CHAPTER FOUR

DISCUSSION

4.1 Summary of key findings

CD44 and CD155 were shown to be highly expressed in glioma cells and both epitopes were closely juxtaposed on their cell membranes. Antibody-mediated blocking and gene knock-down studies of CD44 and CD155 were accompanied by a decrease in invasion, however, no additive or synergistic role of the antigens in curbing invasion was demonstrated. CD44 showed a far greater role in invasion than CD155 as judged by the 'knock-down' studies.

The key findings of the present study are:

1. CD44 was expressed evenly across the cell surface while CD155 sometimes accumulated in 'zones' over the cell surface and at the leading edge of invadopodia in GBM cells.
2. TIRF microscopy revealed close proximity between the two epitopes, albeit at distinct sites on the cell surface.
3. Monoclonal Antibody Blocking and siRNA silencing of the two antigens resulted in a high level of inhibition of invasion. Additionally, blocking and silencing CD44 showed a more substantial decrease in invasion compared to treatment of CD155. However, double MAb blocking and siRNA 'knock-down' of the antigens did not show any further substantial decrease in invasion.
4. Live cell imaging showed reduced motility and velocity in cell movement of 'knockdown' cells.
5. Higher proliferative rates were seen in antibody blocked and silenced cells.

6. siRNA knockdown cells showed a decrease in adhesive potential on various ECMs including fibronectin, laminin, vitronectin, tenascin and collagen I.
7. siRNA silenced cells lowered the expression level of integrins and F-actin and their interactions determined by confocal microscopy
8. The level of Rho GTPase proteins were altered in cells transfected by both siRNA CD44 and siRNA CD155.

Prior to carrying out our experiments, we verified that our cultures were in fact of human origin and not contaminated with rat or mouse cells. Since, MacLeod (1999) highlighted that such species cross-contamination of cultured cells occurs in approximately 20% of all continuous cell lines (Cabrera, 2006); we considered it crucial to show that our cell lines used in the scope of this thesis were of human origin. A PCR based method was used to test the cell lines used and they were all proven to be of human origin and confirmed that no cross-contamination with other species has occurred. However, the limitation of this method was that we could not rule out whether contamination with other human cell lines had happened. Methods such as: isoenzyme analysis for inter-species cross-contamination; HLA typing and DNA fingertyping using short tandem repeats and a variable number of tandem repeats for intra-species cross-contamination (Cabrera, 2006) would additionally have been appropriate. We are now, therefore, planning to set up a new Capillary Electrophoresis platform for DNA sequencing and genotyping assays which will be employed in the cell line fingerprinting screening.

4.2 The expression and close proximity of CD44 and CD155 on glioma cells

Expression and wide distribution of CD44 on the surface of cells of CC-2565, UPAB, UPMC and SNB-19 cultures were demonstrated by ICC and flow cytometry. This was in accordance with previous studies which reported that CD44 is expressed on both normal and reactive cells but is upregulated on neoplastic cells (Pilkington *et al*, 1993). Similarly, previous data have highlighted CD44 expression to be moderate in astrocytes and strong in high grade glioma (Kupper *et al*., 1992 and Ariza *et al*., 1995). The expression of CD44 in areas of cells involved in migration such as the leading edge of cells and processes was consistent with previous studies carried out by Toole and colleagues (1979). The stain was widely distributed over the cells but was more condensed on the leading membrane and ruffles which relates to its function as a cell adhesion molecule and its attachment to hyaluronic acid. However, while the exact mechanism by which CD44 and HA interaction is co-ordinated currently remains unclear, there is speculation that endogenously low levels of HA may inhibit glioma cell proliferation and increase cell-ECM adhesiveness (Stamenkovic *et al*., 1991). On the other hand, substantial concentrations of HA, which have been demonstrated in gliomas, may increase receptor expression to induce cell detachment for migration (Evered & Whelan, 1989; Thomas *et al*., 1992). CD44 is also involved in the uptake of degraded intracellular HA as well as transmitting signals which mediate apoptosis and haematopoiesis (Noar *et al*., 1997). Moreover, the role of HA in cancer cell invasion was described by Knudson and colleagues (1989) who suggested that cancer cells stimulate host fibroblasts to produce and secrete HA by way of a paracrine growth factor mechanism, known as HASA (hyaluronic acid stimulating activity). In the tissue matrix the secreted HA takes on water of hydration and swells to extend the

extracellular spaces and provide a pathway into which neoplastic cells may invade. Invading cells can then adhere to the HA by way of various cell surface receptors including CD44 (Stern *et al*., 2001). Having gained entry to the new host tissue the secretion of hyaluronidase, by invading tumour cells facilitates breakdown of the HA molecules to give rise to a pro-angiogenic product which stimulates the angiogenesis necessary for continued survival of the neoplasm (Pilkington, 1996) as shown in figure 52 below.

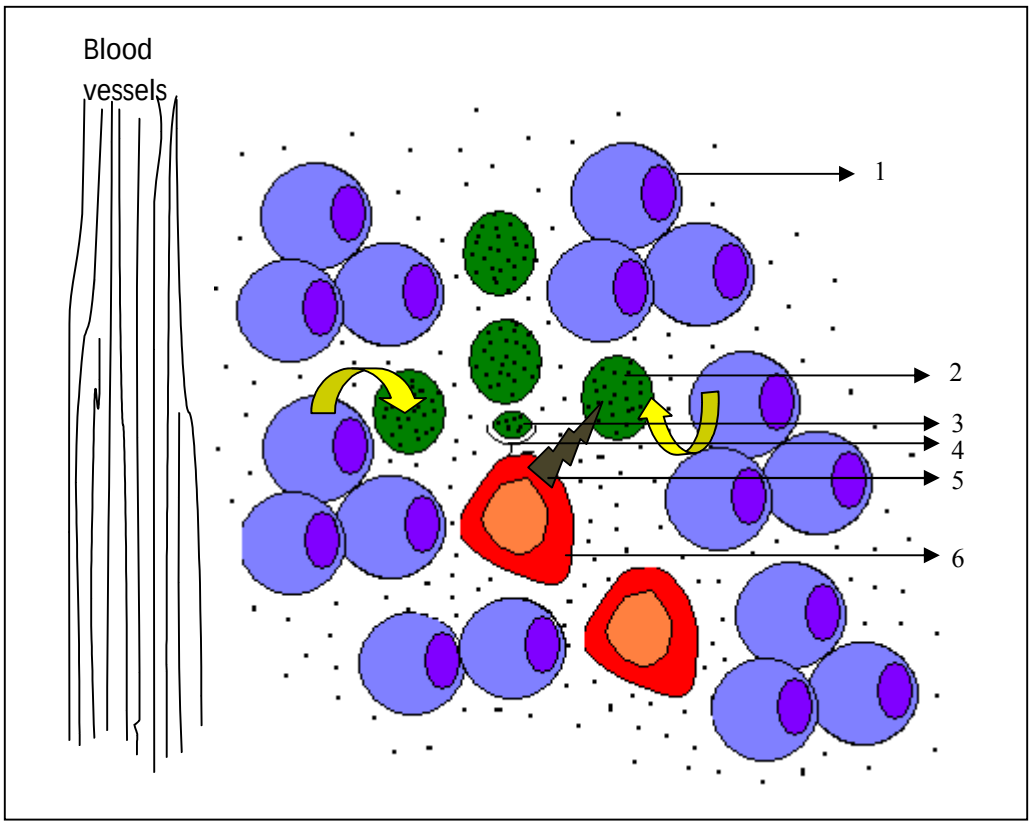

Figure 52: Diagrammatic representation of the role of HA in cancer cell invasion.

Legend: (1) Non-cancer cells are pushed away by the swollen HA to create a passage for cancer cells to move their way through (2) HA takes on water of hydration and swells (3) HA (the main receptor for CD44) produced and secreted by the host normal cells (4) CD44- Invading cells adhere to HA by cell surface receptors including CD44 (5) Hyaluronidase-the secretion of hyaluronidase by invading tumour cells facilitates break down of HA molecules to give rise to pro-angiogenic products (6) tumour cell invading.

Positive and higher expression of CD155 were also evident in GBM cultures (UPAB, UPMC and SNB-19) compared to the normal astrocyte culture (CC-2565). CD155 was well distributed throughout the cells with more condensed expression at cell-cell junctions, established points of adhesion and the prominent filopodia. These results were consistent with findings that CD155 was recruited to the leading edge of migrating cells and that small areas of focal adhesion at these areas were potentially involved in the process of polarization and directional motility (Sloan *et al*., 2005; Bernhardt *et al*., 1994 and Luna and Hitt, 1992). Sloan and co-workers (2004) provided evidence that CD155 co-localised with actin ruffles at the leading edge of migrating cells. This suggested a potential association between CD155 and the actin skeleton of the cell. CD155 also co-localised with an integrin (especially α_v and β_3) known to mediate ECM adhesion by localizing receptors into focal contacts and binding them through interactions with proteins associated with cytoskeletal elements (Merzak and Pilkington, 1997). In general the increased presence of focal adhesions was inversely correlated with the rate of cell locomotion. Therefore, CD155 expressed at the peripheral edges and filopodia of migrating cells may be associated with substrate adhesion and directional motility (Sloan *et al*., 2004). Furthermore, our results were also consistent with previous studies that reported elevated CD155 expression in U87 human glioma cells, primary GBM tumour tissue and high-grade malignant glioma (Merrill *et al*., 2004; Sloan *et al*., 2004). Conversely, Bernhardt and colleagues (1994) stated that, due to the low levels expressed, it was difficult to determine accurate levels of CD155 expression even in cell lines which had been used routinely for the propagation of poliovirus. Since then, consistent expression of CD155 in more recent studies may reflect advanced techniques in detecting expression and

variation of expression among specific glioma cell lines. Better antibodies recognizing the CD155 epitope might have been developed. Additionally various grades of malignant glioma will express different levels of CD155 and affinities for ECM (Merrill *et al*., 2004). Ensuring that gliomas with similar grades and passage numbers are being compared is thus of importance. TIRF microscopy was then used as it gives great clarity and resolution in identifying the exact location of CD44 and CD155 on the cell membranes of glioma cells.

TIRF microscopy which permits high signal, low noise identification of antigen activity within the first 100nm of the sample to glass coverslip interface has helped us in determining how close CD44 and CD155 reside to each other on the cell membrane of brain tumour cells (Mashanov *et al*., 2003) and our results correlated with the current knowledge of CD44 and CD155 expression patterns on the cell surface of human monocytes, which suggested they were closely physically apposed on the cell membrane (Freistadt and Eberle, 1997). CD155 was observed at the leading edges of both cells and cell-cell junctions whereas CD44 was evenly distributed all over the cell surface.

4.3 Inhibition of CD44 and CD155 decreases invasion and increases proliferation in GBM cells.

4.3.1 Invasion

Several previous studies have linked the diffuse local invasion of neoplastic cells, a characteristic of brain tumours, to elevated expression of CD44 (Merzak *et al*., 1994) and it has been reported that CD44 is expressed in many types of migratory cells and metastatic tumour cells (Gunthert *et al*., 1991; Sneath and Mangham, 1998) and has been shown to

promote migratory potential of these cells (Ladeda *et al*., 1998; Okada *et al*., 1996). However, the exact mechanism underlying the phenomenon is obscure which also goes in hand about the relationship of CD155 with invasion.

In the present study, CD44 and CD155 were seen to be expressed on cells which had invaded studied through the Transwell™ Boyden chamber set up and characterisation with Alkaline phosphatase vector red. The results clearly demonstrated that CD44 is involved in human glioma cell invasion *in vitro* and that invasion is probably mediated through its role in interactions between neoplastic cells and ECM proteins (Merzak *et al*., 1994). Likewise, it was previously published that high levels of CD44 are associated with the perceived function of CD44H interaction with HA to induce glioma detachment from the ECM and promote migration and invasion of glioma cells *in vitro* (Koochekpour and Pilkington, 1996). Several lines of evidence support the notion that CD44 promotes tumour progression: for example, overexpression of CD44 promotes tumour growth and metastasis in several experimental models (Sy *et al*., 1991; Bartolazzi *et al*., 1994); and the CD44 variant 6 isoform confers metastatic behaviour on rat pancreatic carcinoma cells (Gunthert *et al*., 1991). In addition there is a good correlation between increased expression of CD44 and enhanced tumour invasiveness by some tumour types. For example, a CD44 proteoglycan can promote the motility and invasiveness of a melanoma cell line (Faassen *et al*., 1992), the expression of CD44 isoforms is correlated with the invasive capacity of various human breast carcinoma cell lines (Culty *et al*., 1994), increased CD44 expression is required for AP-1 mediated tumour invasion (Lamb *et al*., 1997) and CD44 promotes breast cancer invasion and metastasis to the liver (Ouhtit *et al*., 2007). Moreover, CD44 plays an essential role in promoting invasiveness and survival of metastatic breast

carcinoma cells (Yu and Stamenkovic, 1999; Yu *et al*., 1997) and disruption of the CD44 gene has been reported to prevent the metastasis of osteosarcomas (Weber *et al*., 2002). The results obtained in the present study show a strong correlation between CD155 and invasion and are consistent with previous papers which highlighted that the up-regulation of CD155 expression suggested a possible mechanism by which malignant gliomas acquired an invasive phenotype (Sloan *et al*. 2005). Similarly, Goldbrunner and co-workers (1999) reported that the expression of CD155 and its interaction with ECM have been associated with inducing invasive properties in malignant glioma cells.

Two approaches were used in this project to inhibit invasion: firstly by monoclonal antibody blocking and second by transfection against CD44 or CD155 by siRNA. Initial investigation into the effect of blocking monoconal antibody added to the cell culture (applied to the top chamber of the Transwell^{TM} unit) on invasion demonstrated that this treatment causes a substantial decrease in invasion of the GBM cell lines studied. The first conclusion drawn based on the present results is that SNB-19 appears to be the most invasive cell line compared with the other two cell lines used namely, UPAB and UPMC. This corresponds with the flow cytometry data which shows that UPMC had the lowest fluorescence fold and interestingly, invasive potential. The second conclusion is that the monoclonal antibody blocking added to the top chamber drastically decreases invasion, however, whether this is a consequence of the antibody-antigen complex formed that inhibits its passage through the insert or the antibody truly blocks the invasive domain responsible of the antigen rendering him functionless remain to be elucidated. However, the results obtained were consistent with previous papers where Pilkington and colleagues (1993) showed that application of anti-CD44 monoclonal antibody to human glioma

culture impaired their ability to migrate across 8 μm ECM-coated filters in an *in vitro* invasion/motility assay. The possible explanation is that HA synthesis and secretion are stimulated in a paracrine mechanism via sub-populations of heterogenous glioma by release of HA stimulating activity (Pilkington *et al*., 1993). Previous studies have further demonstrated a decrease in glioma invasiveness by a genetically CD44-suppressed human glioma cell line (Radotra and McCormick, 1997). Altogether this proves that CD44 monoclonal antibody blockinghas an effect on invasiveness of a GBM-derived cell line and that CD44 plays an important role in human glioma cell invasion *in vitro* (Merzak *et al*., 1994). Furthermore, Merzak *et al*. (1994) showed that the application of a CD44-specific monoclonal antibody and antisense oligonucleotides to human glioma cell lines *in vitro* were able to disrupt the invasion processes. Moreover, studies in animal models carried out by Stern *et al* (2001) have demonstrated that injection of reagents that disturb CD44-ligand interaction subsequently inhibit local tumour invasion and metastatic spread. The suppression *in vivo* compromised the invasion of human glioma cells transplanted in mice (Berens and Giese, 1999; Quackenbush *et al*., 1990). Similarly, studies have illustrated the inactivation of the CD155 receptor through Fluorophore Assisted Light Inactivation (FALI), which resulted in reduced Transwell^{TM} migration of HT1090 fibrosarcoma cells *in vitro* while siRNA knockdown of CD155 in U87MG GBM cells resulted in similar findings (Sloan *et al*., 2004). Additionally, Enloe and Jay (2010) have reported that inhibition of CD155 may limit the spread of malignant brain tumour cells.

It was possible to achieve effective CD44 and CD155 knock-down (24.27% and 60.83% respectively) at the gene level with the introduction of CD44 siRNA and CD155 siRNA. The delivery of nucleic acids into cells is one of the most commonly used molecular

biology techniques. However, it is the efficient delivery of nucleic acids that is the essential step, which must have a fine balance between maximum transfection efficiency and minimal cytoxicity. RNA interference (RNAi) can be used to suppress the function of a gene in cell lines by introducing siRNA into the cell. This will trigger an endogenous RNAi pathway, which inhibits target gene expression and this whole process is called gene knock-down or gene silencing. One way of increasing the probability of obtaining knock-down is by using a target-gene specific mixture (pooling of siRNA duplexes) of different siRNAs more likely to contain effective sequences. Off-target effects were minimized by using highly purified, low concentration of each individual siRNA in the pool.

Transfection of CD44 and CD155 with the Accell siRNA and delivery media was not toxic to the cells and did induce CD44 and CD155 gene knock-down. It should be noted that *in vivo* the issue of delivering siRNA has been the greatest obstacle to the successful treatment of gliomas and the achievement of intracellular delivery at the therapeutically effective concentrations is still a major challenge (Chekenya and Immervoll, 2006).

However, although RNAi generally occurs within 24 hours of transfection, both onset and duration of RNAi depend on the turnover rate of the protein of interest as well as the rate of dilution and long life of the siRNAs (Mocellin and Provenzano, 2004).

SNB-19 (Homogenous culture) was the glioblastoma cell line used in all the knock-down studies rather than UPAB and UPMC (both heterogeneous cultures). The choice of the cell line was thus determined as studies reported that epigenetic regulations and the state of differentiation may affect the transduction/transfection efficacy and hence, the more

heterogeneous the cell population the more variable the expression of the desired gene (Sarkis *et al*., 2000).

Gene knock-down of CD44 and CD155 were initially evaluated by Western blot. Although semi-quantitative, this method allowed estimations of whether gene knock-down had occurred. The positive control for silencing and validation of experimental design used was GAPD siRNA and the complete ablation of its protein confirmed that transfection was successful. Although a band for CD44 was still obvious after transfection, it was much fainter than the untreated/control CD44, indicating that knock-down was at least partially successful. The probable explanation for the incomplete knockdown of CD44 was that the high turnover rate of CD44 protein, which is 20 hours as stated by Goebeler and co-workers (1996) on aggressive melanoma cells. In addition, Vikesaa and colleagues (2006) which reported by performing a knock-down of all CD44 transcripts using siRNA, the amount of CD44 protein was reduced to approximately 15%. Our results showed that complete knock-down of CD155 was achieved. Other means to quantitate knock-down efficiency such as qRT-PCR and northern blot maybe however more appropriate. Northern blot analysis remains a standard method for the detection and quantitation of mRNA level despite the advent of more sensitive approaches such as real time PCR.

To further confirm the initial investigations into the effect of Monoclonal Antibody blocking of CD44 and CD155 as discussed above, a similar Boyden chamber invasion assay following transfection of siRNA for CD44 and CD155 was carried out respectively. The results obtained yielded in a similar reduction in invasion although the number of cells which had invaded differed. The discrepancy observed was as expected and indeed the gene knock-down technique was seen to be more effective than MAb blocking. As

explained above, these results matched strongly those published previously. Additionally, it was reported that CD44 may be one of the mediators responsible for the differences in invasive behaviour between meningioma and malignant gliomas and may be pivotal in rendering both tumours unable to metastasize (Picker *et al*., 1989; Stamenkovic *et al*., 1991). This could be explained by the fact that CD44s is only weakly expressed in meningioma cells (Rooprai *et al*., 1999) but strongly expressed in glioma cells (Kuppner *et al*., 1992). Additionally, the brain is rich in hyaluronate (Rukta *et al*., 1988), a potent ligand of CD44 (Carter *et al*., 1988; Lesley *et al*., 1990; Aruffo *et al*., 1990;Miyake *et al*., 1990). Moreover, a variant CD44 molecule that seems to confer metastatic potential to non-metastatic tumour cells (Gunthert *et al*., 1991) is present in brain metastases but not in glioblastoma multiforme (Li *et al*., 1993). It is thus possible to postulate that the inability of CD44-poor meningioma cells to bind to hyaluronate would render them incapable of infiltration into the brain whereas CD44-rich glioma cells would successfully migrate by CD44-hyaluronate interaction. This line of thought could be applied to CD44-depleted glioma cells and hypothesize the same effect in terms of invasion as we have been dealing with CD44s throughout the thesis. Silencing CD44 would result in the inability of cells to bind to hyaluronic acid thus impairing their role in invasion as explained in section 4.2 (figure 52).

4.3.2 Proliferation

A proliferation assay was also carried out to determine whether an inverse relationship existed between invasion and proliferation as has been previously reported (Giese *et al*., 1996 a,b). Different approaches to measure proliferation include the identification of mitotic figures. In this thesis, the cellular incorporation of BrdU into the DNA of proliferating cells have been used as it gave a good indication of the 'S' phase of the cell cycle. Two treatment regimes (MAb and siRNA) were used to assess the proliferative rate of cells and compared.

First, cells were blocked with their specific monoclonal antibody; CD44, CD155 and CD44+CD155 respectively. A marked increase in proliferation was observed under all these treatment conditions in comparison to the control cells; 0.341 (MAb CD44), 0.326 (MAb CD155), 0.363 (MAb CD44+CD155) and 0.168 (untreated cells). Secondly, cells were transfected with their siRNA for 120 hours and their proliferative rate assessed afterwards. The results reflected those obtained by blocking the antigens with their respective monoclonal antibodies. The proliferative rate increased substantially in post-transfected cells compared with untreated cells; 0.314 (siRNA CD44), 0.333 (siRNA CD155), 0.375 (siRNA CD44+CD155) and 0.175 (untreated cells). The above findings showed that, irrespective of the technique, the highest proliferative rate was reached when both antigens were blocked/silenced in conjunction rather than singly. Based on the invasion assay data obtained, these results were not unexpected since there have been numerous papers that showed the inverse relationship between proliferation and invasion (Giese *et al*., 1996 a,b; Giese *et al*., 2003; Finn *et al*., 1996; Hatzikirou *et al*., 2010). This may be explained as follows: A cell has to arrest from the cell cycle to be able to invade.

Cells transiently arrest from the cell cycle (become "quiescent") in order to migrate. Once these migratory tumour cells have reached a suitable site within a given tissue (determined by perhaps nutrient supply or O_2) they can stop and return to cycling mode to re-establish a second tumour focus. Additionally, in substantiation of the above hypothesis, Szeto *et al* (2009) reported that a mathematical model had been developed that described invasion in terms of two patient specific variables: the net dispersal rate and proliferation rate. Szeto's study (2009) showed that a high proliferation rate would correspond to a mitotically active tumour with relative low invasiveness and conversely, a low proliferation capacity would correspond to a diffusely invasive tumour. Likewise, Chekenya and colleagues (1999) stated that proliferative cell populations are not considered to be invasive. *In vitro* experimental observations further confirmed the essential decrease in cell motility during cell proliferation. The above results were consistent with previous studies which highlighted the observation that CD44 promotes tumour cell invasion (Toole, 2004). Therefore, it can be postulated that inhibiting CD44 expression would increase cell proliferation. Furthermore, earlier studies have reported that despite the exact mechanism by which CD44 and HA interaction is co-ordinated remain unclear, there is speculation that endogenously low levels of HA may inhibit glioma cell proliferation and increase cell-ECM adhesiveness (Stamenkovic *et al*., 1991). Similarly, HA-induced activation of CD44 and RHAMM has been shown to induce the invasion of multiple cancer cell lines. Enzymes of the matrix metalloproteinase (MMP), serine protease and cysteine protease families have been reported to underpin cancer cell invasion and metastasis (Deryugina and Quigley, 2006; Mohamed and Sloane, 2006; Gocheva and Joyce, 2007; Lopez-Ortin and Matrisian, 2007). There is increasing evidence that there is significant interplay

between these different protease families in promoting tumour invasion. MMP-9 has a high avidity for degradation of type IV collagen, an extracellular matrix protein that is enriched in the basement membrane. MMP-9 has been shown to associate with the large ectodomain of CD44 in murine mammary carcinoma cells (Yu and Stamenkovic, 1999). Consequently, this interaction localizes MMP-9 proteolytic activity to the invasive surface of the leading edges of 'invadopodia', promoting physical degradation of the matrix in the immediate vicinity of the cell. Previous studies have also demonstrated a dose-dependent cell detachment activity and depending on the concentration and cell type, either stimulation or inhibition of glioma cell proliferation was possible (Koochekpour *et al*., 1995; Sy *et al*., 1991). Anti-CD44H monoclonal antibodies have been applied to these cell populations effectively which reversed any proliferative or anti-proliferative effects as a result of CD44H interaction with HA.

4.4 CD44 and CD155 knock-down alter cell morphology and decrease velocity of cell movement

4.4.1 Cell morphology

An increasing number of investigations are being carried out using live cell imaging techniques to provide critical insight into the fundamental nature of cellular and tissue function. Time-lapse microscopy, in which live cells (untreated and post-transfected with siRNA) were monitored under standard culture conditions for 72 hours allowed assessment of the proliferative rate and morphology of cells and quantitative tracking of cells for velocity and motility rate (explained in section 2.13). Throughout the 72 hours,

there was no reduction in cell viability and this proved that all the critical settings were set correctly and that the treatment was not toxic to the cells. At the end of the 72 hours, it was clear that the cells treated with siRNA (CD44, CD155 and CD44+CD155) had a higher proliferative rate than the control cells as confirmed by the high confluency rate in these wells. Additionally, post-transfected cells showed changes in morphology to a much rounder shape which was further confirmed by ICC and flow cytometry. Our results were consistent with those of previous published works which pinpointed that CD44-depleted cells were strikingly devoid of invadopodia (Vikesaa *et al*., 2006). This result therefore confirms that overexpression of CD44 correlates with the invasive capacity of cancer cells (Jothy, 2003). CD155-silenced cells seem to follow the same trend where the cells were round, devoid of any invadopodia and had a fainter granular staining compared to the control cells and this result is in accordance with those published by Sloan *et al* (2005) which reported that depletion of CD155 resulted in increased uniformity of shape in particular the rounding up of cells and absence of ruffles at the leading edge. Similar findings were brought forward by Sloan *et al* (2004) which illustrated that siRNA knock-down of CD155 in cultured glioma cells (U87MG). Additionally, the morphological changes following knock-down of CD155 and CD44 are further supported by the results obtained in figure 50.1 and figure 51 which show a significant decline in expression of F-actin and a marked reduction in RHO GTPases expression such as Rho, Rac and cdc42. Whilst Rho is involved in the bundling of actin filaments into stress fibres and formation of focal adhesion complexes, Rac plays a role in the polymerisation of actin to form lamellipodia and membrane ruffles and cdc42 is involved in the polymerisation of actin to form filopodia protrusion. Thus, reduction in

their expression result in the inability of actin to form protrusion involved in invasion as well as disorganisation of the actin cytoskeleton and therefore leading to a more uniform and rounder morphology of cells.

4.4.2 Motility rate and velocity of cell movement

The motility rate of the cells silenced with siRNA was seen to decrease considerably compared to control cells and our findings demonstrated that when gene knock-down was applied to both genes, the cells achieved the lowest motility rate.

Similarly, the speed by which siRNA knock-down cells moved was substantially reduced in comparison to control cells. The highest velocity reached was in untreated cells and lowest was observed with silencing of the both CD44 and CD155.

However, no literature has been published yet on the topic and thus it is impossible to validate the significance of these findings. The main critical aspect to take into consideration when monitoring transfected cells for cell live imaging experiments is that the effects of introduction of siRNA are usually short-lived (72-96 hours post-transfection) due to the inherent short-life of these molecules within mammalian cells (Mathupala *et al*., 2006).

4.5 Inhibition of CD44 and CD155 decreases adhesiveness of GBM cells.

Matrix adhesion and modulation is an important factor in tumour progression (Duffy *et al*., 2008; Cretu and Brooks 2007). The adhesion assay used consisted of an array of ECM and under two different treatment regimes namely MAb blocking and transfection with siRNA. Under both treatments, levels of adhesion on non-specifically coated surfaces (BSA) were considerably lower than on the ECM-coated wells. The main finding was that both treatments (MAb and siRNA) caused a definite decrease in adhesion of cells to different ECMs compared to control cells with the exception of vitronectin where unexpectedly, MAb CD155 (0.161) and siRNA CD155 (0.168) treatment both resulted in the highest level of adherence in cells over untreated cells. Additionally, irrespective of the treatment condition, cells adhered most effectively to laminin especially when no treatment was given. This was followed by fibronectin, collagen I, vitronectin and tenascin. A similar pattern of adhesion to different ECMs was seen with both siRNA and MAb. Chiquet-Ehrismann and colleagues (1995) reported while laminin is the most important of all ECM proteins in the context of brain tumour invasion, another ECM component, tenascin has been shown to be expressed in relatively high quantities in malignant glial tumours in man. The tenascins are thought to counteract the cell adhesion activity of fibronectin to which they bind thus facilitating cell migration. Tenascin has also been shown to facilitate glioma migration *in vitro* (Giese *et al*., 1995). The high expression levels of the ECMs in the untreated cells could further be justified as stated by Claes *et al* (2007) who reported that glioma cells may create their own microenvironment by synthesizing and depositing ECM molecules such as laminin, vitronectin and tenascin-C. Saitoh and co-workers (1995)

have on the other hand, reported that several ECM proteins such as tenascin, vitronectin and hyaluronic acid are expressed directly by the glioma cells themselves.

CD44 was one of the main proteins under investigation, so hyaluronic acid/hyaluronan would have been the best ECM to compare its adhesiveness as reported by various literature that the major ligand for CD44 is hyaluronan (Toole, 2004; Aruffo *et al*., 1990) and it is one of the major glycosaminoglycans in the brain (Knupfer *et al*., 1999). Unfortunately, it was not commercially available and thus attempting to produce hyaluronic acid in-house would have been costly and labour intensive. CD44 can, however, interact with several additional molecules such as collagen, fibronectin, laminin and fibrinogen (Naor *et al*., 2002). Underhill (1992) reported that all CD44 isoforms contain an HA-binding site in their extracellular domain and thereby serve as a major cell surface receptor for HA. Both CD44v isoforms and HA are overexpressed at sites of tumour attachment (Toole *et al*., 2002). It has furthermore been determined that HA binding to CD44v isoforms is involved in the stimulation of both receptor kinases (Bourguignon *et al*., 2007) and non-receptor kinases (Bourguignon *et al*., 1999) required for a variety of tumour cell-specific functions leading to tumour progression. Previous literature has demonstrated that blockade of CD44 function using neutralizing antibodies or inhibition of CD44 expression using siRNA attenuated the adhesion of the PC3 prostate cancer cell line and the MDA-MB-157 breast cancer cell lines and overexpression of CD44 in the DU145 prostate cancer cell line and the T47 breast cancer cell line increased the capacity for these cells to adhere to bone marrow endothelial cells. Therefore, the capacity with which metastatic cancer cell lines adhere can be dictated by manipulating the expression of CD44 in these cancer cells (Okada *et al*., 1999; Draffin *et al*., 2004).

The above findings were consistent with previous studies that reported laminin and structural collagen were found to be most adhesive substrates for G-112 glioma cells and that fibronectin and vitronectin only slightly promoted cell adhesion (Giese *et al*., 1998). It was also demonstrated that areas of the brain that strongly promoted adhesion expressed at least one of the following matrix proteins; laminin, structural collagens, collagen type IV, fibronectin or vitronectin (Giese *et al*., 1998). Additionally, Koochekpour *et al* (1996) reported that while ECM proteins such as collagen type I, fibronectin, laminin, vitronectin inhibit glioma cell proliferation, they upregulate migration *in vitro*.

Lange *et al* (2001) reported that CD155 binds specifically to vitronectin which is an important component of the extracellular matrix. The vitronectin/CD155 interaction can be distinguished by a rapid complex formation and CD155 and vitronectin are co-expressed on other tissues (Lange *et al*., 2001). Vitronectin was also shown to bind to several cell receptors particularly of the integrin type, therefore plays a role in promoting cell attachment and invasion (Uhm *et al*., 1999 and Gladson *et al*., 1995). Our results, however, show that both treatments (MAb blocking and siRNA knock-down) of CD155 resulted in a higher adhesive potential on vitronectin, thus conflicting with those data published previously (Sloan *et al*., 2005). The probable explanation could be that CD44 is a stronger receptor for vitronectin as shown by our results. The double siRNA knock-down shows that adhesion on vitronectin was higher than that of singly knocking down CD155.

It has also been reported that altered cell-matrix interaction may result in pathological cellular behaviour such as invasion and metastasis of solid tumours (Liotta & Stetler-Stevenson, 1991).

As mentioned above, both treatment regimes (MAb and siRNA) demonstrated a net decrease in adhesiveness of cells to various ECMs used. These results can further be related to those obtained by the live cell imaging data which showed a marked decrease in speed of cell movement and motility of cells under similar treatment. This can be explained as if cells cannot adhere, they cannot move. Furthermore, since no ECM coating for live cell imaging was used, this suggests that glioma cells may produce their own ECM which may be the result of autocrine stimulation with example TGFβ (Pilkington, 1996).

4.6 CD44 and CD155 knockdown is accompanied by changes in integrin expression and RHO GTPases signaling pathways.

4.6.1 Integrin expression

Integrins have a variety of functions such as: cell to substrate and cell-cell adhesion and cell maintenance and signaling (Ivaska, 2010). Integrins are needed in cell movement, but they might also have other roles in cancer invasion. Enzymes such as cathepsins and MMPs play a significant role in brain tumour invasion by remodeling the ECM. In addition, Baronas-Lowell and colleagues (2004) have demonstrated that by hyaluronan binding, CD44 contribute to tumour invasiveness by stimulating the production of matrix metalloproteinase-2 and -9. Integrins are implicated in this behaviour by modulating the activity of such enzymes.

All three GBM cell lines used in the present study (UPAB, UPMC and SNB-19) showed similar staining patterns with single labeling for F-actin, β_1 integrin, $\alpha_v\beta1$ integrin and $\alpha_v\beta_3$ integrin and co-expression with either CD44 or CD155.

SNB-19s were also transfected with siRNA to achieve knock-down of CD44 and CD155. They were then co-labeled with either the above mentioned integrins or F-actin. A consequent decline in expression level of the integrins and F-actin was seen and these results were consistent with the protein levels obtained through Western blotting.

The above findings were consistent with previous papers which reported that integrins are involved in glioblastoma progression (Desgrosellier, 2010), with α_v and β_1 being central to glioma invasion (Hu, 2006). CD44 was shown to promote integrin receptor activation, in order to mediate the arrest of movement and firm adhesion of these cells on endothelium (Siegelman *et al*., 2000; Nandi *et al*., 2004). β_1 integrin is reported to be the most commonly expressed subunit in glioma (Rooprai *et al*, 1999). Furthermore, functional *in vitro* studies on the antibody blocking against β_1 integrin have shown that glioma adhesion, motility and invasion is inhibited to different degrees, dependent upon the ECM substrate the glioma was cultured on, and this thus led to the suggestion that other integrins and β_1 dimers are implicated in glioma invasion (Giese, 1994). Additionally, the α_v subunit appears to be the subunit capable of mediating the putative invasion-enhancing signal (Seftor *et al*., 1992). A marked expression of the α_v subunit has been found in several glioma cell lines (Haugland *et al*., 1997).

Earlier publications have reported that $\alpha_v\beta_1$ subunits are expressed together as a receptor for vitronectin. Vitronectin expression correlates with malignancy again suggesting this is produced by neoplastic cells. GBM migration has also been shown to be inhibited with both anti-β_1 and anti- α_v antibodies (Barcellos-Hoff, 2009). In addition, in embroyonic cells $\alpha_v\beta_1$ integrin was instrumental in cell attachment and spreading on fibronectin only when these cells were made deficient of $\alpha5\beta1$ integrin (Yang & Hynes, 1996) indicating that the

role of $\alpha_v\beta_1$ integrin may be linked to malignant phenotype of cells (Kovisto *et al*., 2000). This statement was further supported by earlier studies which reported that overexpression of $\alpha5\beta_1$ from transfected cDNA increases the deposition of fibronectin matrix (Giancotti and Ruoslahti, 1990) and that malignant transformation often reduces the expression of the $\alpha5\beta_1$ integrin and this is accompanied by a loss of fibronectin matrix around the transformed cells (Plantefaber and Hynes, 1989). The matrix loss is thought to contribute to the migratory and invasive properties of such cells. It was shown that overexpression of the $\alpha5\beta_1$ integrin in CHO cells drastically restricts the ability of these cells to migrate and makes them non-tumourigenic (Giancotti and Ruoslathi, 1990), whereas elimination of the integrin has the opposite effect (Schreiner *et al*., 1991).

Previous studies have also reported that the heterodimer $\alpha_v\beta_3$ is commonly expressed in glioblastoma (Bello, 2001). $\alpha_v\beta_3$, the ligand for vitronectin, tenascin, osteopontin, von Willebrand factor and thrombospondin is involved in glioma invasion (Bellail *et al*., 2004). In particular, $\alpha_v\beta_3$ integrin has been identified as a tumour progression marker (Danen *et al*., 1995). $\alpha_v\beta_3$ has also been found to be expressed in GBM specimens (Paulus *et al*., 1993 and Gingras *et al*., 1995). Earlier correlative observations suggested that the $\alpha_v\beta_3$ integrin is one of the integrins that plays a role in tumorigenicity and metastasis. Thus, elevated expression of this integrin is associated with invasive tumours including melanoma *in vitro* (Albelda *et al*., 1990).

Actin constitutes a major component of the underlying molecular machinery that drives cell shape and protrusions; it adopts different organizational configurations in lamellipodia and filopodia, the two main types of cells processes involved in invasion (Vicente-Manzanares *et al*., 2009). During cell spreading and locomotion the assembly of early cell

contacts to the ECM at the leading edge is driven by actin polymerization (Pollard *et al*., 2003; Ballestrem *et al*., 1998).

The linkage between integrin receptors and the actin cytoskeleton appears to be crucial for the integrity and stability of adhesions. Interestingly, it was highlighted that the $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins have been implicated in interactions with actin cytoskeleton, since these integrins localize to focal contacts when fibroblasts spread on either fibronectin or vitronectin (Singer *et al*., 1988). Similarly, recent data reported that comet-like actin associate with integrins near the leading edge and induce β_1 integrin clustering at the tip of newly formed actin ripples or filopodia (Galbraith *et al*., 2007). Integrin receptors may also regulate different aspects of cell motility, e.g., migration speed and motile phenotype, as has been shown for $\alpha4\beta_1$ and $\alpha5\beta_1$ integrins (Chon *et al*., 1998). The strong co-localisation at the leading edges of cells seen when the GBM cells were stained with CD155 and $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins were consistent with previous papers which reported that $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins which are upregulated on glioma cells are receptors for vitronectin (Claes *et al*., 2007) and that vitronectin is preferentially expressed at the advanced margins of gliomas (Uhm *et al*., 1999). Additionally, recently, CD155 has been proposed as playing a key role in glioma motility and invasion whereby it is recruited to the leading edge of migrating cells and co-localises with α_v integrins and actin (Sloan *et al*., 2004). On the other hand, following siRNA knock-down of CD155, a consequent decrease in its expression and co-localisation with $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrins were observed.

4.6.2 RHO GTPases signaling pathway

The intracellular mechanisms of astrocytoma migration are poorly understood but ultimately require a balance of environmental cues and responsive intracellular signals that lead to dynamic regulation of the interactions between actin microfilaments, microtubules and intermediate filaments (Maidment, 1997). The driving force for cell movement, however, is normally provided by dynamic reorganization of the actin cytoskeleton, directing protrusion at the front of the cell and retraction at the rear (Maidment, 1997; Raftopoulou and Hall, 2004; Sahai and Marshall, 2002). In this regard, the key mediators of actin cytoskeleton reorganization are the Rho family of GTPases which have been previously implicated in cell motility and invasive phenotypes (Raftopoulou and Hall, 2004; Sahai and Marshall, 2002; Schmitz *et al*., 2000).

The preliminary data obtained with the present project show some degree of consistency with previous publications for example, where Okamoto and collegues (1999) reported that when the dominant active mutant of Rac1 was overexpressed in human glioblastoma cells (U251MG), CD44 was shown to redistribute to lamellipodia where it was subsequently cleaved and shed from the surface. Additionally, Murai *et al* (2004) observed that engagement of CD44 induces cytoskeletal rearrangement and activation of the small GTPase Rac at the leading edge of the membrane ruffling areas. This raises the possibility that intracellular signals elicited by ligation of CD44 induce CD44 cleavage and enhance tumour cell migration and invasion through activation of Rac. Oliferenko *et al* (2000) stated that the precise details of Rac1 activation upon HA/CD44 engagement remain to be elucidated but it is tempting to speculate that one of the Rac1 accessory proteins could participate in complex formation with CD44. Thus based on the above observations, it can

be postulated that knock-down of CD44 should reduce the level of Rac and hence, migration. Salhia *et al* (2005) reported that to further examine the role of Rac in astrocytomas, a Rac1-directed siRNA experiment was carried out and the findings confirmed reduction of Rac protein expression in Rac1-depleted U251 cells compared to untransfected and non-targeting siRNA controls. Rac1 depletion with either the Rac1-1 or Rac1-2 siRNA oligo inhibited the migration of U251 cells by 2 fold. Similarly, siRNAs directed against Rac1 or Rac3 have demonstrated that depletion of Rac1 strongly inhibits lamellipodia formation, cell migration and invasion in SNB19 GBM cells (Chan *et al*., 2005).

Several previous studies have confirmed our findings that Rho proteins play a specific role in cancer progression. Studies have shown that RhoA appears to be up-regulated in human carcinoma, specifically in high-grade astrocytomas (Yan *et al*., 2006). RhoA expression and/or activity have also been implicated in tumour invasion and metastasis (Banyard *et al*., 2000; Michiels and Collard, 1999). Forget *et al* (2002) also reported the first astrocytic tumour markers, RhoA and RhoB were present in all brain samples. Therefore, as our results demonstrated, single and double knock-down of CD44 and CD155 resulted in a marked decrease in expression level of RhoA, with a more profound decrease achieved with double knock-down; it could thus be hypothesized that this in turn may inhibit tumour migration and invasion.

Yang *et al* (2006) recently reported that Cdc42-null primary fibroblasts are defective in filopodia induction, directed migration and proliferation while Pankov *et al* (2005) reported impaired migration after siRNA-mediated knockdown of Cdc42. Furthermore, the loss of Cdc42 function in primary mouse fibroblasts has been reported to impair migration

speed (Yang *et al*., 2006). Since we have showed that siRNA CD44 silencing resulted in a marked decrease in expression level of Cdc42, it could therefore, be postulated that migration could be halted in the context of brain tumour invasion.

Monypenny and collegues (2009) reported that combined knockdown of Cdc42, Rac1 and RhoG resulted in far greater inhibition of cell migration than when each protein was knocked down alone, demonstrating that these GTPases function co-operatively during fibroblast formation. In earlier publications, it was reported that the speed of cell migration is dependent on substrate composition, and the relative levels of Rho and Cdc42 activation vary with extracellular matrix composition (Adams and Schwartz, 2000; Ridley, 2000; Wenk *et al*., 2000). Therefore, by reducing the level of extracellular matrix deposition, the level of Rho and Cdc42 activation could be impaired and in turn reduce invasion.

No clear literature has yet however, been published on this topic, especially the involvement of RHO GTPases with CD44 and CD155, thus it is impossible to validate the significance of our initial findings without further investigations.

4.7 Conclusions

The main aim of this study was to demonstrate the expression of CD44 and CD155 in glioma cell lines at different levels of cellular heterogeneity and passage level using a variety of microscopy techniques such as epi-fluorescence, confocal and TIRF. Additionally, we investigated the roles and effects of MAb 'blocking' and siRNA "knock-down" of CD44 and CD155 on brain tumour invasion, proliferation and adhesion.

Our preliminary data confirmed the positive expression of CD44 and CD155 in glioma cells lines. These extracellular antigens showed high levels in GBM cells compared to normal human astrocytes CC-2565 as demonstrated by flow cytometry. ICC showed the localisation and distribution of these antigens in both CC-2565 and GBM cells. Epi-fluorescence microscopy demonstrated close proximity of both antigens which was further confirmed by TIRF microscopy. No co-localisation was observed between the two antigens. CD155 was seen to be mainly recruited at the leading edges of migrating cells and invadopodia whereas CD44 was uniformly expressed throughout the cells. These observations are in accord with those of Sloan *et al* (2004) and Pilkington (1993) on single antigen expression studies for CD155 and CD44 respectively.

Monoclonal blocking antibody studies resulted in a consequent decrease in invasion of GBM cells which was in line with earlier publications (Merzak *et al*., 1994 and Sloan *et al*., 2004). However, blocking of both antigens did not seem to affect greatly the invasive potential of cells. Based on the findings, we could thus stipulate that the key player to inhibit invasion was CD44 and not CD155 although both antigens are closely located on the cell membranes of glioma cells. To validate these results, SNB-19 cells were

transfected with siRNA CD44 and siRNA CD155 to achieve transient knock-down. These cells were then investigated for their invasiveness. As shown by the above technique, substantial decrease in invasive potential was achieved. Live cell imaging on silenced cells have demonstrated a consequent decline in velocity of cell movement and total distance moved compared to untransfected cells where double knock-down of CD44 and CD155 have proved to work conjointly to bring the highest reduction rate of the parameters mentioned above. ICC on knock-down cells demonstrated a change in morphology of cells which had rounder shape and were devoid of filopodia with a low fluorescence intensity for CD44 and CD155 which was further supported by flow cytometry.

Similarly, we have demonstrated that overall, a net decline in adhesive potential of silenced cells over controls was reached. Out of all the ECMs investigated, untreated cells adhered best to laminin whilst cells treated with CD155 (MAb and siRNA) resulted in a highest level of adhesion to vitronectin. The former observation was consistent with previous publication (Giese *et al*., 1998) while the latter showed conflicting evidence with studies undertaken by Lange *et al* (2001).

We have furthermore confirmed the hypothesis stated by Szeto *et al* (2009) which stated that proliferation rate was inversely correlated to invasion. We have proved that silenced cells had a higher proliferative rate over untransfected cells and cells were most proliferative when expression of both CD44 and CD155 were inhibited. This observation fully supports our invasion assay findings.

In line with the proposition made by Sloan *et al* (2004) on CD155, we have shown consistency in our findings. Confocal microscopy showed that CD44 and particularly

CD155 in all GBM cells co-localised with β_1 integrin, $\alpha_v\beta_1$ integrin and $\alpha_v\beta_3$ integrin at the leading edge of cells and in their filopodia. These results confirmed that CD155 might play a key role in glioma motility and invasion, albeit not as significant as that of CD44. Transfection of CD44 and CD155 in SNB-19 cells clearly showed a reduced level of expression of the integrin sub-units and no co-localisation was seen.

Our initial findings with regards to the signal transduction pathway studies through the RHO GTPases have demonstrated that CD44 and CD155 indeed contribute to invasion via similar pathways. Gene silencing of CD44 and CD155 resulted in a decrease in expression level in some of the key mediators of invasion particularly Rac 1/2/3, RhoA and Cdc42. These data are consistent with publications mentioned in section 4.6 for other non-neural neoplasms.

4.8 Future work

4.8.1 Stable siRNA transfection of CD44 & CD155

To achieve stable transfection, CD44 and CD155 genes will be identified. We will then design target sequence for small interfering RNA (siRNA) which will then be checked using the Basic Local Alignment Search Tool (BLAST) program to ensure that non-specific binding of untargeted siRNA does not occur. Subsequently, the appropriate siRNA sequence will be inserted into pre-constructed virus vectors available commercially (e.g; Thermofisher scientific, UK) which will in-turn be used to transfect glioma cell lines to inhibit the expression of CD44 and CD155.

4.8.2 Signal Transduction studies

Following the preliminary signal studies involving the RHO GTPases which is the cytoskeletal signaling pathways, we will be investigating the pathways for migration signaling which include the ERK and PI3-kinase pathways in cells after ligation or blockade of CD155 and/or CD44.

4.8.3 Three-dimensional (3D) "all-human" spheroid confrontation assay

We have recently developed an "all-human" 3D spheroid model to study tumour invasion into non-neoplastic brain (Pilkington and Parker, 2007). An adaptation of the "hanging-drop" method was used to produce multicellular tumour spheroids from brain tumour biopsy tissue then juxtapose these with spheroids produced from astrocyte-rich cultures derived from the brains of patients who had undergone surgical resection for intractable epilepsy. These cultures are also maintained under human serum-supplemented conditions (Biowest Ltd). Invasion of glial neoplasms into "target" brain spheroids is studied using vital cell markers for each population by either confocal microscopy or by live cell (real time) imaging over periods of up to 72 hours. Confrontation cultures may be kept in healthy condition for several days and are studied subsequently, following fixation and processing by light, fluorescence and electron microscopy. We will thus adapt this technique to further investigate the role and effects of CD44 and CD155 and their knock-down in invasion studies.

4.8.4 In vivo studies of knockdown (KD) for CD44/CD155

CD155 KD, CD44 KD, combined CD155 & CD44 KDs, Scrambled KD and Controls for each of two cell lines IN699 (a paediatric GBM with very high CD133 expression) and UPAB (a heterogenous adult GBM with low CD133 expression) will be used to produce tumours in orthotopic brain tumours in nude mice. Cells will be transduced with a lentiviral vector expressing the luciferase gene to allow *in vivo* imaging of tumour progression. Mice will be anesthesized with a mixture of ketamine (10mg/ml) and xylazine (1mg/ml) and fixed in a sterotaxic frame and tumour cells will be slowly injected into the right frontal cortex. Development of tumours will be monitored weekly using bioluminescence imaging. After sacrifice, brains will be removed and prepared for immunohistochemical analysis. Tumour volume at endpoint will be evaluated by IHC analysis to monitor changes in invasion, proliferation (Ki67) and relation to tumour vasculature (CD31).

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