The invasive nature of cancers in general, and malignant gliomas in particular, is a major clinical problem rendering tumors incurable by conventional therapies. Using a novel invasive glioma mouse model established by serial in vivo selection, we identified the p75 neurotrophin receptor (p75NTR) as a critical regulator of glioma invasion. Through a series of functional, biochemical, and clinical studies, we found that p75NTR dramatically enhanced migration and invasion of genetically distinct glioma and frequently exhibited robust expression in highly invasive glioblastoma patient specimens. Moreover, we found that p75NTR-mediated invasion was neurotrophin dependent, resulting in the activation of downstream pathways and producing striking cytoskeletal changes of the invading cells. These results provide the first evidence for p75NTR as a major contributor to the highly invasive nature of malignant gliomas and identify a novel therapeutic target.
Gliomas are highly malignant and invasive tumors with tendrils that extend far from the primary tumor site, rendering conventional therapies ineffective and leading to an invariably poor prognosis. To understand the molecular mechanisms underlying this invasive behavior, we injected immunocompromised mice with human gliomas and compared invasive cells, which left the primary tumor site, to noninvasive cells, which remained at the site of injection. We identified the neurotrophin receptor p75NTR—which normally functions during development to induce neurite outgrowth and promote neuronal cell death—as an important regulator of glioma invasion. We present the first evidence that this neurotrophin receptor can also be a potent mediator of glioma invasion, and we show that the expression of this receptor is sufficient to impart a dramatic invasive behavior on genetically distinct tumors. These data highlight a previously unknown function of this receptor and suggest it may be a novel therapeutic target in the treatment of this devastating cancer.

Results

Establishment of a Malignant Glioma Mouse Model to Study the Molecular Determinants of Glioma Invasion

One of the problems in xenotransplanting human glioma cells into the brains of immunocompromised mice is that the resulting tumors are circumscribed, with very little cell infiltration into the brain parenchyma [41]. To generate an orthotopic model that more closely mimics the human disease and allows for the identification of molecular determinants of glioma invasion in a global and unbiased manner, we used an in vivo-selection procedure to select for highly invasive human glioma cells (Figure 1A). We isolated highly invasive glioma cells from the noninvasive human malignant glioma cell line U87 expressing green fluorescent protein (GFP) (U87GFP) and a neomycin resistance gene. Expression of these genes afforded us the ability to isolate the rare glioma cell that migrated away from the primary tumor site. These “invasive” cells were grown and expanded in tissue culture, and reintroduced into the brains of immunocompromised mice where they formed highly infiltrative tumors with poorly defined edges (Figure 1B). These extremely invasive cells were found vast distances from the main tumor mass, with GFP-positive tumor cells readily identifiable in the contralateral hemisphere. In clear contrast, reimplantation of the noninvasive “tumor” cells led to the formation of large tumors with sharply demarcated edges (Figure 1B). Using this model, we identified gene expression differences between the noninvasive and highly invasive in vivo–selected glioma cells. RNA extracted from tumor and invasive populations was used to prepare labeled cDNA that was hybridized to 14,000-gene human oligonucleotide microarrays (produced by the Southern Alberta Microarray Facility, University of Calgary). Genes up- or down-regulated in the invasive population were compared to the tumor population, and genes that showed consistent gene expression changes of 2-fold or greater are outlined in Figure 2A. To ensure the integrity of the microarray data, we chose seven arbitrary genes for validation, the expression of five of which are shown in Figure 2B and 2C. Semiquantitative real-time polymerase chain reaction (RT-PCR) confirmed the expression of all seven genes, including granulocyte colony-stimulating factor (G-CSF), interleukin-8 (IL-8), DZFKp434B204 (unknown hypothetical protein), tissue inhibitor of metalloproteinases-3 (TIMP-3), and p75NTR (Figure 2B and 2C). The semiquantitative RT-PCR indicates that our microarray data is an under-representation of the fold changes in RNA expression. Based on the reproducibility of the data, previous implication in tumorigenesis in other cancers (e.g., melanoma and prostate) [42–47], and the novelty of the finding in brain tumors, we chose p75NTR for further study. Importantly, we confirmed the up-regulation of p75NTR was not only at the mRNA level, but that a dramatic alteration in abundance of p75NTR was seen in the invading cells (Figure 2C). A number of invasive lines were generated by serial in vivo selection and microarray analysis using a second independent U87 invasive line validated the presence of p75NTR by microarray that was confirmed by RT-PCR and Western blot (unpublished data). In addition, using the in vivo–selection paradigm outlined in Figure 1, we isolated both tumor and invasive cells from a second human glioma cell line, U251N. These in vivo–selected invasive U251N cells also expressed high levels of endogenous p75NTR (Figure S1).

In Vivo Selection Identifies p75NTR as a Mediator of Glioma Invasion

Although p75NTR and its ligands, the neurotrophins, are expressed throughout the nervous system, particularly during development, a role for p75NTR in central nervous system tumors has not been described previously. We therefore assessed whether the up-regulation of p75NTR found in the invasive glioma cells had a functional consequence (i.e., increased their migration and invasion). The noninvasive and highly invasive cells were treated with the p75NTR ligand NGF, and migration and invasion were measured. The addition of NGF to invasive cells significantly increased the number of cells able to invade through matrigel, but had no effect on the
invasive ability of the tumor cells (which had no detectable p75NTR; Figure 2D). Because neurotrophins are also ligands for the Trk receptors, RT-PCR and immunoprecipitation experiments were performed. No detectable mRNA or protein for the Trk receptors was found in the invading glioma cells (unpublished data). In addition, we tested the effect of the unprocessed or proform of NGF (pro-NGF), a high-affinity ligand for p75NTR [33,39] that is unable to activate Trk [16]. Accordingly, treatment of the invasive cells with cleavage-resistant pro-NGF enhanced their migration at concentrations as low as 1 ng/ml while having no effect on the tumor cells (Figure S2). Although we found that neurotrophin could enhance invasion of the p75NTR-positive invasive cells (Figure 2D), we also observed a significant increase in the absence of ligand. Signals from p75NTR can arise both in the absence and presence of ligand; however, these signals often evoke opposing biological responses. Because the outcome of both neurotrophin-dependent and neurotrophin-independent signaling was the same, we considered the possibility that the glioma cells were producing and secreting neurotro-
Figure 2. Microarray Experiments Were Performed to Compare the Gene Expression Differences between the In Vivo–Selected Noninvasive (Tumor) and Invasive Glioma Cells

(A) Table lists results from a representative set of lineage experiments. Four independent microarray experiments were performed, each containing a pair of dye-flipped hybridizations. Genes that displayed consistent gene expression changes (>2-fold change in at least five out of eight hybridizations) are listed. The indicated ratios represent the fold change in gene expression in the invasive compared to the noninvasive cells. Genes chosen for validation are indicated in red.

(B and C) Seven genes were chosen for validation; the expression of five are shown. (B) RT-PCR confirmed the expression of granulocyte colony-stimulating factor (G-CSF), interleukin-8 (IL-8), an unknown hypothetical protein DZFKp434B204 (DZFK), and tissue inhibitor of metalloproteinases-3 (TIMP-3) in the invasive population. Expression levels of GAPDH (unchanged) are shown for comparison. (C) RT-PCR and Western blot confirm

### Genes Upregulated in the Invasive Cells compared to the Tumour Cells

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| Cytokines, Chemokines, Growth Factors and their Receptors |       |
| colony stimulating factor 3, granulocyte (G-CSF) | 10.89 |
| nerve growth factor receptor, TNFR superfamily, member 16 (p75) | 7.38 |
| interleukin 1, beta (IL-1B) |       |
| interleukin 8 (IL-8) | 5.45 |
| insulin-like growth factor 2 (somatomedin A) | 3.76 |
| pre-B-cell colony-enhancing factor (PECF) | 3.31 |
| connexin 31 | 3.24 |
| tumor necrosis factor | 2.1 |

### Proteas and Protease Inhibitors

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| tissue factor pathway inhibitor 2 | 5.09 |
| tissue inhibitor of metalloproteinases 3 (TIMP-3) | 3.87 |
| cystatin B (stefin B) | 2.95 |
| cathepsin K (pymyodysosostosis) | 2.49 |

### Tumour-Associated Antigens

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| G antigen 2 (GAGE-2) | 3.63 |
| G antigen 4 (GAGE-4) | 3.69 |

### Small GTP-Binding Proteins (Cytoskeletal Effects)

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| ras homolog gene family, member E (RhoE) | 3.38 |
| Ras-related associated with diabetes (Ran) | 2.85 |

### Cell Cycle

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| putative lymphocyte G0/G1 switch gene (G0S2) | 2.41 |

### Adhesion

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor) | 2.37 |

### Gene Expression Modulators

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| high-mobility group (nonhistone chromosomal) protein isoforms I and Y | 2.23 |

### Other

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| sequestosome 1 (p52) | 2.62 |
| annexin A1 (annexin X) | 2.57 |
| coated vesicle membrane protein (p24A) | 2.51 |
| 5' nucleotide (CD73) | 2.49 |
| unknown hypothetical protein (from clone DZFKp434B204) | 2.46 |
| eukaryotic translation elongation factor 1 alpha 1-like 14 | 2.45 |
| teratin, light polypeptide | 2.26 |

### Genes Downregulated in the Invasive Cells compared to the Tumour Cells

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| Angiogenesis-Related                        |       |
| heme oxygenase (decatropyl) | 0.22 |

### Apoptosis-Related

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| BCL-2/adenovirus E1B 19kD-interacting protein 3 (Bip3) | 0.4 |

### Adhesion

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| syndecan 2, heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan (HSPG) | 0.37 |

### Other

| Genes                                      | Ratio |
|---------------------------------------------|-------|
| carbonic anhydrase IX | 0.17 |
| glutaryl-Coenzyme A dehydrogenase | 0.26 |
expression of p75 in the invasive population (Inv) but not the tumor cell population (T). RT-PCR analysis of GAPDH levels and Western blot analysis of pyruvate kinase levels were included as loading controls. Human dorsal root ganglia (DRG) were used as a positive control.

(D) Addition of NGF (200 ng/ml) enhanced the migratory ability of the invasive glioma cells in matrigel-coated invasion chambers, but had no significant effect on invasion of the tumor cells. Values shown are the mean ± SEM from three independent experiments. Triple asterisks (****) indicate p < 0.001 versus control (two-way ANOVA with Bonferroni post-tests).

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phosphin(s), thus activating an autocrine loop. We assessed the expression of several neurotrophins and found that BDNF was present in both the conditioned media and the cell lysate of all glioma cells tested (unpublished data). Furthermore, we found that the presence of p75NTR shifted the localization of BDNF from the conditioned media to the cell membrane (Figure S3), supporting the notion of autocrine/paracrine activation of the p75NTR receptor.

To directly test the hypothesis that elevated expression of p75NTR is necessary for neurotrophin-induced glioma migration and invasion, we surveyed a panel of human glioma cell lines for p75NTR protein expression. We found that the human glioma cell line SF767 endogenously expressed high levels of p75NTR, as detected by Western blot (Figure 3A) and fluorescence-activated cell-sorting (FACS) analysis (unpublished data). Using RNA interference (RNAi), we down-regulated p75NTR in the SF767 cell line using an expression vector containing a p75NTR-specific small interfering ribonucleic acid (siRNA) and confirmed the down-regulation by RT-PCR and Western blot (Figure 3A). A random, nonspecific siRNA sequence was used as a control. Down-regulation of p75NTR levels in SF767 was sufficient to reduce its migration in vitro and rendered the cells nonresponsive to addition of NGF in both migration and invasion assays (Figure 3B and 3C). Similarly, down-regulation of p75NTR by siRNA in the original in vivo–selected U87 invasive cells significantly blocked migration and invasion (Figure S4).

Since down-regulation of p75NTR in SF767 cells and U87 in vivo–selected invasive cells inhibited glioma invasion, we assessed whether ectopic expression of p75NTR alone was sufficient to increase glioma migration and invasion in a cell line without detectable p75NTR (the original U87 cell line). To this end, we stably transfected the full-length cDNA of human p75NTR into the U87 glioma cell line, using stable transfection of the empty pcDNA vector as a control. Expression levels of p75NTR in these cells were confirmed by RT-PCR and Western blot (Figure 3D). Expression of p75NTR caused a significant increase in migration and invasion in vitro (Figure 3E and 3F). Treatment of these cells with NGF had no further enhancement on their migration or invasion consistent with the idea that when p75NTR is expressed, an autocrine loop is completed, leading to enhanced migration and invasion.

Expression of p75NTR Increases Invasion In Vivo in Genetically Distinct Glioblastomas

Malignant gliomas clinically show extensive infiltration away from the main tumor and into the surrounding normal brain tissue. To determine whether the expression of p75NTR was important for glioma cell invasion in vivo, we implanted the U87 human glioma cell line ectopically expressing p75NTR into the brains of severe combined immunodeficiency (SCID) mice. U87 cells stably transfected with the empty pcDNA vector were implanted for comparison as a control. Twenty-eight days after implantation, the mice were sacrificed and the brains prepared for immunohistochemical staining using antibodies directed against human nuclei and p75NTR.

Implantation of U87 glioma cells stably transfected with pcDNA led to the formation of well-circumscribed tumors that were p75NTR negative (Figure 4A). In sharp contrast, implantation of U87 glioma cells stably expressing p75NTR resulted in the formation of tumors with highly infiltrative edges (Figure 4B). Isolated p75NTR–positive human glioma cells could be detected in regions vastly distant from the main tumor mass (Figure S5).

Because malignant gliomas are an extremely heterogeneous group of tumors and the in vivo–selected U251N cells also expressed high levels of p75 (Figure S1), we determined whether the sole expression of p75NTR was sufficient to impart an invasive phenotype, not only on the U87 cells, but also on the genetically distinct U251N cells. U251N cells ectopically expressing p75NTR (U251Np75), along with empty vector–transfected cells as a control (U251NpcDNA), were implanted into the brains of SCID mice as described above. As we have observed previously, the U251N control cell line (U251NpcDNA) was inherently more invasive than U87pcDNA in vivo, with finger-like projections extending from the main tumor mass into the surrounding normal brain (compare Figure 4A and 4C). Nevertheless, ectopic expression of p75NTR (U251Np75) dramatically enhanced the inherent invasive ability, with p75NTR–positive cells being found at locations distinct from the main tumor mass (compare Figure 4C and 4D). Thus, up-regulation of p75NTR is sufficient to allow glioma cells of diverse genetic backgrounds to invade into the surrounding normal brain. Because p75NTR can have effects on several physiological responses, we also evaluated the effect of p75NTR expression on cell cycle, proliferation, and survival, and observed no significant change (unpublished data).

p75NTR–Mediated Glioma Invasion Is Neurotrophin Dependent

In order to test whether neurotrophin was important in the invasive behavior of these cells, we constructed two p75NTR mutants, p75CRD105 and p75CRD130, containing a four–amino acid insertion in the cysteine-rich domain (CRD) following amino acids 105 and 130 (CRD 105 and CRD 130), respectively. Insertions at these locations disrupt the normal spacing of the cysteine residues within the164 ligand-binding domain and create p75NTR proteins that are unable to bind to mature neurotrophin [48]. These constructs were stably transfected into U87 glioma cells, and cell surface expression for the mutant p75NTR proteins was confirmed by FACS analysis (Figure 5A). To verify that the mutant p75NTR do not bind neurotrophin, BDNF expression in the conditioned medium and total cell lysates of U87 cells expressing CRD105 and CRD130 were performed. Unlike the wild-type p75NTR–expressing glioma cells in which expression of p75NTR causes a shift in BDNF localization from the medium to the cell lysate, cells expressing the mutant alleles (CRD105 and CRD130) did not result in a shift of BDNF localization, confirming that...
these mutants do not bind endogenous BDNF (Figure 5B).

Figure 3. p75NTR-Induced Invasion in Glioma

(A) Down-regulation of p75NTR using RNAi decreases glioma migration/invasion. RT-PCR (GAPDH used as a loading control) and Western blot (pyruvate kinase used as a loading control) for p75NTR confirm down-regulation of p75NTR in the glioma cell line SF767 transfected with a p75-specific siRNA. Untransfected cells, cells transfected with a random siRNA, and the in vivo–selected tumor and invasive cells are shown for comparison.

(B) Treatment with NGF (200 ng/ml) enhanced migration of SF767 cells transfected with the random (control) siRNA, but had no significant effect on migration of SF767 cells in which p75NTR expression was inhibited by p75NTR-specific siRNA. Values shown are the mean ± SEM from three independent experiments. A single asterisk (*) indicates $p < 0.05$, and double asterisks (**) indicate $p < 0.01$ versus control-treated random siRNA-transfected cells; triple asterisks (***) indicate $p < 0.001$ versus NGF-treated random siRNA-transfected cells (two-way ANOVA with Bonferroni post-tests).

(C) Treatment with NGF (200 ng/ml) enhanced invasion of SF767 cells transfected with random siRNA, but had no significant effect on invasion of SF767 cells in which p75NTR expression was inhibited by p75NTR-specific siRNA. Values shown are the mean ± SEM from a single experiment. Similar results were seen in two independent experiments. Double asterisks (**) indicate $p < 0.01$ versus control-treated random siRNA-transfected cells, and double pluses (++) indicate $p < 0.01$ versus NGF-treated random siRNA-transfected cells (two-way ANOVA with Bonferroni post-tests).

(D) Ectopic expression of p75NTR induces glioma migration/invasion. RT-PCR (GAPDH used as a loading control) and Western blot (pyruvate kinase used as a loading control) for p75NTR confirm expression of p75NTR in U87 cells stably transfected with pcDNA3.1 encoding human p75NTR (U87p75). Cells stably transfected with the empty pcDNA3.1 vector (U87pcDNA), as well as in vivo–selected tumor and invasive cells are shown for comparison.

(E) Migration of U87 glioma cells is enhanced by ectopic expression of p75NTR. No additional increase was seen following treatment with NGF (200 ng/ml). Values shown are the mean ± SEM from three independent experiments. Triple asterisks (***) indicate $p < 0.001$ versus pcDNA-transfected cells (two-way ANOVA with Bonferroni post-tests).

(F) Similarly, invasion of U87p75 glioma cells in matrigel-coated invasion chambers was significantly increased compared to controls. No further increase was seen with exogenous NGF (200 ng/ml). Values shown are the mean ± SEM from four independent experiments. A single asterisk (*) indicates $p < 0.05$, and double asterisks (**) indicate $p < 0.01$ versus pcDNA-transfected cells (two-way ANOVA with Bonferroni post-tests).

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Expression of p75NTR in tumor specimens and normal human brain using immunohistochemical staining (Figure 6A), RT-PCR, and Western blot (Figure 6B). Expression of p75NTR protein was detected in 20 of 40 human glioma patient specimens (50%) (one of 11 low-grade astrocytomas [8%], two of nine mid-grade astrocytomas [22%], and 17 of 20 glioblastoma multiforme (GBM) specimens [85%]) and was undetectable in normal human brain (zero of five). Thus, expression of p75NTR is a common event in GBM. To demonstrate that the presence of p75NTR in these patient specimens confers an increased migratory ability, short-term cultures of these samples were analyzed in transwell motility assays. The percentage of cells positive for p75NTR in the original population was determined by immunostaining and compared to the percentage of p75NTR-positive cells in the migratory population (i.e., those cells that migrated to the underside of the transwell chamber during the assay). As a positive control for this assay, a mixture of 25% U87p75 cells and 75% U87pcDNA cells were used as input. At completion of the control assay, the migratory population contained approximately 50% p75NTR-positive cells (Figure 6C), as expected from initial experiments that demonstrated that p75NTR-positive cells migrate at a greater

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**Figure 4.** Expression of p75NTR in the U87 and U251N Glioma Cell Lines Dramatically Increases Invasion In Vivo

U87 or U251N human glioma cells stably transfected with the empty pcDNA vector (U87pcDNA [A] or U251NpcDNA [C]) or the p75NTR-expression vector (U87p75 [B] or U251NpcDNA [D]) were implanted into the brains of SCID mice and allowed to grow for 28 d. The mice were sacrificed, and frozen brain sections were stained with antibodies against human nuclei (left) and human p75NTR (right). Boxed areas indicate the region shown in the panel below; thus magnification increases from top to bottom; scale bars in (A) and (B) represent 100 µm, 50 µm, and 25 µm; and scale bars in (C) and (D) represent 200 µm and 100 µm. Implantation of U87 glioma cells stably transfected with the empty pcDNA vector led to the formation of well-circumscribed tumors that were p75NTR-negative (A). In sharp contrast, implantation of U87 glioma cells ectopically expressing p75NTR led to the formation of tumors with highly infiltrative edges (B). Similar results were seen in three independent experiments with six animals in each group. U251NpcDNA glioma cells were generally more invasive than U87pcDNA cells upon implantation into the brains of SCID mice, and formed tumors with finger-like projections extending into the surrounding normal brain (C); nevertheless, ectopic expression of p75NTR in U251N cells dramatically increased the invasiveness of these cells in vivo with isolated individual tumor cells being found distant from the main tumor mass (D). Similar results were seen in all ten animals in each group.

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rate than the p75NTR-negative cells (Figure 3E and 3F). Similar effects were observed with the glioma patient specimens. The percentage of p75NTR-positive cells in the migratory population compared to the original population was increased by 40%–100% (Figure 6C), demonstrating that the p75NTR-positive cells within the glioma patient samples are more migratory than the p75NTR-negative glioma cells.

p75NTR Expression Results in Cytoskeletal Rearrangement and Changes in RhoA Activity

During the in vitro growth stage of the serial in vivo–selection procedure, we observed that the invasive glioma cells had striking morphological differences to the "tumor" cells. To examine the morphology of these cells, fluorescent staining of the actin cytoskeleton was performed. Staining of the actin cytoskeleton using rhodamine phalloidin revealed cells with numerous filamentous protrusions present only in the invading population (Figure 7A). Similarly, we found that expression of p75NTR alone induced structural rearrangement of the actin cytoskeleton similar to that of the in vivo–selected invasive cells (Figure 7B). Because the small molecular weight GTPase RhoA is a potential downstream readout from p75NTR that may help contribute to the distinct phenotype, we examined the effect of RhoA. Expression studies in HEK293 cells demonstrated that in the absence of ligand, p75NTR constitutively activated Rho, whereas ligand binding leads to a decrease in the levels of active Rho [27]. In addition, Gehler et al. [49] have shown that neurotrophin-bound p75NTR induces growth cone filopodia through the modulation of RhoA and that neurotrophin binding is necessary and sufficient to regulate filopodia dynamics. We found that concomitant with the changes in actin cytoskeleton, cells expressing p75NTR had reduced RhoA activity (Figure 7C and 7D).

Discussion

Human malignant gliomas are highly invasive tumors. This highly invasive nature associates theses tumors with an extremely poor prognosis owing to recurrence of the tumor outside the margin of therapeutic resection [50]. Invasion of
glioma cells into the normal surrounding brain requires changes that make these cells distinct from their noninvasive counterparts. Specifically, these glioma cells activate a number of coordinate cellular programs that involve the regulation of many molecules, including adhesion molecules, extracellular matrix constituents, proteases, cytoskeleton components, and signaling molecules. Altered regulation of any of these constituents may lead to changes in glioma cell migration and invasion. Although numerous molecules have been implicated in the migration and invasion of gliomas, what triggers glioma cells to leave the main tumor mass and invade into the normal brain is not well understood. To this end, we have developed a serial in vivo–selection paradigm to isolate highly invasive glioma cells from a human glioma cell...
In recent years, there has been a growing importance of the neurotrophin signaling axis in cancer. Specifically, there is increasing evidence that the neurotrophic receptor tyrosine kinase TrkB, sometimes in conjunction with its primary ligand BDNF, is over-expressed in a variety of human cancers, ranging from neuroblastomas to pancreatic ductal adenocarcinomas [51–55]. Here, we present data that the pan-neurotrophin receptor p75NTR is expressed in malignant glioma and is a major contributor to their highly invasive nature. Although a universal role for p75NTR in cancer has not been established, recent studies implicate p75NTR in the metastatic progression of melanoma, and specifically in those tumors that metastasize to the brain [43,46,56]. Conversely, p75NTR expression has been linked to the progression of prostate cancer, but in this cancer, p75NTR, which is expressed in normal prostate epithelia, is lost upon transformation [45]. The divergence observed in the tumor progression of these two distinct tumors can likely be explained by the presence of Trk. In prostate tumor cells, Trk expression is retained and mediates proliferation [42,57], whereas p75NTR-induced invasion in melanoma is independent of Trk expression [58]. Thus the recurring theme emerges that p75NTR function is cell-type specific (even in cancer) and must be independently determined for each cellular context. Here, we have shown that p75NTR-induced glioma invasion is also Trk independent with neither mRNA nor protein for the Trk receptors expressed by the invading glioma cells. Further supporting the Trk independence of p75NTR-mediated glioma invasion is the finding that treatment of the invasive cells with cleavage-resistant pro-NGF (which cannot bind Trk; Figure S2) also enhanced the migration of invading glioma cells.

Tumor cells can survive by means of an autostimulatory (autocrine) signaling loop, such as that mediated by TrkB and BDNF, or through a paracrine cross-communication with their environment. In brain metastatic melanoma, normal brain tissue adjacent to the melanoma displays increased neurotrophin expression [56], making it tempting to speculate that the metastatic melanoma uses the neurotrophin-rich nervous system as a paracrine mediator of invasion. It has similarly not escaped our attention that the neurotrophin environment of the brain may provide an extremely advantageous milieu for an invading glioma cell. Our data show that the p75NTR-expressing glioma cells are ligand responsive and may therefore use neurotrophins available in the brain environment to their advantage. In addition, we show that the invasive nature of glioma cells expressing p75NTR is negated when these cells express mutant p75NTR receptors that no longer bind to neurotrophin.

The concept of p75NTR playing a role in migration is not unprecedented. Neural crest cells, the most extensively studied population of migrating cells in the nervous system, express p75NTR even before they commit to any cell differentiation lineage [59]. In addition, Anton et al. [35] showed that stimulation of the p75NTR by NGF allowed Schwann cells to migrate on peripheral nerves, and examination of p75−/− mice showed severe impairment of Schwann cell migration, with no response to NGF [26]. More recently, the Hempstead laboratory [46] has shown that activation of p75NTR with NGF or pro-NGF (the unprocessed, precursor form of NGF) caused migration of melanoma cells and increased expression of p75NTR correlated with advanced stages and invasive poten-

**Figure 7. Ectopic Expression of p75 Results in Actin Cytoskeletal Rearrangement and Decreased RhoA Activity**

(A) Actin staining of tumor (left) and invasive cells (right) shows striking cytoskeletal rearrangement in the invading glioma cells. Actin cytoskeleton was visualized by staining fixed and permeabilized cells with rhodamine phalloidin (red), and cell nuclei were visualized with DAPI (blue). Numerous filamentous protrusions are seen in the invading glioma cells.

(B) p75NTR is sufficient to induce cytoskeletal rearrangement of glioma cells. U87pcDNA (left) and U87p75 (right) cells were fixed, permeabilized, and stained with rhodamine phalloidin (red) and DAPI to visualize the nucleus (blue).

(C) p75NTR expression results in decreased RhoA activity. RhoA activity was determined in U87pcDNA (pcDNA) and U87p75 (p75NTR) by RhoA pulldown assay using a GST fusion protein containing RBD-rhotekin that binds only to activated (GTP-bound) RhoA. Western blots using total cell lysates were performed for p75NTR, RhoA, and β-actin (used as a protein loading control).

(D) Bar graph shows quantitation of activated RhoA (RhoA-GTP) as compared to total RhoA in both U87pcDNA and U87p75. Values shown are the mean ± SEM from four independent experiments.

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tial of melanoma brain metastasis [60]. At present, the underlying mechanism of p75NTR-induced migration of melanoma cells is not understood; however, p75NTR has been shown to interact with the actin cytoskeleton [46]. The small GTPase RhoA is a downstream effector of p75NTR [27,28]. The capability of p75NTR to modulate the activity of RhoA provides a reasonable explanation as to how p75NTR regulation might result in changes in cellular architecture of glioma cells. We found that concomitant with increased glioma invasion, glioma cells expressing p75NTR showed reduced RhoA activity and striking actin rearrangement.

Previous molecular characterization has defined genetic changes between low-grade and high-grade glioma [61–67]. In addition, molecular signatures of glioblastoma subtypes have been identified, including profiles of primary and secondary glioblastoma subgroups [68–70]. On the other hand, very little is known with respect to the transcriptional profiles of invading glioma cells. Studies have been performed using laser capture microdissection in patient specimens to collect the invasive cells and the cells from the main tumor mass. Although this approach has been used successfully to identify invasion-related genes [9], these experiments make the assumption that the invasive cells at the leading edge of the tumor have distinct profiles from the main tumor mass and that only tumor cells at the invading edge express genes important for migration and invasion. Yet, within the highly heterogeneous environment of a glioblastoma, in which there are many hypoxic and necrotic regions, it would be easy to envision that tumor cells experiencing oxidative stress would activate mechanisms enabling them to move to a more favorable environment. As such, some genes may not be identified using such an approach. Indeed, our data show that, in addition to p75NTR-expressing glioma cells at the invasive edge of patient tumors, histological analysis identified p75NTR-positive glioma cells in regions of the tumor not adjacent to normal brain parenchyma. An alternative explanation for the appearance of p75NTR-positive glioma cells is that p75NTR promotes survival of glioma cells in vivo, though we did not find that p75NTR conferred a survival advantage in vitro. Additionally, reports of “stem-like” cells in brain tumors suggest that brain tumors arise from the transformation of neural stem cells [71–73], and when implanted into the brains of SCID mice, these cells form highly invasive tumors [16,74,75]. Whether these brain tumor stem cells express p75NTR is an important question for future studies, especially given that nestin-positive, p75NTR-positive cells have been identified in the subventricular zone of the adult brain [76].

Identification of key regulatory proteins of glioma invasion is extremely important clinically because this will be used to provide therapeutically relevant targets to prevent malignant glioma recurrence at the invasive margin of gliomas [4]. Herein, we present the first evidence that p75NTR is important in glioma migration, and the mere expression of p75NTR is sufficient to impart a dramatic invasive behavior on genetically distinct glioblastomas. Because p75NTR has also been implicated in the progression of melanoma, and specifically in those tumors that metastasize to the brain [53,46,77], therapies that target p75NTR, p75NTR downstream effectors, or their ligands may not only be beneficial for malignant glioma, but may target other metastatic diseases.

Materials and Methods

Cell culture. The human glioma cell line U87 was obtained from the American Type Culture Collection (http://www.atcc.org). The human glioma cell lines U251N and SF767 were kind gifts from V. W. Young (University of Calgary, Alberta, Canada) and M. Berens (Barrow Neurological Institute, Phoenix, Arizona, United States), respectively. All cells were maintained in complete medium (Dulbecco’s Modified Eagle’s Medium [DMEM] F12 supplemented with 10% heat-inactivated fetal bovine serum, 1% antibiotic/antimycotic, 0.1 mM nonessential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate [GIBCO BRL, http://www.invitrogen.com]) at 37 °C in a humidified 5% CO2 incubator. Cells were passaged by harvesting with trypsin when they reached 80%–90% confluence. Stable transfectants of U87 and SF767 cells were maintained in the same medium, with the addition of 490 μg/ml G418 or 200 μg/ml hygromycin, respectively. Invitrogen, http://www.invitrogen.com.

Generation of plasmids. The GFP expression vector was pGFP-N1 from Clontech (http://www.clontech.com). The human p75NTR expression vector was constructed as described previously [46]. The expression plasmids containing the p75NTR mutants were constructed either by subcloning of PCR fragments containing the desired p75NTR sequences (for p75CRD105) or by PCR-based site-directed mutagenesis (for the p75CRD130 constructs). Primers used for the construction of the mutants were: p75CRD105 primers (sense: 5′-CGG GCT CGG GCC GCT CGA GCG GCC TCG TGT TC–3′; antisense: 5′-GAA GAC CAC GGC GCC GCT CAC TTC TTC TGT C–3′); p75CRD130 primers (sense: 5′-GAA GAC CAC GGC GCC GCT CAC TTC TTC TGT C–3′; antisense: 5′-CTC ACT ATA GCA GCG CCA GAA TTC G–3′) and template p75WT [46]; p75CRD130 (primers: sense: 5′-GAA GAT CTC CAA GGA GAG TTC CAC CCC CAC AGG CC–3′; antisense: 5′-CTC ACT ATA GCA GCG CCA GAA TTC G–3′) and template p75L17p7a. The original templates were from B. Hempstead (p75WT; Cornell University Medical College) and M. Chao (p75CRD130; New York University School of Medicine, New York, New York, United States). The sequences of all the mutation expression plasmids were confirmed prior to stable transfection. The p75NTR-specific siRNA expression vector was constructed by ligating a double-stranded hairpin oligonucleotide: 5′-GAT CAG AAT TCA GAT TCT CAG GAA GCA GGC GCT CAC TTC TTC GAG AGA TGA TCA GAA GCC TTC GAT CCT CTT TTT TGG AAA–3′, containing a p75NTR-specific siRNA sequence (underlined), into the pSilencer 2.1-U6 hygro vector (Ambion, http://www.ambion.com). The negative control pSilencer vector, containing a random siRNA with limited homology to any known human, mouse, or rat sequences, was obtained from Ambion.

Transfection of glioma cell lines. Cells to be transfected were seeded at 2 × 105 cells/well of a six-well plate and incubated at 37 °C overnight in complete media. Vector DNA was introduced to the cells using FuGENE 6 transfection reagent (Roche Diagnostic, http://www.roche.com) according to the manufacturer’s instructions. The cells were then incubated at 37 °C overnight; and the following day, the medium was changed to fresh complete media containing an antibiotic (concentration determined by toxicity curve for cell line) to select for those cells that had taken up the vector. The cells were then grown under antibiotic selection until the wells were confluent. For GFP transfection, transfected cells were identified by fluorescent microscopy and GFP expression of greater than 95% was obtained by fluorescence-activated cell sorting. For p75NTR, p75CRD105, p75CRD130, and p75NTR-siRNA transfection, transfected cells were identified by RT-PCR and Western blot.

Animals. Six- to 8-wk-old female SCID mice were purchased from Charles River Laboratories (http://www.criver.com). The animals were housed in groups of three to five, maintained on a 12-h light/dark schedule with a temperature of 22 °C ± 1 °C and a relative humidity of 50% ± 5%. Food and water were available ad libitum. All procedures were reviewed and approved by the University of Calgary Animal Care Committee.

In vivo selection of invasive glioma cells. Actively growing U87 cells expressing GFP and neomycin resistance genes (U87GF) were harvested by trypsinization, washed, and resuspended in sterile PBS (137 mM NaCl, 8.1 mM Na2HPO4, 2.68 mM KCl, and 1.47 mM KH2PO4 [pH 7.5]). These cells were implanted intracerebrally into the right parietal of SCID mice (1 × 105 cells/well) in a depth of 3 mm through a scalp incision and a 0.5-mm burr hole made 1.5–2 mm right of the midline and 0.5–1 mm posterior to the coronal suture. Stereotactic techniques were described previously [77]. Tumor formation was allowed to proceed for 21+ days, depending on the health of the mouse and the type of cells injected. The mice were then sacrificed and the brain examined using fluorescence. The brain was divided in half coronally; one half was used for frozen sections and the other used for tissue culture. For tissue culture, the hemisphere
containing the main tumor mass was separated from the contralateral hemisphere, and the two pieces were treated individually. The tissue was minced into small pieces and dissociated with trypsin and DNase I at 37 °C. The tissue suspension was then forced through a 100-μm mesh, and the resulting cell suspension was centrifuged and resuspended in complete medium containing 400 μg/ml G418 to select for the G4P-transfected tumor cells. Cells obtained from the tumor mass were labeled as “tumor” cells, and those from the contralateral hemisphere were labeled as “invasive” cells. Tumor and invasive cells were then resuspended into SCID mice, and the procedure was repeated.

**RT-PCR.** Total cellular RNA was extracted from subconfluent cells using Trizol Reagent (Invitrogen) and DNase-treated using DNA-free (Ambion). The reverse transcription reaction took place in a buffer of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, and 1.5 mM MgCl₂, and contained 3 μg of total RNA, 25 units of RNase inhibitor, 1 mM each of deoxynucleoside triphosphates, 100 ng of pd(N)₆ random hexanucleotide primers (Amersham Biosciences, http://www.amersham.com), and 200 units of Superscript II reverse transcriptase (Invitrogen). The PCR amplification reaction was carried out in the same buffer and contained 1 μl of p75NTR-specific primers (forward: 5′-GGG ACC GAC GGC GAA TGC-3′; reverse: 5′-GGA CAC GAC GCC GGT CGG CGA GCC CGA GGC CG-3′), p75CRD-specific primers (forward: 5′-GGA TAT CTC CAA GGA GGC ATG CCC CAC AGG CC-3′; reverse: 5′-GAA CAC GAC GCC GGT CGG CGA GCC CGA GGC CG-3′), p75CRD⁎ specific primers (forward: 5′-GAA GAT CTC CAA GGA GGC ATG CCC CAC AGG CC-3′; reverse: 5′-GCA CAC GCC GGT CGG CGA GCC CGA GGC CG-3′), the initial area of the cells. Western blotting. Total cellular protein extracts were obtained by gentle rocking in lysis buffer (20 mM Tris (pH 8.0), 157.5 mM NaCl, 10% glycerol, 1% Nonidet P-40, 25 μg/ml aprotinin, 10 μg/ml leupetin, 5 mM sodium orthovanadate, 1 mM PMSF) at 4 °C. Protein extracts of human glioma biopsies were obtained by immersing the samples in ice-cold extraction buffer (50 mM Tris (pH 7.6), 290 mM NaCl, 10 mM CaCl₂, 1% Triton X-100) followed by homogenization on ice. Cellular debris was removed by centrifugation, and protein quantification was performed using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, http://www.piercenet.com). Proteins were resolved on 10% SDS-PAGE gels, and Western blots were performed using the following primary antibodies: rabbit polyclonal anti-human p75NTR (Promega, http://www.promega.com), goat polyclonal anti-pan-rabbit (Chemicon, http://www.chemicon.com), mouse monoclonal anti-neurofilament, 70 kDa (Chemicon), mouse monoclonal anti-glial fibrillary acid protein (Chemicon, http://www.chemicon.com), mouse monoclonal anti-p75NTR (Promega, http://www.promega.com), and mouse monoclonal anti-human nuclear (Chemicon) in blocking buffer overnight at 4 °C. Following washing with PBS, the appropriate biotinylated secondary antibody (Vector Laboratories, http://www.vectorlabs.com) was applied. Avidin-biotin peroxidase complexes (Vector Laboratories) and detected by addition of SIGMAFAST DAB (3,3′-diaminobenzidine tetrahydrochloride) (Sigma-Aldrich). The SIGMAFAST DAB was converted to a brown reaction product by the enzyme, Hematoxylin (for paraffin sections) and toluidine blue (for frozen sections) were used as nuclear counterstains. Sections were then dehydrated in an ethanolation series and mounted with Entellan (Electron Microscope Sciences).

**Immunohistochemistry.** Frozen sections were air dried at room temperature, fixed with cold acetone, and then rinsed with PBS. Paraformaldehyde (2%) was added with two changes of medium, layered onto an 8-μm pore-size transwell chamber (BD Bioscience, http://www.bdbsciences.com), and incubated at room temperature for 1 h. The wells were then rinsed with serum-free medium. The coated chambers were placed into the wells of a 24-well tissue culture plate containing 500 μl of media with or without the desired treatment. Cells (2.5 × 10⁴) were then added in each chamber, in a volume of 500 μl of the same medium contained in the bottom of the well, and incubated at 37 °C for 48 h. The medium was then removed from the chambers and cells scraped off the top of the membrane using a PBS-soaked cotton-tipped swab. Cells were then placed into the bottom of the chambers, fixed with 4% formaldehyde, incubated in hematoxylin, and mounted on slides. Invasion was quantified by counting the stained cells adherent to the lower side of the membranes in ten fields (at 10⁵ magnification) for each of three chambers for each condition.

**In vivo studies of p75NTR overexpression in an intracranial glioma model.** Actively growing U87pcDNA, U87p75, U87CRD 105, U87CRD 130, U251NpcDNA, and U251Np75 cells were implanted intracerebrally into SCID mice as described previously [77]. Mice were sacrificed weekly from day 1 to 42. At each time point, the brains were removed, frozen in OCT compound (Tissue-Tek; Electron Microscopy Sciences, http://www.emsdiasum.com), and cryosectioned for examination by immunohistochemistry.

**Immunocytochemistry.** Coverslips were coated with a Collagen I (3 mg/ml; Vitrogen 100; Cohesion Technologies, http://www.vwrinternational.com) and incubated overnight at 37 °C. Excess collagen solution was aspirated, and cells were plated at 2 × 10⁵/ml in DMEM culture medium (DMEM with 10% FBS, 6 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 40 μg/ml G418) and allowed to equilibrate overnight at 37 °C, 5% CO₂. Coverslips were then fixed in 4% paraformaldehyde (2%, 30 min in PBS for 10 min, and rinsed twice with PBS. Unperoxidized action was extracted for 3 min in CSK buffer (10 mM MES [pH 6.1], 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 320 mM sucrose, 0.1% Triton X-100)
followed by two rinses with PBS. Alexa Fluor 568 phalloidin (Invitrogen) was diluted 1:40 in 1% BSA/PBS and 200 µl of this solution was added to each coverslip for 20 min at room temperature. Coverslips were rinsed twice with PBS, counterstained with a 500 nM solution of DAPI for 3 min, mounted in glycerol, and imaged with an Olympus IX70 Delta Vision RT Microscope (http://www.olympus.co.jp) and the SoftWoRx software package.

**Tumor tissue.** Tumor and normal tissues were obtained from the Canadian Brain Tumor Tissue Bank in London, Ontario, and Foothills Hospital, Calgary, Alberta. Briefly, tissue was taken during surgery while patients were under a general anesthetic, and was placed immediately in liquid nitrogen and stored at –80 °C or placed in culture medium for establishment of short-term cultures. An institutional ethics board approved the collection and use of all of the surgical tissue used, and all of the patients gave signed informed consent. The following tissues were studied: 20 GBMs, eight anaplastic astrocytomas, one anaplastic oligodendroglioma, five astrocytomas, five mixed oligoastrocytomas, one oligodendroglioma, and five controls obtained during nontumor brain surgery.

**Short-term culture of primary human glioma cells.** Operative samples of human gliomas were obtained during brain tumor surgery and transported to the laboratory in culture medium. Short-term cultures were then established. Briefly, necrotic and connective tissue and any visible clot were removed using dissecting tools, and tissue was washed in PBS and cut into pieces of approximately 1 mm². The tissue was then incubated for 30 min at 37 °C in an enzyme cocktail of trypsin (0.25%/g) and DNase I (10 µg/ml) in PBS. The digested tissue was strained through a 100-µm mesh and washed with PBS. The cells were then pelleted and washed with PBS, pelleted, and resuspended in complete media containing 20% FBS, and plated.

**Transwell motility assay of primary human glioma cells.** Primary human glioma cells, less than 5 wk were “serum-starved” by incubating them in medium containing only 1% FBS for 2 h at 37 °C and 5% CO₂. Cells were then released from the culture dish using Puck’s EDTA (1 mM EDTA, 10 mM HEPES, 5 mM KCl, 140 mM NaCl, 8 mM NaHCO₃, and 6 mM dextrose [pH 7.3]) at 37 °C for 5 min. Cells were then pelleted and then thawed to washout the culture dish. Following lysis of red blood cells, the remaining cells were washed with PBS, pelleted, and resuspended in complete media containing 20% FBS, and plated.

**Supporting information**

**Figure S1.** In Vivo–Selected U251 Invasive Cells Express p75NTR. U251 human glioma GFP-expressing cell line was implanted into the brains of SCID mice; and 4–6 wk later, the mice were sacrificed. The ipsilateral side of the brain (containing a grossly visible tumor) was separated from the contralateral side (containing only isolated invasive cells and both were grown in culture). These noninvasive (U251T1) and highly invasive (U251R) cell lines were reimplanted and the process repeated to select for increasingly noninvasive or invasive glioma cells. Through the serial in vivo selection, highly invasive U251 glioma cells were isolated. Western-blot analysis of p75NTR expression in tumor and invasive U251 human glioma cells shows a dramatic increase in endogenous p75NTR expression in the U251 invasive cells as compared to the U251 tumor cells. U87 tumor (U87T) and invasive (U87R) cells were used for comparison (A). The invasive U251 cells, which endogenously express p75NTR, showed a significant increase in migration (B) and invasion (C) compared to U251 tumor cells. Double asterisks (**) indicate p < 0.001 (tumor vs. invasive, paired t-test). Values shown are the mean ± standard error of the mean (SEM) from three independent experiments. Found at doi:10.1371/journal.pbio.0050212.sg001 (136 KB TIF).

**Figure S2.** Invasive Glioma Cells Expressing p75NTR Increase Migration in Response to Cleavage-Resistant Form of pro-NGF. Treatment of invasive cells with a cleavage-resistant form of pro-NGF significantly increased migration at concentrations as low as 1 ng/ml. Values shown are the mean ± SEM for a single experiment. Similar results were seen in three independent experiments Double asterisks (**) indicate p < 0.01, and triple asterisks (***) indicate p < 0.001 versus control (one-way analysis of variance [ANOVA], Bonferroni post-test). Statistics were done on the experiment shown. Found at doi:10.1371/journal.pbio.0050212.sg002 (58 KB TIF).

**Figure S3.** Autocrine BDNF Is Bound to the Cell Surface of p75NTR-Expressing U87 Cells. BDNF protein expression was confirmed by RT-PCR (A). The amount of BDNF protein contained in the conditioned medium (B) or cell associated before and after treatment with a cell surface-stripping reagent (C) was measured by ELISA in cells expressing either empty vector (pcDNA) or a p75NTR-expression vector (p75). U87 pcDNA and U87p75. Values shown are the mean ± SEM from a single experiment; double asterisks (**) indicate p < 0.01 versus control compared to pcDNA; triple pluses (++++) indicate p < 0.001 U87p75 before and after cell surface stripping. Similar results were seen in three independent experiments. Found at doi:10.1371/journal.pbio.0050212.sg003 (153 KB TIF).

**Figure S4.** Down-Regulation of p75 NTR Expression Using RNAi Decreases Migration and Invasion of In Vivo–Selected U87 Invasive Glioma Cells. (A) RT-PCR (GAPDH used as a loading control) and (B) Western blot (pyruvate kinase used as a loading control) confirm knock-down of p75NTR in the in vivo–selected invasive glioma cells transiently transfected with a p75NTR-specific siRNA. (C) Down-regulation of p75NTR levels by siRNA in the in vivo–selected invasive glioma cells significantly reduces their circular migration and transwell invasion. Values shown are the mean ± SEM from four independent experiments. Double asterisks (**) indicate p < 0.01 versus random siRNA transfected cells (paired t-test). Found at doi:10.1371/journal.pbio.0050212.sg004 (136 KB TIF).

**Figure S5.** U87p75 Glioma Cells Are Found Substantial Distances from the Main Tumor Mass. To further illustrate the invasive nature of the p75NTR-expressing glioma cells, serial sections of brains from animals implanted with either pcDNA or p75NTR-expressing U87 glioma cells were stained using a human nuclear-specific antibody. U87p75 glioma cells were found substantially distances from the main tumor mass; right panel shows whole-brain sections posterior to the main tumor. (First two panels are the same as Figure 4). Found at doi:10.1371/journal.pbio.0050212.sg005 (1.1 MB TIF).

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**Author contributions.** SMR, PAF, and DLS conceived and designed the experiments. ALMJ, XL, JJR, AL, and LW performed the experiments. ALMJ, XL, JJR, AL, LW, SMR, PAF, and DLS analyzed the data. MGH, IFP, BLH, and SMR contributed reagents/materials/analysis tools. ALMJ and DLS wrote the paper. MGH and IFP supplied clinical surgical tissue for investigation.

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