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Publication Date
2015

DOI
10.1007/978-1-4939-1714-3_39

Peer reviewed

HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2018 July 25.

Published in final edited form as:

Methods Mol Biol. 2015 ; 1229: 507–516. doi:10.1007/978-1-4939-1714-3_39.

Measuring sulfatase expression and invasion in glioblastoma

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Summary

Extracellular sulfatases (SULF1 and SULF2) selectively remove 6-O-sulfate groups (6OS) from heparan sulfate proteoglycans (HSPGs) and by this process control important interactions of HSPGs with extracellular factors including morphogens, growth factors and extracellular matrix (ECM) components. The expression of SULF1 and SULF2 is dynamically regulated during development and is altered in pathological states such as glioblastoma (GBM), a highly malignant and highly invasive brain cancer. SULF2 protein is increased in an important subset of human GBM and it helps regulate receptor tyrosine kinase (RTK) signaling and tumor growth in a murine model of the disease. By altering ligand binding to HSPGs SULF2 has the potential to modify the extracellular availability of factors important in a number of cell processes including proliferation, chemotaxis, and migration. Diffuse invasion of malignant tumor cells into surrounding healthy brain is a characteristic feature of GBM that makes therapy challenging. Here, we describe methods to assess SULF2 expression in human tumor tissue and cell lines and how to relate this to tumor cell invasion.

Keywords

GBM; glioma; invasion; sulfatase; SULF2; HSPG; tumor microenvironment; NPC

1. Introduction

Heparan Sulfate Proteoglycans (HSPGs) comprise a protein core attached to one or more glycosaminoglycan chains. Plasma membrane-associated HSPGs or those secreted into the extracellular environment participate in a diverse array of interactions mediating many important functions, such as adhesion, migration and morphogen/growth factor signaling (1,2). An important determinant of these interactions is the extent of HSPG sulfation, particularly 6O-sulfation, which can be regulated during HSPG biosynthesis or extracellularly by the sulfatases, SULF1 and SULF2 (3). The SULFs specifically remove sulfates from the 6-O position of glucosamine (4,5). This modification changes the ability of HSPGs to bind specific growth factors and morphogens, modulating HSPG-dependent signaling in a temporally and spatially controlled manner e.g. Wnts, fibroblast growth factor (FGF2), glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) (6,4,7–9).

Consistent with the important roles for extracellular sulfatases in signaling, SULF2 expression is increased in a variety of human cancers including brain, lung, breast, head and neck, and pancreatic cancer (10–16,9). In human and murine models for GBM, loss of SULF2 results in decreased signaling through the platelet-derived growth factor receptor-alpha (PDGFRα) and decreased cell proliferation (11). Furthermore, ablation of Sulf2 expression in tumor cells prolongs survival in vivo, demonstrating a functional role for Sulf2 in malignant glioma (11). Interestingly, SULF2 loss is also associated with decreased activation of EPHA2 and IGF1Rβ, demonstrating SULF2 can modulate multiple signaling pathways simultaneously.

Invasion of tumor cells into the adjacent brain is a hallmark of GBM and severely complicates therapy (17). Mechanisms of tumor cell invasion are complex, involving multiple extracellular and intracellular cues (18). As demonstrated in development, wound repair, and in cancer, the SULFs can help to regulate extracellular migratory cues and alter cell migration (7,19,20). SULF2 can promote glioma development (11,21) but its role in glioma invasion is unclear. Similar to the role of SULF2 in tumor development, the specific molecular alterations present in a tumor may be an important determinant of its function in tumor invasion.

Post-synthetic modifications of HSPGs are increasingly being recognized for their importance in tumor biology and as potential therapeutic targets. Here we describe methods to assess SULF2 mRNA and protein expression and to quantify tumor cell invasion of human and murine glioma cells in vitro.

2. Materials

2.1 SULF2 mRNA expression analysis

1. Reagents to isolate mRNA from cultured cells, such as the Qiagen RNeasy Mini Kit which includes RLT lysis buffer
2. DEPC-treated water
3. Superscript III First-Strand Synthesis System for RT-PCR (Life Technologies, Grand Island, NY).
4. FastStart Universal SYBR Green Master
5. Primers:

   Human SULF2 forward primer: ACAGGGATGTCCTCAACCAG

   Human SULF2 reverse primer: CTTCCCACAGTTGTCCCAGT

   Expected size of amplified product is 204 bp [Homo sapiens sulfatase 2 (SULF2), mRNA transcript variant 1 (NM_018837.3); and transcript variant 3, (NM_001161841.1)]; and 195 bp [mRNA transcript variant 2 (NM_198596.2)].

   Mouse SULF2 forward primer: GAAAGACCACAAGCTGCACA

   Mouse SULF2 reverse primer: GGAGCCTTTGTGCTTGAGAC

Expected size of amplified product is 169 bp [Mus musculus sulfatase 2 (Sulf2), mRNA transcript variant 3 (NM_001252579.1); transcript variant 2 (NM_028072.5); transcript variant 1 (NM_001252578.1)]

6. Control primers are essential and can include primers to GAPDH, β-tubulin, cyclophilin, actin, elongation factor 1, adenine phosphoribosyl transferase (aprt), and cytoplasmic ribosomal protein L2. We commonly normalize expression of SULF2 to the expression of GAPDH.

   Human GAPDH forward primer: CGACAGTCAGCCGCATCTT

   Human GAPDH reverse primer: CCGTTGACTCCGACCTTCA

   Mouse GAPDH forward primer: AGGTCGGTGTGAACGGATTTG

   Mouse GAPDH reverse primer: TGTAGACCATGTAGTTGAGGTCA

7. 7900 HT Fast Real Time PCR machine

2.2 SULF2 protein expression analysis

1. Protein lysis buffer: 1x of 10X Lysis buffer (Cell Signaling Technology, Boston, MA), 1x of 100x Protease Inhibitor Cocktail (Sigma Chemical Company, St. Louis, MO, USA), and 1x of 100X HaltTM Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific Inc, Waltham, MA) in milli-Q water.
2. Cell scraper (Falcon, Corning Life Sciences, Corning, NY)
3. Dulbecco's PBS (D-PBS), ice-cold
4. 22½ gauge needle or sonicator Diagenode Biorupter®
5. 1x Sample Buffer plus reducing agents: 1x of 4X LDS Sample Buffer Tris(2-carboxyethyl)phosphine) (TCEP) and Dithiothreitol (DTT).
6. NuPAGE® 4–12% Bis-Tris Gels (Life Technologies, Grand Island, NY)
7. NuPAGE® MOPs SDS Running Buffer (Life Technologies, Grand Island, NY)
8. Invitrogen XCELL II Blot Module system (Life Technologies, Grand Island, NY, #EI9051)
9. Polyvinylidene fluoride (PVDF) 0.45 μm pore size membrane.
10. Transfer buffer 10X stock (1 L): 75 g Tris-base, 187.5 g Glycine, mili-Q water.
11. Transfer buffer 1x: Transfer buffer 10X stock, 20% Methanol, mili-Q water.
12. BIO-RAD Mini-PROTEAN Tetra-System (Bio-Rad Laboratories Inc, Hercules, CA, #165-8000)
13. Tris-buffered saline with tween (TBST): Tris-buffered saline plus 0.05% Tween.
14. Blocking buffer: 5% Bovine serum albumin (BSA) (w/v) in TBST.
15. Mouse anti-SULF2 antibody 2B4 (Novus Biologicals, Littleton, CO)
16. Loading control antibody: mouse anti-GAPDH antibody

17. Anti-mouse IgG horseradish peroxidase linked F(ab′)2 fragment from sheep
18. Electrochemical luminescence (ECL) detection system

2.3 Method to assess invasion in SULF2 expressing systems – 3D invasion assay

1. 96 well round bottom ultra-low attachment plate
2. 96 well flat bottom tissue culture plate
3. BD Growth Factor Reduced Matrigel™ (BD Biosciences, San Jose, CA)
4. ImageJ (http://rsbweb.nih.gov/ij/)

2.4 Method to assess invasion in SULF2 expressing systems – transwell migration assay

1. BD BioCoat™ Growth Factor Reduced Matrigel™ Invasion chamber (BD Biosciences, San Jose, CA #354483)
2. 24 well tissue culture treated plate
3. Growth factor-free/serum-free media
4. Chemoattractant media
5. 4% paraformaldehyde (PFA) in PBS
6. Crystal Violet
7. Methanol

3. Methods

3.1. RNA extraction, cDNA synthesis and quantitative real time PCR (qRTPCR)

1. Perform all steps on ice.
2. Cell culture media is removed, RLT lysis buffer is added, and cell lysates are scraped and transferred to a 1.5 mL eppendorf on ice (See Note 1).
3. Samples in RLT buffer can be immediately processed for RNA extraction or transferred to −80°C for long-term storage. The Qiagen RNeasy Mini Kit is used to extract RNA from cells following the manufacturer's protocol and RNA is resuspended in DEPC-treated water.
4. cDNA synthesis with Superscript III First-Strand Synthesis System following manufacturer's protocol and 50 μM oligo(dT) primer. Use the same amount of RNA (200–300 ng) across all samples. Perform the additional RNase H step from the manufacturer's protocol after cDNA synthesis to remove RNA template from the reaction mix.

[1]Volume of RLT lysis buffer added is dependent on cell number. For example, one confluent 10 cm^2 plate of adherent U251 cells is lysed with 600 μl of RLT buffer. For murine neural progenitor cell (NPC) cultures, 2–4 confluent wells of a 6 well plate are pooled, centrifuged at 100× g for 4 minutes and resuspended in 350 μl of RLT buffer.

5. Prepare mastermix for PCR: 450 nM of forward and reverse primers, 7.5 μL of FastStart Universal SYBR Green Master, and DEPC-treated water to a final reaction volume of 10 μL. Add 5 μL of cDNA from step 4 to each well. Run each sample in triplicate (Figure 1A).

6. Use 7900 HT Fast Real Time PCR machine standard cycling conditions 10 minutes at 95°C for cDNA denaturation, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, for primer annealing and amplicon elongation respectively.

3.2 SULF2 protein expression analysis

1. Protein lysate buffer prepared immediately before use and stored on ice. Perform all steps on ice unless otherwise noted.

2. Adherent cells: Remove cell culture media, gently wash cells with ice-cold D-PBS, add lysis buffer directly to cells, collect cells using a cell scraper with plate on ice, and transfer lysate to a ice-cold eppendorf tube (see Note 2).

3. Non-adherent cells: Collect cells by centrifugation, wash cells once by re-suspending in ice-cold D-PBS, repeat the centrifugation at 4°C, remove D-PBS and re-suspend cells in lysis buffer on ice.

4. Extract proteins on ice for 20 minutes

5. To aid cell lysis and shred chromosomal DNA, either pass samples 12 times through 22½ gauge needle or sonicate sample using the Diagenode Biorupter® with an on/off interval of 30 seconds with high intensity ultrasonic waves for 10 minutes.

6. Spin homogenized protein lysate at 21,000x g to pellet cell debris.

7. Transfer protein lysate supernatant to a fresh eppendorf and store at −80°C.

8. Measure protein concentration per standard protocol.

9. Prepare 20 μg of protein with 1x Loading Buffer plus reducing agents (see Note 3).

10. Boil samples for 5 minutes, place immediately on ice for 3 minutes, pulse spin, and leave on ice.

11. Run the samples into the stacking gel at 110 mV and then increase the voltage to 125 mV for 3–4 hours.

12. Activate PVDF membrane with sequential 5 minute washes in methanol, mili-Q water, and transfer buffer.

13. Transfer protein from PAGE gel to PVDF membrane using BIO-RAD Mini-PROTEAN Tetra-System. Transfer overnight at 4°C at constant current 20 mA.

[2]Adjust cell lysate buffer volume according to the number of cells being processed. Approximately 500 μl per 10 cm dish of confluent cells and 150 μl per 6 well plate.
[3]The use of fresh reducing agents is important for detecting the 75 kD SULF2 band.

14. Wash membranes with TBST and clip bottom right corner of membrane for orientation.
15. Block non-specific binding to the membrane with Blocking Buffer for 1 h at room temperature.
16. Incubate membrane with primary SULF2 antibody at 1:500 diluted in Blocking Buffer overnight at 4°C.
17. Wash the membrane 5 times for 5 minutes with 25 mL TBST.
18. Incubate membrane with secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP) at 1:5000 diluted in Blocking Buffer for 1 hour at room temperature.
19. Wash the membrane 5 times for 5 minutes with 25 mL TBST.
20. Electrochemical luminescence (ECL) detection system to visualize SULF2 protein (Figure 1B, See Note 4).

3.3 Method to assess invasion in SULF2 expressing systems - 3D spheroid invasion assay

1. Prepare single cell suspension of cells of interest in media (see Note 5).
2. To form spheroids plate 500–1000 cells in 100 μL of standard growth media per well of 96 well round bottom plate. Centrifuge at 200x g for 3 minutes at room temperature.
3. Allow spheres to form over 3 days under standard growing conditions. Plate a minimum of 3 wells per condition to be tested.
4. Take care handling matrigel. Thaw matrigel on ice, use cooled tips (stored at −20°C) to transfer and mix matrigel and perform all steps on ice to prevent spontaneous polymerization and in tissue culture hood to keep sterile.
5. Coat 96 well flat bottom plate with a matrigel plug to prevent migration of cells along the plastic of the dish. Mix matrigel with pre-cooled full growth media at a 1:1 ratio. Add 50 μL matrigel:media mix to each well and transfer to 37°C for 30 minutes to promote matrigel polymerization.
6. Cool plates on ice. Transfer spheroids in 50 μL of media from the round bottom plate to the matrigel plug-coated plate using cooled and blunted p200 pipette tips.
7. Gently add 50 μL of matrigel to each well and incubate for 1 hour at 37°C for matrigel polymerization.
8. Add 50 μL of warmed full growth media to the top of each well containing a spheroid in matrigel.

[4]The 2B4 antibody recognizes both human and mouse SULF2, binding to an epitope in the C-terminus to resolve the C-terminal 50 kD fragment and the full-length/unprocessed, SULF2 at 135–140 kD (22). In some human and murine cells, the antibody detects a non-specific band at ~155 kD, which is not altered by ablation of SULF2 expression.
[5]The 3D spheroid assay can be used with adherent cells such as U251 or with non-adherent cells such as NPCs.

9. Use inverted tissue culture microscope with low magnification objective (2.5 X) to take picture of spheroid size at 0 hour (Figure 2A) and at specific time points afterward such as 16 hour, 24 hour, 48 hour (Figure 2B; see Note 6).

10. Quantify cell invasion using ImageJ. Overall spheroid outgrowth can be determined and the fold change in area can be compared across wells after normalizing spheroid outgrowth to the spheroid size at 0 hour. To do this: In 'Analyze', 'Set Measurements' choose 'Area' and ' Limit to Threshold'. Adjust the threshold so the spheroid is highlighted, and select 'Analyze', 'Measure.' If there are issues with uneven illumination across the field of view, the polygon tool can be used to limit the area analyzed to the spheroid area of interest.

3.4 Method to assess invasion in SULF2 expressing systems - Transwell migration assay

1. Transfer the appropriate number of chambers into a 24 well tissue culture plate in tissue culture hood and thaw for 5 minutes. Each condition should be tested in triplicate.

2. To rehydrate the chamber inserts, add 500 μL of warm media and incubate for at least 2 hours at 37°C.

3. Prepare cells as a single cell suspension in a growth factor-free/serum-free media (see Note 7).

4. In a new 24 well place add 750 μL of media containing a chemo-attractant of interest to each well (see Note 8).

5. Remove the media used to rehydrate the inserts, and transfer inserts into wells containing chemo-attractant media. Be careful not to trap air bubbles beneath the membrane.

6. Immediately add 500 μL of cell suspension into the inserts.

7. Incubate the Invasion Chambers for 16 hours at 37°C 5% CO_2 (see Note 9).

8. Carefully remove media from each insert and transfer inserts into wells containing 500 μL of 4% PFA for 10 minutes on ice.

9. Transfer inserts into wells containing 500 μL of 0.1% crystal violet diluted in methanol at room temperature for 10 minutes.

10. Wash inserts by immersing in 3 changes of milli-Q water, 2x dips in each.

11. Wick off excess water by touching edge of insert with Kimwipe.

12. Gently rinse interior of chamber with 100 μL of milli-Q water.

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[6]The optimum time points to assess invasion will vary between different cell types and their ability to invade.
[7]The plating density needs to be optimized according to the cell types used. For murine NPC cultures, dilute cells to 2×10^5 cells/mL in growth factor-free/serum-free media and use 1×10^5 cells per insert.
[8]Epidermal growth factor (EGF)/ Fibroblast Growth factor (FGF) for NPC or serum-containing media for U251 cells.
[9]The invasion chamber incubation time will vary across cells lines but must be minimized to reduce confounding cell proliferation.

13. Wipe the inside of one-half of the membrane with one swipe of a cotton swab moistened with water. The area of the membrane left un-wiped is later used to determine the depth of focus (Figure 2C and 2D).
14. Let inserts dry for at least 1hr
15. Invert insert and remove membrane using a scalpel blade cutting along the membranes edge.
16. Place membrane upper chamber-side down on a drop of immersion oil on a microscope slide.
17. Place another drop of immersion oil on top of the membrane and mount a coverslip on top
18. Identify the cells that have invaded through the membrane. Take 6 photos at 20x magnification per insert (Figure 2D). Count the number of invading cells and average the results across replicate wells.

References

1. Bernfield M, Gotte M, Park PW, et al. Functions of cell surface heparan sulfate proteoglycans. Annu Rev Biochem. 1999; 68:729–777. [PubMed: 10872465]
2. Esko JD, Lindahl U. Molecular diversity of heparan sulfate. J Clin Invest. 2001; 108(2):169–173. [PubMed: 11457867]
3. Habuchi H, Habuchi O, Kimata K. Sulfation pattern in glycosaminoglycan: does it have a code? Glycoconj J. 2004; 21(1–2):47–52. [PubMed: 15467398]
4. Dhoot GK, Gustafsson MK, Ai X, et al. Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science. 2001; 293(5535):1663–1666. [PubMed: 11533491]
5. Ai X, Do AT, Lozynska O, et al. QSulf1 remodels the 6-O sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J Cell Biol. 2003; 162(2):341–351. [PubMed: 12860968]
6. Freeman SD, Moore WM, Guiral EC, et al. Extracellular regulation of developmental cell signaling by XtSulf1. Dev Biol. 2008; 320(2):436–445. [PubMed: 18617162]
7. Ai X, Kitazawa T, Do AT, et al. SULF1 and SULF2 regulate heparan sulfate-mediated GDNF signaling for esophageal innervation. Development. 2007; 134(18):3327–3338. [PubMed: 17720696]
8. Fujita K, Takechi E, Sakamoto N, et al. HpSulf, a heparan sulfate 6-O-endosulfatase, is involved in the regulation of VEGF signaling during sea urchin development. Mech Dev. 2010; 127(3–4):235–245. [PubMed: 20036737]
9. Lui NS, van Zante A, Rosen SD, et al. SULF2 expression by immunohistochemistry and overall survival in oesophageal cancer: a cohort study. BMJ Open. 2012; 2(6)doi: 10.1136/bmjopen-2012-001624
10. Wade A, Robinson AE, Engler JR, et al. Proteoglycans and their roles in brain cancer. FEBS J. 2013; 280(10):2399–2417. DOI: 10.1111/febs.12109 [PubMed: 23281850]
11. Phillips JJ, Huillard E, Robinson AE, et al. Heparan sulfate sulfatase SULF2 regulates PDGFRalpha signaling and growth in human and mouse malignant glioma. J Clin Invest. 2012; 122(3):911–922. DOI: 10.1172/JCI58215 [PubMed: 22293178]
12. Dai Y, Yang Y, MacLeod V, et al. HSulf-1 and HSulf-2 are potent inhibitors of myeloma tumor growth in vivo. J Biol Chem. 2005; 280(48):40066–40073. [PubMed: 16192265]
13. Rosen SD, Lemjabbar-Alaoui H. Sulf-2: an extracellular modulator of cell signaling and a cancer target candidate. Expert Opin Ther Targets. 2010; 14(9):935–949. DOI: 10.1517/14728222.2010.504718 [PubMed: 20629619]

14. Morimoto-Tomita M, Uchimura K, Bistrup A, et al. Sulf-2, a proangiogenic heparan sulfate endosulfatase, is upregulated in breast cancer. Neoplasia. 2005; 7(11):1001–1010. [PubMed: 16331886]
15. Lemjabbar-Alaoui H, van Zante A, Singer MS, et al. Sulf-2, a heparan sulfate endosulfatase, promotes human lung carcinogenesis. Oncogene. 2010; 29(5):635–646. DOI: 10.1038/onc.2009.365 [PubMed: 19855436]
16. Nawroth R, van Zante A, Cervantes S, et al. Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells. PLoS One. 2007; 2(4):e392. [PubMed: 17460759]
17. Preusser M, de Ribaupierre S, Wohrer A, et al. Current concepts and management of glioblastoma. Ann Neurol. 2011; 70(1):9–21. DOI: 10.1002/ana.22425 [PubMed: 21786296]
18. Calvo F, Sahai E. Cell communication networks in cancer invasion. Curr Opin Cell Biol. 2011; 23(5):621–629. DOI: 10.1016/j.ceb.2011.04.010 [PubMed: 21570276]
19. Maltseva I, Chan M, Kalus I, et al. The SULFs, Extracellular Sulfatases for Heparan Sulfate, Promote the Migration of Corneal Epithelial Cells during Wound Repair. PLoS One. 2013; 8(8):e69642.doi: 10.1371/journal.pone.0069642 [PubMed: 23950901]
20. Peterson SM, Iskenderian A, Cook L, et al. Human Sulfatase 2 inhibits in vivo tumor growth of MDA-MB-231 human breast cancer xenografts. BMC Cancer. 2010; 10:427.doi: 10.1186/1471-2407-10-427 [PubMed: 20707913]
21. Johansson FK, Goransson H, Westermark B. Expression analysis of genes involved in brain tumor progression driven by retroviral insertional mutagenesis in mice. Oncogene. 2005; 24(24):3896–3905. [PubMed: 15750623]
22. Tang R, Rosen SD. Functional consequences of the subdomain organization of the sulfs. J Biol Chem. 2009; 284(32):21505–21514. DOI: 10.1074/jbc.M109.028472 [PubMed: 19520866]

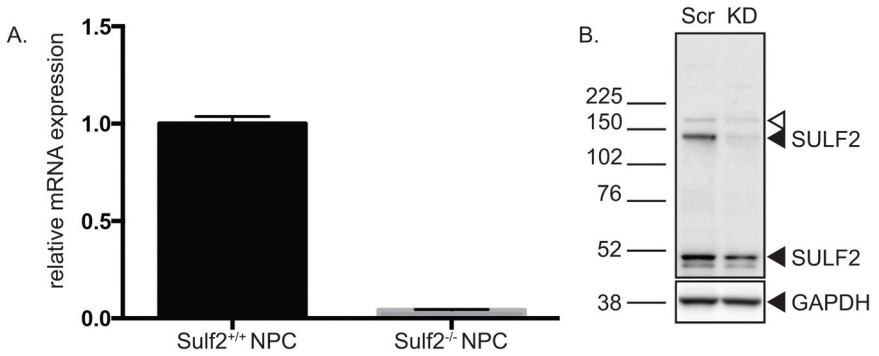

Figure 1. Assessment of SULF2 mRNA and protein expression

There is an absence of Sulf2 mRNA expression in NPC isolated from $Sulf2^{-/-}$ mice demonstrating the specificity of the primers for Sulf2 (**A**). Bars represent the mean +/− SEM of triplicate samples. For each sample the GAPDH Ct was subtracted from the Sulf2 Ct to generate a Delta Ct, this was averaged across technical triplicates, normalized to Sulf2 mRNA expression in Sulf2 wild type NPC ($Sulf2^{+/+}$), and expressed as relative quantification [2^-(normalized DeltaCt). In (**B**) specificity of the 2B4 mouse anti-Sulf2 antibody is demonstrated by the decrease in full length 140kD and C-terminal fragment 50kD SULF2 (black arrows) in U251 cells which have shRNA knockdown of SULF2 (KD) compared to a scrambled shRNA control (Scr) U251 cells. Equivalent quantities of protein are demonstrated with the use of GAPDH as a loading control. A high molecular weight non-specific band is indicated by the white arrow. The shRNA construct has been reported previously (16) (see Note 10).

[10]We would like to acknowledge and thank Drs. Steven D. Rosen and Mark S. Singer for their helpful advice and for providing the SULF2 knockdown constructs.

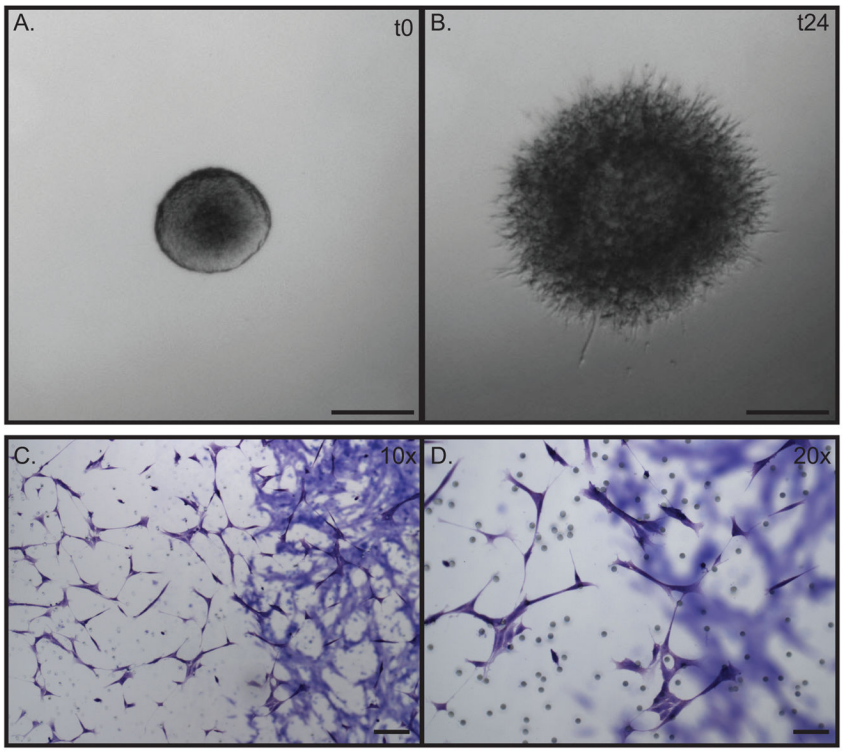

Figure 2. Examples of assays used to measure invasion in vitro

NPC spheroids imaged immediately after plating in matrigel are compact spheres (t 0) but 24 hours later (t 24) cells have invaded into the surrounding matrix (**A, B**). NPC migrating across the matrigel membrane toward the chemoattractant-containing lower chamber are stained purple in (**C**) and (**D**) at both 10x and 20x magnification. The non-wiped area of the membrane is evident on the right-hand-side and is not in the same focal plane as the cells that have migrated through the membrane. Scale bars are 50 μm (**A**) and (**B**), 100 μm (**C**) and 50 μm (**D**).