Published in final edited form as:

Cell Signal. 2012 January ; 24(1): 272–281. doi:10.1016/j.cellsig.2011.09.011.

Integrin $\alpha 9 \beta 1$ -mediated cell migration in glioblastoma via SSAT and Kir4.2 potassium channel pathway

Krishna Kumar Veeravalli^a, Shivani Ponnala^a, Chandramu Chetty^a, Andrew J. Tsung^b, Meena Gujrati^c, and Jasti S. Rao^{a,b,*}

^aDepartment of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, Peoria, IL 61656, U.S.A.

^bDepartment of Neurosurgery, University of Illinois College of Medicine at Peoria, Peoria, IL 61656, U.S.A.

^cDepartment of Pathology, University of Illinois College of Medicine at Peoria, Peoria, IL 61656, U.S.A.

Abstract

The $\alpha 9 \beta 1$ integrin accelerates cell migration through binding of the $\alpha 9$ cytoplasmic domain to SSAT, which catalyzes the catabolism of higher order polyamines, spermidine and spermine, to the lower order polyamine, putrescine. SSAT levels were downregulated at both the mRNA and protein levels by shRNA-mediated simultaneous knockdown of MMP-9 and uPAR/cathepsin B. In addition, we noted a prominent reduction in the expression of SSAT with MMP-9 and uPAR/cathepsin B knockdown in the tumor regions of 5310 injected nude mice brains. Further, SSAT knockdown in glioma xenograft cells significantly reduced their migration potential. Interestingly, MMP-9, uPAR and cathepsin B overexpression in these xenograft cells significantly elevated SSAT mRNA and protein levels. The migratory potential of MMP-9/uPAR/cathepsin B-overexpressed 4910 and 5310 cells was not affected by either glybenclamide (Kir 6.x inhibitor) or tertiapin-Q (Kir 1.1 and 3.x inhibitor) but instead was significantly inhibited by either barium or Kir4.2 siRNA treatments. Co-localization of $\alpha 9$ integrin with Kir4.2 was observed in both 4910 and 5310 xenograft cells. However, MMP-9 and uPAR/cathepsin B knockdown in these cells prominently reduced the co-localization of $\alpha 9$ with Kir4.2. Taken together, our results clearly demonstrate that $\alpha 9 \beta 1$ integrin-mediated cell migration utilizes SSAT and the Kir4.2 potassium channel pathway, and inhibition of the migratory potential of these glioma xenograft cells by simultaneous knockdown of MMP-9 and uPAR/cathepsin B could be attributed to the reduced SSAT levels and co-localization of $\alpha 9$ integrin with Kir4.2 inward rectifier potassium channels.

Keywords

Migration; Integrin; Glioma; Knockdown; Xenograft; Potassium channel

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author at : Department of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, One Illini Drive, Peoria, IL 61605, U.S.A.; phone (309) 671-3445; fax (309) 671-3442; jsrao@uic.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Cell migration is a fundamental process, which plays a central role in tumor metastasis. To migrate, cells must form leading and trailing edges, apply coordinated force in the direction of movement, and both adhere and release their hold on the substrate as they travel [1–5]. To perform these feats, migrating cells respond to a variety of factors such as extracellular matrix (ECM) molecules and growth factors, which engage cell surface receptors to initiate and maintain migration. One such family of receptors, the integrins, plays an important role in migration. The term integrin refers to a member of a family of matrix and cell-cell adhesion receptor proteins that exists at the cell surface as a dimer composed of an α and β subunit. The various combinations of α and β subunits exhibit ligand specificity and interact with various ECM molecules including fibronectin, collagens, laminins, and proteoglycans as well as intercellular adhesion molecules [6,7]. The classic role of integrins is to anchor cells to the ECM. However, they are known to participate in a variety of signaling pathways and to play important roles in fetal development, morphogenesis, cell migration, wound healing, and malignant transformation.

Most members of the integrin family are able to mediate cell migration; however, their mechanism for enhancing cell migration differs. Unlike other integrins, $\alpha 9 \beta 1$ has been proposed to utilize inducible nitric oxide synthase (iNOS)-nitric oxide and spermidine/spermine- N^1 -acetyl transferase (SSAT)-inward rectifier potassium channel (Kir) pathways along with common integrin signaling pathway proteins such as Src and FAK to transduce cell migration. $\alpha 9 \beta 1$ mediates enhanced cell migration, an effect that specifically depends on the $\alpha 9$ cytoplasmic domain [8,9]. The $\alpha 9$ subunit cytoplasmic domain requires binding of the enzyme SSAT in order to accelerate cell migration [8]. SSAT specifically catalyzes catabolism of the higher order polyamines, spermidine and spermine to the lower order polyamine, putrescine [10], thereby increasing intracellular levels of putrescine and decreasing those of spermidine and spermine [11,12]. Numerous studies have suggested that K^+ efflux is a critical factor in modulating cell migration [13–16]. Spermine and spermidine are potent blockers of outward potassium (K^+) currents from inward-rectifier K^+ (Kir) channels [17–19]. The long, positively charged polyamines, spermine (+4) and spermidine (+3) mediate rectification by binding to negatively charged residues in the channel pore [19,20]. The catabolized polyamine putrescine (+2) is less effective in blocking outward flow of K^+ ions [18,19].

Except for endothelial cells of vasculature, normal human brain tissue does not show the expression of $\alpha 9 \beta 1$ integrin in astrocytes, oligodendrocytes, and neurons [21]. The percentage of positively labeled anti- $\alpha 9$ antibody increased in correlation with glioma grade and was the highest in glioblastoma multiforme (GBM) tissue [21]. Recently, we have demonstrated the presence of $\alpha 9 \beta 1$ integrin expression in clinical glioblastoma samples and the involvement of $\alpha 9 \beta 1$ integrin in the migration potential of glioma xenograft cells [22]. We have clearly shown that blockade of the $\alpha 9 \beta 1$ integrin abolished the MMP-9/uPAR/cathepsin B-mediated cell migration in glioma xenograft cells. However, the molecular mechanisms underlying the MMP-9/uPAR/cathepsin B-mediated glioma cell migration via the $\alpha 9 \beta 1$ integrin are still unclear. Our recent study has also demonstrated that the simultaneous downregulation of MMP-9 and uPAR/cathepsin B using bicistronic constructs (MU-sh and MC-sh) effectively inhibited glioma xenograft cell migration when compared to their individual knockdowns [22]. In the present study, we aimed to investigate the involvement of SSAT-Kir channel pathway in the inhibition of glioma xenograft cell migration mediated by simultaneous downregulation of MMP-9 and uPAR/cathepsin B.

2. Materials and Methods

2.1. Ethics statement

The Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria, Peoria, IL approved all surgical interventions and post-operative animal care.

2.2. Chemicals and reagents

Glybenclamide (Glyb) and tertiapin-Q-trifluoroacetate (TPNQ) were obtained from Sigma (St. Louis, MO, USA). Anti-SSAT, anti- $\alpha 9$ integrin and anti-Kir4.2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti- $\beta 1$ integrin was obtained from Millipore (Billerica, MA, USA). Anti-uPAR was obtained from R&D Systems (Minneapolis, MN, USA). Anti-cathepsin B was obtained from Athens Research and Technology (Athens, GA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Novus Biologicals (Littleton, CO, USA).

2.3. Construction of shRNA- and gene-expressing plasmids

Plasmid shRNAs for MMP-9-uPAR (MU-sh) and MMP-9-cathepsinB (MC-sh) designed in our laboratory [23,24] were used to transfect the xenograft cells. Briefly, a pCDNA-3 plasmid with a human cytomegalovirus (CMV) promoter was used to construct the shRNA-expressing vectors. A pCDNA3-scrambled vector with an imperfect sequence, which does not form a perfect hairpin structure, was used as a control (SV-sh). MMP-9-expressing (M-fl) plasmid in the pDNR-CMV vector was designed and constructed in our laboratory whereas uPAR-expressing (U-fl) and cathepsin B-expressing (C-fl) plasmids were purchased from Origene (Rockville, MD, USA).

pSilencer™ 4.1-CMV vector obtained from Ambion (Austin, TX, USA) was used in the construction of the SSAT shRNA (SSAT-sh) and Kir4.2 shRNA (Kir-sh) expressing plasmids. Human SSAT target sequence (AAAGATCTGCTAGAAGATGGTTT) and Kir4.2 target sequence (AACTCCCTTCAAACACAAAGATT) were used to design the shRNA sequences of SSAT and Kir4.2, respectively. Inverted repeat sequences were synthesized for both SSAT and Kir4.2. The inverted repeats were laterally symmetrical making them self-complimentary with a 9-bp mismatch in the loop region. This 9-bp mismatch would aid in the loop formation of the shRNA. Oligonucleotides were heated in a boiling water bath in 6X SSC for 5 min and self-annealed by slow cooling to room temperature. The resulting annealed oligonucleotides were ligated to pSilencer at the BamHI and HindIII sites.

2.4. Cell culture and transfection conditions

The xenograft cell lines (4910 and 5310) were kindly provided by Dr. David James at the University of California, San Francisco. These xenografts were generated and maintained in mice and are highly invasive in the mouse brain [25]. 4910 and 5310 xenografts were maintained in RPMI 1640 buffer supplemented with 10% fetal bovine serum, 50 μ g/mL streptomycin and 50 U/mL penicillin at 37°C in a humidified atmosphere containing 5% CO₂. Xenografts were transfected with SV-sh, MU-sh, MC-sh, Kir-sh, SSAT-sh, M-fl, U-fl, or C-fl using Fugene® HD reagent obtained from Roche Diagnostics, (Indianapolis, IN, USA) according to the manufacturer's instructions.

2.5. Western blot analysis

Xenograft cells were transfected with SV-sh, MU-sh, MC-sh, M-fl, U-fl and C-fl for 72 h. Cells were collected and lysed in RIPA buffer [50 mmol/mL Tris-HCl (pH 8.0), 150 mmol/

mL NaCl, 1% IGEPAL, 0.5% sodium deoxycholate, 0.1% SDS] containing 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 µg/mL aprotinin, 10 µg/mL leupeptin and resolved via SDS-PAGE. After overnight transfer onto nitrocellulose membranes, blots were blocked with 5% non-fat dry milk in PBS and 0.1% Tween-20. Blots were then incubated with primary antibody, followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were visualized using chemiluminescence ECL Western blotting detection reagents on Hyperfilm-MP autoradiography film obtained from Amersham (Piscataway, NJ, USA). Membrane fractions were obtained from controls and cells transfected with SV-sh, MU-sh or MC-sh using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit obtained from Thermo Scientific (Rockford, IL, USA) and subjected to Western blot analysis. GAPDH (housekeeping gene) antibody was used to verify that similar amounts of protein were loaded in all lanes.

2.6. Immunoprecipitation assay

Immunoprecipitation assays were carried out by incubating approximately 900 µg of membrane fractions with antibody (anti-uPAR or anti-cathepsin B) overnight at 4°C on a rotating shaker. Protein A/G agarose beads (Milenyi Biotec, Auburn, CA) were added to the above complex and incubated for 1 hour on ice. Immunoprecipitates were eluted using µMac columns according to the manufacturer's instructions and were processed for Western blot (immunoblot) analysis with appropriate primary (anti-α9 integrin, anti-β1 integrin, and anti-SSAT) and secondary antibodies.

2.7. Reverse transcription PCR analysis

Total cell RNA was isolated from untreated 4910 and 5310 cells and from those transfected with SV-sh, MU-sh, MC-sh, M-fl, U-fl, or C-fl. Approximately 1 µg of total RNA from each sample was synthesized into cDNA following the manufacturer's instructions using the Transcriptor First Strand cDNA Synthesis Kit obtained from Roche Diagnostics (Indianapolis, IN, USA). Forward and reverse primer sequences used in the present study are listed in Table 1. Reverse transcriptase-PCR (RT-PCR) was set up using the following PCR cycle: 95°C for 5 min, (95°C for 30 sec, 55–60°C for 30 sec, and 72°C for 30 sec) × 30 cycles, and 72°C for 10 min. PCR products were resolved on a 1.6% agarose gel, visualized, and photographed under UV light.

2.8. Wound healing assay

To study cell migration, we seeded xenograft cells (4910 and 5310) at a density of 2×10^6 in a 6-well plate and transfected the cells with M-fl, U-fl, or C-fl for 72 hours. Then, a straight scratch was made in individual wells with a 200 µL pipette tip. This point was considered the "0 hour," and the width of the wound was photographed under the microscope. After 16–24 hours, the cells were checked for wound healing and photographed under the microscope. Wound healing was measured by calculating the reduction in the width of the wound after incubation. The effect of barium, Glyb, or TPNQ treatments on M-fl-, U-fl- or C-fl-mediated migration in these xenograft cells was assessed by adding barium (16 µM final concentration), Glyb (1 µM final concentration) and TPNQ (100 nM final concentration) at "0 h" to the appropriate wells containing xenograft cells transfected with M-fl, U-fl, or C-fl. We also studied the effect of Kir-sh on M-fl-, U-fl- or C-fl-mediated migration by transfecting the xenograft cells with Kir-sh at 24 hours after the M-fl, U-fl, or C-fl transfection. In another set of experiments, we performed this wound healing assay in control and SSAT shRNA-treated 4910 and 5310 cells.

2.9. Immunocytochemistry

4910 and 5310 cells (1×10^4) were seeded on 2-well chamber slides, incubated for 24 hours, and transfected with SV-sh, MU-sh, or MC-sh for 72 hours. Then, cells were fixed with 10% buffered formalin phosphate and incubated with 1% bovine serum albumin in PBS at room temperature for 1 hour for blocking. After the slides were washed with PBS, primary antibodies were added at a concentration of 1:100. The slides were incubated overnight at 4°C and washed three times with PBS to remove excess primary antibody. Cells were then incubated with Alexa Fluor® 594 (goat anti-rabbit IgG, red), Alexa Fluor® 488 (goat anti-rabbit IgG, green), or Alexa Fluor® 488 (donkey anti-goat IgG, green) fluorescent-labeled secondary antibodies for 1 hour at room temperature. The slides were then washed another three times with PBS and covered with glass coverslips, and fluorescent photomicrographs were obtained.

2.10. Intracranial administrations in nude mice

The Institutional Animal Care and Use Committee of the University of Illinois College of Medicine at Peoria, Peoria, IL approved all surgical interventions and post-operative animal care. 4910 and 5310 xenografts were trypsinized and resuspended in serum-free medium at a concentration of 0.2×10^5 cells/ μ L. Nude mice were injected intracerebrally with a 10 μ l aliquot (0.2×10^5 cells/ μ L) under isoflurane anesthesia with the aid of a stereotactic frame. After two weeks, mice were separated into three groups in each cell line. The first, second, and third groups served as the control, MU-sh-treated (150 μ g), and MC-sh-treated (150 μ g) groups, respectively. MU-sh and MC-sh plasmid DNAs were injected into the brains of nude mice using Alzet mini pumps at the rate of 0.2 μ L/h. The concentration of the plasmid solution was 2 μ g/ μ L (100 μ l per mouse, six mice in each group). After five weeks, the mice were sacrificed by intracardiac perfusion, first with PBS and then with 4% paraformaldehyde in normal saline. The brains were removed, stored in 4% paraformaldehyde, processed, embedded in paraffin, and sectioned (5 μ m thick) using a microtome. Paraffin-embedded sections were processed for immunohistochemical analysis.

2.11. Immunohistochemistry

Paraffin-embedded brain sections (5 μ m thick) from control and treatment groups were deparaffinized following standard protocol. The sections were rinsed with PBS and treated with 1% BSA in PBS to prevent non-specific staining and incubated with anti-SSAT antibody (1:50 dilution) at 4°C overnight. The sections were then washed in PBS and incubated with appropriate HRP-conjugated secondary antibody for 1 hour at room temperature. After 1 hour, sections were washed in PBS and incubated in DAB for 30 min. The slides were further washed with sterile water and dehydrated. The slides were then covered with glass cover slips and photomicrographs were obtained.

2.12. Densitometry

Densitometry was performed using Image J Software (National Institutes of Health) to quantify the band intensities obtained from Western blot analysis and RT-PCR. Data represent average values from 3 separate experiments.

2.13. Statistical analysis

Statistical comparisons were performed using Graph Pad Prism software (version 3.02). Quantitative data from Western blot analysis and wound healing assays was evaluated for statistical significance using one-way ANOVA. Bonferroni's *post hoc* test (multiple comparison tests) was used to compare any statistical significance between groups. Differences in the values were considered significant at $p < 0.05$.

3. Results

3.1. Effects of silencing or overexpressing proteases on SSAT levels

Silencing MMP-9 and uPAR or MMP-9 and cathepsin B using bicistronic shRNA-expressing plasmid constructs (MU-sh and MC-sh, respectively) significantly reduced SSAT expression at both mRNA and protein levels in 4910 and 5310 glioma xenograft cells (Fig. 1A, 1B). Immunocytochemical analysis of 4910 and 5310 xenograft cells transfected with MU-sh and MC-sh bicistronic constructs also showed the reduced expression of SSAT as compared to control and SV-sh-transfected cells (Fig. 1C). Similar changes in SSAT protein expression were noticed in the membrane fractions of 4910 and 5310 cells transfected with either MU-sh or MC-sh (Fig. 2A). Further, translation of these *in vitro* results were noticed *in vivo* where MU-sh and MC-sh treatments prominently reduced SSAT expression in pre-established intracranial tumors in nude mice (Fig. 2B). As expected, overexpression of MMP-9, uPAR, and cathepsin B in these xenograft cells significantly increased SSAT expression at both the mRNA and protein levels (Fig. 3B, 3C).

3.2. $\alpha 9\beta 1$ integrin-mediated migration via SSAT-Kir4.2 channel

Our recent study has clearly demonstrated that MU-sh and MC-sh treatments inhibit adhesion, migration, invasion, and proliferation of 4910 and 5310 xenograft cells *in vitro* and reduce tumor growth *in vivo* [22]. We also demonstrated the critical role of the $\alpha 9\beta 1$ integrin in MMP-9/uPAR/cathepsin B-mediated migration [22]. In agreement with our earlier findings, in the present study, we noticed reduced protein expression of the $\alpha 9$ integrin and the $\beta 1$ integrin in the membrane fractions of MU-sh- and MC-sh-treated 4910 and 5310 glioma xenograft cells (Fig. 2A). The interaction between the $\alpha 9$ cytoplasmic domain and SSAT is crucial for $\alpha 9\beta 1$ -dependent enhancement of cell migration [26]. As expected, silencing SSAT using SSAT shRNA significantly reduced the migration potential of 4910 and 5310 xenograft cells (Fig. 4A, 4B). It was reported that the $\alpha 9\beta 1$ integrin enhances cell migration by polyamine-mediated modulation of an inward-rectifier potassium channel [26]. In the present study, RT-PCR analysis of cDNAs followed by agarose gel electrophoresis demonstrated the presence of several inward-rectifier potassium channels (Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.2, Kir3.3, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1, and Kir6.2) except Kir1.1 in 4910 and 5310 glioma xenograft cells (Fig. 3D). We investigated the effects of glybenclamide, an inhibitor of ATP-activated K^+ channels [27,28], which exist as heteromultimers of sulfonylurea receptor subunits and Kir6.1 or Kir6.2 subunits [29], or tertiapin-Q-trifluoroacetate (TPNQ), an inhibitor of Kir1.1 and Kir3.x channels [30] on the migration potential of MMP-9/uPAR/cathepsin B-overexpressed 4910 and 5310 xenograft cells. Neither of these inhibitors had any effect on migration of these MMP-9/uPAR/cathepsin B-overexpressing 4910 and 5310 xenograft cells, suggesting that Kir1.1, Kir3.x, Kir6.1 and Kir6.2 potassium channels do not play any role in $\alpha 9\beta 1$ -dependent enhancement of migration (Fig. 5A, 5B). However, barium (Ba^{2+}), at concentrations that specifically inhibit Kir channel function [31], and knockdown of Kir4.2 (by using Kir4.2 shRNA) in MMP-9/uPAR/cathepsin B-overexpressed 4910 and 5310 xenograft cells significantly reduced the migration potential of these xenograft cells compared to their respective controls, indicating the role of Kir4.2 inward-rectifier potassium channel in glioma cell migration (Fig. 5A, 5B). These results are in agreement with our earlier findings, wherein blockade of the $\alpha 9\beta 1$ integrin by anti- $\alpha 9\beta 1$ antibody significantly reduced the migration potential of MMP-9/uPAR/cathepsin B-overexpressed 5310 xenograft cells [22].

3.3. Interactions among uPAR, cathepsin B, MMP-9, $\alpha 9\beta 1$ integrin, SSAT and Kir4.2

Previously, we have reported on the interaction or association among uPAR, MMP-9 and cathepsin B [24,32–34]. Immunoprecipitation studies revealed the interaction among these

molecules in 4910 and 5310 glioma xenograft cells (data not shown). The expression of one of these molecules has a direct or indirect influence on the expression of other molecules in these glioma xenograft cells [22]. In agreement to this observation, the simultaneous downregulation of two of these molecules was found to be more efficacious than their individual knockdowns in reducing the adhesion, migration, proliferation, and invasion potential of 4910 and 5310 xenograft cells [22]. uPAR lacks transmembrane and intracellular domains and so requires transmembrane co-receptors for signaling. Integrins are essential uPAR signaling co-receptors [35]. uPAR localizes to integrin-containing adhesion complexes [36] and co-immunoprecipitates with integrins [37–41]. Immunoprecipitation of the membrane fractions of 4910 and 5310 xenograft cells (control and transfected with SV-sh, MU-sh and MC-sh) with anti-uPAR and anti-cathepsin B antibodies followed by immunoblot analysis revealed that MU-sh and MC-sh treatments reduced $\alpha 9$ integrin and $\beta 1$ integrin levels along with reductions in SSAT protein expression (Fig. 3A). This finding supports the interaction between the $\alpha 9$ cytoplasmic domain and SSAT, which is crucial for $\alpha 9\beta 1$ -dependent enhancement of cell migration [26]. However, association of SSAT with the $\alpha 9$ cytoplasmic domain to functionally modulate Kir4.2 inward-rectifier potassium channel by altering local concentrations of polyamines, $\alpha 9$ and Kir4.2 would need to be in close physical apposition. The $\alpha 9$ integrin and Kir4.2 co-distribute in focal adhesions at the leading edge of migrating cells [26]. In the present study, we noticed co-localization of the $\alpha 9$ integrin with Kir4.2 in untreated 4910 and 5310 xenograft cells (Fig. 6). However, the co-localization of these molecules in the same xenograft cells was prominently reduced with the simultaneous knockdown of MMP-9 and uPAR/cathepsin B (Fig. 6).

4. Discussion

Integrins play an important role in cell migration, in part by adhering to the ECM and also by activating intracellular cascades that promote actin polymerization involved in lamellipodial extension [1–5]. The integrin $\alpha 9$ subunit has been identified as a heterodimer, only in association with the $\beta 1$ subunit (Palmer et al., 1993). The α subunit of this integrin consists of a single polypeptide, whereas most α subunits composed of two chains. Integrin $\alpha 9\beta 1$ is a relatively new addition to the $\beta 1$ integrin subfamily [42] and through homologous sequence it forms a unique subfamily with $\alpha 4\beta 1$ [43]. The $\alpha 9\beta 1$ integrin is widely distributed in the human body and is expressed on many types of cells including epithelial cells, muscle cells, neutrophils and endothelial cells [42,44–46]. Most members of the integrin family are able to mediate cell migration, but two related members, $\alpha 4\beta 1$ and $\alpha 9\beta 1$ integrins, are able to both increase migration and inhibit cell spreading [9,47]. Recently, we have demonstrated the presence of $\alpha 9\beta 1$ integrin expression in clinical glioblastoma samples and the involvement of $\alpha 9\beta 1$ integrin in the migration potential of glioma xenograft cells [22]. Our recent study has shown that the treatment of glioma xenograft cells with anti- $\alpha 9\beta 1$ antibody significantly reduced MMP-9/uPAR/cathepsin B-mediated migration in these cells and thereby indicated the role of $\alpha 9\beta 1$ integrin signaling in cell migration mediated by these molecules [22]. Unlike $\alpha 4\beta 1$, $\alpha 9\beta 1$ has been shown to be paxillin-independent [9]. $\alpha 9\beta 1$ utilizes the SSAT-Kir potassium channel pathway along with common integrin signaling pathway proteins, such as Src and FAK, to transduce cell migration. SSAT was necessary and sufficient for $\alpha 9\beta 1$ -mediated enhanced cell migration. Although the role of $\alpha 9\beta 1$ mediated potassium channel rectification in tumor cells is not yet clear; gene expression profiling has shown ~3-fold increase in SSAT expression in a late stage (metastatic) colon carcinoma cell line, compared to early stage carcinomas (Futschik et al., 2002). Overexpression of SSAT enhanced migration, and siRNA knockdown of SSAT inhibited $\alpha 9\beta 1$ -dependent migration without affecting cell adhesion or migration mediated by other integrins [8]. MU-sh and MC-sh treatments significantly reduced the migration potential of glioma xenograft cells along with $\alpha 9\beta 1$ integrin levels [22]. In addition, in the present study,

MU-sh and MC-sh treatments significantly reduced SSAT levels in the glioma xenograft cells *in vitro* and *in vivo* in nude mice with pre-established tumors. MMP-9/uPAR/cathepsin B-mediated cell migration in these xenograft cells [22] is associated with increases in SSAT protein expression (Fig. 3B). Furthermore, SSAT knockdown in these xenograft cells significantly reduced their migration potential. Taken together, our results suggest that $\alpha 9\beta 1$ integrin-mediated cell migration in glioma xenograft cells is via SSAT. The interaction of SSAT with the $\alpha 9$ cytoplasmic domain is required for $\alpha 9\beta 1$ -mediated migration, although whether this involves increased local recruitment of SSAT or increased activity of the enzyme is not known [8]. In the present study, reductions of $\alpha 9$ and $\beta 1$ integrin levels along with SSAT in the membrane fractions of MU-sh- and MC-sh-treated glioma xenograft cells immunoprecipitated with uPAR and cathepsin B indicated the existence of physical interactions among these molecules.

How does activation of SSAT enhance cell migration? By using mutant SSAT, researchers have shown that catalytically inactive SSAT does not enhance migration, indicating that the enzymatic activity of SSAT is required [26]. SSAT specifically catalyzes catabolism of the higher order polyamines, spermidine and spermine, to the lower order polyamine, putrescine [10]. In their study, deHart, *et al.*, [26] unveiled a new mechanism of cellular migration that involves integrin and polyamines coupling to inward-rectifier potassium (Kir) channels. Kir channels are blocked by intracellular polyamines and Mg^{2+} . Higher-order polyamines such as spermine and spermidine are more potent blockers of these Kir channels compared to lower-order polyamine, putrescine [19,48,49]. The cytoplasmic tail of $\alpha 9$ interacts with SSAT and facilitates a localized enzymatic processing of spermine and spermidine into putrescine, thereby creating a spatially restricted impairment of K^+ ion channel rectification leading to increased K^+ ion efflux, and enhances cell migration [8,26]. In the present study, we noticed the presence of several Kir channels in glioma xenograft cells. MMP-9/uPAR/cathepsin B-mediated migration in glioma xenograft cells was not affected by either glybenclamide or tertiapin-Q treatments, suggesting that Kir1.1, Kir3.x, or Kir6.x potassium channels do not play any role in $\alpha 9\beta 1$ -dependent enhancement of migration. However, barium (Ba^{2+}), a non-specific inhibitor of Kir channels, significantly inhibited MMP-9/uPAR/cathepsin B-mediated migration. Similar kinds of reductions in migration potential were noticed in Kir4.2 knocked down xenograft cells. These results support the earlier reported data wherein the authors have shown the inhibition of $\alpha 9$ -dependent cell migration in Kir4.2 knocked down cells [26]. As mentioned earlier, association of SSAT with the $\alpha 9$ cytoplasmic domain to functionally modulate Kir4.2, $\alpha 9$ and Kir4.2 would need to be in close physical apposition. Kir4.2 was co-localized with $\alpha 9$ integrin in untreated xenograft cells whereas this colocalization was absent in MU-sh- and MC-sh-treated xenograft cells.

5. Conclusion

ShRNA-mediated silencing of MMP-9 and uPAR/cathepsin B (bicistronic constructs) reduce SSAT expression both at mRNA and protein levels *in vitro* in glioma xenograft cells and *in vivo* in nude mice pre-injected with xenograft cells. Bicistronic construct treatment reduces the physical interaction of $\alpha 9\beta 1$ and SSAT with uPAR/cathepsin B. SSAT levels upregulate after uPAR, MMP-9 or cathepsin B overexpression in glioma xenograft cells and SSAT knockdown in xenograft cells reduce their migration potential. Kir4.2 inward rectifier potassium channel knockdown inhibits uPAR, MMP-9 or cathepsin B-mediated glioma cell migration. Further, bicistronic construct treatment reduces the co-localization of $\alpha 9$ integrin with Kir4.2 in these xenograft cells. In summary, the results of our study identify a novel mechanism of integrin-dependent glioma cell migration ($\alpha 9\beta 1$ integrin–SSAT–Kir4.2 potassium channel pathway) and its control by the simultaneous knockdown of MMP-9 and uPAR/cathepsin B.

Highlights

- ShRNA-mediated silencing of MMP-9 and uPAR/cathepsin B (bicistronic constructs) reduce SSAT expression both at mRNA and protein levels *in vitro* in glioma xenograft cells and *in vivo* in nude mice pre-injected with xenograft cells.
- Bicistronic construct treatment reduces the physical interaction of $\alpha 9\beta 1$ and SSAT with uPAR/cathepsin B.
- SSAT levels upregulate after uPAR, MMP-9 or cathepsin B overexpression in glioma xenograft cells and SSAT knockdown in xenograft cells reduce their migration potential.
- Kir4.2 inward rectifier potassium channel knockdown inhibits uPAR, MMP-9 or cathepsin B-mediated glioma cell migration.
- Bicistronic construct treatment reduces the co-localization of $\alpha 9$ integrin with Kir4.2 in glioma xenograft cells.

Acknowledgments

We thank Noorjehan Ali for technical assistance, Shellee Abraham and Marie McWhirter for manuscript preparation, and Diana Meister and Sushma Jasti for manuscript review.

This research was supported by a grant from National Institute of Neurological Disorders and Stroke (NINDS), NS047699 (to J.S.R.). The contents are solely the responsibility of the authors and do not necessarily represent the official views of National Institute of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Berrier AL, Yamada KM. J Cell Physiol. 2007; 213:565–573. [PubMed: 17680633]
2. Morgan MR, Humphries MJ, Bass MD. Nat Rev Mol Cell Biol. 2007; 8:957–969. [PubMed: 17971838]
3. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Science. 2003; 302:1704–1709. [PubMed: 14657486]
4. Rose DM, Alon R, Ginsberg MH. Immunol Rev. 2007; 218:126–134. 126–134. [PubMed: 17624949]
5. Vicente-Manzanares M, Webb DJ, Horwitz AR. J Cell Sci. 2005; 118:4917–4919. [PubMed: 16254237]
6. Arnaout MA, Goodman SL, Xiong JP. Curr Opin Cell Biol. 2002; 14:641–651. [PubMed: 12231361]
7. Humphries JD, Byron A, Humphries MJ. J Cell Sci. 2006; 119:3901–3903. [PubMed: 16988024]
8. Chen C, Young BA, Coleman CS, Pegg AE, Sheppard D. J Cell Biol. 2004; 167:161–170. [PubMed: 15479742]
9. Young BA, Taooka Y, Liu S, Askins KJ, Yokosaki Y, Thomas SM, Sheppard D. Mol Biol Cell. 2001; 12:3214–3225. [PubMed: 11598204]
10. Casero RA Jr, Pegg AE. FASEB J. 1993; 7:653–661. [PubMed: 8500690]
11. McCloskey DE, Coleman CS, Pegg AE. J Biol Chem. 1999; 274:6175–6182. [PubMed: 10037702]
12. Vujcic S, Halmekyto M, Diegelman P, Gan G, Kramer DL, Janne J, Porter CW. J Biol Chem. 2000; 275:38319–38328. [PubMed: 10978316]
13. Cruse G, Duffy SM, Brightling CE, Bradding P. Thorax. 2006; 61:880–885. [PubMed: 16809411]
14. Da Silva-Santos JE, Santos-Silva MC, Cunha FQ, Assreuy J. J Pharmacol Exp Ther. 2002; 300:946–951. [PubMed: 11861802]

15. Rao JN, Platoshyn O, Li L, Guo X, Golovina VA, Yuan JX, Wang JY. *Am J Physiol Cell Physiol.* 2002; 282:C885–C898. [PubMed: 11880277]
16. Schwab A, Reinhardt J, Schneider SW, Gassner B, Schuricht B. *Cell Physiol Biochem.* 1999; 9:126–132. [PubMed: 10494026]
17. Ficker E, Taglialatela M, Wible BA, Henley CM, Brown AM. *Science.* 1994; 266:1068–1072. [PubMed: 7973666]
18. Lopatin AN, Makhina EN, Nichols CG. *Nature.* 1994; 372:366–369. [PubMed: 7969496]
19. Oliver D, Baukrowitz T, Fakler B. *Eur J Biochem.* 2000; 267:5824–5829. [PubMed: 10998040]
20. Bichet D, Haass FA, Jan LY. *Nat Rev Neurosci.* 2003; 4:957–967. [PubMed: 14618155]
21. Brown MC, Staniszevska I, Lazarovici P, Tuszynski GP, Del Valle L, Marcinkiewicz C. *Neuro Oncol.* 2008; 10:968–980. [PubMed: 19074980]
22. Veeravalli KK, Chetty C, Ponnala S, Gondi CS, Lakka SS, Fassett D, Klopfenstein JD, Dinh DH, Gujrati M, Rao JS. *PLoS One.* 2010; 5:e11583. [PubMed: 20657647]
23. Lakka SS, Gondi CS, Yanamandra N, Olivero WC, Dinh DH, Gujrati M, Rao JS. *Oncogene.* 2004; 23:4681–4689. [PubMed: 15122332]
24. Lakka SS, Gondi CS, Dinh DH, Olivero WC, Gujrati M, Rao VH, Sioka C, Rao JS. *J Biol Chem.* 2005; 280:21882–21892. [PubMed: 15824107]
25. Giannini C, Sarkaria JN, Saito A, Uhm JH, Galanis E, Carlson BL, Schroeder MA, James CD. *Neuro-oncol.* 2005; 7:164–176. [PubMed: 15831234]
26. deHart GW, Jin T, McCloskey DE, Pegg AE, Sheppard D. *Proc Natl Acad Sci USA.* 2008; 105:7188–7193. [PubMed: 18480266]
27. Akar F, Uydes-Dogan BS, Buharalioglu CK, Abban G, Heinemann A, Holzer P, van de Voorde J. *Eur J Pharmacol.* 1999; 374:461–470. [PubMed: 10422791]
28. Delaey C, van de Voorde J. *Eur J Pharmacol.* 1997; 325:41–46. [PubMed: 9151937]
29. Mikhailov MV, Campbell JD, de Wet H, Shimomura K, Zadek B, Collins RF, Sansom MS, Ford RC, Ashcroft FM. *EMBO J.* 2005; 24:4166–4175. [PubMed: 16308567]
30. Jin W, Lu Z. *Biochemistry.* 1999; 38:14286–14293. [PubMed: 10572003]
31. Quayle JM, Standen NB, Stanfield PR. *J Physiol.* 1988; 405:677–697. 677–697. [PubMed: 3267155]
32. Gondi CS, Kandhukuri N, Kondraganti S, Gujrati M, Olivero WC, Dinh DH, Rao JS. *Mol Cancer Ther.* 2006; 5:3197–3208. [PubMed: 17172424]
33. Lakka SS, Gondi CS, Rao JS. *Brain Pathol.* 2005; 15:327–341. [PubMed: 16389945]
34. Rao JS. *Nat Rev Cancer.* 2003; 3:489–501. [PubMed: 12835669]
35. Smith HW, Marshall CJ. *Nat Rev Mol Cell Biol.* 2010; 11:23–36. [PubMed: 20027185]
36. Myohanen HT, Stephens RW, Hedman K, Tapiovaara H, Ronne E, Hoyer-Hansen G, Dano K, Vaheri A. *J Histochem Cytochem.* 1993; 41:1291–1301. [PubMed: 8394852]
37. Aguirre-Ghiso JA, Kovalski K, Ossowski L. *J Cell Biol.* 1999; 147:89–104. [PubMed: 10508858]
38. Bohuslav J, Horejsi V, Hansmann C, Stockl J, Weidle UH, Majdic O, Bartke I, Knapp W, Stockinger H. *J Exp Med.* 1995; 181:1381–1390. [PubMed: 7535337]
39. Carriero MV, Del Vecchio S, Capozzoli M, Franco P, Fontana L, Zannetti A, Botti G, D'Aiuto G, Salvatore M, Stoppelli MP. *Cancer Res.* 1999; 59:5307–5314. [PubMed: 10537314]
40. Degryse B, Resnati M, Czekay RP, Loskutoff DJ, Blasi F. *J Biol Chem.* 2005; 280:24792–24803. [PubMed: 15863511]
41. Wei Y, Eble JA, Wang Z, Kreidberg JA, Chapman HA. *Mol Biol Cell.* 2001; 12:2975–2986. [PubMed: 11598185]
42. Vlahakis NE, Young BA, Atakilit A, Sheppard D. *J Biol Chem.* 2005; 280:4544–4552. [PubMed: 15590642]
43. Hibi K, Yamakawa K, Ueda R, Horio Y, Murata Y, Tamari M, Uchida K, Takahashi T, Nakamura Y, Takahashi T. *Oncogene.* 1994; 9:611–619. [PubMed: 8290272]
44. Palmer EL, Ruegg C, Ferrando R, Pytela R, Sheppard D. *J Cell Biol.* 1993; 123:1289–1297. [PubMed: 8245132]
45. Tarui T, Miles LA, Takada Y. *J Biol Chem.* 2001; 276:39562–39568. [PubMed: 11514539]

46. Staniszewska I, Zaveri S, Del Valle L, Oliva I, Rothman VL, Croul SE, Roberts DD, Mosher DF, Tuszyński GP, Marcinkiewicz C. *Circ Res*. 2007; 100:1308–1316. [PubMed: 17413041]
47. Liu S, Slepak M, Ginsberg MH. *J Biol Chem*. 2001; 276:37086–37092. [PubMed: 11477105]
48. Lu Z. *Annu Rev Physiol*. 2004; 66:103–129. 103–129. [PubMed: 14977398]
49. Nichols CG, Lopatin AN. *Annu Rev Physiol*. 1997; 59:171–191. 171–191. [PubMed: 9074760]

Fig. 1.

Effect of treatment with bicistronic constructs (MU-sh and MC-sh) on spermidine/spermine- N^1 -acetyl transferase (SSAT) levels *in vitro* in glioma xenograft cells. (A) mRNA and protein expression of SSAT in 4910 and 5310 glioma xenograft cells. RT-PCR of 4910 and 5310 cells transfected with scrambled vector (SV-sh), MMP-9/uPAR plasmid shRNA (MU-sh), or MMP-9/cathepsin B plasmid shRNA (MC-sh) was performed following standard protocols. The effect of SV-sh, MU-sh and MC-sh treatments on SSAT protein levels in xenograft cells was also evaluated by Western blot analysis (n=3). GAPDH was used as a loading control in both RT-PCR and Western blot analyses. (B) Quantification of the bands by densitometry analysis using Image J software revealed significant reductions in SSAT

protein expressions after MU-sh and MC-sh treatments (n=3). Values shown are the mean ($\pm$ SEM). * p <0.05 vs. control. (C) The effect of SV-sh, MU-sh and MC-sh treatments on 4910 and 5310 cells was also evaluated by immunocytochemical analysis. Green fluorescence indicates SSAT protein expression.

Fig. 2.

Effect of treatment with bicistronic constructs (MU-sh and MC-sh) on $\alpha 9$ integrin, $\beta 1$ integrin and spermidine/spermine- N^1 -acetyl transferase (SSAT) levels *in vitro* and on SSAT levels *in vivo* in nude mice pre-injected with 5310 cells. (A) The effect of scrambled vector (SV-sh), MMP-9/uPAR plasmid shRNA (MU-sh) and MMP-9/cathepsin B plasmid shRNA (MC-sh) treatments on $\alpha 9$ integrin, $\beta 1$ integrin and SSAT protein levels in the membrane fractions of 4910 and 5310 xenograft cells was evaluated by Western blot analysis (n=3). (B) Immunohistochemical comparison of the expression of SSAT in control, MU-sh- and MC-sh-treated nude mice, which were pre-injected (intracerebrally) with 5310 cells

(0.2×10^6 cells). SSAT expression (brown color) was noticed in the brain sections of control animals (n=6).

Fig. 3.

Immunoprecipitation and RT-PCR and Western blot analysis of 4910 and 5310 cells. (A) Membrane fractions from control, scrambled vector (SV-sh)-, MMP-9/uPAR plasmid shRNA (MU-sh)-, and MMP-9/cathepsin B plasmid shRNA (MC-sh)-transfected 4910 and 5310 cells were immunoprecipitated with either uPAR or cathepsin B (Cath B) and then subjected to Western blot analysis with $\alpha 9$ integrin, $\beta 1$ integrin, and/or spermidine/spermine- N^1 -acetyl transferase (SSAT) ($n=3$). The presence of protein bands in control and SV-sh-transfected 4910 and 5310 cells indicate the physical interactions among these molecules. (B) Effect of uPAR, MMP-9 and cathepsin B overexpression on SSAT levels *in vitro* in 4910 and 5310 glioma xenograft cells. RT-PCR of 4910 and 5310 cells transfected

with full-length uPAR (U-fl), MMP-9 (M-fl) and cathepsin B (C-fl) plasmid DNAs was performed following standard protocol (n=3). The effect of U-fl, M-fl and C-fl treatments on SSAT protein levels in glioma xenograft cells was also evaluated by Western blot analysis (n=3). β -actin and GAPDH were used as loading controls in RT-PCR and Western blot analyses, respectively. (C) Quantification of the protein bands by densitometry analysis using Image J software revealed significant increases in SSAT protein expression after U-fl, M-fl and C-fl treatments (n=3). Values shown are the mean ($\pm$ SEM). * p <0.05 vs. control. (D) Expression of various inward- rectifier potassium channels at the mRNA level in normal glioma xenograft cells. Kir1.1, Kir2.1, Kir2.2, Kir2.3, Kir2.4, Kir3.1, Kir3.2, Kir3.3, Kir3.4, Kir4.1, Kir4.2, Kir5.1, Kir6.1 and Kir6.2 expression at the mRNA level in normal 4910 and 5310 glioma xenograft cells was determined by RT-PCR analysis performed following standard protocol (n=3).

Fig. 4.

Effect of SSAT knockdown on the migration potential of 4910 and 5310 cells. (A) 4910 and 5310 cells were cultured in six-well plates and transfected with spermidine/spermine- N^1 -acetyl transferase (SSAT) shRNA (SSAT-sh) plasmid. 72 hours after transfection, a straight scratch was made in individual wells with a 200- μ L pipette tip. This point was considered as "0 hr," and the width of the wound was photographed under a microscope. Between 16 to 24 hours, the cells were checked for wound healing and again photographed under a microscope. (B) Quantification of wound healing assay results (n=3). Error bars indicate SEM. * p <0.05 vs. control.

Fig. 5.

Wound healing assay in 4910 and 5310 glioma xenograft cells. (A) 4910 and 5310 cells were cultured in six-well plates and transfected with full-length uPAR (U-fl), MMP-9 (M-fl) and cathepsin B (C-fl) plasmid DNAs. 24 hours after transfection, one group from each cell line transfected with M-fl, U-fl and C-fl was again transfected with Kir4.2 plasmid shRNA (Kir-sh). 72 hours after transfection, a straight scratch was made in individual wells with a 200- μ L pipette tip. This point was considered as "0 hr," and the width of the wound was photographed under a microscope. At this point, three more groups (other than control and Kir-sh-transfected groups) from each cell line transfected with M-fl, U-fl and C-fl were treated with barium (Ba^{2+}), glybenclamide (Glyb) or tertiapin-Q (TPNQ) at 16 μ M, 1 μ M

and 100 nM concentrations, respectively. Between 16 to 24 hours, the cells were checked for wound healing and again photographed under a microscope. (B) Quantification of wound healing assay results (n=3). Error bars indicate SEM. * $p < 0.05$ vs. control.

Fig. 6.

Effect of treatment with bicistronic constructs (MU-sh and MC-sh) on the co-localization of the $\alpha 9$ integrin with Kir4.2 inward-rectifier potassium channel. The effect of scrambled vector (SV-sh), MMP-9/uPAR plasmid shRNA (MU-sh), and MMP-9/cathepsin B plasmid shRNA (MC-sh) treatments on the co-localization of the $\alpha 9$ integrin with Kir4.2 in 4910 and 5310 cells was evaluated by immunocytochemical analysis (n=3). Red fluorescence indicates the expression of the $\alpha 9$ integrin and green fluorescence indicates the expression of the Kir4.2 channel. Nuclei were stained with DAPI. Yellow fluorescence in the merged figures indicates the co-localization of the $\alpha 9$ integrin with Kir4.2.

Fig. 7.

Schematic presentation of MMP-9/uPAR plasmid shRNA (MU-sh)- and MMP-9/cathepsin B plasmid shRNA (MC-sh)-mediated reduction in glioma cell migration via spermidine/spermine-*N*¹-acetyl transferase (SSAT) and the Kir4.2 potassium channel pathway. Intracellular higher-order polyamines (spermidine and spermine) block the Kir4.2 potassium channel that results in a decrease in outward current at positive membrane potentials, which is a phenomenon known as inward rectification. MU-sh and MC-sh treatments reduce α9β1 integrin levels, and thereby reduce SSAT, an enzyme which converts spermidine and spermine to a lower-order polyamine, putrescine, that cannot block the Kir4.2 channel.

Table 1

Genes analyzed by RT-PCR

Gene	Accession Number	Primer	Sequence (5'–3')	Product size
Kir1.1	NM_000220	Forward Primer	GTCGGAATGTGTTTGACACG	159
		Reverse Primer	CCTCCACATTGCCAAATTCT	
Kir2.1	NM_000891	Forward Primer	CGGTGGATGCTGGTTATCTT	234
		Reverse Primer	GAAAACAGCAATTGGGCATT	
Kir2.2	NM_021012	Forward Primer	CCAGTGCAACATTGAGTTCG	165
		Reverse Primer	GCGATGACCCAGAAGATGAT	
Kir2.3	NM_152868	Forward Primer	CCATCATCATTGTCCACGAG	197
		Reverse Primer	GAAGACCACAGGCTCAAAGC	
Kir2.4	NM_170720	Forward Primer	GCTAAGGAGCTGGATGAACG	205
		Reverse Primer	CCAGGGTTGGTGTGAGAACT	
Kir3.1	NM_002239	Forward Primer	CGTCCCCTTTAATAGCACCA	236
		Reverse Primer	GTTGCCCAGAACTGAACCTTA	
Kir3.2	NM_002240	Forward Primer	GCTACCGGGTCATCACAGAT	163
		Reverse Primer	ACTGCATGGGTGGAAAAGAC	
Kir3.3	NM_004983	Forward Primer	CCTGGTAGACGAGGTGCTGT	185
		Reverse Primer	TGGGGATGGACCAGTAGAGA	
Kir3.4	NM_000890	Forward Primer	CAACTTGCTCGTCTTCACCA	165
		Reverse Primer	GAGAACAGGAAAGCGGACAC	
Kir4.1	NM_002241	Forward Primer	CAAGGACCTGTGGACAACCT	221
		Reverse Primer	GGGATTCAAGGGAGAAGAGG	
Kir4.2	NM_170736	Forward Primer	GGAATGTCCTCATGCCATCT	159
		Reverse Primer	TTCTGCTTGGTGATGACTGC	
Kir5.1	NM_018658	Forward Primer	TCCACTGGAACATCTCACCA	232
		Reverse Primer	ACGTGCAGGATTCTCGAACT	
Kir6.1	NM_004982	Forward Primer	CTATCATGTGGTGGCTGGTG	196
		Reverse Primer	GGGCATTCTCTGTCATCAT	
Kir6.2	NM_000525	Forward Primer	CTGATCCTCATCGTCAGAA	172
		Reverse Primer	CACCCACACGTAGCATGAAG	
SSAT	NM_002970.2	Forward Primer	CCGTGGATTGGCAAGTTATT	218
		Reverse Primer	CTCCAACCCTCTTCACTGGA	
GAPDH	NM_002046	Forward Primer	GAGTCAACGGATTGGTCGT	185
		Reverse Primer	GACAAGCTTCCCGTTCTCAG	
β-actin	NM_001101	Forward Primer	GGCATCCTCACCTGAAGTA	203
		Reverse Primer	GGGGTGTGAAGGTCTCAA	