

SYNDECAN-2 IS EXPRESSED IN THE MICROVASCULATURE OF GLIOMAS AND REGULATES ANGIOGENIC PROCESSES IN MICROVASCULAR ENDOTHELIAL CELLS

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Running Title: The Role of Syndecan-2 in Angiogenic Processes

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Angiogenesis is the formation of new blood vessels from the existing vasculature and is necessary for tumor growth. Syndecan-2 (S2) is highly expressed in the microvasculature of mouse gliomas. When S2 expression was downregulated in mouse brain microvascular endothelial cells (MvEC), this inhibited cell motility and reduced the formation of capillary tube-like structures in vitro. Pro-angiogenic growth factors and enzymes upregulated during glioma tumorigenesis, stimulated shedding of the S2 ectodomain from endothelial cells in vitro. The effect of shed S2 on angiogenic processes was investigated by incorporating recombinant S2 ectodomain (S2ED) into in vitro angiogenesis assays. S2ED promoted membrane protrusion, migration, capillary tube formation, and cell-cell interactions. We therefore propose that S2 is necessary for angiogenesis of MvEC, pro-angiogenic factors expressed during glioma progression regulate S2 shedding, and shed S2 ectodomain may increase endothelial cell angiogenic processes.

Syndecan-2 is one of a family of transmembrane heparan sulfate proteoglycans. Syndecan-1, -2, -3, and -4 (S1, S2, S3, S4)¹ have divergent ectodomains with covalently bound heparan sulfate glycosaminoglycan chains, conserved transmembrane domains, and short cytoplasmic tails (1). The ectodomains can be shed from the cell surface both constitutively and in response to stress or injury, although the precise mechanism is not known (2,3). The cytoplasmic tails contain conserved sequences (C1 and C2) which mediate interactions with the actin cytoskeleton and PDZ-domain-containing proteins, respectively (1). Located between the C1 and C2 domains, is the variable (V) region (1); the function of this domain in S1 and S3 is not known. The V region in syndecan-4 is pivotal in focal adhesion formation in fibroblasts (4,5). The V

domain in S2 regulates laminin and fibronectin matrix assembly in CHO-K1 cells (6) and left-right asymmetry in *Xenopus* (7).

Syndecan-2 may modulate tumorigenesis. Its expression is increased in human ovarian carcinoma biopsies (8) and in various cancer cell lines, and it can modulate colon cancer cell adhesion, motility, and proliferation (9-12). Overexpression in colorectal cancer-derived cells decreased cell-cell interactions, increased lamellipodial and filopodial membrane protrusions, and increased motility (13), and competitive inhibition of S2 with a recombinant S2 ectodomain decreased cancer cell growth on soft agar and reduced cell adhesion (10). Similarly, a reduction in S2 expression by anti-sense cDNA in colon cancer cells inhibited anchorage independent growth (14).

Syndecan-2 is expressed by the cells of the vasculature (1), and a deficiency in S2 inhibits developmental angiogenesis in zebrafish (15). Angiogenesis is the formation of new blood vessels from the existing vasculature and is necessary for tumor growth. Syndecan-2 interacts with cytokines and growth factors that stimulate angiogenesis (e.g. interleukin-8, VEGF, bFGF and TGF β), and in the case of VEGF, can potentiate its pro-angiogenic intracellular signaling (15-18).

The mechanisms which regulate S2 expression and distribution during tumorigenesis and angiogenesis have not been described, but S1 and S4 can be shed from the cell surface, resulting in soluble ectodomains (2,3). Soluble S1 is present in the extracellular matrix of myeloma biopsies and in patient serum (19,20), and the shedding of both S1 and S4 is regulated in response to cancer or stress (2). Injury also stimulates the shedding of syndecans (3) as do factors such as epidermal growth factor (EGF) and plasmin (3,21), which are expressed during tumor progression and wound healing (22,23).

Gliomas are high grade brain neoplasms characterized by cell proliferation, necrosis, and extensive angiogenesis (24). The role of S2 in these tumors has not been investigated. In this study, the expression and localization of S2 was determined in mouse gliomas and its function in microvessel formation was investigated. We propose that S2 shedding that is regulated by pro-angiogenic growth factors and enzymes results in a soluble ectodomain that promotes angiogenic processes.

EXPERIMENTAL PROCEDURES

Materials- Purified bovine fibronectin (Sigma, St. Louis, MO), growth factor reduced matrigel (BDBiosciences, Bedford, MA), rabbit polyclonal anti-S2 (R1891) and rat rec-S2ED have been previously described (25). Mouse MAB3538 anti-lamin A and C antibody (Chemicon, Temecula, CA), rabbit polyclonal anti-S1 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit Alexa Fluor-488 and goat anti-mouse Alexa Fluor-594 (Invitrogen, Carlsbad, CA), mouse anti-GAPDH (Chemicon), anti-mouse CD31 antibody (PharMingen, San Diego, CA), Texas Red conjugated phalloidin (Molecular Probes, Eugene, OR), active human MMP-2 and MMP-9 (Calbiochem, San Diego, CA), human recombinant EGF, bFGF and VEGF (Upstate, Lake Placid, NY) were also used.

Immunohistochemistry- Mouse glioma cells, GL261, obtained from the National Tumor Repository at the NIH were harvested and injected (1×10^5) into the mouse brain, as described previously (26). After 10 days, animals were euthanized, the brains harvested, fixed in buffered formalin, and paraffin embedded. Tissue sections (5 μ m) were cut, de-paraffinized, rehydrated, permeabilized with 0.1% pepsin and 0.05% saponin, and endogenous peroxidases were blocked with 1% H₂O₂. Sections were incubated with anti-S2 (R1891; 1:750), 5 μ g/ml of anti-mouse CD31 IgG, or non-specific rabbit serum. Staining was performed per manufacturers (Biogenex, San Ramon, CA) instructions using biotinylated secondary antibody, streptavidin conjugated to HRP, and 3,3'-diaminobenzidine substrate. The tissue was counterstained with hematoxylin.

Cell Culture- Immortalized MvEC were a kind gift from Drs. Candace L. Gladson and Tika

Benveniste (UAB), and were isolated from temperature-sensitive SV40 large T antigen transgenic mice (27). They were cultured in Ham's F-12 media with 10% FBS, penicillin/streptomycin and amphotericin-B on 1% gelatin-coated tissue culture plates in a humidified chamber (33°C, 5% CO₂).

FACS analysis- MvEC were trypsinized, centrifuged (1300 rpm, 3 min, 4°C), and 300,000 cells were resuspended in Ham's F-12 with 1% BSA, and treated with primary antibody (45 min, 4°C). Cells were centrifuged, washed, and treated with secondary antibody conjugated to Alexa Fluor-488 or Alexa Fluor-594 (Invitrogen) for 30 min at 4°C. Cells were centrifuged, washed, resuspended in PBS and analyzed at the UAB Flow Cytometry Core Facility.

siRNA Experiments- Duplex siRNA directed toward S2 (accession number: NM_008304) was purchased (Dharmacon, Boulder, CO); the sense sequence was GAACAGAGCUGACAUCGGAUU, and the antisense sequence was UCGGAUGUCAGCUCUGUUCUU. Lamin A/C was used as a positive control for siRNA transfection and gene downregulation. MvEC were plated (300,000 cells/well) in Ham's F-12 with 10% FBS in 6-well plates overnight, followed by incubation with siRNAs complexed with lipofectamine 2000 (Invitrogen) per manufacturers instructions for 48 h. Culture medium was removed, cells were lysed (30 min, 4°C) in RIPA buffer (50 mM Tris-HCl, 1% deoxycholate, 0.2% SDS, 1% Triton-X-100, protease inhibitors), and the lysate was centrifuged and subjected to dot blotting.

Dot Blotting- Conditioned media or whole cell lysates were dotted onto a nitrocellulose membrane in a Biorad apparatus and washed once with PBS. The membrane was then blocked in TBST with 5% milk, washed, incubated with primary antibody for 1 h, washed, incubated with secondary antibody conjugated to HRP (DAKO, Carpinteria, CA) for 1 h, and developed for chemiluminescence (Amersham, Bucks, UK).

Migration Assays- Transwell 8 μ m-pore filters (Costar, Corning, NY) were coated on the upper surface with 10 μ g/ml fibronectin for 1 h (37°C, 10% CO₂), then washed with sterile PBS. Cells were trypsinized, centrifuged, resuspended in assay buffer (Ham's F-12 + 1% BSA,

penicillin/streptomycin and amphotericin-B), and plated (15,000 cells/well) in the upper well. MvEC were allowed to migrate toward Ham's F-12 with 5% FBS for 5 h (33°C, 5% CO₂), cells on the top of the filter were removed with a cotton swab, and migrated cells on the underside were fixed with 4% buffered paraformaldehyde, stained with crystal violet, and counted.

Capillary Tube Formation Assays- MvEC were trypsinized, resuspended in Ham's F-12 with 0.25% BSA, penicillin/streptomycin and amphotericin-B and were plated (15,000/well) on the top of growth factor-reduced matrigel for 6 or 24 h. Capillary tube-like structures were photographed with a Nikon Diaphot microscope at the UAB Imaging Core Facility. Capillary tube length was measured as arbitrary units.

Syndecan-2 Shedding Assays- MvEC were trypsinized, centrifuged, resuspended in culture medium, and plated (15,000 cells/well) in 96-well plates overnight, washed, and incubated for 24 h in Ham's F-12 with 5% FBS ± treatment agents. Media were removed, protease inhibitors were added, and samples were dot blotted.

Cell Morphology Assays- Sterile glass coverslips were coated with 10 µg/ml fibronectin for 1 h (37°C, 10% CO₂), rinsed with PBS, and blocked with 1% BSA for 30 min (37°C, 10% CO₂). Cells were trypsinized, centrifuged, resuspended in migration assay buffer, and plated (30,000 cells/coverslip) ± S2ED for 6 h. Cells were fixed with 3% glutaraldehyde (Sigma), washed three times with serum-free Ham's F-12, and mounted with PBS. Random fields were photographed using phase contrast microscopy.

Immunofluorescence- Sterile glass coverslips were coated with a 1:4 dilution of growth factor-reduced matrigel for 1 h, rinsed with PBS, and blocked with 1% BSA for 30 min (37°C, 10% CO₂). MvEC were trypsinized, centrifuged, resuspended in assay buffer, and plated (30,000 cells/coverslip) ± S2ED or S4ED for 3 h. Cells were fixed with 4% paraformaldehyde, washed with PBS, permeabilized with 0.15% Triton-X-100, and incubated with phalloidin conjugated to Texas Red. Coverslips were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Random fields were photographed.

Statistics- A two-sample *t* test was used for data analysis. *P* < 0.05 was required for statistical significance.

RESULTS

Syndecan-2 is expressed in the microvasculature of mouse glioma tumors. Immunohistochemistry demonstrated that S2 was highly expressed in gliomas propagated in mouse brain, in the vasculature of both the tumor (Fig. 1A arrows) and normal brain tissue (Fig. 1A, arrowheads) but was generally not expressed in the normal brain parenchyma (Fig. 1A). Syndecan-2 staining was also associated with the tumor cells (Fig. 1A, T). Its localization to microvessels was confirmed by immunostaining for the endothelial cell marker, CD31 (Fig. 1B).

siRNA downregulation of S2 inhibits angiogenic processes in vitro. Flow cytometry indicated that both S2 and S4 were expressed on the surface of MvEC, but S1 was not detected. Syndecan-2 siRNA downregulated S2 protein in MvEC by approx. 70% as assessed by dot blotting of cell lysate and subsequent densitometric analysis using GAPDH as a control for protein loading (data not shown). Lamin A and C were similarly reduced by siRNA and used as a positive control for gene downregulation (data not shown). No decrease in cell viability was seen with Trypan blue staining of siRNA-transfected cells (data not shown). MvEC transfected with siS2 exhibited a 60% decrease in cell migration on fibronectin compared to cells transfected with control siLamin A/C (Fig. 1C, *p* ≤ 0.05). SiS2 transfected MvEC also showed a 3-fold decrease in capillary tube formation on growth-factor reduced matrigel, as assessed by tube length, compared to siLamin A/C transfected MvEC (Fig. 1D, *p* ≤ 0.05).

Pro-angiogenic molecules stimulate the shedding of S2 ectodomain from MvEC cells. EGF can accelerate the shedding of S1 and S4 from the surface of SVEC4-10 venous endothelial cells (21). We, therefore, investigated whether EGF and other pro-angiogenic growth factors that are upregulated in gliomas could stimulate S2 shedding from brain MvEC. When MvEC were incubated in reduced serum-containing media for 24 h, minimal levels of shed S2 and S4 ectodomains were detected in the conditioned medium (Fig. 2A). However, 20 ng/ml purified EGF, bFGF, or VEGF induced S2 shedding (Fig. 2A). EGF and bFGF treatment increased S2 shedding by approx. 8-fold and VEGF induced a 4-fold increase (Fig. 2A,B, *p* ≤ 0.05). Addition of EGF did not significantly increase S4 shedding,

although bFGF and VEGF both increased this by 3-fold (Fig. 2A,B, $p \leq 0.05$).

Matrix metalloproteinases (MMPs) may regulate growth-factor-mediated shedding of syndecans, as broad spectrum MMP inhibitors and tissue inhibitor of MMP-3 (TIMP-3) can abrogate EGF-mediated shedding from epithelial cells (2). Similarly, stimulants of MMP production, such as phorbol esters, enhance S1 and S4 shedding, and this can be inhibited by MMP inhibitors and TIMP-3 (2,21). Since MMP-2 and MMP-9 are upregulated in the microvasculature of human glioma biopsies and can promote angiogenesis, (28,29), we tested whether purified active MMP-2 and MMP-9 induced syndecan shedding (Fig. 2A,B). 20 ng/ml of MMP-2 and MMP-9 increased S2 shedding by more than 11-fold and 9-fold, respectively, and both increased S4 shedding by 3-fold (Fig. 2A,B, $p \leq 0.05$). No S1 shedding could be detected in response to growth factors or MMPs, consistent with the negative FACS analysis for S1 (data not shown).

Recombinant S2ED promotes angiogenic processes in vitro. Since pro-angiogenic factors increased S2 and S4 ectodomain shedding, we investigated whether exogenously added S2 and S4 ectodomains affected angiogenic processes in vitro. Although many interactions of proteoglycans are via their glycosaminoglycan chains (1), recent evidence suggests that the ectodomain of the core protein may have a direct role(s) (1,10,18). When a bacterial recombinant S2ED, which lacks glycanation, was incorporated into in vitro assays, there was a dose-dependent increase in MvEC migration on fibronectin, with 100nM resulting in a more than 3-fold increase (Fig. 3A, $p \leq 0.05$). Addition of S4ED resulted in a different biphasic pattern of migration with increasing concentration (Fig. 3A). S2ED at 100nM also promoted the formation of capillary tube-like structures on fibronectin, while no change was observed with S4ED (data not shown). To investigate further the ability of S2ED to promote angiogenic processes, MvEC were plated \pm S2ED onto growth factor-reduced matrigel. Incubation of MvEC with 100nM S2ED increased capillary tube formation, with a 1.5-fold increase in length at both 6 and 24 h when tube length was compared to control (Fig. 4B,C, $p \leq 0.05$). There was also an apparent reduction in tube branching, as indicated by increased cell number at the branch

points. (Fig. 3C). S4ED had no effect on capillary tube formation at either time point (data not shown). MvEC were also allowed to attach and spread on coverslips coated with diluted growth factor-reduced matrigel \pm 100nM S2ED. Staining with phalloidin indicated that 100nM S2ED increased cell-cell interactions with cells appearing as groups (Fig. 3E arrows), whereas control cells were mostly present as single cells (Fig. 3D). MvEC plated in the presence of S4ED resembled control cells (data not shown).

DISCUSSION

Syndecans have been investigated in some other types of cancers and in cancer cells (8,10,12), but this is the first report to indicate a role for S2 in glioma angiogenesis, particularly a role for the shed ectodomain. Syndecan-2 is highly expressed in the microvasculature of mouse glioma tumors, and downregulation in MvEC cells inhibits cell migration and capillary tube formation in vitro, processes necessary for angiogenesis. Furthermore, pro-angiogenic growth factors and enzymes that are increased in glioma tumors (e.g. EGF, bFGF, VEGF, MMP-2, and MMP-9) (22,24,28-30) can stimulate the shedding of S2 ectodomain from endothelial cells. This may promote angiogenesis, since recombinant soluble S2ED increased brain microvascular endothelial cell motility, capillary tube formation, and cell-cell interactions in vitro.

Syndecan-1 and -4 are constitutively shed from the surface of mouse epithelial and SVEC4-10 cells in vitro, and factors released during stress, injury, and cancer may upregulate this process (2,3,19-21). Syndecan-2 shedding has not been investigated. Epidermal growth factor, which is increased in some gliomas (22), can accelerate S1 and S4 shedding (21). Basic fibroblast growth factor and VEGF can also be upregulated in gliomas (24,30). These three growth factors stimulated S2 shedding from MvEC cells with much less effect on S4. Neither bFGF nor VEGF accelerated S1 or S4 shedding from SVEC4-10 cells (21), suggesting that syndecan shedding in response to growth factors may differ with cell type or each syndecan family member.

Addition of exogenous active MMP-2 and MMP-9, proteases highly upregulated in the angiogenic microvasculature of human gliomas (28,29), stimulated S2 and S4 shedding from brain

MvEC. Growth factors, such as EGF, bFGF, and VEGF increase MMP-2 and -9 activities in human anaplastic astrocytoma cells (31). Thus, EGF, bFGF, and VEGF may increase S2 shedding through an upregulation of MMPs, which could in turn cleave the ectodomain from the endothelial cell surface.

To investigate the possible effects of S2 shedding on angiogenic processes, we mimicked the presence of soluble S2 or S4 ectodomain by adding recombinant ectodomain to brain MvEC. Syndecan-2ED stimulated a dose-dependent increase in MvEC migration on fibronectin. Syndecan-4ED also had an affect on migration, but this was biphasic with concentration, consistent with its role in focal adhesion formation². Soluble S1 secreted by transfected ARH-77 B lymphoid cells increased cell invasion through collagen type I (32), and increased S1 ectodomain shedding correlated with increased HT1080 cell migration on collagen (33). Soluble S2ED, but not S4ED, increased MvEC cell-cell

interactions on growth factor-reduced matrigel. This may promote the specific increase in capillary-tube formation and decreased branching in the presence of S2ED.

Syndecan-2 localization to the microvasculature of gliomas, its necessity for angiogenic processes in brain MvEC in vitro (shown here), and requirement for embryonic vascular sprouting (15), strongly implicate S2 as a regulator of neovascularization. Further research is needed to elucidate the specific mechanism(s) involved. Syndecan-2 may exert its effect via regulation of matrix assembly (e.g. basement membrane laminin) as previously shown (6), generation of a chemotactic gradient in a complex with growth factors (as shown for S1) (34,35), or by protecting pro-angiogenic factors from inactivation. Whatever the mechanism, recombinant soluble S2ED that lacks glycanation is biologically effective, and may be a target for novel anti-tumor/anti-angiogenic cancer therapies.

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FOOTNOTES

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¹The abbreviations used are: S1, S2, S3, S4, syndecans-1, 2, 3, 4; C1 and C2, conserved sequences in syndecans; V, variable region in syndecans; CHO, Chinese hamster ovary cells; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; TGF β , transforming growth factor β ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; MvEC, mouse brain microvascular endothelial cells; UAB; FACS, fluorescent activated cell sorter; TIMP-3, tissue-inhibitor of MMP-3; SVEC, small vein endothelial cells; S2ED, recombinant syndecan-2 ectodomain; S4ED, recombinant syndecan-4 ectodomain.

² Nunes, S.S., Klass, C.M., Morandi, V., Woods, A. In preparation

FIGURE LEGENDS

Fig. 1. Syndecan-2 is expressed in the microvasculature of mouse glioma tumors and is needed for angiogenic processes. *A,B*, Mouse glioma tumors (denoted T) were immunostained for S2 (*A*) or the endothelial cell marker, CD31 (*B*). Arrows and arrowheads denote vessel staining in the tumor, and in the normal brain, respectively. Bar = 50 μ m. *C*, MvEC transfected with siLamin A/C (siLamin) or siS2 were allowed to migrate on fibronectin-coated transwell filters for 5 h. Migrated cells were stained, counted, and the percent of migrated cells plotted as mean \pm S.E.M, $p \leq 0.05$. *D*, MvEC transfected with siS2 or siLamin A/C were allowed to form capillary tubes on growth factor-reduced matrigel for 24 h. Random fields were photographed and capillary tube length assessed as arbitrary units. The data are plotted as the mean \pm S.E.M, $p \leq 0.05$.

Fig. 2. Pro-Angiogenic molecules stimulate the shedding of S2 and S4 ectodomain. *A*, MvEC were incubated \pm 20 ng/ml EGF, bFGF, VEGF, MMP-2, or MMP-9 for 24 h. The conditioned media was dot blotted onto a nitrocellulose membrane, and probed with antibodies against S2 or S4 ectodomain. *B*, Quantitation of S2 and S4 ectodomain shedding was performed using densitometric analysis of dot blots. The data are plotted as the mean \pm S.E.M, $p \leq 0.05$.

Fig. 3. Recombinant S2ED promotes angiogenic processes in vitro. *A*, MvEC in serum-free media \pm S2ED or S4ED (2.5, 25, 50, 100 nM) were allowed to migrate on fibronectin-coated filters in transwell assays for 5 h. Migrated cells were stained, counted, and the percent of migrated cells plotted \pm S.E.M, $p \leq 0.05$. *B,C*, MvEC were allowed to form capillary tubes on growth factor-reduced matrigel in the absence (*B*) or presence of 100 nM S2ED (*C*) for 24 h. Random fields were photographed, bar= 100 μ m. Arrowheads indicate branch points. *D,E*, MvEC in serum-free media were plated on growth factor reduced matrigel-coated coverslips for 3.5 h in the absence (*D*) or presence (*E*) of S2ED at 100 nM and stained with phalloidin. Random fields were photographed, bar = 50 μ m. Arrows denote groups of cells.

Figure 1

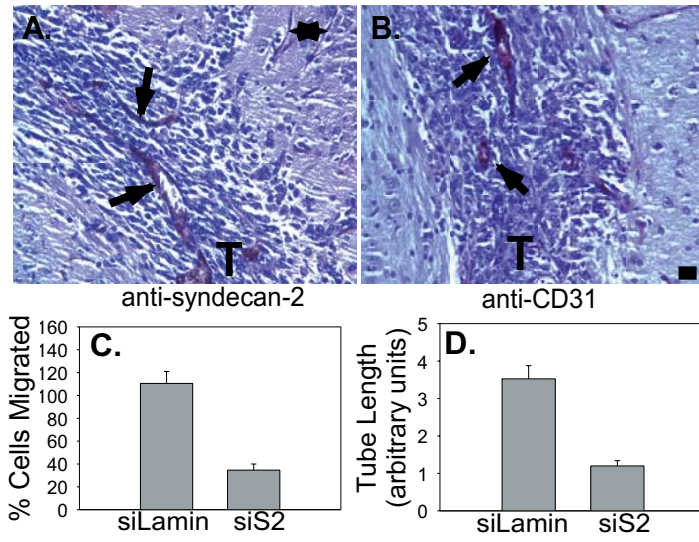


Figure 2

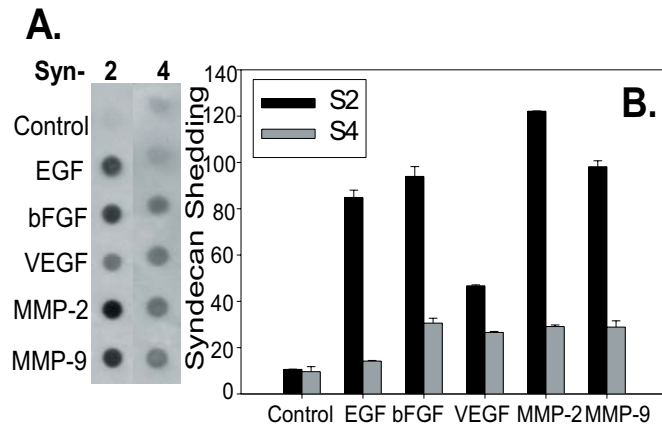


Figure 3

