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ANALYSIS OF THE MECHANISM BY WHICH YKL-40 PROMOTES  
GLIOMA CELL MIGRATION

A thesis submitted in partial fulfillment of the requirements for the degree Master  
of Science at Virginia Commonwealth University.

By

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May, 2011

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# **Analysis of the Mechanism by which YKL-40 Promotes Glioma Cell Migration**

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## List of abbreviations:

cDNA	complementary DNA
DMEM	Dulbecco`s modification of Eagle`s medium
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
IL-1	Interleukin-1
MAPK	mitogen-activated protein kinase
GBM	glioblastoma multiforme
HEK	human embryonic kidney
mRNA	messenger RNA
OSM	oncostatin M
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative PCR
WB	western blot



## Abstract

# **Analysis of the Mechanism by which YKL-40 Promotes Glioma Cell Migration**

By Bahiya Mohammed Osrah

A Thesis submitted in partial fulfillment of the requirements for the degree of  
Master at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Tomasz Kordula, Ph.D.  
Associate Professor, Department of Biochemistry and Molecular Biology

This thesis elucidates the crucial role of YKL-40 in enhancing glioma cell migration and invasion *in vitro*. Increased levels of YKL-40 are specifically associated with the increased invasive capacity of glioma multiforme (GBM) tumors and lower survival rate of GBM patients. In order to examine the effects of YKL-40 on the migration and invasion of GBM cells, we overexpressed YKL-40 in three different glioma cell lines. The overexpression of YKL-40 significantly enhanced glioma cells migration and invasion *in vitro* and also increased ERK phosphorylation, which is believed to enhance glioma cell survival, and invasiveness. Although receptors for YKL-40 are still unknown, YKL-40 induces interactions between integrin  $\alpha v \beta 3$  and syndecan-1 in endothelial cells. However, syndecan-1 does not mediate YKL-40-induced migration and invasion of glioma cells since it is expressed at very low levels, in comparison to

other syndecans. In contrast, we found that syndecan-4 is expressed at high levels in all glioma cells we tested. Importantly, down-regulation of syndecan-4 dramatically reduced YKL-40-induced migration of U373 cells, suggesting that syndecan-4 may mediate the effect of YKL-40.

Since inflammation has been associated with the progression of many cancers, including GBM, we studied the effect of major pro-inflammatory cytokines on the expression of both YKL-40 and syndecans. Interestingly, OSM and IL-1 synergistically enhanced both YKL-40 and syndecan-4 expression in glioma cells. This suggests that this synchronous induction of YKL-40 and syndecan-4 by OSM and IL-1 may enhance invasion of GBM *in-vivo*.

In summary, we propose a mechanism through which YKL-40 may function under pro-inflammatory conditions. Increased expression of YKL-40 and syndecan-4 in glioma cells leads to the subsequent activation of the MAPK/ERK pathway and results in glioma cell invasion.

# 1. INTRODUCTION

## 1.1 GBM

### **Glioblastoma:**

Gliomas comprise the majority of the primary tumors of the central nervous system [1] and are classified into four different classes based on the ability for cells to infiltrate and invade the surrounding brain tissue. Grade I astrocytoma is considered to be a non-malignant and less aggressive brain tumor. Grade II tumors have a low level of malignancy and the ability to diffuse to nearby brain tissue, which makes them susceptible to surgery and most patients with this tumor can survive 5-10 years. Grade III astrocytoma is more aggressive and malignant, with patients surviving for a period of 2-3 years. Finally, Grade IV or glioblastoma multiforme (GBM), exhibit high levels of malignancy, which includes significant invasion, proliferation, angiogenesis, and necrosis. GBM is the most aggressive form of primary brain tumor, with an average patient survival period of 9-12 months post diagnosis [1]. The symptoms of GBM include deficits in neurocognitive functions, including information processing, working memory and executive functioning, and epileptic seizures [2]. Despite the current treatment regimens, including surgical removal of the tumor, radiation therapy and chemotherapy, GBM is incurable due to the high rate of recurrence. The recurrence rate is very high because GBM cells aggressively infiltrate nearby brain tissues. GBM is a tumor of astroglia origin (astrocyte), which is the most abundant cell type in the brain comprising at least 50% of human brain cells [3].

Many genetic mutations contribute to the complex network of molecular events that lead to tumor formation and progression. Mutations in many cell cycle regulatory genes, including p53, PTEN, and RB contribute to tumor progression. Furthermore, these mutations lead to the

constitutive activation of many mitogenic signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide-3-kinase (PI3K) pathways [1]. PI3Ks catalyze the phosphorylation of phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P<sub>2</sub>] into [PtdIns(3,4,5)P<sub>3</sub>], which provides a binding site for many signaling proteins and then activates Akt via T308 and S473 phosphorylation [1]. Moreover, changes in the expression of many genes are associated with GBM tumor progression [1]. Recently, YKL-40, a secreted glycoprotein associated with extracellular matrix, was identified as the most highly up-regulated protein in the serum of GBM patients. Furthermore, a microarray analysis of tumors has shown that YKL-40 is one of the most overexpressed genes compared to normal brain tissue [4]. However, the regulation and role of YKL-40 in GBM tumor progression remains elusive.

## **1.2. Chitinase-3 like1 (CHI3L1) or YKL-40**

YKL-40, also known as Chitinase-3 like (CHI3L1), human cartilage glycoprotein-39 (HC gp-39), the mouse homologue breast regression protein-39 (BRP-39), and chondrex is a secreted glycoprotein. The gene encoding YKL-40 is located on chromosome 1q32.1. Chitinases, including YKL-40 are endo- $\beta$ -1, 4-N-acetylglucosamidases that are a part of the innate immune response, involved in the host defense against infections from organisms that contain chitin. Chitin is a polymer of  $\beta$ -(1-4)-poly-N-acetyl-D-glucoseamine, which is found in the cell walls of many bacteria and fungi [5]. The chitinase-like enzymes, including YKL-40, can bind to chitin, but do not display catalytic activities due to sequence differences in their active sites compared to chitinase. YKL-40 is expressed in several cell types, including macrophages, neutrophils, endothelial cells, vascular smooth muscle cells, chondrocytes, and fibroblasts [5]. In normal individuals, expression of YKL-40 is induced in the late stages of macrophage differentiation and also during astrocyte differentiation from neural progenitors [5, 46]. The YKL-40 expression

is also up-regulated in many chronic inflammatory conditions, such as rheumatoid arthritis, osteoarthritis and in many cancers, including glioblastoma, melanoma, and myeloid leukemia. A high level of serum YKL-40 protein is used as a biological marker for many inflammation associated pathological conditions, including rheumatoid arthritis, multiple sclerosis and Alzheimer's disease [5,6,7]. In consonance with its association with inflamed tissues, YKL-40 expression is regulated by many inflammatory cytokines, such as IL-1, TNF- $\alpha$ , IL-6, and IL-13 [5, 8]. Furthermore, its expression is also controlled by hormones, such as vasopressin, and parathyroid hormone-related protein in both macrophages, and epithelial cells [5]. Despite, the high expression of YKL-40 associated with many diseases, its biological function is unknown. YKL-40 knock-out mice are normal, except that these animals lack Th2-dependent immune responses upon antigen challenge, suggesting its role in immunological functions [9].

Increased expression of YKL-40 in GBM tumors is associated with the invasive phenotype of GBMs. Interestingly; YKL-40 expression is attenuated by the p53 tumor suppressor that is often mutated in GBM, which likely leads to YKL-40 overexpression [10]. Many studies have indicated that YKL-40 stimulates signaling pathways that have critical roles in enhancing the malignant phenotype of GBM and progression of these tumors. Furthermore, a positive association between YKL-40 and both AKT and MAPK signaling has been found, indicating that YKL-40 activates these signaling pathways and may lead to GBM progression [34, 16]. Many studies have also shown that YKL-40 inhibits apoptosis of inflammatory cells by suppressing Fas expression and enhancing the phosphorylation of Akt. However, the mechanism by which YKL-40 activates these signaling pathways is still under investigation. Further studies have shown that YKL-40 interacts with many components of the extracellular matrix (ECM), including hyaluronic acid, and collagen I, II and III [17-19]. Adhesion to the extracellular matrix

is important for glioma cell migration and invasion [20]. On the other hand, suppression of YKL-40 expression in U87 glioma cells reduces angiogenesis and adhesion to ECM proteins, such as hyaluronic acid, fibronectin, and collagen IV [21]. However, the receptors through which YKL-40 functions to regulate these processes are still not known. Recent studies indicated that YKL-40 can bind the heparin binding domain of syndecan-1, which is a transmembrane heparin sulfate proteoglycan in endothelial cells. However, it is not clear if syndecans are required for YKL-40 induced downstream signaling in GBM pathogenesis.

### **1.3. Syndecans:**

#### **1.3.1 General information:**

Syndecans are transmembrane proteoglycans, which have heparin sulfate glycosaminoglycan (GAG) chains in their extracellular domains (ectodomains). This family is composed of four different members: syndecan-1, syndecan-2, syndecan-3 (N-syndecan), and syndecan-4. Syndecans contain three functional domains, ectodomain, transmembrane domain, and cytoplasmic domain. The ectodomain is composed of serine-glycine sequences that covalently bind to glycosaminoglycan chains and involved in the syndecans interaction with the heparin binding domain in most extracellular matrix (ECM) protein and growth factors [22]. The cytoplasmic domain is divided into C1, C2, and V domains. The ectodomain and the V domain imply functional and structural specificities among the family members. Syndecans ectodomain differ in size and amino acid sequences, which indicate different functions and specific interactions with extracellular matrix. The variable region (V) is distinct among the syndecans and in syndecan-4 specifically enhances the formation of focal adhesion via regulating the PKC $\alpha$  activity. PKC $\alpha$  is a serine/threonine kinase that plays important roles in cell migration,

proliferation, and cell adhesion. On the other hand, C1 and C2 domains are conserved in all syndecans among several species, and are important for linking syndecans' cytoplasmic domain to the actin cytoskeleton, further enhancing focal adhesion formation. Finally, the extracellular domain of syndecans plays a critical role in regulating syndecan-ligand interactions and many downstream signaling pathways, including FAK-MAPK signaling pathway [23]. Moreover, these signaling pathways are important for the regulation of many biological processes, including wound healing, inflammation, and angiogenesis [24, 25].

Syndecans are expressed differently among many cell types. Syndecan-1 is expressed in epithelial and plasma cells, but are not expressed in normal brain [26]. Syndecan-2 is originally expressed in developing neural tissue. On the other hand, syndecan-3 is expressed by oligodendrocyte progenitors, but not differentiated oligodendrocytes. However, it is not expressed by astrocytes [26, 27]. Syndecan-4 is more widely distributed and expressed in many cell types compared to the other syndecans [28]. A Semi-quantitative qRT-PCR analysis revealed that only syndecans-2 and -4 are expressed in the hippocampus [29].

### **1.3.2 Syndecan-4**

Syndecan-4 is the most ubiquitous member of the syndecan family that is widely expressed at focal adhesions sites. The heparin sulfate motif of syndecan-4's in the ectodomain regulates glioma cell adhesion to ECM proteins, and therefore regulates cell proliferation, focal adhesion, and spreading [32]. Cell adhesion to ECM also regulates many downstream signaling molecules, including Rho proteins, GTPases, Tyr Kinases, and MAP kinases [30]. Overexpression of the full length syndecan-4 increased the focal adhesion formation in Chinese hamster ovary (CHO-K1) cells, while the expression of its cytoplasmic core with either full or partial deletion causes reduction in the focal adhesion formation [31]. Therefore, the

intracellular domain of syndecan-4 is required for its outside-in signaling and subsequent focal adhesion formation. In parallel, integrins have also vital role in focal adhesion formation. In fact, the extracellular domain of integrins can bind to different ECM molecules, while its cytoplasmic domain interacts with many components that connect it to the actin cytoskeleton and further enhances focal adhesion and migration. A recent study has shown a cooperative interaction between  $\alpha 5\beta 1$  integrin and syndecan-4 that increases focal adhesion of astrocytes that further enhances their spreading on fibronectin [32].

The invasion and migration of tumor cells are multi-functional processes, which require cooperative interactions between syndecans and integrins in the presence of ECM proteins. Importantly, all ECM proteins have binding sites for integrins and syndecans. Moreover, both integrins and syndecans present in close proximity are both required for full cell adhesion [33]. Rong Shao et al. indicated that YKL-40 induces a cooperative interaction between integrin  $\alpha v\beta 3$  and syndecan-1 in endothelial cells [24].

#### **1.4. Cytokines.**

Many inflammatory cells and inflammation mediators are associated with tumor progression of many cancers [35]. Cytokines have pleiotropic biological implications, including inflammation, tumorigenesis, and invasiveness of cancer cells. The expression of many pro-inflammatory cytokines, including IL-1, IL-6, OSM, and TNF $\alpha$  is up-regulated in inflammation-associated cancer cells [36, 37, 38]. Cytokines are involved in activating many downstream signaling pathways that play a pivotal role in tumor progression.

Interleukin-1 (IL-1) is a pleiotropic cytokine, which activates various signaling pathways, including IKK-NF- $\kappa$ B and MAPK in many cell types. NF- $\kappa$ B is regarded as one of the major



players in inflammation and is associated with tumorigenesis. Moreover, activation of NF- $\kappa$ B further activates downstream factors, including IL-6 and IL-23, which in turn activate STAT-3 signaling. Oncostatin M (OSM), a member of the IL-6 family of cytokines, is a secreted glycoprotein that is expressed normally in monocytes, macrophages, and microglia [39]. Interestingly, OSM is not detected in normal brain and its expression has been increased in many brain tumors, including GBM. However, the role of OSM in brain tumors has not been defined. Recently, we have shown that OSM induces the expression of YKL-40 by activating STAT-3 in primary astrocytes and glioma cells.

### **1.5. Hypothesis/Aim.**

YKL-40 is highly up-regulated in many inflammation-associated tumors and promotes migration of several cell types. We hypothesize that increased expression of YKL-40, in response to pro-inflammatory cytokines, promotes the invasiveness of glioma cells. We also hypothesize that a member of the syndecan family may be required for this YKL-40-induced migration and invasion. To test these hypotheses, YKL-40 was overexpressed in several glioma cell lines and their migration and invasion *in vitro* was analyzed. We also analyzed the expression of syndecans and tested the role of syndecan-4 in mediating the effect of YKL-40 on migration and invasion of glioma cells. Finally, the expression of YKL-40 and syndecans in response to pro-inflammatory cytokines was examined.

## **2. MATERIALS AND METHODS:**

### **2.1 Cell culture.**

The human glioma cell lines, U1242, U373MG and LN229, and 293 HEK cells were obtained from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% CO<sub>2</sub> incubator at 37 ° C.

### **2.2 Western blotting.**

Cells were lysed in 10 mM Tris pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, 1 mM sodium orthovanadate, 0.2 mM PMSF, and protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were resolved using SDS-PAGE, and electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The anti-YKL-40, anti-pERK1/2, anti-pAKT, and anti-β-tubulin antibodies were from Santa Cruz Biotechnology. Antigen-antibody complexes were visualized by enhanced chemiluminescence using Immobilon Western blotting kit (Millipore, Temecula, CA).

### **2.3 RNA isolation and quantitative PCR.**

Total cellular RNA was prepared by Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Subsequently, 1 µg of total RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). YKL-40, Syndecan-1, Syndecan-2, Syndecan-3, Syndecan-4 and GAPDH mRNA levels were measured using pre-mixed primer-probe sets, and TaqMan Universal PCR Master Mix according to the supplier's instructions (Applied Biosystems, Foster City, CA). The cDNAs were diluted 10-fold (for the

target genes) or 100-fold (for GAPDH), and amplified using the ABI 7900HT cyclor. Gene expression levels were normalized to GAPDH mRNA levels, and presented as a fold induction with mean values +/- standard deviation. Statistical analysis was performed by one-way analysis of variance.

#### **2.4. Plasmids.**

pc DNA3.1 was obtained from Invitrogen Life Technologies and it has ampicillin and neomycin resistances. pCMVS-PORT YKL-40 plasmid, was obtained from Open Biosystem. YKL-40 DNA was excised out from the parent pCMV SPORT YKL-40 plasmid using SalI and XbaI enzymes. Subsequently, pcDNA3.1-YKL-40 plasmid was generated using the excised YKL-40 DNA which is sub-cloned into the pcDNA3.1 plasmid digested with XbaI and XhoI.

#### **2.5. Transfections and creation of stable cell lines.**

HEK Cells were transiently transfected in 12 well clusters using FuGENE6 transfection reagent (Roche, Indianapolis, IN), according to the supplier's instructions. One day after transfection the cells were selected by neomycin antibiotic. Furthermore, the cells were harvested, protein extracts were prepared, and the protein concentration was determined by the BCA method (Sigma Chemical Co., St. Louis, MO). To generate stable clones, the PcDNA3.1-YKL-40 construct was transfected into 10 cm dishes of three different cell lines, U1242, LN229, and U373 MG and after one day they have been selected by neomycin antibiotic.

#### **2.6. Wound Healing Assay.**

Cells were plated at 300,000 per well in a six well plate. Next day, scratch was made by using 20  $\mu$ l sterile pipet tips. Cells were allowed to migrate to the wounded area for 24 h or 48 h. Pictures of the migrated cells in the wound area were taken. Subsequently, the number of migrated cells in the wounded area was manually counted. Each data point is the average number of cells in eight random fields.

### **2.7. Matrigel invasion assay.**

The invasion of the cells was measured in a modified Boyden chamber using polycarbonate filters (25  $\times$  80mm, 12  $\mu$ m pore size) coated with Matrigel (BD). The cells were added to the upper chamber at 50,000 cells per well. After 7 h, non-migratory cells on the upper membrane surface were mechanically removed, and the cells that traversed and spread on the lower surface of the filter were fixed and stained with Diff-Quik (Fisher Scientific). The invading cells were counted with an inverted microscope and a 10 $\times$  objective. Each data point is the average number of cells in six random fields

### **2.8. Cytokines and Cell Stimulation**

Cells were stimulated with 25 ng/ml OSM, or 25 ng/ml IL-6 and 10 ng/ml IL-1 (R&D, Systems, Inc., Minneapolis, MN).

### **2.9. Down-regulation of target genes.**

Expression of syndecan-4 mRNA was down-regulated using SmartPool siRNAs (si-SDC-4) from Dharmacon (Dharmacon, Int., Lafayette, CO).

### 3. RESULTS:

#### 3.1 YKL-40 overexpression increases glioma cells migration *in vitro*.

It has been shown that the YKL-40 expression is up-regulated in cancers, including GBM. However, its biological roles are still under investigation. In order to examine the YKL-40 role in glioma cell invasion and migration *in vitro*, we stably overexpressed this protein in U373, LN229, and U1242 glioma cells and examined its effect on migration. First, we subcloned the YKL-40 cDNA into pcDNA3.1 plasmid as described in Materials and Methods. The YKL-40 cDNA construct was transiently transfected into 293 HEK cells, which do not express YKL-40, and the protein expression was analyzed in both lysates and media (Fig. 3.1 a.). Subsequently, we transiently transfected the YKL-40 construct into U373 MG cells and the protein expression was verified in lysates and media (Fig. 3.1 b.). These experiments show that both 293 HEK and U373 cells efficiently express YKL-40. In order to analyze the effects of increased expression of YKL-40 on glioma cells, we generated stable glioma cells lines, LN229, U1242, and U373 overexpressing YKL-40. U373 cells are PTEN<sup>-/-</sup>, and exhibit high basal AKT activity and LN229 cells are PTEN<sup>+/+</sup>. Both of these cell lines are non-invasive *in vivo*. On the other hand, U1242 cells are invasive *in vivo*. We verified the expression of YKL-40 on the protein and mRNA levels in the three generated stable glioma cell lines by western blotting and qRT-PCR respectively (Fig. 3.2 a, and b). We investigated the effect of YKL-40 in glioma cell migration *in vitro* by wound healing and trans-well migration assays. Overexpression of YKL-40 significantly enhanced the migration of all three different glioma cell lines by 1.5 to 2 fold (Fig. 3.3 a, and b). Because invasiveness of GBM is a major clinical problem, we asked whether YKL-40 affects GBM invasiveness *in vitro*. It was previously shown that U373 cells are non-invasive *in vivo*. YKL-40 overexpression in U373 cells significantly enhanced its invasiveness *in vitro* in matrigel

invasion assay (Fig. 3.4). These data demonstrate that YKL-40 affects glioma cell migration and invasion.

### **3.2. YKL-40 activates MAPK signaling in glioma cell.**

Since overexpressing YKL-40 in glioma cells increases their migration and invasion, we asked whether YKL-40 affected signaling in these cells. YKL-40 has previously been shown to activate several signaling pathways including PI3K-AKT and MAPK-ERK in breast cancer cells and chondrocytes [40]. Activation of these pathways in glioma cells may lead to increased migration and invasion. Fibronectin, and laminin are extracellular matrix proteins that are expressed in the brain and they are believed to stimulate glioma cell migration [48]. YKL-40 has been shown to interact with many components of extracellular matrix (ECM) that are expressed in the brain, including hyaluronic acid, and collagen I, II and III by using affinity chromatography experiments with purified YKL-40 [17-19]. Down-regulation of YKL-40 reduced cell-matrix adhesion via impairing the cell adhesion to many ECM in glioma cell [47]. For this reason, cells were cultured on these ECM components and ERK and AKT activation was analyzed using phosphospecific antibodies. YKL-40 overexpression efficiently activated ERK phosphorylation regardless of the ECM used. (Fig. 3.5 a,b).

### **3.3. Syndecan-4 is critical for YKL-40 induced glioma cells migration.**

A recent study proposed a mechanism, by which YKL-40 induces the interaction of syndecan-1 with integrin  $\alpha v \beta 5$  in U87 glioma cells [40]. We analyzed the expression of syndecan-1 in the generated glioma cell lines (Fig. 3.6 a). Both U1242 and LN229 cells express very low amounts of syndecan-1, suggesting that syndecan-1 is not a mediator of YKL-40 induced migration and invasion of these cells. Subsequently, we analyzed the expression of the other syndecans (-2, -3, and -4). These syndecans were expressed at various levels in all glioma

cell lines (Fig. 3.6 b, c, d). Syndecan-4 caught our attention because it has been previously shown to increase migration and adhesion of glioma cells to ECM proteins [31]. In order to test whether syndecan-4 mediates YKL-40-induced glioma cell migration, we knocked-down its expression in either vector or YKL-40 U373 cell line (Fig. 3.7.). Syndecan-4 down-regulation diminished migration of the U373-vec cells by 25%. Significantly, syndecan-4 down-regulation in U373-YKL-40 cells dramatically reduced migration of these cells (Fig. 3.7.). These results suggest that syndecan-4 mediates YKL-40-induced cell migration of glioma cells.

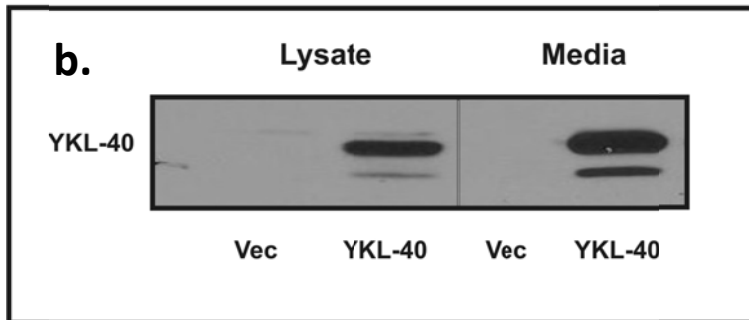
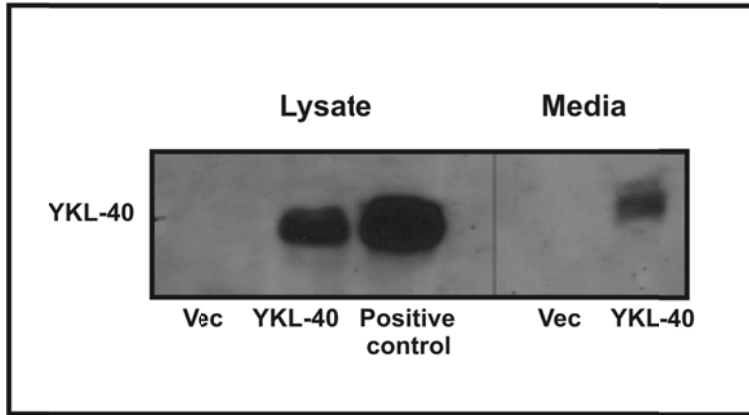
### **3.4. IL-1 and OSM cytokines simultaneously activate the expression of syndecan-4 and YKL-40 in glioma cells.**

Both IL-1 and OSM are expressed in minute amounts in normal brain, however, their expression is increased in some brain tumors and it is believed that they regulate tumor growth and invasiveness. Therefore, we examined whether these cytokines affect YKL-40 expression in glioma cell (Fig. 3.8 a). IL-1 and OSM synergistically increased YKL-40 expression in U373 cells. We have also examined the effect of OSM and IL-1 on the expression of syndecans. We found that syndecan-4 expression was increased by both IL-1 and OSM in both U373 and U1242 cells (Fig. 3.9.d). In contrast, Syndecan-1 expression was induced by OSM and IL-1 only in U373 (Fig. 3.9 a). While, Syndecan-2 expression was down-regulated in both cell types by OSM (Fig.3.9.b). The expression of syndecan-3 was not changed by these cytokines in both cell lines (Fig. 3.9.c). Taken together, we can conclude that IL-1 and OSM simultaneously induce the expression of both syndecan-4 and YKL-40. These data suggest that synchronous induction of YKL-40 and syndecan-4 by OSM and IL-1 may enhance migration and invasion of GBM *in vivo*.

**Fig. 3.1. YKL-40 overexpression in 293 HEK and U373 cells**

(a.) 293 HEK cells were transiently transfected with pcDNA 3.1-YKL-40 construct. The YKL-40 expression was analyzed in lysates and media by Western blotting. U373 NFIX3 lysate was used as a positive control. (b.) YKL-40 has been overexpressed in U373 cells. Lysates were prepared and the expression of YKL-40 was detected by Western blotting.

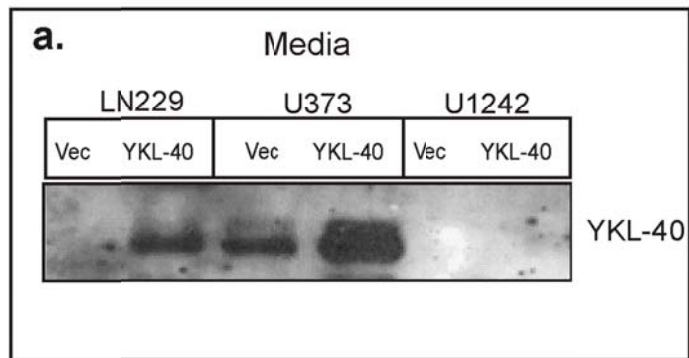




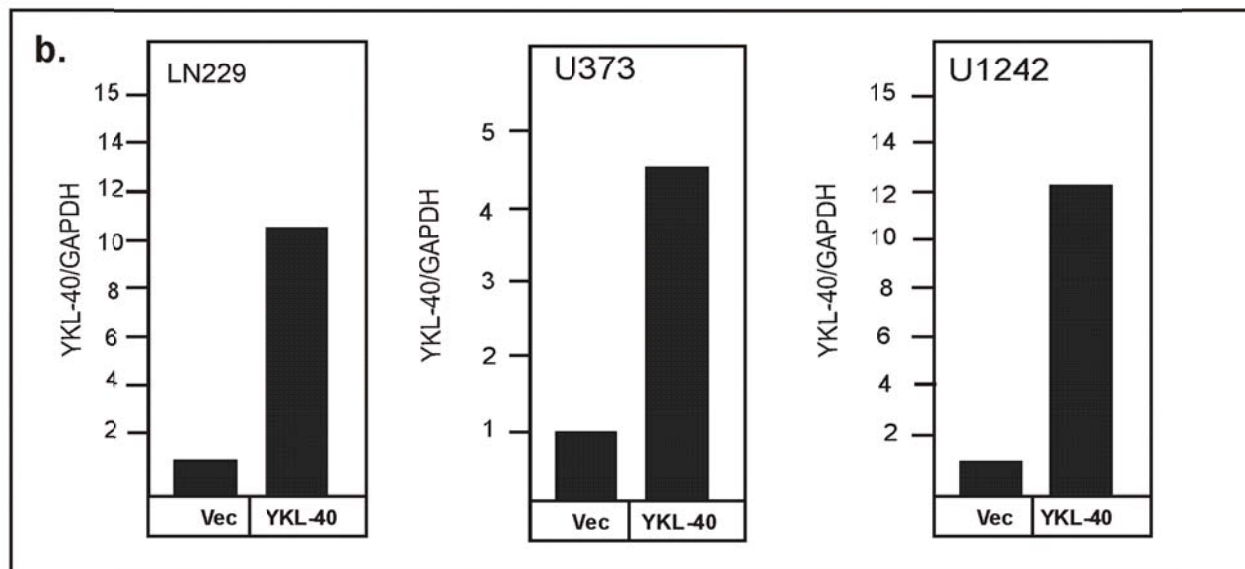
**Fig. 3.2. Stable overexpression of YKL-40 in three different glioma cells.**

(a.) The protein levels of YKL-40 in the media were detected by western blotting for U373, U1242, and LN229 cells. (b.) RNA was prepared from LN229, U1242, and U373 glioma cells overexpressing YKL-40 or empty vector. The expression of YKL-40 mRNA was analyzed by qRT-PCR, and normalized to GAPDH.

## YKL-40 Expression by Western Blot



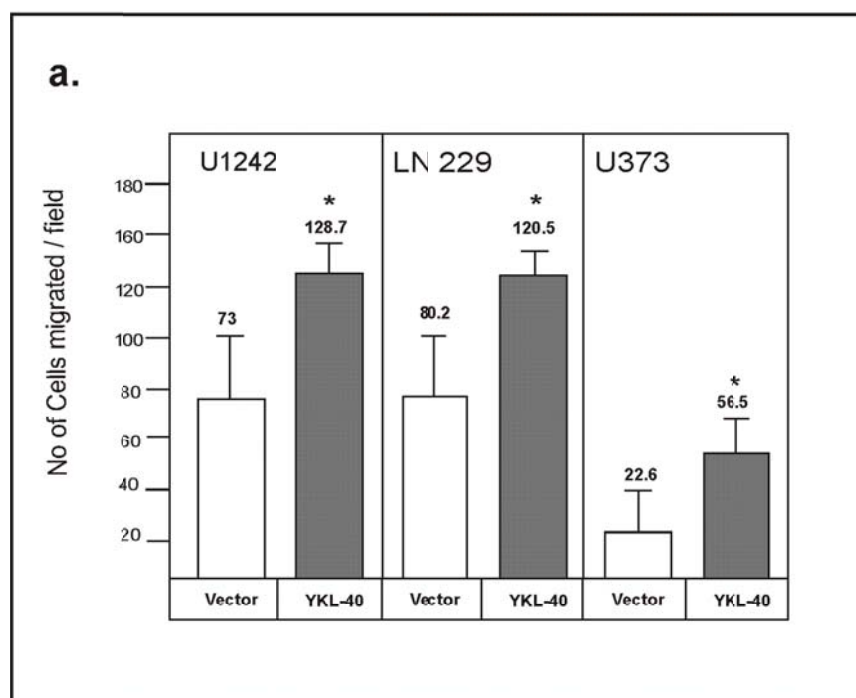
## YKL-40 Expression by qRT-PCR assay



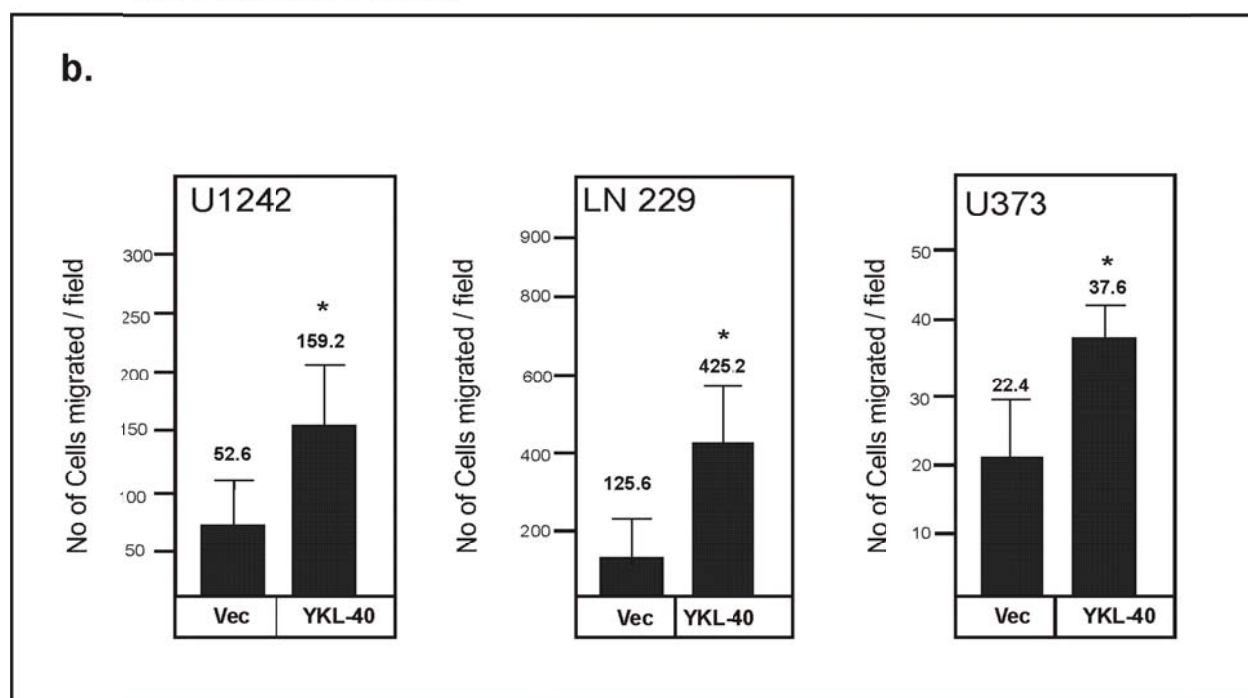
**Fig. 3.3. YKL-40 overexpression enhances glioma cells migration *in vitro*.**

(a.) A wound healing assay for U373, U1242, and LN229 stable cells, which overexpress YKL-40. The number of cells that migrated to the wound area was counted in 8 different fields after 24 hours (b.) Transwell migration assay was done for U1242, LN229, U373 cells as it described in material and methods. The number of migrated cells was counted after 12 hours. Data are expressed as mean  $\pm$  SE. \*p <0.05.

## Wound Healing Assay



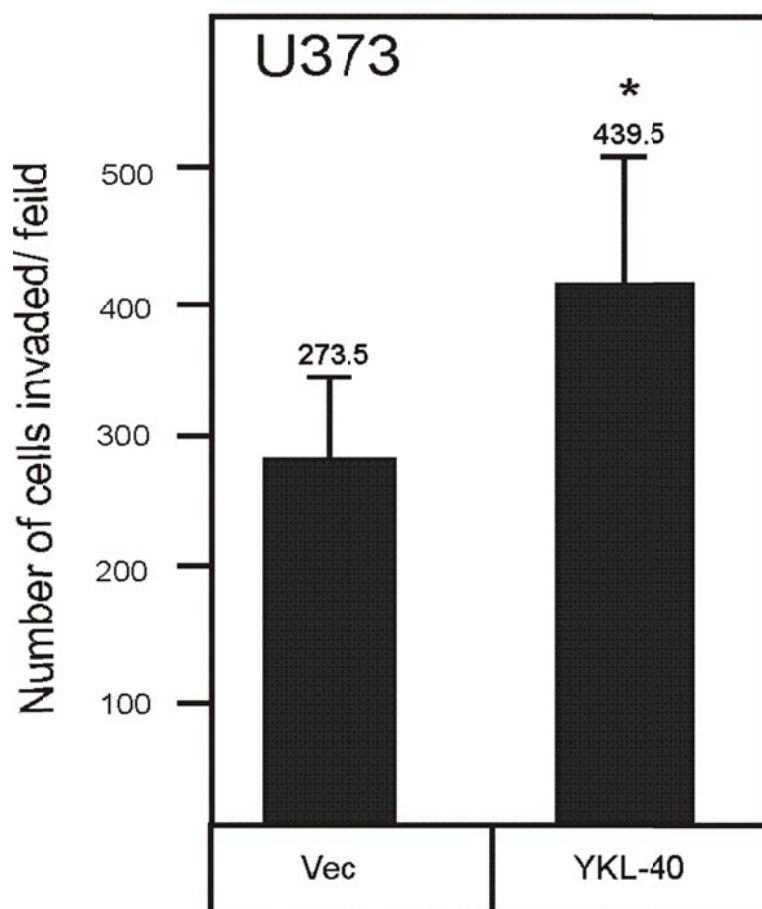
## Transwell migration assay



**Fig. 3.4. YKL-40 overexpression enhances invasion of U373 cells *in vitro*.**

A matrigel invasion assay was done with U373 cells overexpressing YKL-40 using boyden chamber. Cells were allowed to invade through the matrigel for 7 hours. The invaded cells were counted in six random fields per experiment. Invasion is represented as number of cells invaded per field. Data are expressed as mean  $\pm$  SE. \*p <0.05.

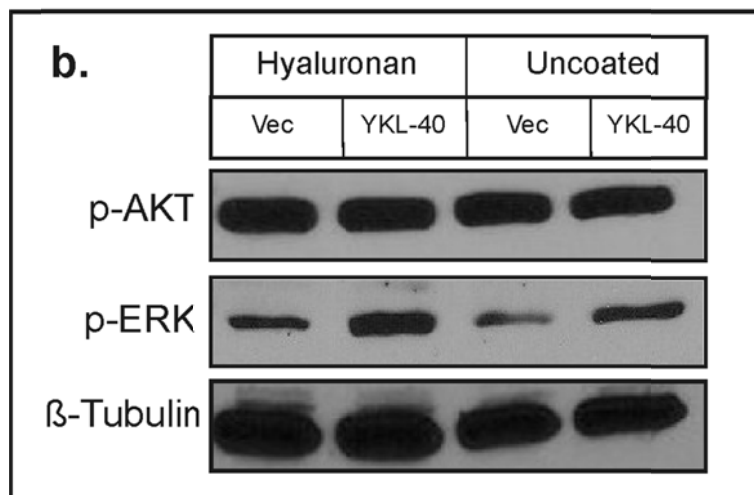
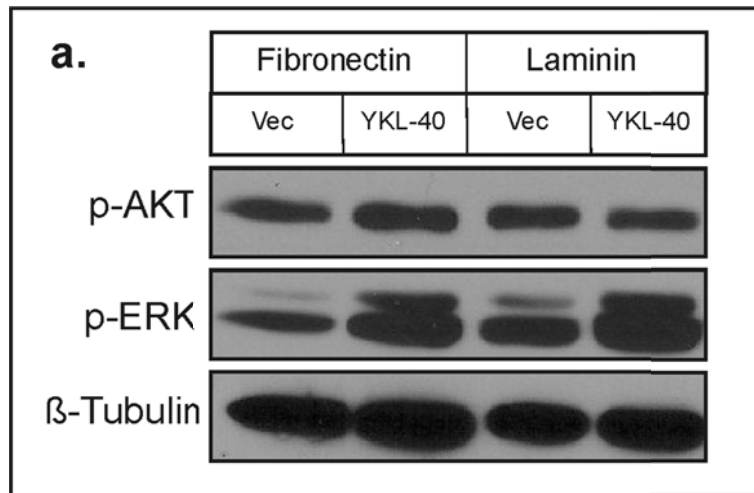
## Matrigel Invasion Assay



**Fig. 3.5. Overexpression of YKL-40 activates MAPK signaling in glioma cells.**

U373 glioma cells were grown on fibronectin, laminin, and hyaluronan coated 6-well plates. Cell lysates were prepared and the levels of pERK and pAKT were analyzed by Western Blotting (Fig. 3.5 a, b).

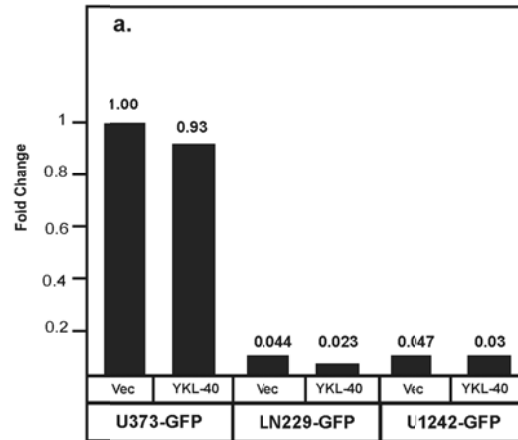




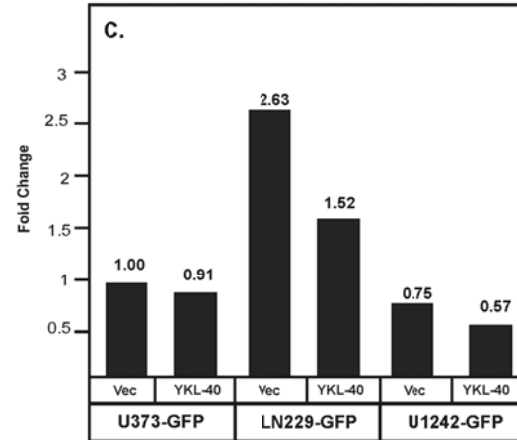
**Fig. 3.6. Syndecan expressions in glioma cell lines.**

RNA was isolated from the indicated stable glioma cell lines. The mRNA expression of syndecan-1, syndecan-2, syndecan-3, and syndecan-4 was analyzed by qRT-PCR, and normalized to GAPDH.

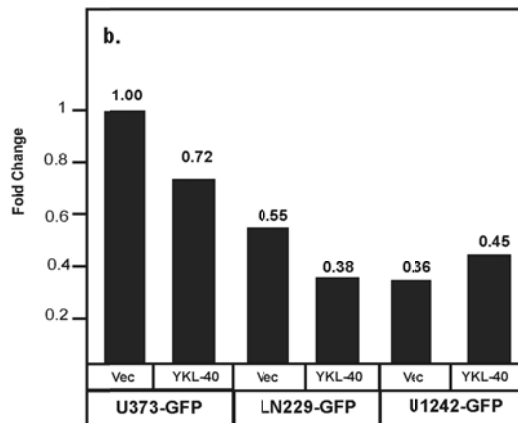
**Syndecan-1 Expression in U373, U1242, and LN 229**



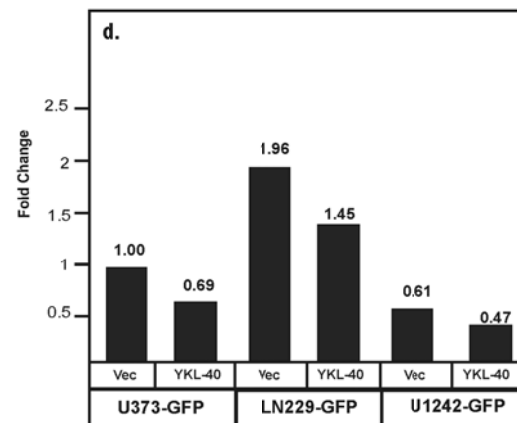
**Syndecan-3 Expression in U373, U1242, and LN 229**



**Syndecan-2 Expression in U373, U1242, and LN 229**



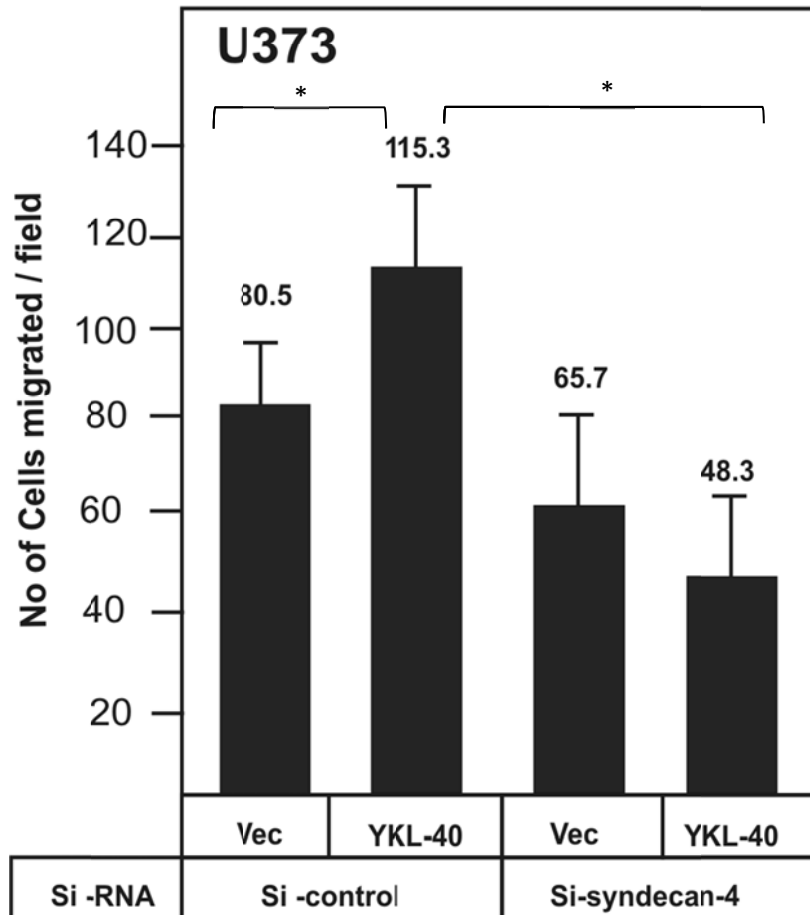
**Syndecan-4 Expression in U373, U1242, and LN 229**



**Fig. 3.7. Syndecan-4 affects YKL-40-induced migration of glioma cells.**

U373-vector and U373-YKL-40 cells were transfected with control siRNA or (Si-control) and siRNA targeting syndecan-4, (si-syndecan-4). Scratches were made 48 hours after the transfection. The migrated cells were counted after 48 hours. Data are expressed as mean  $\pm$  SE. \*p <0.05.

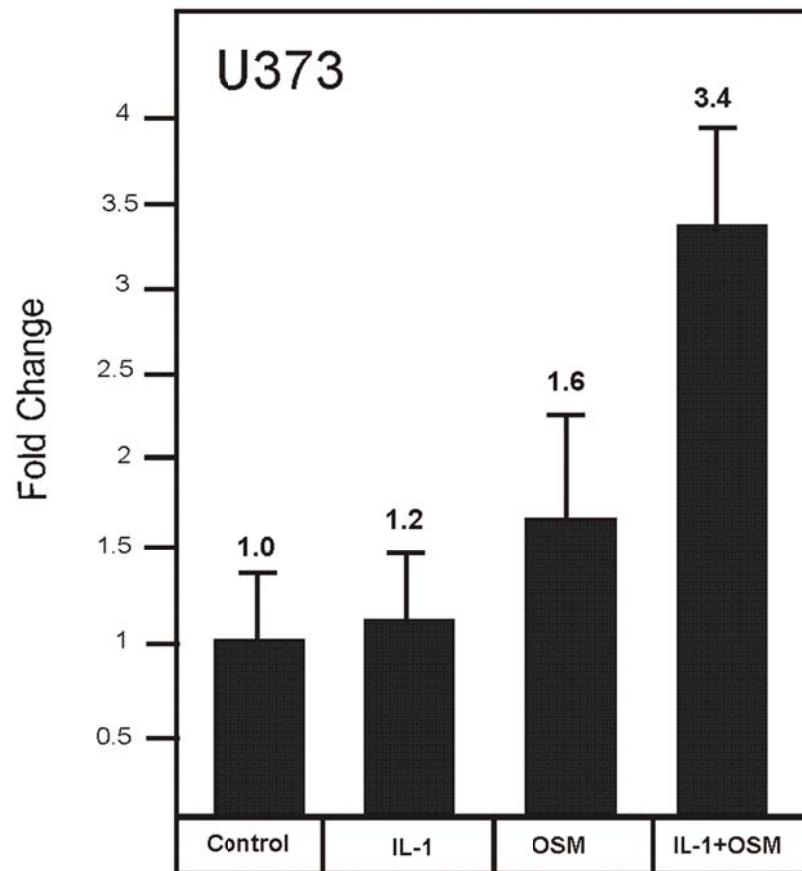
## Wound Healing Assay



**Fig. 3.8. Analysis of YKL-40 expression after cytokines stimulation.**

U373 cells were stimulated with IL-1 or OSM for 24 hours. RNA was isolated, and YKL-40 expression was analyzed by qRT-PCR. Expression was normalized to GAPDH and represented as fold change.

## YKL-40 expression in U373 by qRT-PCR

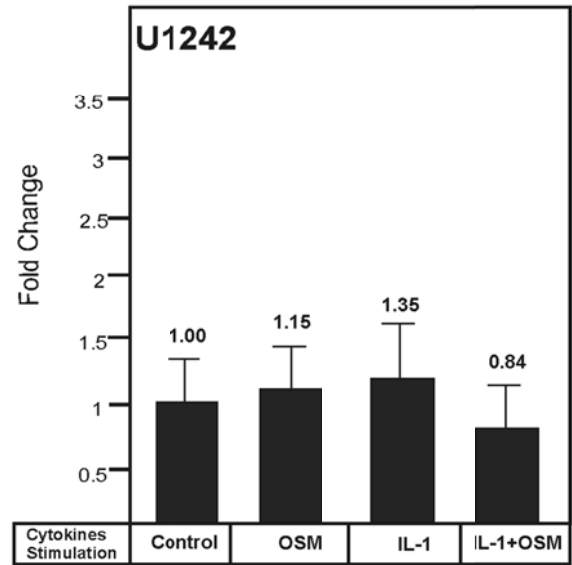
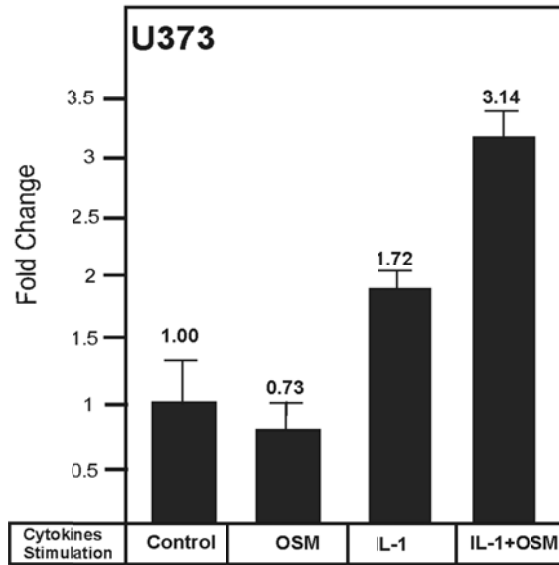


**Fig. 3.9. Analysis of syndecan expressions in U373 and U1242 stable cell lines after cytokines stimulation.**

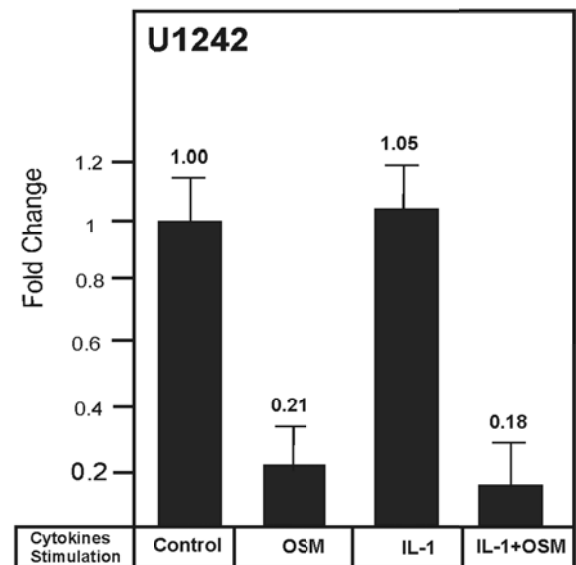
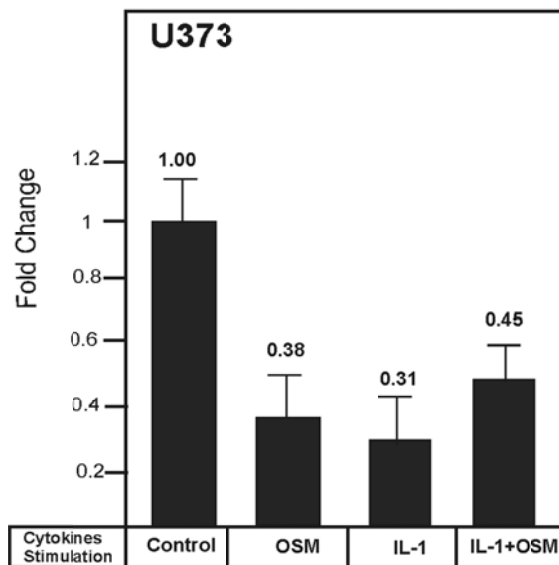
U373 and U1242 cells were stimulated with IL-1 or OSM, for 24 hours. RNA was isolated, the expressions of Syndecan-1 (a.), Syndecan-2 (b.), Syndecan-3 (c.), and Syndecan-4 (d.) were analyzed by qRT-PCR. The expressions were normalized to GAPDH and represented as fold change.



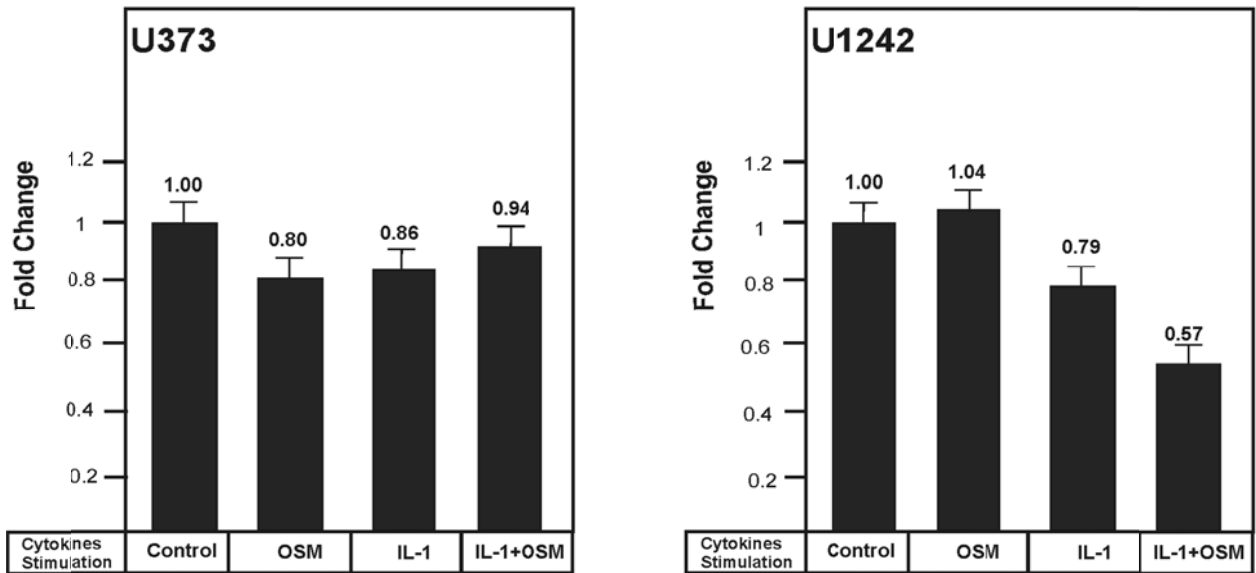
**a. Syndecan-1 Expression in U373 and U1242 with cytokines stimulation**



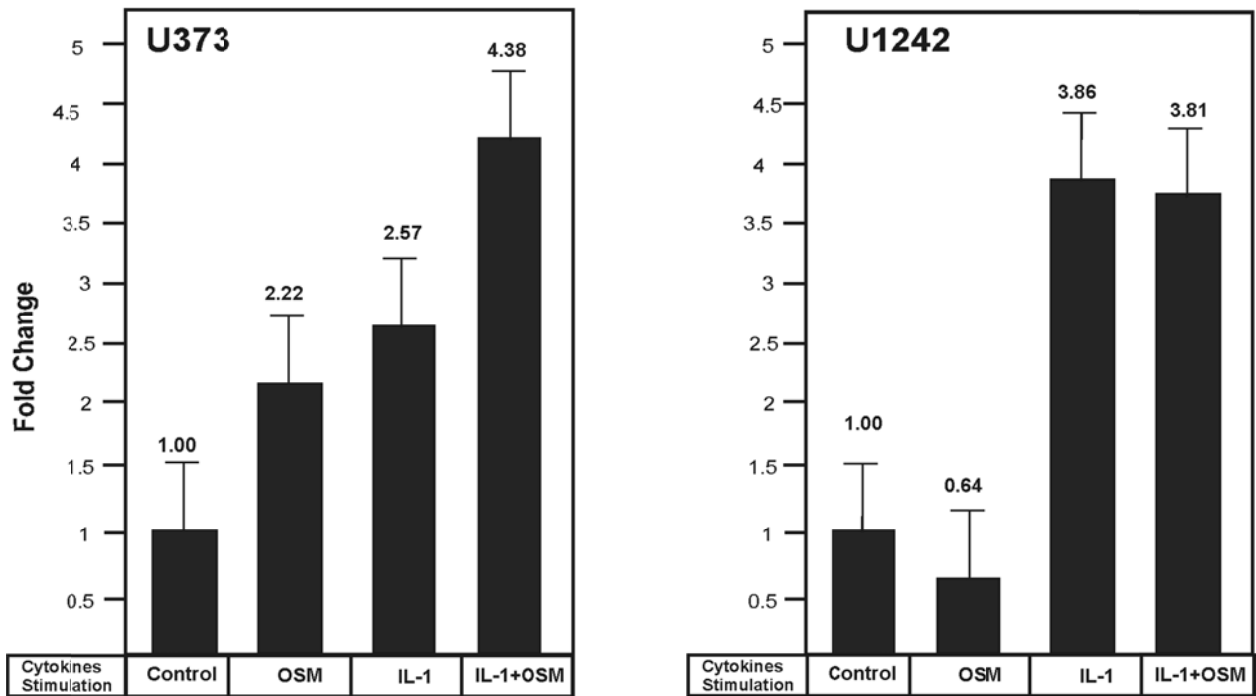
**b. Syndecan-2 Expression in U373 and U1242 with cytokines stimulation**



**c. Syndecan-3 Expression in U373 and U1242 with cytokines stimulation**



**d. Syndecan-4 Expression in U373 and U1242 with cytokines stimulation**



#### 4. Discussion:

YKL-40 is a biomarker of many chronic inflammatory conditions and is expressed in many cancers, including GBM [5]. Increased levels of YKL-40 are specifically associated with the highly invasive capacity of GBM tumors and lower survival rate of GBM patients [4]. However, the exact role of YKL-40 and its mechanism of action in promoting glioma migration and invasion remain unknown. In this study, we investigated the mechanism by which YKL-40 enhances glioma cell migration and invasion. We showed that YKL-40 overexpression in glioma cell significantly increased the migration (Fig. 3.3. a, b) and invasion (Fig. 3.4. a) *in vitro*. This is supported by the fact that YKL-40 overexpression in transformed human astrocytes enhances their migration and invasion [41]. Very recently, our findings were also corroborated in U373 cells, they overexpressed YKL-40 in U373 glioma cell and they examined their invasiveness by using Matrigel assay. They concluded that YKL-40 overexpression increased U373 invasion through matrigel [47].

We have also studied the impact of YKL-40 overexpression on MAPK pathway activation. We have shown that YKL-40 enhanced ERK phosphorylation in U373 cells, which is believed to enhance glioma cell survival, and invasiveness [11-15]. Our data showed that YKL-40 enhanced ERK phosphorylation in U373 cells (Fig. 3.4. b.). We did not observe activation of pAKT in U373 cells overexpressing YKL-40. This is expected since these cells contain mutation of the PTEN gene and as a result exhibit high AKT activity. However, YKL-40 has been shown to activate AKT in other glioma cell lines [34].

Subsequently, we sought to understand the mechanism by which YKL-40 enhances glioma cell migration. A recent study has indicated that YKL-40 induces a cooperative

interaction between  $\alpha v\beta 3$  and syndecan-1 in endothelial cells, which may form a receptor for YKL-40 [24]. However, we found very low expression of syndecan-1 in LN229 and U1242 cells, which led us to examine the expression of other syndecans. We found that syndecan-4 was expressed at relatively high levels by all glioma cells we analyzed (Fig. 3.6.). In order to investigate the possible role of syndecan-4 in glioma cell migration, we down-regulated syndecan-4 in U373 cells. This knockdown significantly decreased YKL-40-enhanced migration *in vitro* (Fig. 3.6). Our data suggest that syndecan-4 is required for glioma cell migration and are supported by the finding that syndecan-4 is an important regulator of focal adhesion formation, cell morphology, and migration in chinese hamster ovary (CHO-K1) [31].

YKL-40 is overexpressed in many chronic inflammatory conditions, such as rheumatoid arthritis, osteoarthritis and cancers, including GBM, melanoma, and myeloid leukemia. It is believed that inflammatory mediators, such as pro-inflammatory cytokines of the IL-1 and IL-6 families, regulate YKL-40 expression in these inflammatory conditions. The inflammatory cytokines, IL-1, IL-6, and OSM are all expressed by malignant gliomas [43]. IL-1 and IL-6 are known to play a major role in GBM invasiveness [44, 45]. Moreover, OSM have also been detected in many brain tumors, including GBM and astroglomas. Nevertheless, it is still not well understood how inflammation contributes to brain tumor progression, growth, and survival. We stimulated glioma cells with IL-1 and OSM. Our data suggest that IL-1 and OSM can synergistically increase YKL-40 expression in U373 cells (Fig. 3.7). Interestingly, both of these cytokines also induce expression of syndecan-4 in these cells (Fig. 3.8). Therefore, in GBM cells, YKL-40 may function through syndecan-4 to activate migration of glioma cells. In this thesis we proposed the mechanism by which YKL-

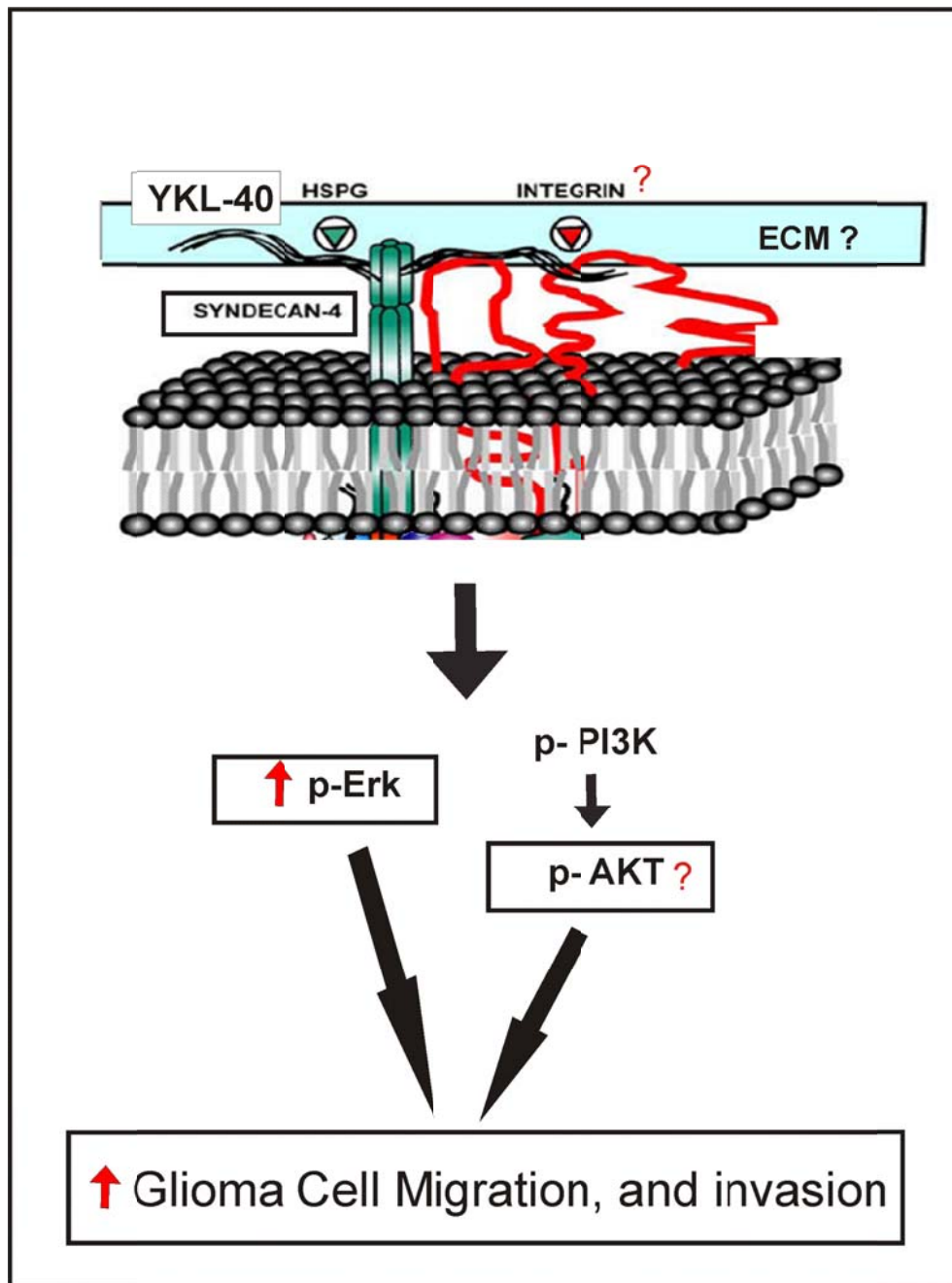
40 promotes glioma cell migration (fig. 3.10). YKL-40 binds to the ectodomain of syndecan-4 by its heparin sulfate binding domain, and activates downstream signaling, including, ERK, and maybe AKT that lead to enhance cell migration and invasion. A cooperative interaction between syndecan-4 and integrin is suggested in this model.

However, it remains to be resolved whether syndecan-4 is required for YKL-40-induced downstream signaling in GBM. This thesis demonstrates that YKL-40 promotes glioma cell migration and invasion *in vitro*. More importantly it implies that syndecan-4 may play a role in YKL-40-induced migration and invasion. Moreover, it demonstrates that both YKL-40 and syndecan-4 expression is promoted by inflammatory cytokines in glioma cells.

In the future, it will be important to investigate the role of YKL-40 in enhancing migration and invasion of glioma cells *in vivo*. For this purpose, we have recently generated U373-RFP tagged cells which are also expressing luciferase, and growth and invasion of these cells can be easily monitored *in vivo*.

**Fig. 3.10. Proposed Mechanism by which YKL-40 Promotes Glioma Cell Migration.**

Inflammatory cytokines enhance expression of syndecan-4 and YKL-40 by glioma cells. YKL-40 subsequently binds to syndecan-4 by its heparin sulfate binding domain, and activates downstream signaling, including, ERK, and AKT that enhance cell migration and invasion. A cooperative interaction between syndecan-4 and integrin is suggested in this model.



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## **PUBLICATIONS**

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