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YKL-40, a secreted glycoprotein, promotes tumor angiogenesis

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Abstract

Tumor angiogenesis is of paramount importance in solid tumor development. Elevated serum levels of YKL-40, a secreted heparin-binding glycoprotein have been associated with a worse prognosis from a variety of advanced human cancers. Yet the role of YKL-40 activity in these cancers is still missing. Here, we have shown that ectopic expression of YKL-40 in MDA-MB-231 breast cancer cells and HCT-116 colon cancer cells led to larger tumor formation with an extensive angiogenic phenotype than did control cancer cells in mice. Affinity purified recombinant YKL-40 protein promoted vascular endothelial cell angiogenesis *in vitro*, the effects similar to the activities observed using MDA-MB-231 and HCT-116 cell conditioned medium after transfection with YKL-40. Further, YKL-40 was found to induce the coordination of membrane-bound receptor syndecan-1 and integrin $\alpha_v\beta_3$ and activate an intracellular signaling cascade including focal adhesion kinase and MAP kinase Erk1/2 in endothelial cells. Also, blockade of YKL-40 using siRNA gene knockdown suppressed tumor angiogenesis *in vitro* and *in vivo*. Immunohistochemical analysis of human breast cancer revealed a correlation between YKL-40 expression and blood vessel density. These findings provide novel insights into angiogenic activities and molecular mechanisms of YKL-40 in cancer development.

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Introduction

The development of human cancers involves multiple pathological events through which cancerous cells gain the ability to overcome adverse stress imposed by adjacent normal tissue. During this process, the intrinsic genomic instability of cancerous cells induces a number of genetic and epigenetic alterations that enable them to proliferate, survive, and invade. Once they have expanded to a critical level, cancerous cells find a way to promote new vasculature development, a process known as tumor angiogenesis in order to progress and metastasize (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2000). Tumor angiogenesis, an integral part of solid tumor development, provides nutrients and oxygen in a hypoxic microenvironment formed in the centers of growing tumors and facilitates tumor cell proliferation by enhancing oxygenation and the removal of metabolic wastes that would normally induce necrosis. A number of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have been demonstrated to play critical roles in the development of tumor vasculature (Fahmy et al., 2003; Kim et al., 1993; Yancopoulos et al., 2000). However, much more remains to be understood about the molecular nature of unidentified factors that are also believed to have the ability to promote angiogenesis in the development of a variety of human cancers.

YKL-40 (also known as human cartilage-glycoprotein 39 or Chitinase 3-like 1) is a 40-kDa secreted glycoprotein that was discovered as a heparin-binding protein in the conditioned medium of human synoviocytes, chondrocytes, and MG-63 osteosarcoma cell line (Hakala et al., 1993; Johansen et al., 1992; Shackelton et al., 1995). Although YKL-40 falls into the chitinase gene family that can bind oligosaccharide, it does not have chitinase activity due to the substitution of an essential glutamic acid with leucine in the chitinase-3-like catalytic domain (Fusetti et al., 2003; Renkema et al., 1998). Therefore, its physiological or pathological function remains enigmatic. Given the evidence that serum levels of YKL-40 were elevated in patients with multiple chronic inflammatory diseases (Johansen et al., 2000; Sharif et al., 2006), its function is speculated to be associated with tissue remodeling.

Over the past decade, considerable attention has been focused on a potential role of YKL-40 in the development of a variety of human cancers. Gene microarray analyses and serial analysis of gene expression (SAGE) database showed significantly higher expression levels of YKL-40 in carcinoma tissues from ovary, brain, and breast than those seen in adjacent normal tissues (Lal et al., 1999; Lau et al., 2006). A multitude of clinical studies have revealed that serum levels of YKL-40 were elevated in patients with a series of carcinomas (Cintin et al., 2002; Jensen et al., 2003; Johansen et al., 2004). For instance, clinical data studying brain tumors indicate that glioblastomas which exhibit striking angiogenesis, contain considerably elevated YKL-40 levels both in tumor tissue and in the blood (Hormigo et al., 2006; Nigro et al., 2005). Taken together, these data suggest that serum levels of YKL-40 may serve as a cancer biomarker. Nonetheless, little is known regarding YKL-40 activity in human cancers.

In the current study, we tested the hypothesis that YKL-40 acts as an angiogenic factor to promote tumor growth and angiogenesis. We found that YKL-40 ectopically expressed by breast and colon cancer cells induces tumor angiogenesis and tumor development. The

molecular mechanisms underlying YKL-40-induced angiogenesis involve the co-activation of membrane receptor syndecan-1 (S1) and integrin $\alpha_v\beta_3$ and downstream signaling effectors focal adhesion kinase (FAK) and MAP Kinase. Furthermore, elevated YKL-40 expression in human breast cancer is associated with blood vessel formation. These findings may shed light on the angiogenic signature of YKL-40 in the pathogenesis of a wide range of human cancers.

Materials and Methods

Generation of cancer cell lines stably expressing YKL-40

Full length of YKL-40 cDNA was subcloned into a retroviral pCMV-neo-vector. 293T retroviral packaging cells were transfected with YKL-40 or vector control DNA in the presence of pCL 10A1 vector using Fugene 6 as the delivery vehicle. Forty-eight hours after transfection, the supernatant was harvested and filtered through 0.45-μm pore size filter and the virus-containing medium was used to infect to tumor cells. Selection with 800 μg/ml of G418 was started 48 hr after infection and the drug-resistant cell populations were used for subsequent studies.

S1 and YKL-40 Gene knockdown

DNA oligos (19 bp) specifically targeting ectodomain of S1 (Beauvais et al., 2004), N-terminal (siRNA 1) or C-terminal (siRNA 2) region of YKL-40, were selected and then templates (64 oligo nucleotides) containing these oligos were subcloned into a retroviral pSUPER-puro-vector. Retroviral media were generated as described above and the resulting medium was infected to HMVEC or U87 cells to establish the lines stably silencing S1 or YKL-40 gene, respectively.

Generation of recombinant YKL-40

Full-length human YKL-40 cDNA with a His-tag was subcloned into pFastBac1 vector (Life Technologies, MD). Following transformation and amplification in DH10Bac E. coli, bacmid DNA containing YKL-40 was transfected into Sf9 insect cells by using CellFECTIN reagent (Invitrogen, CA) and baculoviral medium was produced. A Ni-NTA column was used to purify recombinant YKL-40 according to manufacture's instruction (Invitrogen) and pure YKL-40 was finally produced through a PD-10 desalting column (Amersham Biosciences).

Heparin-Sepharose affinity binding assay

Recombinant YKL-40 (1 μg) was loaded into a heparin-Sepharose affinity column (Amerham Biosciences) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 25 mM NaCl. After washing with 5 volume of the loading buffer, the column was eluted with 20 mM sodium phosphate buffer containing 1 M NaCl. All collected samples were concentrated with centrifugal concentrators prior to immunoblotting.

Migration assays

HMVEC (2×10^5) were pre-incubated with serum-free medium for 12 hrs and transferred onto transwells (24-well plates) pre-coated with collagen IV (100 μg/ml). The lower chamber of transwells included YKL-40 (100 ng/ml), ySP (100 ng/ml) or VEGF (10 ng/ml). After 4 hours of incubation, cells in the top chamber of the transwells membrane were removed using Q-tip. Average cell numbers were calculated from five different fields in each sample. In some experiments, a neutralizing anti-VEGF monoclonal antibody (Sigma Co.) was used.

Tube formation assays

HMVEC (0.1×10^5cells) were transferred onto 96-well Matrigel in the presence of YKL-40 (100 ng/ml), ySP (100 ng/ml) or VEGF (10 ng/ml) (Becton Dickson Lab, MA). After 12 hours of incubation, tube-forming structures were analyzed.

Immunoprecipitation and Western blot

Cell lysates were prepared as described before (Yan et al., 2008). The samples were then incubated with anti-integrin β_3 or β_5 antibody at 4°C for overnight followed by incubation with protein A sepharose beads at 4°C for 2 hr. The immunocomplex was extensively washed and the samples were subjected to immunoblots. The primary antibodies against S1, Erk 1/2, pErk 1/2, FAK (Santa Cruz Inc, CA), FAK pY^{397}, FAK pY^{861} (Biosource Inc.), integrin β_3 and β_5 (Chemicon International, CA), and rAY were used to examine protein expressions. Specific signals were detected using an ECL kit.

Tumor xenografts in mice

All animal experiments were performed under Institutional Animal Care and Use Committee approval of University of Massachusetts. SCID/Beige mice were subcutaneously injected with HCT-116 (4.5×10^6), MDA-MB-231 (1.5×10^6) or U87 (2×10^6) cells in 0.2 ml of Hank's balance buffer without calcium and magnesium. Tumor growth from these injected cells was monitored weekly for 6 weeks before the animals were sacrificed. The tumors were measured and calculated as follows: volume = length x $\text{width}^2 \times 0.52$.

Immunohistochemistry

The study of breast cancer samples was approved by University of Massachusetts Institutional Review Board. Frozen tumor tissues from animals were cut to 6 μm thickness and processed for the staining of CD31. In brief, the samples were blocked with 3% H_2O_2 to block endogenous peroxidase activity for 30 min followed by incubation with blocking buffer containing 10% goat serum for 1 hr. Then, rat anti-CD31 monoclonal antibody (1: 500, BD Biosciences) was incubated at room temperature for 2 hr and goat anti-rat secondary antibody (1: 100) conjugated with HRP were added. Finally, DAB substrate (Dako Inc) was introduced for several minutes and after washing, methyl green will be used for counterstaining. Paraffin-embedded human cancer specimens were processed similarly as above for staining of YKL-40 (1:400) and CD34 (1:200) (Dako Inc). YKL-40 staining was evaluated as combined scores of percent and intensity of positive staining cells as following 1) percent: no staining is 0 points; <10% of cells stained is 1 point; 11–50% of

cells stained is 2 points; and >50% of cells stained is 3 points; 2) intensity: no staining is 0 points, weak staining is 1 point, moderate staining is 2 points and strong staining is 3 points. Thus, the valid range of scores was 0 to 6. Cancer cases were classified into three groups: negative/low (0–2 points), medium (3–4 points), and high (5–6 points) of YKL-40 staining.

Results

YKL-40 promotes tumor growth and blood vessel formation

To explore a possible role for YKL-40 in cancer development, we created a full-length YKL-40 cDNA from the osteosarcoma line MG-63 (Supplemental Figure 1). We began to evaluate endogenous expression levels of YKL-40 in a number of cell lines, including immortalized human microvascular endothelial cells (HMVEC), 293T cells, breast cancer cells MCF-7, T47D and MDA-MB-231, and colon cancer cells HCT-116. In the pre-screening of those lines by both PCR and Western blot analysis, YKL-40 expression was found to be restricted to MG-63 cells (Figure 1A). The absence of endogenous YKL-40 in the other lines provided a null background against which to examine the biological activities of YKL-40. To monitor its functional behavior in cancer cells, colon cancer cell line HCT-116 and breast cancer cell line MDA-MB-231 were engineered to ectopically express YKL-40 through a retroviral infection system. In this way, we generated populations of cancer cells that stably express ectopic YKL-40. As demonstrated by Western blot analysis using cell cultured medium (Figure 1B), YKL-40 was released from these cell lines and the levels of YKL-40 in cell lysates were below the limit of detection (data not shown), supporting that YKL-40 is a secreted protein.

To determine whether YKL-40 can enhance proliferation of HCT-116 and MDA-MB-231 cells once they acquired the expression of YKL-40, we monitored ^{3}H-thymidine incorporation into DNA of dividing cells. Unexpectedly, proliferation was not significantly altered by the introduction of YKL-40 gene into either cell type (Supplemental Figure 2). The inability to increase cell division was also observed in the testing of these parental cancer cell lines treated with recombinant protein YKL-40 (data not shown). We next wanted to determine if YKL-40 could influence tumor growth and development *in vivo*. HCT-116 and MDA-MB-231 cells over-expressing YKL-40 were subcutaneously injected into SCID/Beige mice and tumor growth was monitored weekly for up to six weeks (Figure 2A). No palpable tumors were discernable until week two. From the third week, both YKL-40-expressing cancer cell lines rapidly developed palpable tumors relative to control tumor cells. By week six, YKL-40-expressing MDA-MB-231 and HCT-116 cells gave rise to 4 and 8-fold larger tumors than ones formed from their corresponding control cells. Interestingly, the tumors developed from YKL-40-expressing cells displayed an extensive arbor of ramified blood vessels on the tumor surface as compared to relatively few vessels observed on the control tumors (Figure 2B), suggesting that YKL-40 may induce tumor angiogenesis. To confirm this angiogenesis, we stained tumors with CD31, a marker of vascular endothelial cells. Consistent with the augmented blood vessels observed in YKL-40-expressing tumors, immunohistochemical analysis showed that the levels of vasculature formed in YKL-40-expressing HCT-116 and MDA-MB-231 tumors were 1.8–2-fold greater than those in control tumors (Figure 2C and 2D, Supplemental Figure 3). These

results indicate that YKL-40 does not directly influence tumor cell proliferation. Rather, it appears to have the ability to promote tumor angiogenesis by which YKL-40 enhances tumor growth.

Recombinant YKL-40 protein stimulates endothelial cell angiogenesis

To monitor whether YKL-40 exerts angiogenesis through direct activation on vascular endothelial cells, we first employed a standard baculoviral expression system to express and isolate recombinant protein YKL-40 (Supplemental Figure 4). YKL-40 tagged with 6xhis was specifically detectable in Western blot analysis using an anti-YKL-40 (named as rAY, generated from our lab) and an anti-his polyclonal antibody. The affinity-purified recombinant protein YKL-40 was then engaged to test its activities in endothelial cell angiogenesis. Unlike VEGF, recombinant YKL-40 had a modest effect on proliferation of HMVEC (∼25% increase in cell proliferation, data not shown). However, YKL-40 resembled VEGF angiogenic properties, one of the most potent angiogenic factors, by inducing endothelial cell migration and tube formation (Figure 3A & 3B). The migration and tube formation induced by recombinant YKL-40 were approximately three to four times greater than were seen in control cells, similar to VEGF. A short peptide of YKL-40 (ySP) that contains 20 amino acids of YKL-40 failed to induce angiogenesis, serving as an isotype control. These data support the hypothesis that YKL-40 has the ability to stimulate angiogenic responses in endothelial cells.

In an attempt to determine if YKL-40 derived from cancer cells promotes endothelial cell angiogenesis via a paracrine manner in tumor *in vivo*, we utilized a cell co-culture system. Conditioned media derived from both MDA-MB-231 and HCT-116 cells ectopically expressing YKL-40 or vector were introduced to HMVEC and then endothelial cell angiogenic activity including cell migration and tube formation was analyzed. As shown in Figure 3C, the media from both cancer cells expressing ectopic YKL-40 displayed the same effects as recombinant YKL-40 on the induction of endothelial cell angiogenesis (Figure 3A & 3B). Moreover, an inclusion of the anti-VEGF antibody failed to protect YKL-40 activities (Figure 3C). Collectively, all the data demonstrate the novel angiogenic activity for YKL-40, independent of VEGF.

YKL-40 induces coupling of S1 with integrin $\alpha_v\beta_3$.

To test if YKL-40 can bind to heparin, we employed a heparin-Sepharose affinity binding assay and we found that YKL-40 directly bound to the affinity column (Figure 4A), consistent with previous reports (Fusetti et al., 2003; Shackelton et al., 1995). We next questioned whether YKL-40 acts on endothelial cells through activating the transmembrane receptor S1, a proteoglycan that is the major source of cell surface heparan sulfate (HS). HS is known to function as a key mediator connecting membrane-associated receptors with extracellular heparin-binding proteins such as extracellular matrix protein (ECM, *e.g.* vitronectin) and angiogenic factors (*e.g.* bFGF and VEGF) (Beauvais et al., 2004; Bernfield et al., 1999). Moreover, there is compelling evidence demonstrating that endowed with the HS chain on its ectodomain, S1 acts as a matrix co-receptor with adjacent membrane-bound receptors such as integrins to mediate cell adhesion and/or spreading (McQuade et al., 2006). In an attempt to test whether YKL-40 has the ability to induce the cooperation of S1

with co-membrane receptor integrins, we investigated the physical interaction between S1 and integrin $\alpha_v\beta_3$ or $\alpha_v\beta_5$ upon YKL-40 stimulation using co-immunoprecipitation and Western blot analysis. Protein levels of both S1 and integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in HMVEC were detectable (data not shown). Exposure of HMVEC to YKL-40 resulted in a dramatic increase in the association of S1 with integrin β_3 but this event did not occur in the treatment with ySP as a control (Figure 4B). As expected, this interaction was prevented by pre-treatment with heparitinase (Hep) and chondroitinase ABC (Chon) that function to remove cell surface glycosaminoglycans. S1 did not collaborate with integrin β_5 when an anti-integrin β_5 antibody replaced β_3 antibody in the co-immunoprecipitation, validating the specificity of receptor coordination between integrin $\alpha_v\beta_3$ and S1 in response to YKL-40. To further define the functional role of this co-receptor interaction in YKL-40-induced angiogenesis, we examined cell adhesion and spreading that are directly regulated by the coupling of syndecans with integrins (Beauvais et al., 2004). As shown in Figure 4C, HMVEC plated in the absence of serum had greater potential to adhere to and spread on wells coated with YKL-40 than on ySP-coated wells. However, incubation with either a heparitinase/chondroitinase ABC or neutralizing anti-integrin $\alpha_v\beta_3$ antibody (LM609) abrogated YKL-40 effects on cell spreading; whereas an anti-integrin $\alpha_v\beta_5$ antibody (P1F6) failed to block this activity. Again, the analysis on tube formation displayed the same profile as the one tested in cell spreading (Figure 4D), highlighting the essential role of the collaboration of S1 with integrin $\alpha_v\beta_3$ in YKL-induced endothelial cell angiogenesis.

We then sought to test whether only S1, but not other family members of syndecans, mediates YKL-40-induced angiogenesis. The small-interfering RNA (siRNA) approach designed to specifically target the ectodomain of S1 (S1 siRNA) (Beauvais et al., 2004) was engaged in HMVEC. Transfection with S1 siRNA efficiently silenced S1 expression in the cells (Figure 5A). The suppression of S1 gene resulted in a significant decrease in S1 coupling with integrin $\alpha_v\beta_3$ compared to their receptor association seen in control cells (Figure 5B). As with the deficiency in the membrane-associated receptor collaboration in siRNA cells, functional analysis with tube formation showed that silencing of the S1 gene abolished the effects of YKL-40 on endothelial cell activation (Figure 5C), confirming that S1 plays a key role in mediating endothelial cell angiogenesis induced by YKL-40.

Activities of focal adhesion kinase and MAP kinase are required for YKL-40-induced angiogenesis

As one key factor mediating integrin signaling, FAK is shown to transmit signaling to downstream effectors in cells to evoke angiogenic responses (Hood et al., 2003). To test whether FAK is activated by YKL-40, we stimulated HMVEC with YKL-40 and cell lysates were measured for the expression levels of phosphorylated FAK and subsequent downstream mediators MAP Kinase Erk1/2. Consistent with previous findings that FAK pY^{861}, but not FAK pY^{397}, mediates angiogenic responses, (Yan et al., 2008), FAK pY^{861} was found to act as a key mediator to trigger angiogenic signaling cascades in endothelial cells as the activated form of FAK pY^{861} was increased by YKL-40 in as early as 5–10 min (Figure 5D). As well, downstream effectors pErk1/2 were similarly induced. Furthermore, this signaling initiated through the activation of integrin $\alpha_v\beta_3$ other than $\alpha_v\beta_5$ was validated by the study using anti-integrin neutralizing antibodies (Figure 5D). To identify the function

role of Erk1/2 in YKL-40-induced angiogenesis, we examined the effects of YKL-40 on tube formation in the presence of PD98059, an inhibitor of Erk1/2 (Figure 5E). Introduction of PD98059 abrogated tube formation enhanced by YKL-40, supporting Erk1/2 as an important downstream effector.

siRNA knockdown of YKL-40 inhibits tumor angiogenesis

In the prescreening of several cancer lines, we found a few cell lines expressing endogenous YKL-40 such as osteoblastoma MG-63 cells and glioblastoma U87 cells. Given that MG-63 failed to develop tumor in mice as reported previously (Naruse et al., 2006), we utilized U87 cells for the study of YKL-40 gene inhibition. As demonstrated in Figure 6A, siRNA1 and siRNA2 knockdown of YKL-40 reduced its expression by 80% compared with the level expressed by control cells. To test the effects of YKL-40 gene knockdown on endothelial cell angiogenesis *in vitro*, conditioned medium of U87 cells expressing vector, YKL-40 siRNA1 or siRNA2 was transferred to HMVEC and tube formation was subsequently analyzed (Figure 6B). Consistent with the suppression of YKL-40 gene, the cell conditioned medium containing either YKL-40 siRNA1 or siRNA2 inhibited tube formation by more than 50% in comparison with the effect seen by the control medium (the same effects as VEGF inhibition, Supplemental Figure 5). The inhibitory activity of YKL-40 siRNA1 and siRNA2 was also observed in the testing of endothelial cell migration (Figure 6B). These results indicate that U87-secreted YKL-40 similar to the affinity-purified recombinant protein YKL-40 described earlier is capable of inducing endothelial cell angiogenesis.

Next, we questioned whether the YKL-40 gene knockdown can influence tumor growth and angiogenesis *in vivo*. To test this, we analyzed tumor development by injection of U87 cells expressing vector, YKL-40 siRNA1, or siRNA2 into SCID/Beige mice (Figure 6C). As expected, both siRNA1 and siRNA2 knockdown of YKL-40 in U87 cells notably suppressed tumor growth as tumor volume was reduced to approximately 30% of that observed in the control animals on week 6 (Figure 6C). Moreover, immunohistochemical analysis showed that the vessel density of siRNA1 and siRNA2 tumors was around 56% of vasculature that was found in the control tumors (Figure 6D). All the results were consistent with *in vivo* data from earlier xenograft models in which tumor cells were enforced to express ectopic YKL-40, validating the angiogenic activity of YKL-40 in tumor development *in vivo*.

Expression levels of YKL-40 are associated with blood vessel density in breast cancer

Finally, to exploit the relationship between expression levels of YKL-40 and vasculature in human breast cancer, we performed immunochemistry to stain YKL-40 and CD34, a marker of human vascular endothelial cells in thirty-eight cases of infiltrating ductal carcinoma. Nine of thirty-eight cancers (23.7%) contained high expression levels of YKL-40 and nine cases (23.7%) displayed medium levels of YKL-40; whereas twenty cases (52.6%) were negative or low (Figure 7A). Accordingly, these three groups with different expression levels of YKL-40 were found to contain different degrees of vascularization in the tumor sections (Figure 7A). The blood vessel density of the two groups that demonstrated high and medium levels of YKL-40 were 2.1 and 1.6-fold greater than the group expressing low YKL-40, respectively (Figure 7B). Furthermore, correlation analysis showed that YKL-40 expression levels directly correlated with CD34 density (Figure 7C). Taken together, all the

lines of evidence identifying the angiogenic signature of YKL-40 from both *in vivo* and *in vitro* studies have strongly supported our hypothesis that YKL-40 acts as an angiogenic factor to promote tumor growth and development.

Discussion

Our studies have used a multidisciplinary approach to demonstrate a novel angiogenic signature for YKL-40 and identify mechanisms by which YKL-40 promotes tumor growth and development. These findings reveal a fundamental mechanism for the phenomena reported previously that elevated serum levels of YKL-40 are associated with poorer prognosis and shorter disease-free survival in patients with a broad type of cancers, including breast (Jensen et al., 2003), colorectal (Cintin et al., 2002), ovarian (Hogdall et al., 2003), prostate (Brasso et al., 2006), small cell lung cancer (Johansen et al., 2004), malignant melanoma (Schmidt et al., 2006); glioma (Pelloski et al., 2005), and acute myeloid leukemia (Bergmann et al., 2005). In addition, SAGE database also shows that YKL-40 transcript is over-expressed in a number of cancer tissues compared to adjacent normal controls such as mammary, ovary, and brain tissue (http://cgap.nci.nih.gov/). Immunohistochemical analysis of breast cancer tissue in the current study which displays a correlation of YKL-40 expression levels with blood vessel density has provided direct evidence indicating the pathological role of YKL-40 in the development of human cancers. It would be quite interesting to know whether there is an intimate association between tissue expression levels and serum concentrations of YKL-40 in the same cancer patients. Should YKL-40 expression in cancer tissue be the determinant of its concentrations in the blood, the testing of serum levels of YKL-40 as a cancer biomarker might have immense value in the cancer diagnosis and prognosis.

Recent attention has been focused on the relationship between YKL-40 expression and clinical outcome in breast cancer although the data are inconsistent as some evidence shows the correlation between increased expression levels of YKL-40 and decreased disease-free survival but the other does not (Kim et al., 2007; Roslind et al., 2007). An epidemiological study with a substantial sample size appears to be necessary in order to elucidate the association of expression levels of YKL-40 with metastasis, severity or outcome of breast cancer. Our current results presented here, however, have provided a valuable insight into molecular mechanisms by which YKL-40 stimulates tumor development.

The first 22 amino acid residues at N-terminal sequence of YKL-40 constitute the signal peptide that ensures the secretion of mature protein YKL-40 (Johansen et al., 1992; Nyirkos and Golds, 1990). Because YKL-40 does not have an RGD domain, it can not directly bind to integrins on cell membrane. However, YKL-40, a heparin-binding protein (Fusetti et al., 2003; Hu et al., 1996; Shackelton et al., 1995), contains putative arginine-lysine (RK) rich domain(s), the motif that has been shown to be important for heparin binding in other angiogenic factors such as aFGF, bFGF and VEGF (Albini et al., 1996; Higashiyama et al., 1991; Maglione et al., 1993). Like heparin-binding protein vitronectin (VN) and bFGF whose activities largely depend on the presence of HS (Beauvais et al., 2004; Bernfield et al., 1999), YKL-40 was found to induce the collaboration of membrane receptor S1 with integrin $\alpha_v\beta_3$ through binding to HS and then trigger intracellular signaling cascades.

Several lines of evidence have demonstrated that syndecans act as matrix co-receptors with integrins to mediate cell binding and signaling on ligands that contain heparan sulfate (Beauvais et al., 2004; Beauvais and Rapraeger, 2003; McQuade et al., 2006). Our results, as with these findings, strongly suggest that S1 in collaboration with integrins is a critical regulator of cell motility in vascular endothelial cells whose functions are dependent on the activity of integrin $\alpha_v\beta_3$ and/or $\alpha_v\beta_5$. It is also quite possible that there are receptors specific for YKL-40 binding present on endothelial cells which are currently unknown. But, the heparin-binding property of YKL-40 may also be indispensable in the affinity binding and activation of these specific receptors if they also contain HS chains on the ectodomains. The current evidence identifying signaling pathways through the activation of membrane receptors further strengthens a paradigm that the cross-talk between adjacent membrane-anchored receptors plays a key role in transmitting “outside-in” signaling to the cells, leading to a diverse array of intracellular signaling events that participate in cell adhesion, spreading, and migration (Hood et al., 2003; Yan et al., 2008). Consistent with the evidence reported previously that MAPK and AKT mediate YKL-40-induced mitogenic signaling in connective tissue cells (Recklies et al., 2002), our results showed that YKL-40 utilizes intracellular FAK-MAPK signaling pathways for endothelial cell angiogenesis.

While YKL-40 is expressed by some tumor lines, the lack of its expression in several breast and colon cancer lines may reflect the fact that selection pressures for establishing these cell lines do not require angiogenesis, and thus the lack of YKL-40 expression exists as an artifact of different cell culture conditions. Furthermore, this deficiency is not an isolated and rare event since a number of other tumor angiogenesis-promoting factors such as periostin and SPARC were similarly reported in literature (Jendraschak and Sage, 1996; Yan and Shao, 2006). Nevertheless, the fact that a substantial fraction of primary breast tumors express YKL-40 in our study suggests that it is a common pathway *in vivo*.

A wealth of tumorigenic evidences from both human cancers and animal tumor models indicate that elevated levels of angiogenic factors in cancer tissue correlate with tumor angiogenesis and tumor progression (Kim et al., 1993; Millauer et al., 1996; Polnaszek et al., 2003). Anti-angiogenic intervention has received considerable effectiveness in the suppression of cancer growth and development, which includes monoclonal antibodies against angiogenic factors and synthetic small molecule inhibitors specific for membrane receptor kinases or intracellular mediators (Fahmy et al., 2003; Kim et al., 1993). The present findings that YKL-40 functions tumor angiogenesis may hold promise for developing novel therapeutic agents targeting YKL-40 that is over-expressed in a broad type of human cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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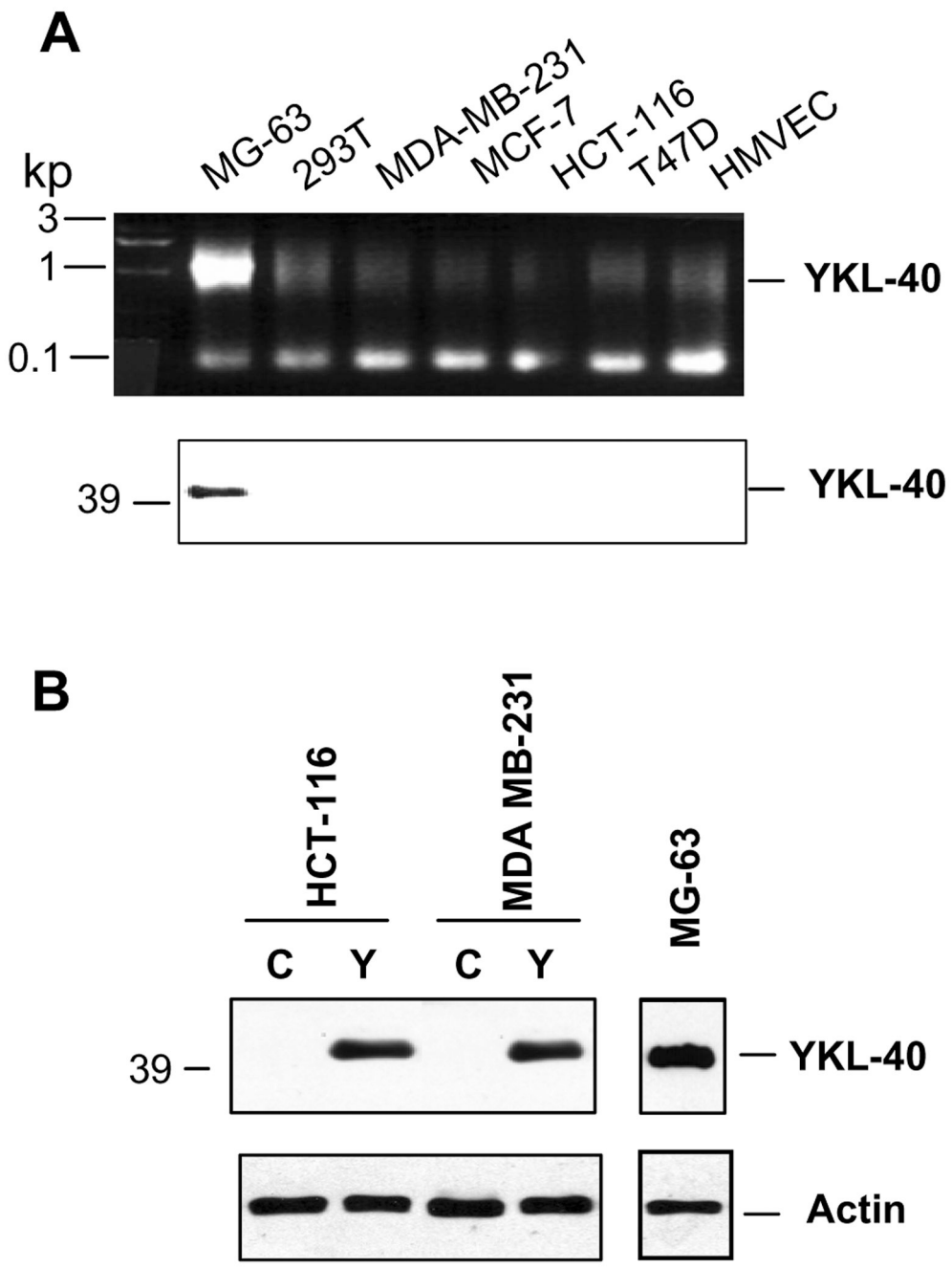

Figure 1. Expression of YKL-40 in a variety of cell lines

A. Once all the cells were grown to be confluent, culture medium was replaced with serum-free medium and cultured for 24 hr. Conditioned medium was used for immunoblotting using polyclonal anti-YKL-40 antibody (rAY) and cells were lyzed for measuring mRNA levels of YKL-40 by RT-PCR. **B**. By retroviral infection, HCT-116 and MDA-MB-231 cells were engineered to express ectopic YKL-40 (Y) or control vector (C) and after 24-hr culture, the conditioned media in the absence of serum were collected for analyzing the

levels of YKL-40 secreted from these cells by immunoblotting. Cell lysates were subjected to the testing of actin expression. MG-63 cells were used for the positive control.

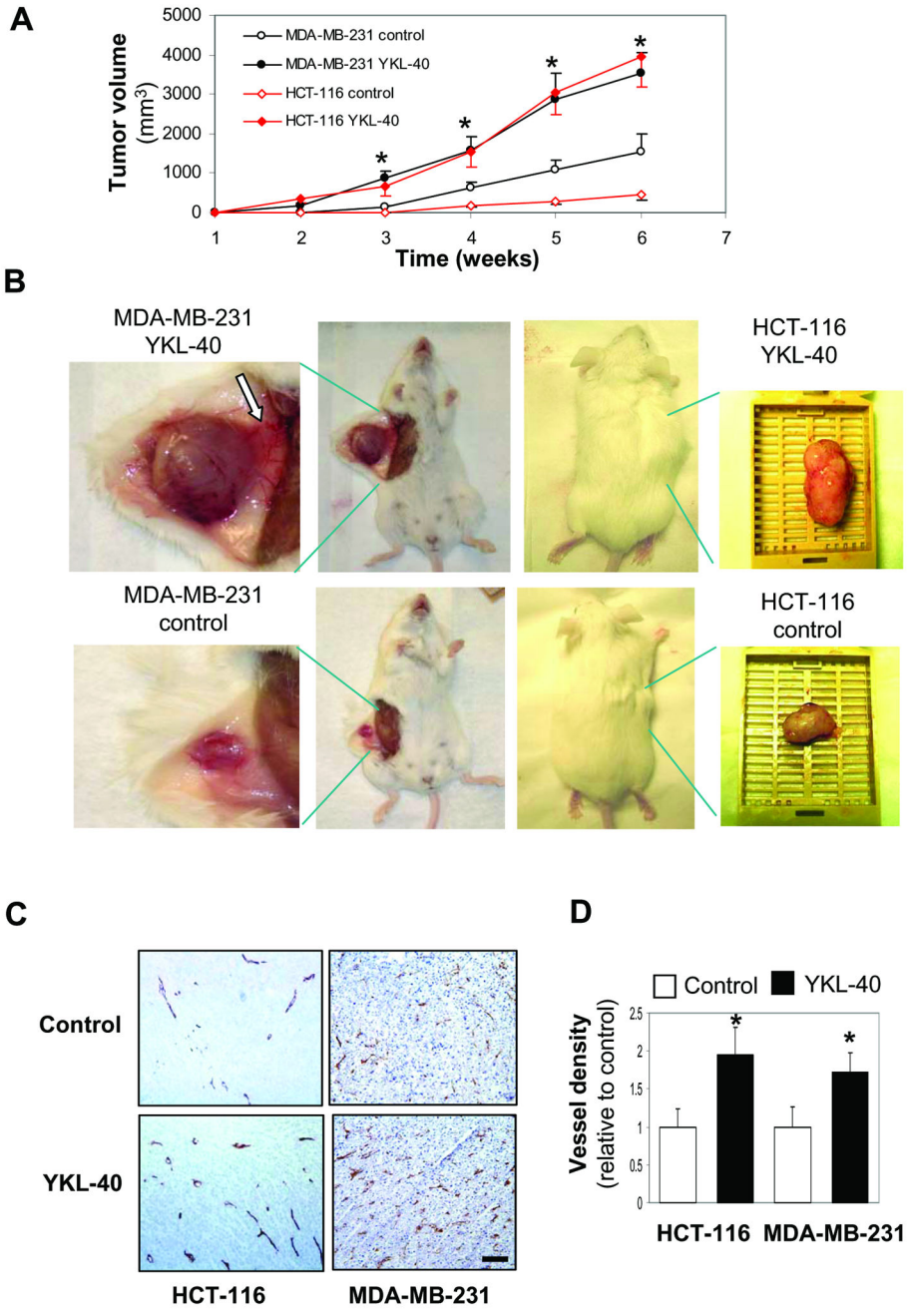

Figure 2. Tumor cells expressing ectopic YKL-40 promote tumor growth and angiogenesis in xenograft models

A. HCT-116 and MDA-MB-231 cells over-expressing YKL-40 or vector were subcutaneously injected into right flank of mice and tumor growth was monitored for 6 weeks. n=6. *P<0.05 compared with the control group. **B**. A representative sample of each group from HCT-116 and MDA-MB-231 tumor models indicated that larger tumors and more blood vessels developed in mice bearing YKL-40-expressing cells relative to these seen in control animals. An arrow indicates blood vessels extended from host vessels. n=6.

C and D. Blood vessel density in tumor tissue was evaluated by IHC staining with anti-CD31 antibody and quantified with an average of CD31 density from six to eight fields in each section using an NIH image analysis program. n=6. *P<0.05 compared with the control group. Bar:100 μm.

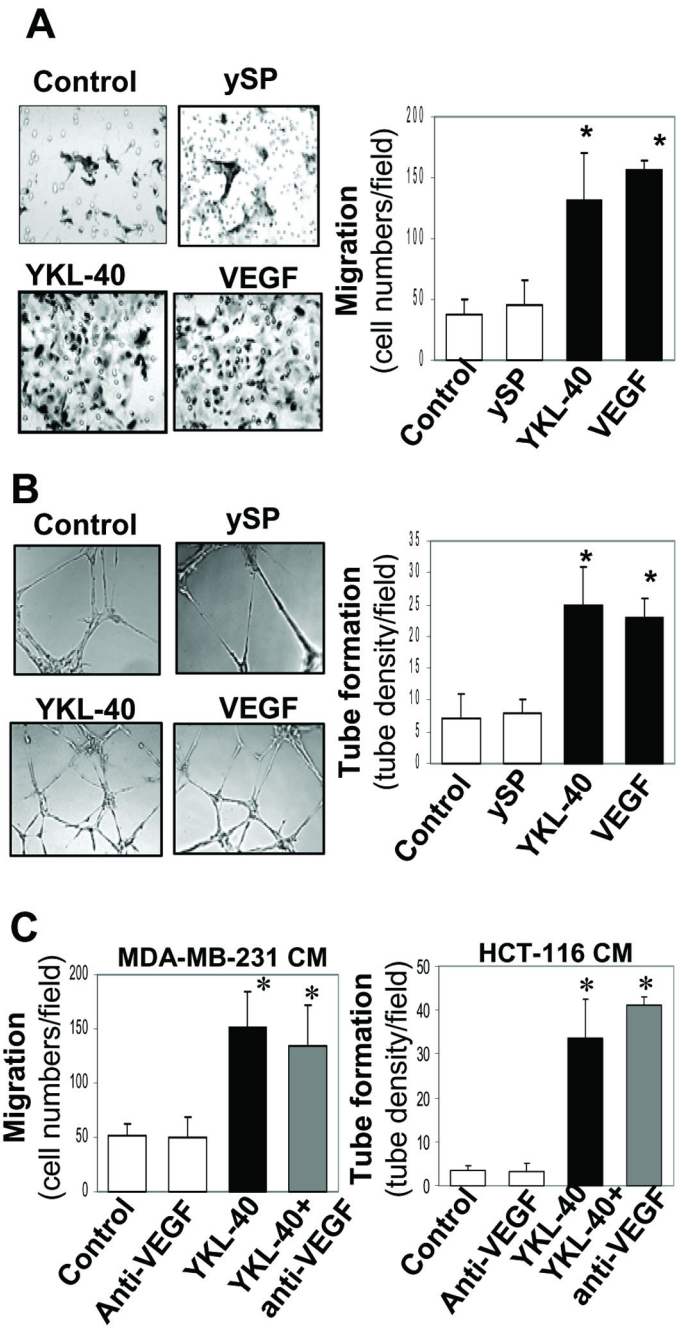

Figure 3. YKL-40 stimulates endothelial cell angiogenesis in culture

A. HMVEC were loaded into the upper chamber of a transwell and ySP (100 ng/ml), YKL-40 (100 ng/ml), or VEGF (10 ng/ml) was introduced into the lower chamber for migration assay. Cells migrated into the membrane were quantified. n=5. *P<0.05 compared with the control or ySP group. **B**. HMVEC were loaded on a layer of Matrigel and cultured for overnight in the presence of ySP (100 ng/ml), YKL-40 (100 ng/ml), or VEGF (10 ng/ml) for tube formation analysis. Vessel-like network was quantified. n=4–5. *P<0.05 compared with control or ySP group. **C**. Conditioned media (CM) derived from MDA-MB-231 and

HCT-116 cells expressing YKL-40 or control vector were transferred into HMVEC culture in the presence of an anti-VEGF antibody (1 μg/ml) to evaluate their effects on endothelial cell migration and tube formation. n=6. *P<0.05 compared with the control.

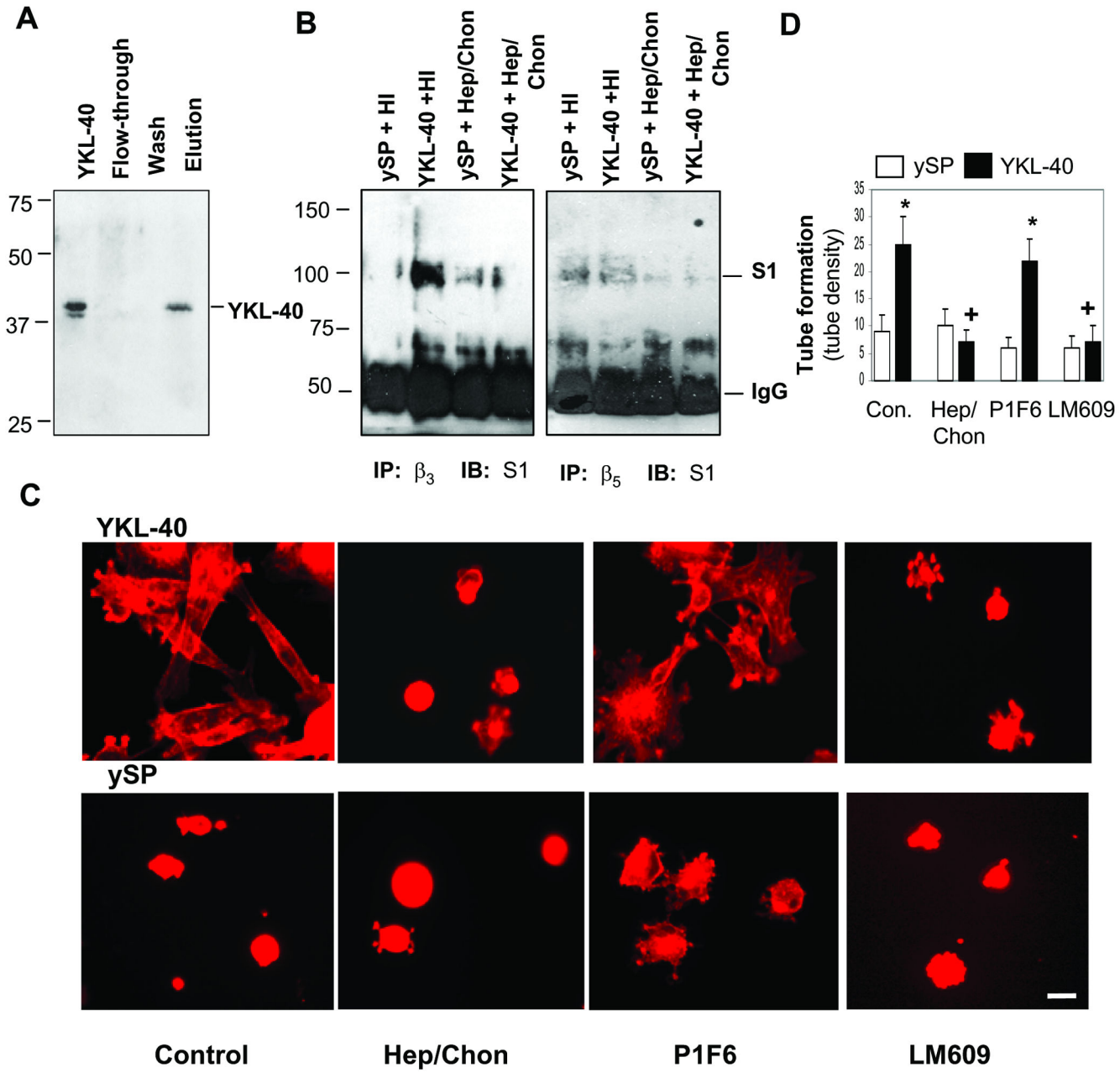

Figure 4. Coordination between S1 and integrin $\alpha_v\beta_3$ is required for YKL-40-induced angiogenesis

A. YKL-40 (1 μg) was used for heparin-Sepharose affinity binding assay. YKL-40 was finally eluted with sodium phosphate buffer containing 1 N NaCl. The first lane indicates YKL-40 recombinant protein used in immunoblot. **B**. HMVEC were treated with heparitinase (Hep 0.1U/ml)/chondroitinase (Chon 0.1 U/ml) or those heat-inactivated enzymes (HI) for 2 hr prior to stimulation with YKL-40 or ySP (100 ng/ml) for 10 min. Cell lyastes were subjected to imunoprecipitation with anti-integrin β_3 or β_5 antibody followed by immunoblotting using anti-S1 antibody. **C**. After treatment with Hep/Chon or HI as indicated in B, cells were introduced to the plates pre-coated with YKL-40 or ySP (10

μg/ml) in the absence or presence of LM609 or P1F6 (10 μg/ml, Chemicon) for 2 hr. The cells were then fixed and stained with rhodamine-conjugated phalloidin. Bar: 10 μm. **D**. After treatment with Hep/Chon or HI as indicated in B, cells were applied to Matrigel for analysis of tube formation in the absence or presence of YKL-40 (100 ng/ml), ySP (100 ng/ml), LM609 (10 μg/ml), or P1F6 (10 μg/ml). n=4. *$P<0.05$ compared with corresponding control cells treated with ySP. $^{+}P<0.05$ compared with cells treated with YKL-40 alone.

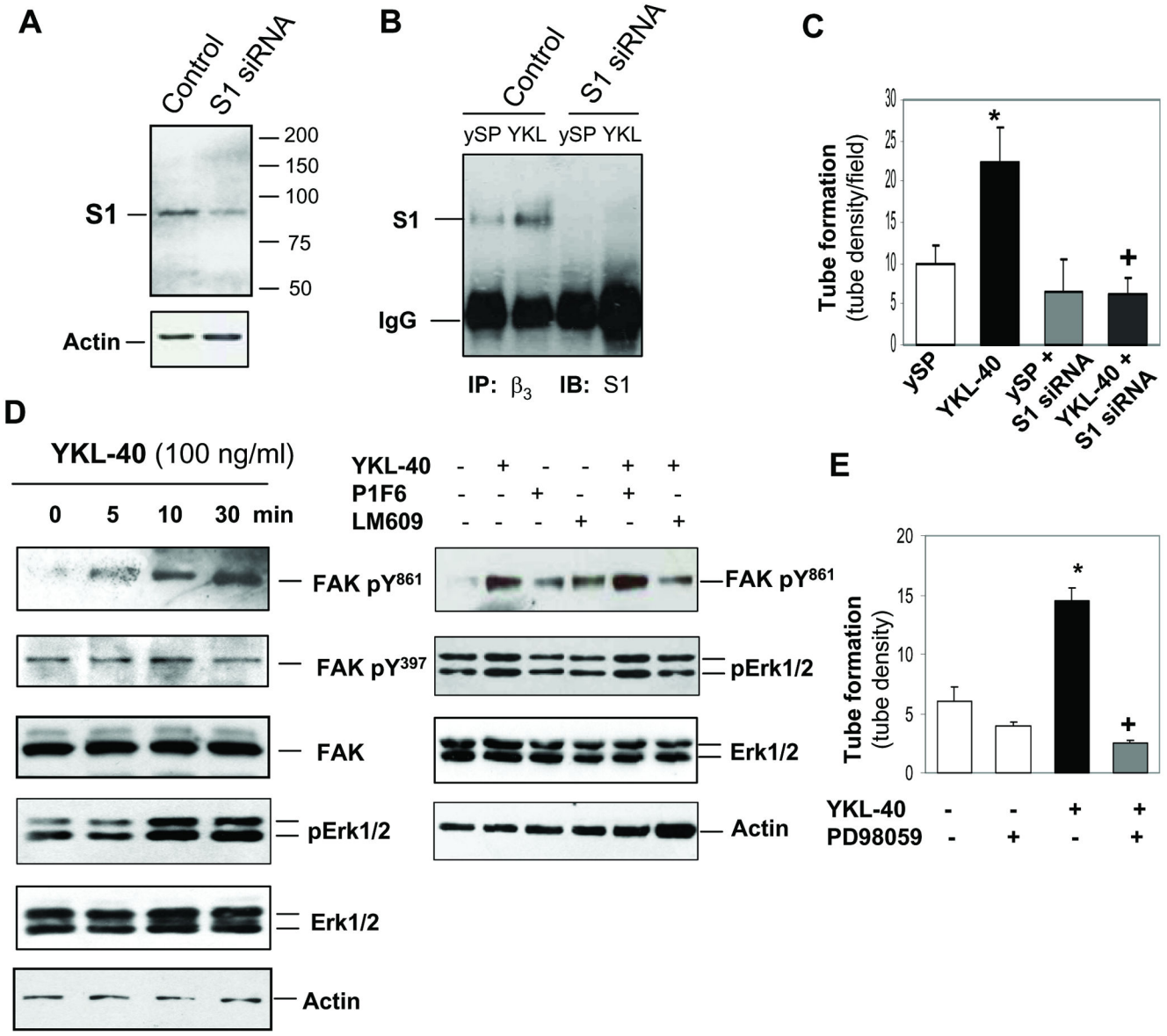

Figure 5. siRNA knockdown of S1 and angiogenic signaling cascade in endothelial cells

A. Western blot analysis showed S1 gene knockdown in the cells. siRNA targeted the ectodomain of S1. **B**. Control and siRNA S1 endothelial cells were stimulated with YKL-40 or ySP (100 ng/ml) for 10 min and then cell lysates were subjected to immunoprecipitation and immunoblotting as described in Figure 4. **C**. Control and siRNA S1 cells were used for tube formation in the presence of YKL-40 or ySP (100 ng/ml). n=3. *P<0.05 compared with control cells treated with ySP. $^{+}$P<0.05 compared with cells treated with YKL-40 alone. **D**. HMVEC pre-treated with serum-free medium were stimulated with YKL-40 (100 ng/ml) from 5 to 30 min. Cell lysates were collected for analyzing phosphorylated levels of FAK and Erk1/2 by immunoblotting using anti-FAK, FAK pY^{397}, FAK pY^{861}, Erk, and pErk1/2. In some conditions, HMVEC were pre-treated with LM609 or P1F6 (10 μg/ml) for 15 min and then stimulated with YKL-40 (100 ng/ml) for 10 min. **E**. HMVEC were measured for

tube formation in the presence of YKL-40 (100 ng/ml) and/or PD98059 (10 μM). n=3. *P<0.05 compared with control cells. $^{+}$P<0.05 compared with cells treated with YKL-40 alone.

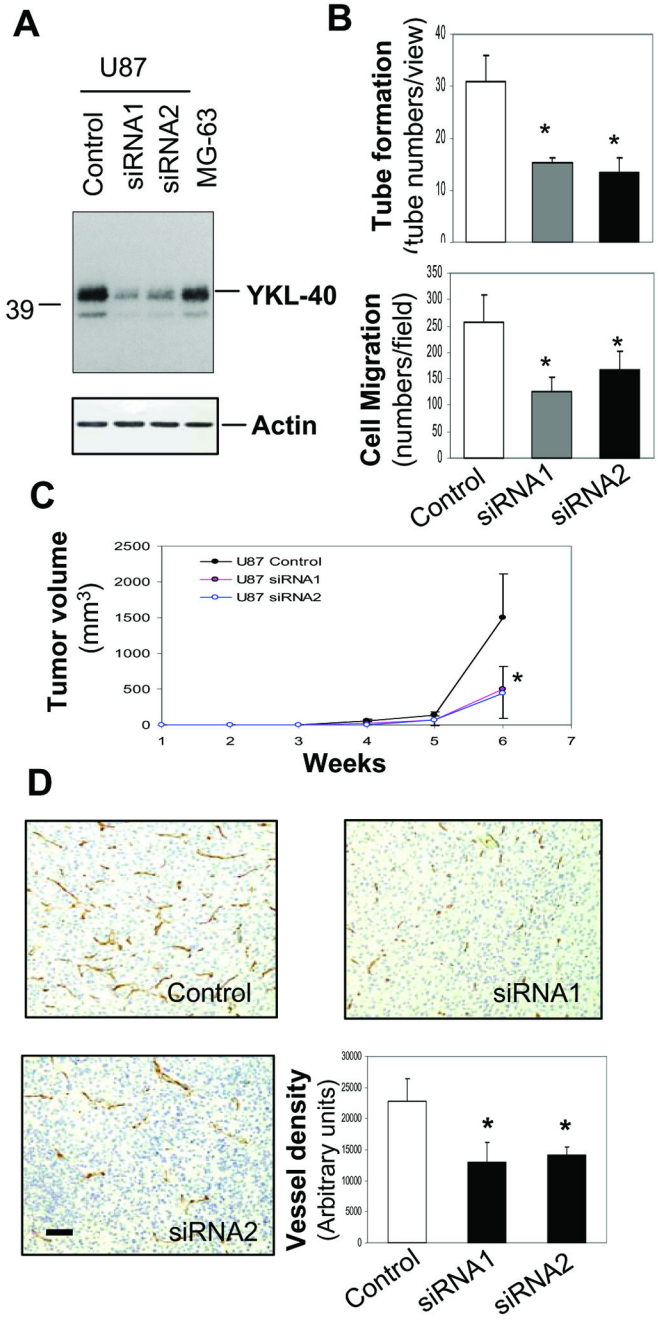

Figure 6. YKL-40 gene knockdown inhibits angiogenesis

A. Western blot analysis displayed YKL-40 gene knockdown in U87 cells. Cell cultured serum-free media were collected for immunoblotting. MG-63 cells as a positive control. **B**. The conditioned medium of U87 cells was transferred to HMVEC for measuring tube formation and migration. *P<0.05 compared with the control. n=4. **C**. The time course for the development of xenografted tumors in mice. U87 cells expressing vector control, YKL-40 siRNA 1 or siRNA 2 were subcutaneously injected into SCID/Beige mice. *P<0.05 compared with control group. n=5. **D**. Immunohistochemistry with CD31 staining showed

higher levels of microvasculature in YKL-40 control tumors than those seen in YKL-40 siRNA1 and siRNA2 tumors. *P<0.05 compared with control group. n=5. Bar: 100 μm.

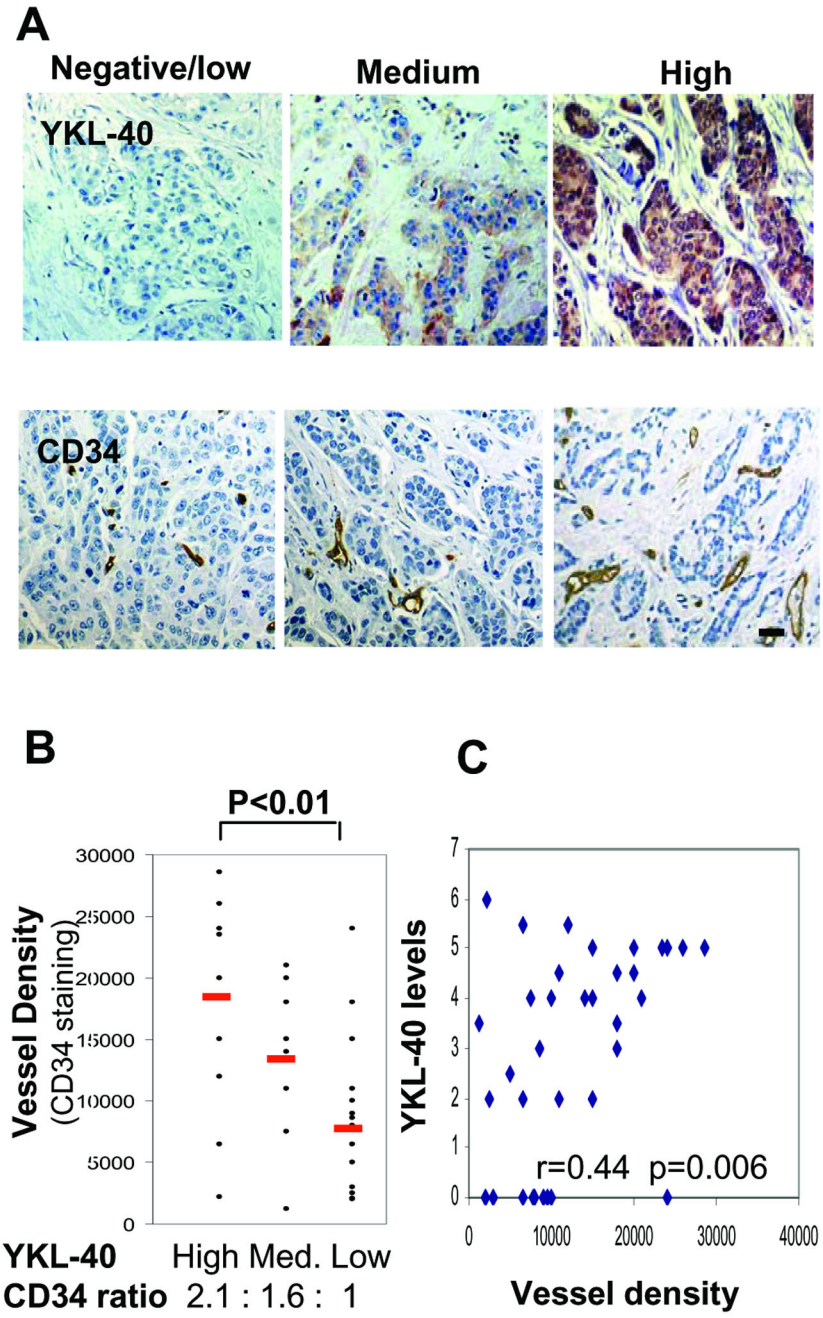

Figure 7. YKL-40 expression correlates with blood vessel density in breast cancer tissue

A. Human breast cancer specimens were performed for IHC YKL-40 (top panel) and CD34 staining (low panel). The images from the left to the right represent the levels of YKL-40 and CD34 from negative/low to high in cancer tissues. Bar: 100 μm. **B**. Cancer cases were categorized into three groups according to the expression levels of YKL-40 (negative/low: n=20; medium: n=9; and high: n=9) as described in the Methods. Blood vessel density in each case was quantified with an average of CD34 arbitrary density numbers from six to eight fields using an NIH image analysis program. Red bars indicate the average levels of

CD34 density. **C**. All of cancer cases were plotted to analyze the relationship between expression levels of YKL-40 and CD34 density by the software of Significance of a Correlation Coefficient.