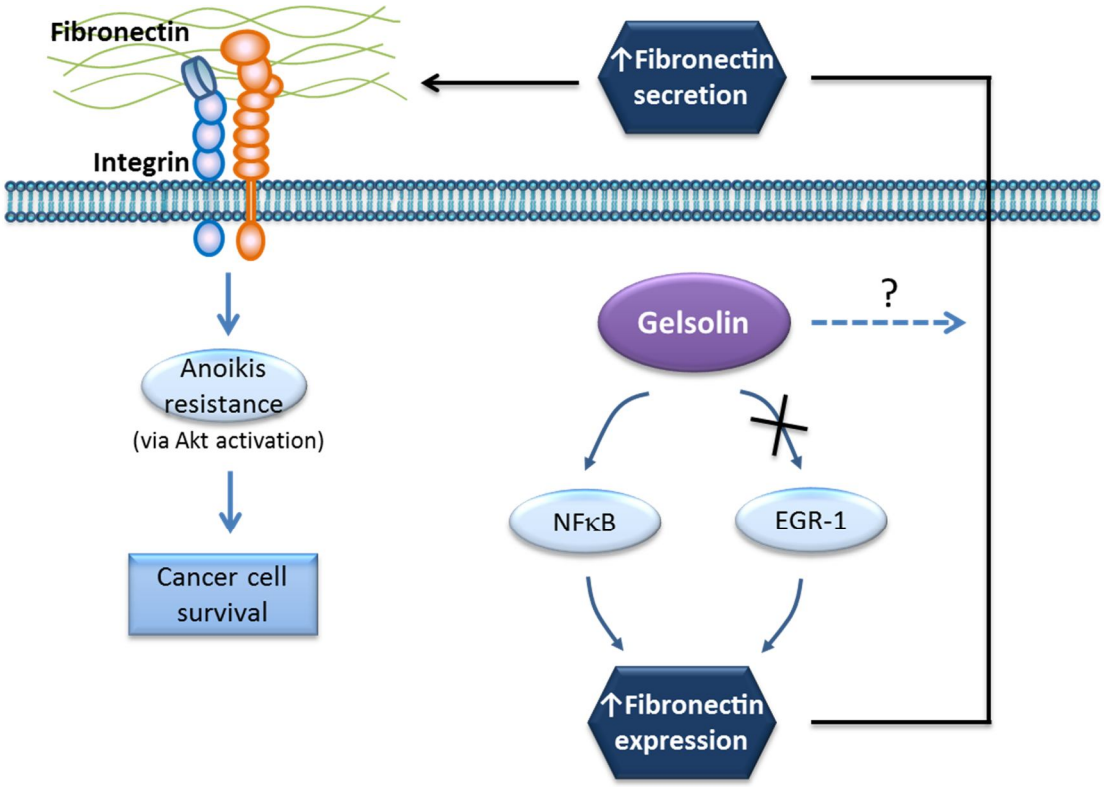


Figure 5.1 Schematic illustration showing the summary of the key findings presented in this thesis. In HCT 116 cells, gelsolin stimulates NFκB activity, which then upregulates fibronectin expression and secretion. Secreted fibronectin binds to integrin receptors, stimulates Akt activation, resulting in anoikis resistance and increased survival of cancer cells. Fibronectin expression is also upregulated by EGR-1 activation, but the effects of gelsolin on fibronectin expression is unlikely via EGR-1.

5.3. LIMITATIONS OF THE STUDY AND FUTURE WORK

5.3.1. Limitations of the study

In investigation of the effect of gelsolin on fibronectin expression and secretion, besides using gelsolin knockdown, it would be good if gelsolin is deactivated by either using inactive forms of gelsolin or using gelsolin activity inhibitors. These experiments would substantiate our findings that gelsolin regulates fibronectin expression and secretion. However, to our knowledge, there are no such inactive forms or activity inhibitors of gelsolin, and hence using gelsolin knockdown is the only choice at the moment.

The second limitation of the study is that possible compensatory effects of other actin-binding proteins have not been studied. Despite the important roles of gelsolin in regulating different aspects of cellular biology, gelsolin knockout had no lethal effects on experimental animals, suggesting that gelsolin's functions were compensated by other factors, such as other actin-binding proteins. Our results also suggest similar possibility. When about 80% of gelsolin was knocked down, there was only about 40% suppression in fibronectin expression and secretion (Figure 3.4A&B). Therefore, when gelsolin is knocked down, there are probably compensatory effects of other actin-binding proteins, which have not been addressed in this study and deserve further investigations.

In addition, we have shown that gelsolin promotes expression of fibronectin via NFκB activation. However, it is not clear whether gelsolin enhances fibronectin secretion by only upregulating fibronectin expression or by promoting both fibronectin expression and exocytosis of fibronectin secretory vesicles. Given that gelsolin plays an important role in exocytosis of

insulin secretory vesicles due to its effects on actin dynamics, it is possible that gelsolin may influence exocytosis of fibronectin vesicles. Future studies could be extended to confirm this hypothesis.

5.3.2. Future work

The following are key areas of interest for future investigations:

(1) To investigate the underlying mechanisms of NFκB activation by gelsolin:

We have provided evidence showing that gelsolin activated NFκB activity in HCT 116 cells. However, the mechanisms underlying gelsolin-regulated NFκB activity need to be elucidated. As gelsolin did not affect NFκB expression, the increased NFκB activity by gelsolin is possibly a result of other reported mechanisms such as: (a) enhanced nuclear translocation of NFκB, (b) suppression of NFκB inhibitors or (c) direct stimulatory effects on nuclear NFκB transcriptional activity [133]. We have detected increased gelsolin translocation in the nucleus in gelsolin-overexpressing cells (data not shown), suggesting possible involvement of gelsolin in nuclear translocation of NFκB. To investigate this possibility, gelsolin expression in cells will be modulated by either overexpression or siRNA knock-down. Nuclear translocation of NFκB will be measured by cytoplasmic and nuclear fractionation followed by Western blot. The nuclear translocation of NFκB will also be visualized by live-cell imaging. If gelsolin exerts its effect on NFκB by either mechanism (a) or (b), NFκB translocation to the nucleus will be increased in gelsolin-overexpressing cells and attenuated in gelsolin-knockdown cells. Nuclear translocation of NFκB as well as possible co-localization of gelsolin with NFκB can also be studied by immunofluorescence microscopy. To investigate if gelsolin stimulates NFκB by suppressing the NFκB inhibitory proteins, IκB (b), the expression,

phosphorylation and ubiquitination of IκB will be measured. If gelsolin overexpression leads to decreased IκB expression or increased IκB phosphorylation or ubiquitination, this means that gelsolin activates NFκB by increasing IκB degradation. To investigate whether gelsolin directly enhances nuclear NFκB transcription activity (c), further studies on post-translational modification of nuclear NFκB upon modulation of gelsolin can be conducted. Phosphorylation of NFκB at Ser-529 and Ser-536 were shown to stimulate NFκB transactivation, and acetylation of NFκB at Lys-218, -221 and -310 enhanced DNA binding of NFκB [133]. If overexpression of gelsolin induces phosphorylation or acetylation at the positive regulatory of NFκB, whilst gelsolin knock-down exhibits the opposite effects, it means that gelsolin stimulates NFκB activity via direct post-translational modification of NFκB, which induces enhanced DNA binding capacity or transactivation of NFκB.

(2) To investigate if the actin-regulating functions of gelsolin are involved in regulating fibronectin secretion:

Actin filaments at the proximity of plasma membrane serve as barriers against exocytosis of secretory vesicles. By severing actin filaments, gelsolin possibly disrupts the barriers for enhanced exocytosis. Thus, our study can be extended to examine whether gelsolin enhances fibronectin secretion by severing the actin cytoskeleton to facilitate the transport of fibronectin to plasma membrane for exocytosis. Deletion mutants of the gelsolin gene can be constructed, including deletion of domain G1, which is responsible for actin-severing function and deletion of domains G1-3, which are responsible for

both actin-severing and capping functions of gelsolin. Cells will be transfected with plasmids containing either of the deletion mutants. Cells transfected with plasmids containing wild-type gelsolin and plasmids with empty vector will be used respectively as positive and negative controls. If secreted fibronectin is reduced in mutant gelsolin-expressing cells compared to wild-type gelsolin, it means that the actin-severing function of gelsolin is important in the secretion of intracellular fibronectin. Immunofluorescence studies will be conducted on cells transfected with either mutant or wild-type gelsolin to confirm the findings. The vesicle-SNARE protein synaptobrevin (VAMP2), which is normally coupled on secretory vesicles, will be used as a secretory vesicle marker. F-actin, fibronectin and secretory vesicles will be respectively stained with fluorescence-tagged phalloidin, fibronectin antibodies and VAMP2 antibodies. Secretory vesicles of fibronectin will be identified by the co-localization of fibronectin with VAMP2. If decreased depolymerization of actin filaments coincident with less secretory vesicles of fibronectin are observed at the proximity of plasma membrane of the mutant gelsolin cells compared to wild-type gelsolin cells, it indicates that gelsolin enhances fibronectin secretion by severing actin filaments and promoting actin turnover.

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APPENDICES

APPENDIX I. PLASMID CONSTRUCT FOR GELSOLIN OVER-EXPRESSION

1. Sequence of pIRES2-EGFP-gelsolin recombinant plasmid:

Black: pIRES2-EGFP sequence; blue: gelsolin sequence cloned (2196 nucleotides); underlined in red: EcoR I and Sac II sequences; start (ATG) and end (TGA) codons. The cytoplasmic gelsolin sequence matches that of Genbank accession #BC026033.1.

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA
GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCC
CGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCC
ATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGT
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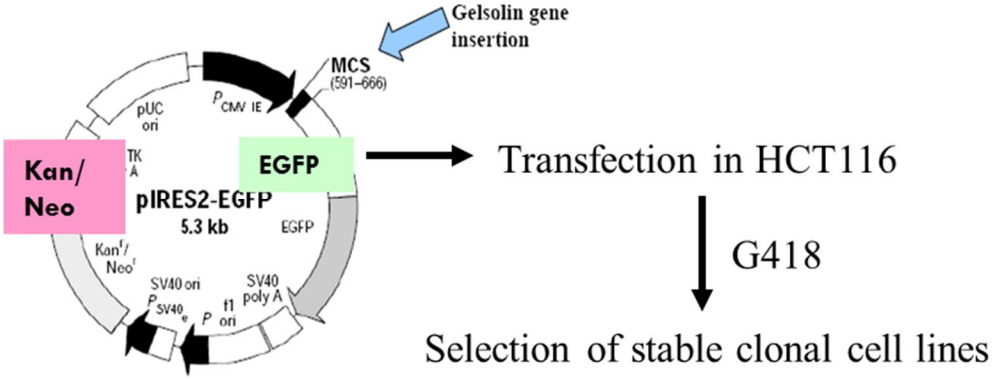
2. Plasmid construct for stable expression of gelsolin in HCT 116 cells:



APPENDIX II. PREPARTION OF REAGENTS AND CHEMICALS

Cell culture materials

McCoy's 5A Medium (1L)

McCoy's 5A powder (M4892, Sigma-aldrich)

2.2g $NaHCO_3$,

100mL Fetal Bovine Serum

Top up to 900mL MiliQ water and filter through 0.22µm filter membrane.

RPMI-1640 (1L)

RPMI-1640 powder (R4130, Sigma Aldrich)

2g $NaHCO_3$,

100mL Fetal Bovine Serum

Top up to 900mL MiliQ water and filter through 0.22µm filter membrane.

Freezing media

95% Fetal bovine serum

5% DMSO

Western blot materials

Resolving gel for SDS-PAGE

Distilled water

30% bis-acrylamide

1.5M Tris (pH 8.8)

10% SDS

10% APS

TEMED

4% Stacking gel for SDS-PAGE

Distilled water

30% bis-acrylamide

1M Tris (pH 6.8)

10% SDS

10% APS

TEMED

10X SDS-PAGE running buffer

25mM Tris base

192mM Glycine

0.1% SDS

10X transfer buffer for SDS-PAGE

25mM Tris base,

192mM Glycine

0.025% SDS

APPENDIX III. LIST OF PUBLICATIONS

MANUSCRIPTS PREPARED FOR PUBLICATION

- Bao Hua Huang, Ser Yue Loo, Arpita Datta, Yan Lin Yap, Benedict Yan, Chia Huey Ooi, **Thuy Duong Dinh**, Jingli Zhuo, Lalchhandami Tochhawng, Suma Gopinadhan, Patrick Tan, Manuel Salto-Tellez, Wei Peng Yong, Richie Soong, Khay Guan Yeoh, Yaw Chong Goh, Alan Prem Kumar, Sutherland K. Marciver, Jimmy B.K. So, Celestial T. Yap. Gelsolin mediates HGF-dependent cell scattering and invasion of gastric cancer cells *(In submission)*

- **Thuy Duong Dinh**, Deng Shuo, Sutherland Maciver, Yaw Chong Goh, Celestial T Yap. Gelsolin upregulates fibronectin expression and secretion to promote survival of colorectal cancer cells. *(In preparation)*

CONFERENCE PRESENTATIONS

- **Thuy Duong Dinh**, Lalchhandami Tochhawng, Yaw Chyn Lim, Yaw Chong Goh, Sutherland Maciver, Shazib Pervaiz, Celestial T Yap (2013). The role of gelsolin in remodeling the tumour microenvironment. Beatson International Cancer Conference July 2013, Glasgow, Scotland.

- **Thuy Duong Dinh**, Jingli Zhuo, Lalchhandami Tochhawng, Deng Shuo, Suma Gopinadhan, Sutherland Maciver, Yaw Chong Goh, Celestial T Yap (2012). Gelsolin modulates the expression of extracellular matrix components in colon cancer. CSH Asia / ICMS Joint Conference on Tumor Microenvironment 2012, Suzhou, China.

- Baohua Huang, Jingli Zhuo, **Thuy Duong Dinh**, Benedict Yan, Yan Lin Yap, Chia Huey Ooi, Lalchhandami Tochhawng, Suma Gopinadhan, Tao Jiong, Manuel Salto-Tellez, Patrick Tan, Richie Soong, Jimmy B.K. So, Celestial Yap (2011). Gelsolin regulates gastric cancer intercellular adhesion and contributes to gastric cancer dissemination. Cancer Models and Novel

Therapies, Glasgow, Scotland, July 2011.

- Huang BH, Zhuo J, **Dinh TD**, Yan B, Yap YL, Ooi CH, Tochhawng L, Gopinadhan S, Jiong T, Salto-Tellez M, Tan P, Soong R, So JBK, Yap CT (2011). Gelsolin is a novel negative regulator of E-cadherin and contributes to the dissemination of gastric cancer. Singapore Gastric Cancer Consortium, 4th Annual Scientific Meeting, Singapore, July, 2011.