CHAPTER THREE

RESULTS
3.1 Human Authentication PCR

Cells used in the scope of thesis were first confirmed as being of human origin and uncontaminated by mouse and rat DNA. DNA known to exist in human DNA only and from mouse and rat tails were run on a 1.5% agarose gel with the results shown below. The human, mouse and rat DNA were used as positive controls.

Three glioma cell lines: UPAB; UPMC; SNB-19 and one non-neoplastic astrocyte cell culture: CC-2565 were analysed and confirmed to be of human origin when compared to the control, as bands found were at 500, 300 and 200 bps.

![Human authentication of cell lines by PCR.](image)

B =blank, L = 100bp DNA ladder, HC, MC, RC = human, mouse and rat controls respectively. HC revealed bands at the desired points of 500, 300 & 200bps for human DNA, with the mouse control DNA revealing bands at 500, 350 & 150bps and rat control DNA revealing bands at 500, 450 & 250bps. Samples H1 UPAB, H2 UPMC, H3 SNB-19 & H4 CC2565.
3.2  **Cellular morphology of Non-Neoplastic Astrocyte and Glioma cells**

The study of cultured glioma and non-neoplastic astrocytes cultures by phase contrast microscopy revealed a selection of cells in monolayer culture which illustrates morphological differences according to culture and passage number. The monitoring of cell morphology and confluency also ensured that bacterial or fungal contaminations in cell cultures could be detected and optimum cell growth achieved through regular sub-culture and media changes.

Figures 15.1-15.4 are representative images obtained through monitoring of the cells throughout the study.

The non-neoplastic astrocyte cell culture CC-2565 at passage 4 (P4) was seen to display slender cells with elongated processes with a tendency to grow in streams. Additionally figure 15.1 belows show a very low number of dividing cells.

![Figure 15.1](image)

Figure 15.1: Cellular morphology of CC-2565- A human non-neoplastic astrocytic cell line. (A) P4, 80% confluent elongated and stellate cells, few of which are retractile and dividing. (B) P4, 30% confluent long, polygonal cells with thin and elongated intercommunating processes at either end. Scale bar = 20µm and 10µm respectively.
UPAB is a cell line derived from biopsy material cultured in-house and is used at early passage so that it retains most of its original features of the primary cell culture. GBMs are known for their cell heterogeneity and this can be seen in UPAB which shows mixed cell populations with some elongated cells with many processes at the end, some short more rounded cells and some big flat cells. Figure 15.2 shows many retractile cells indicative of rapid cell division.

![Figure 15.2: Cellular morphology of UPAB- A human biopsy derived Grade IV GBM. (A) P12, 90% confluent showing heterogenous nature with many processes at the end and some short, more rounded cells with predominantly fusiform cells. Several retractile and dividing cells could be seen. (B) P12, 40% confluent illustrating some flat, polygonal cells with long processes. The images were obtained using the x4 and x10 objectives under the phase contrast microscope with scale bars 20µm and 10µm respectively.](image)

UPMC is a cell line derived from biopsy material cultured in-house and is used at early passage. These cells still retained their heterogenous nature, a characteristic of gliomas. Again they have a complex and varied morphology with some fusiform, slender cells with elongated processes and whilst some cells look like a spindle with their tapering ends. Some flatter and shorter cells could also be seen occasionally. Figure 15.3 shows some retractile dividing cells.
Figure 15.3: Cellular morphology of UPMC- A human biopsy derived Grade IV GBM. (A) P10, 80% confluent elongated, fusiform predominantly stellate cells. Few retractile and dividing cells. (B) P10, 20% confluent, interconnecting stellate cells with slender long processes. Some small rounder cells could be seen. Images obtained using the x4 and x10 objectives under the phase contrast microscope with scale bars 20µm and 10µm respectively.

SNB-19 (also known as U251MG) is a high passage homogenous cell line from the DSMZ brain bank that is characterized by its short and smaller cells that lack the long and thin filamentous processes seen for example in UPAB. Single nucleotide polymorphism (SNP) array analysis have demonstrated that at later passages, SNB-19 and U251MG lines are in fact derived from the same individual (Garraway, 2005). Therefore, cross contamination was presumed to have occurred at some stages of culturing but these cells were authenticated as of human origin. Figure 15.4 shows many retractile cells indicating rapid cell division.
Figure 15.4: Cellular morphology of SNB-19- A human biopsy derived Grade IV GBM. (A) P48, 60% confluent small predominantly stellate cells. Many retractile cells indicate rapidly dividing cells. Additionally, the cells tend to aggregate with a mass of cells at the centre with shorter fatter processes. (B) P48, 10% confluent flattened irregular shape with tiny slender processes. Images obtained using the x4 and x10 objectives under the phase contrast microscope with scale bars 20µm and 10µm respectively.

3.3 Growth Kinetics of Cell Lines

It is important to determine the amount of cells within a flask before beginning experimentation, as it is usually necessary to use pre-determined quantities of cells for certain procedures therefore it is essential to establish the population doubling times of individual cell lines.

To determine the population doubling time (PDT) for each cell line, increase in cell number on a semi-log scale was plotted against time from seeding. The population doubling time could then be derived from the exponential part of the graph by calculating the time taken for the cell population to double.

Figure 16 shows an example of a growth curve for UPAB.
Figure 16: Growth curve fit for UPAB at seeding density of $1\times10^5$ cells. Population doubling time (PDT) derived from the exponential part of the semi-log graph, is 21 hours.

The population doubling times for each cell line used in this project are summarized in table 9.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Population doubling time (PDT) (Hours)</th>
<th>Seeding density (cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC-2565</td>
<td>31</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>UPAB</td>
<td>21</td>
<td>$1 \times 10^5$</td>
</tr>
<tr>
<td>UPMC</td>
<td>24</td>
<td>$1 \times 10^4$</td>
</tr>
<tr>
<td>SNB-19</td>
<td>18</td>
<td>$1 \times 10^5$</td>
</tr>
</tbody>
</table>

Table 9 illustrates the population doubling times and seeding densities used throughout the project (unless stated otherwise) for CC-2565, UPAB, UPMC and SNB-19 cells. Results are representative of three independent experiments carried out in triplicate with the more appropriate seeding densities chosen from three different concentrations.
The results above show that PDT is a function of passage number. The higher passage culture has a shorter PDT as seen by SNB-19 followed by UPAB, UPMC and normal human astrocytes CC-2565.

3.4 Antigenic Characterisation of Cell lines by Immunocytochemistry and Flow cytometry

All cell lines were cultured in 10% FCS as the serum supplement and evaluated for their antigenic expression profiles. Cell lines evaluated by both techniques are CC-2565, UPAB, UPMC and SNB-19.

Immunostaining allows visualization of the cellular localisation of specific antigens in relation to cell organelles and/or other antigens also named as co-localisation/co-expression. It also enables a qualitative assessment of the relative level and distribution of antigen present in the individual cell and cell population.

Levels of expression of antigens were quantified using flow cytometry. Flow cytometry allows the analysis of both the mean percentage of cell population expressing an antigen through the percentage gated expression and the mean intensity of fluorescence emitted by the particular antigen by the cell through the fluorescence fold. Analysis of fluorescence fold is crucial in the scope of this thesis to differentiate between the differences of antigens expressed by between normal and neoplastic glial cells.
3.4.1 Antigenic characterisation of CC-2565 at P5

The CC-2565 cells used in this project were characterised prior to use in experiments by staining with anti-GFAP and analysing expression by ICC and flow cytometry to confirm their astrocytic lineage by demonstrating the high expression of filamentous proteins such as GPAP. GFAP, an intermediate filament protein, is located throughout astrocytes. The experiment was carried out by Dr Suzanne Birks, PhD (2010) and her results show an expression of over 75% of the population was positive for GFAP in this particular cell culture.

The non-neoplastic astrocytic cell culture, CC-2565 was then characterised for single labeling of CD44 and CD155 through both ICC and flow cytometry and double staining of CD44 and CD155 through ICC.

3.4.1.1 Single ICC staining

A strong positive staining was observed for CD44 (A) and CD155 (B) as shown in figure 17. Both CD44 and CD155 displayed a uniform staining across the cell surface with an increase in fluorescence at the edges of the cells.
Figure 17: CC-2565 ICC images

Expression of CD44 (A) and CD155 (B) on CC-2565 at x40 objective stained by Alexa Fluor 488 (Green) with Hoechst Blue nuclei counterstain (Blue). Figure A shows clear and even CD44 staining across the cell surface with particular intense staining at the edges of the cell. Figure B shows distinct and granular staining of CD155 dispersed across the cell surface with a relatively higher expression at the edges of the cell. Scale bar is 40µm.

3.4.1.2 Flow cytometry results

The expression of CD44 and CD155 was assessed quantitatively by flow cytometry. Flow cytometry allows for the analysis of both the mean percentage of cell population expressing an antigen (% gated expression) and the mean amount of antigen expressed by the cells (fluorescence fold).

Figure 18 shows positive expression of CD44 (A) and CD155 (B) in CC-2565 cells analysed by flow cytometry.
Figure 18: Flow cytometric histogram plot for CD44 (A) and CD155 (B) expression in CC-2565 cells. The solid black line is the control sample and the colored lines (green, blue and pink) which show a shift to the right indicate the positive expression of CD44 (A) and CD155 (B). Results are representative of three independent experiments carried out in triplicate (n=3). The data is summarized in figures 29.1 and 29.2.

3.4.1.3 Double ICC staining

The co-staining of CD44 and CD155 in CC-2565 cells clearly shows that both antigens are present on the cell surface but no co-localisation is seen. CD44 (green fluorescence) is evenly distributed across the cell surface while CD155 (red fluorescence) is faintly expressed across the cell surface with a particular intense staining at the edge of the cell (arrow) as shown in figure 19.
Figure 19: Co-localisation of CD44 (Green) and CD155 (Red) with Hoechst Blue nuclei counterstain (Blue) on CC-2565 at x40 objective with a 10µm scale bar. The figure clearly demonstrates faint staining of both CD44 and CD155. CD44 is uniformly present on the cell membrane whereas CD155 shows relatively intense staining at the edge of the cell (arrow).

3.4.2 Antigenic characterisation of UPAB at P13

UPAB cells (GBM) cultured in FCS-supplemented medium were characterised for single labeling of CD44 and CD155 through ICC and flow cytometry. The double staining of CD44 and CD155 was analysed through ICC.
3.4.2.1 Single ICC staining

Positive staining of CD44 (A) and CD155 (B) are observed in UPAB cells as demonstrated in figure 20 below. CD44 is universally expressed on all cells with higher fluorescence intensity observed at the edges of the cells. Granulated CD155 expression is seen across the cell surfaces with greater expression at the peripheral edges of the cells (arrow).

![UPAB ICC Images](image)

**Figure 20: UPAB ICC Images**

Localisation of CD44 (A) and CD155 (B) in UPAB cells using x40 objective and scale bar 40µm stained by Alexa Fluor 488 (Green) with Hoechst Blue nuclei counterstain (Blue). The green fluorescence shows CD44-positive cells (A). CD44 is present on all cells with a diffuse stippled staining on their cell surface but also at cell edges and clustered perinuclearly. Figure B shows CD155-positive cells. CD155 is consistently observed on the cell surfaces with higher expression at some peripheral edges of the cells (arrow).
3.4.2.2 Flow cytometry results

Figure 21 shows positive expression of CD44 (A) and CD155 (B) in UPAB cells illustrated by the bell-shaped flow cytometry histogram which shifts to the right.

Figure 21: Flow cytometric histogram plot for CD44 (A) and CD155 (B) expression in UPAB cells. The solid black line is the control sample and the colored lines (green, blue and pink) which show a shift to the right indicate the positive expression of CD44 (A) and CD155 (B). Results are representative of three independent experiments carried out in triplicate (n=3). The data is summarized in figures 29.1 and 29.2.

3.4.2.3 Double ICC staining

Figure 22 below shows positive co-expression of CD44 and CD155 in UPAB. There is more CD44 (green fluorescence) than CD155 (red fluorescence). CD44 is evenly distributed across the cell surface. Granulated expression of CD155 was seen sparsely scattered across the cell surface and often seen as ‘clumps’ within the end of the process (arrow).
Figure 22 shows co-expression of CD44 (Green)-AlexaFluor 488 IgG2a and CD155 (Red)-AlexaFluor 568 IgG1 with Hoechst Blue nuclei counterstain (Blue) on UPAB at x40 objective with a 10µm scale bar. CD44 is present on the cell and evenly distributed on the cell surface whilst the red fluorescence shows CD155 that is faintly expressed throughout the cell surface with an exceptionally high expression within the tapering process (arrow).

3.4.3 Antigenic characterisation of UPMC at P12

UPMC cells (GBM) were characterised for single labeling of CD44 and CD155 through both ICC and flow cytometry. The double staining of CD44 and CD155 was carried out by ICC and analysed using an epi-fluorescence microscope.
3.4.3.1 Single ICC staining

Positive staining of CD44 (A) and CD155 (B) are observed in UPMC cells as demonstrated in figure 23 below. CD44 is uniformly distributed on all cells with higher fluorescence intensity observed at the edges of the cells. Granulated CD155 expression is seen across the cell surfaces with greater expression at the peripheral edges of the cells (arrows).

**Figure 23: UPMC ICC Images**

Localisation of CD44 (A) and CD155 (B) in UPMC cells using x40 objective and scale bar 40µm stained by Alexa Fluor 488 (Green) with Hoechst Blue nuclei counterstain (Blue). The green fluorescence shows both CD44 and CD155-positive cells. CD44 is present on virtually all cells and are well distributed on the cell surface. Particular intense expression was detected at the edges of the cells. CD155 is consistently distributed throughout the cells with preferential localisation forming ‘clumps’ to the leading edges of cells (arrows). Staining at some cell-cell contacts can also be seen
3.4.3.2 Flow cytometry results

Figure 24 shows positive expression of CD44 (A) and CD155 (B) in UPMC cells illustrated by the bell-shaped flow cytometry histogram which shifts to the right.

![Flow Cytometry Histograms](image)

**Figure 24:** Flow cytometric histogram plot for CD44 (A) and CD155 (B) expression in UPMC cells. The solid black line is the control sample and the colored lines (green, blue and pink) which show a shift to the right indicate the positive expression of CD44 (A) and CD155 (B). Results are representative of three independent experiments carried out in triplicate (n=3). The data is summarized in figures 29.1 and 29.2.

3.4.3.3 Double ICC staining

Figure 25 below shows positive co-expression of CD44 and CD155 in UPMC. There is an even distribution of both antigens across the cell surface. Some CD44 ‘clumping’ was seen at some areas on the cells (yellow arrows). CD155 tends to be more expressed at the leading edges of the cells (white arrows).
Figure 25 shows co-expression of CD44 (Green)-AlexaFluor 488 IgG\textsubscript{2a} and CD155 (Red)-AlexaFluor 568 IgG\textsubscript{1} with Hoechst Blue nuclei counterstain (Blue) on UPMC at x40 objective with a 10µm scale bar. Both CD44 and CD155 are present on the cell and evenly distributed on the cell surface. CD155 is also seen to be mainly recruited to the leading edges of the cells (white arrows).

3.4.4 Antigenic characterisation of SNB-19 at P49

SNB-19 cells (GBM) were characterised for single labeling of CD44 and CD155 through both ICC and flow cytometry. Double staining of CD44 and CD155 was carried out by ICC and visualized by an epi-fluorescence microscope.
3.4.4.1 Single ICC staining

Positive staining of CD44 (A) and CD155 (B) are observed in SNB-19 cells as demonstrated in figure 26 below. CD44 is uniformly distributed on all cells. CD155 expression is seen across the cell surfaces with greater expression at the peripheral edges of the cells (arrow).

![SNB-19 ICC Images](image)

**Figure 26: SNB-19 ICC Images**

Localisation of CD44 (A) and CD155 (B) in SNB-19 cells using x40 objective and scale bar 40µm stained by Alexa Fluor 488 (Green) with Hoechst Blue nuclei counterstain (Blue). CD44 is well distributed on the cell surface. CD155 is sparsely distributed throughout the cell with higher expression at the peripheral edge (arrow).
3.4.4.2 Flow cytometry results

Figure 27 shows positive expression of CD44 (A) and CD155 (B) in SNB-19 cells illustrated by the bell-shaped flow cytometry histogram which shifts to the right.

Figure 27: Flow cytometric histogram plot for CD44 (A) and CD155 (B) expression in SNB-19 cells. The solid black line is the control sample and the colored lines (green, blue and pink) which show a shift to the right indicate the positive expression of CD44 (A) and CD155 (B). Results are representative of three independent experiments carried out in triplicate (n=3). The data is summarized in figures 29.1 and 29.2.
3.4.4.3 *Double ICC staining*

Figure 28 below shows positive co-expression of CD44 and CD155 in SNB-19 cells. There is an even distribution of both antigens across the cell surface.

Figure 28 shows co-expression of CD44 (Green)-AlexaFluor 488 IgG\textsubscript{2a} and CD155 (Red)-AlexaFluor 568 IgG\textsubscript{1} with Hoechst Blue nuclei counterstain (Blue) on SNB-19 at x40 objective with a 20\textmu m scale bar. Both CD44 and CD155 are uniformly distributed on the cell surface. There was more CD44 than CD155 with some degree of co-localisation at the edges of the cells (arrow).
3.4.5  Level of expression of antigens across all cell lines

3.4.5.1  Level of expression of CD44 across cell lines

Expression of CD44 was assessed in CC-2565, UPAB, UPMC and SNB-19 cells thus allowing us to make comparisons in terms of its percentage expression and mean fluorescence fold between the normal astrocytes and GBM cell lines. Figure 29.1 below shows a marked increase in both percentage gated expression and mean fluorescence fold in glioma cells (UPAB, UPMC and SNB-19) compared to the control, CC-2565. The percentage expression of CD44 across the GBM cell lines is fairly consistent despite the differences in heterogeneity and passage number.

![Graph](image)

Figure 29.1: Percentage gated expression and mean fluorescence fold of cell surface antigen CD44 on different cell lines. Results are representative of three independent experiments carried out in triplicate (n=3). *** indicates statistically significant (P<0.001) and * indicates statistically significant (P<0.05) difference compared with the CC-2565 cell line.
There is very strong evidence that the nature of the cell line affects the percentage of the cell population that expresses CD44 ($P < 0.0001$) and its mean fluorescence intensity ($P < 0.0001$). CC-2565 showed an expression of 75.97% with a mean fluorescence intensity of 34.66. A consequent increase in both percentage of cells expressing CD44 and its mean fluorescence intensity was observed in all the GBM cell lines namely UPAB, UPMC and SNB-19. The highest expression of CD44 (98.93%) and mean fluorescence intensity (139.55) was shown by UPAB. 97.63% of cells expressing CD44 with a mean fluorescence intensity of 98.2 was observed in UPMC. SNB-19 showed that 95.88% of its cell population expresses CD44 with a mean fluorescence intensity of 104.26.

### 3.4.5.2 Level of expression of CD155 across cell lines

Expression of CD155 was assessed in CC-2565, UPAB, UPMC and SNB-19 cells thus allowing us to make comparisons in terms of its percentage expression and mean fluorescence fold between the normal astrocytes and GBM cell lines. Figure 29.2 below shows a marked increase in percentage gated expression in UPAB and SNB-19 compared to the control, CC-2565. The percentage expression of cells expressing UPMC was fairly low although still higher than the normal astrocytes, CC-2565. Again, the mean fluorescence fold of UPAB and SNB-19 was much higher compared to CC-2565 and UPMC.
Figure 29.2: Percentage gated expression and mean fluorescence fold of cell surface antigen CD155 in CC-2565, UPAB, UPMC and SNB-19 cells determined by flow cytometry. Results are representative of three independent experiments carried out in triplicate (n=3). Statistical significance was assessed by one-way ANOVA and the Tukey’s post-hoc test (p≤0.05). *** indicates statistically significant (P<0.001) and ** indicates statistically significant (P<0.01) difference compared with the CC-2565 cell line.

There is statistical evidence to suggest that there is a difference in cell population expressing CD155 (P < 0.0001) and its mean fluorescence fold (P < 0.0001) among the cell lines as shown by figure 28.2. All the three GBM cell lines showed an increase in percentage population expressing CD155 and mean fluorescence fold when compared to the normal astrocytes CC-2565. UPAB has the highest percentage of cell population expressing CD155 (95.27%) with a mean fluorescence intensity of 32.17. A rather low expression of CD155 (77.65%) and mean fluorescence fold (9.24) was observed in UPMC.
whereas SNB-19 showed a higher expression CD155 of 95.73% with a mean fluorescence intensity of 28.2. The lowest expression was seen in CC-2565 with 66.49% of cells expressing CD155 with a mean fluorescence intensity of 9.

3.5 Total Internal Reflection Fluorescence (TIRF) Microscopy

TIRF microscopy selectively excites fluorophores in a cell within less than 100nm without exciting fluorescence from regions further from the surface, in other words, the contact area between a cell and a coverslip. This microscope was used to confirm the literature that ‘CD155 resides proximal to CD44 on the cell membrane’ in this case on glioma cells rather than on monocytes as previously reported (Freidast and Eberle, 1997).

UPAB, UPMC and SNB-19 cells all cultured under 10% FCS supplementation were subjected to double labeling for both CD155 and CD44 to assess their respective distribution and staining pattern using the TIRF microscope. This method permitted high-signal, low-noise fluorescence imaging of the CD155 and CD44 epitopes within the tumour cell membranes with great clarity and resolution.
3.5.1  **Co-expression of CD44 and CD155 on UPAB cells (P13)**

The figure below shows double staining of CD44 and CD155 using a TIRF microscope. Figure B gave a clearer image when one of the epitope was “TIRF-ed” in this case CD155. CD155 (green) was more prominent on the extending processes whereas CD44 (red) was clearly distinct across the cell surface. We can thus confirm that CD44 resides in close proximity to CD155 in UPAB cells.

![Figure A](image1.png) ![Figure B](image2.png)

**Figure 30** shows the co-expression of CD44 and CD155 epitopes on UPAB cells through TIRF microscopy using objective x100 (oil immersion) with scale bars 10µm.

Co-expression of CD155 (Green)-AlexaFluor 488 IgG₂a (Goat anti-mouse) and CD44 (Red)-AlexaFluor 568 IgG₁ (Goat anti-mouse) were visualised with normal fluorescence Argon lasers (A). CD44 is present on the surface of the cells and CD155 is mainly expressed on the invadapodia (arrows). The TIRF-laser (B) gave a clearer image showing the precise location of CD155 in respect to CD44. CD155 once again was seen to be distinctly present on the processes of the migrating cells (arrow). It should be noted that TIRF laser was applied only to CD155.
3.5.2  **Co-expression of CD44 and CD155 on UPMC cells (P11)**

Figure 31 below shows double staining of CD44 and CD155 using a TIRF microscope. Figure B gave a clearer image when one of the epitope was “TIRF-ed” in this case CD44. Higher expression of CD44 (green) was seen across the cell surface whereas CD155 (red) was seen to be more prominent on the extending processes. We can thus confirm that CD44 resides in close proximity to CD155 in UPMC cells.

![Fluorescence-based lasers](image1.png) ![TIRF-based lasers](image2.png)

**Figure 31** shows the co-expression of CD44 and CD155 epitopes on UPMC cells through TIRF microscopy using objective x100 (oil immersion) with scale bars 10µm.

Co-expression of CD44 (Green)-AlexaFluor 488 IgG2a (Goat anti-mouse) and CD155 (Red)-AlexaFluor 568 IgG1 (Goat anti-mouse) were visualised with epi-fluorescence Argon lasers (A). CD44 and CD155 are evenly distributed on the surface of the cell with CD155 being expressed within the ‘invadapodia’. The evanescent wave illumination (B) revealed a more precise localisation of CD155 with respect to CD44. Both CD44 and CD155 were closely localised on the cell but were not co-expressed on the same sites. CD155 is highly expressed on the ‘invadapodia’ of the cells. It should be noted that TIRF laser was applied only to CD44.
3.5.3 Co-expression of CD44 and CD155 on SNB-19 cells (P50)

Figure 32 below shows double staining of CD44 and CD155 using a TIRF microscope. Figure B gave a clearer image when one of the epitope was “TIRF-ed” in this case CD44 and shows that CD44 and CD155 are closely located on SNB-19 cells. Intense expression of CD155 was seen in the hair-like tiny processes.

![Fluorescence-based lasers](image1) ![TIRF-based lasers](image2)

**Figure 32** shows the co-expression of CD44 and CD155 epitopes on SNB-19 cells through TIRF microscopy using objective x100 (oil immersion) with scale bars 10µm.

Co-expression of CD44 (Green)-AlexaFluor 488 IgG2a (Goat anti-mouse) and CD155 (Red)-AlexaFluor 568 IgG1 (Goat anti-mouse) were visualised with epi-fluorescence Argon lasers (A). CD44 and CD155 are diffusely spread on the surface of the cell. CD155 staining is particularly prominent on the filopodia of the cell. The evanescent wave illumination (B) demonstrates the proximal location of CD155 in relation to CD44 expression. A clearer image with less noise was obtained. CD44 is sparsely scattered on the cell surface with a considerate CD155 staining on the filopodia of the cell. It should be noted that TIRF laser was applied only to CD44.
3.6 Invasion assay

3.6.1 Characterisation with alkaline phosphatase vector red staining

The modified Transwell™ Boyden chamber assay is an effective method to determine the invasive potential of cell lines. Each cell line (UPAB, UPMC and SNB-19) was labeled for CD44 and CD155 through Alkaline phosphatase vector red staining to show that both CD44 and CD155 were expressed on invasive cells. The images represented below are representative of the random images obtained by capturing 5 fields of each Transwell™ unit in the study. Each experiment was carried out three times in triplicate.

3.6.1.1 Characterisation of CD44 and CD155 on UPAB cells (P13)

The figure below shows that the invaded cells express both CD44 (A) and CD155 (B). CD44 seems to be highly expressed in the invaded cells compared to a lower CD155 expression in figure B.
Figure 33: Invasion of CD44-positive (A) and CD155-positive (B) cells on UPAB using Transwell™ Boyden chamber. PDGF<sub>AB</sub> (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded after 5 hours onto the lower side of the filter insert were stained via Alkaline phosphatase vector red. Cells positive for CD44 and CD155 were stained red with blue nuclear counterstaining with Haematoxylin and viewed with an Olympus inverted brightfield Microscope with objectives x20 and scale bars 20µm. Both figures A and B show that CD44 and CD155 are expressed in the invasive cells. Cells invaded in figure A and B appear to be elongated with prominent fine processes.

3.6.1.2 Characterisation of CD44 and CD155 on UPMC cells (P12)

The figure below shows that the invaded cells express both CD44 (A) and CD155 (B). CD44 was more expressed in the invasive cells compared to CD155. This reflect the flow cytometry results obtained which showed a low percentage gated expression and mean fluorescence fold of CD155 in UPMC cells.
Figure 34: Invasion of CD44-positive (A) and CD155-positive (B) cells on UPMC using Transwell™ Boyden chamber. PDGF<sub>AB</sub> (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded on the lower side of the filter insert were stained via Alkaline phosphatase vector red. Cells positive for CD44 and CD155 were stained red, with blue nuclear counterstaining with Haematoxylin and viewed with an Olympus inverted brightfield Microscope with objective x20 and scale bars 20µm. CD155 was less expressed than CD44 in the invasive cells. Cells which had invaded (figure A and B) show a heterogeneous nature. Some cells are elongated with prominent fine processes while some are short and polygonal.

3.6.1.3 Characterisation of CD44 and CD155 on SNB-19 cells (P52)

The figure below shows that the invaded cells express both CD44 (A) and CD155 (B). Figure A shows that all the cells which had invaded expressed CD44 while Figure B shows that not all cells which had invaded expressed CD155. This is demonstrated by the blue nuclear staining with no red CD155 expression.
Figure 35: Invasion of CD44-positive (A) and CD155-positive (B) cells on SNB-19 using Transwell\textsuperscript{TM} Boyden chamber. PDGF\textsubscript{AB} (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded on the lower side of the filter insert were stained via Alkaline phosphatase vector red. Cells positive for CD44 and CD155 were stained red, with blue nuclear counterstaining with Haematoxylin and viewed with an Olympus inverted brightfield Microscope with objective x20 and scale bars 20µm. CD44 and CD155 were expressed in the invaded cells. Cells which had invaded (figure A and B) show a homogenous nature with small cells with short fine processes.

3.6.2 Comparison of invasive potential of CD44 and CD155 across glioma cell lines

A very high expression of both CD44 and CD155 was seen in the invasive cells as demonstrated by the red staining (figures 33 and 25). This result thus confirms those obtained in ICC and flow cytometry where 98.93% of cells expressed CD44 with a fluorescence intensity of 139.55 and 95.27% of cells expressed CD155 with a fluorescence intensity of 32.17 in UPABs. SNB-19s showed that 95.88% and 95.73% of its cell population expressed CD44 and CD155 respectively with mean fluorescence folds of 104.26 and 28.2. The high invasive potential of CD44 and CD155 in UPAB and SNB-19 cells could also be explained as both antigens were uniformly expressed across the cell
membrane with more intense expression at the peripheral edges particular towards the processes of the cells. This statement supports that of previous papers which stated that overexpression of CD44 and CD155 promote invasion in glioma cells (Kupper et al., 1992 and Sloan et al., 2005). The low invasive potential of CD155 in UPMC cells as shown in figure 34 could be explained by the lowest expression level demonstrated by flow cytometry where only 77.65% of its population expressed the antigen with a very low fluorescence fold of 9.24.

3.7 Monoclonal Antibody Blocking Assay via the Transwell™ Boyden Chamber

UPAB, UPMC and SNB-19 cells were subjected to CD44 MAb, followed by CD155 MAb and finally both antibodies and left to incubate for 8 hours at 37°C. Images were captured with an Olympus inverted brightfield Microscope. The images represented below are representative of the random images obtained by capturing 5 fields of each Transwell™ unit in the study. To statistically validate the results, each sample and parameter was assayed in triplicate (1 control + 3 positives) in at least three independent experiments.
3.7.1. **Monoclonal Antibody blocking assay of CD44, CD155 and (CD44+CD155) on UPAB cells (P12)**

Figure 36.1 below shows a marked decrease in invasion is reached when blocking with either CD44 or CD155 Mab alone or when double treatment is given. Double blocking of CD44 + CD155 shows that CD44 reduces the effectiveness of CD155 to invade, thus CD44 appears to be a stronger blocker than CD155.

![Figure 36.1: Invasive potential of UPAB cells after treatment with their specific Monoclonal Antibody Blocking regime via Transwell™ Boyden chambers. PDGF\textsubscript{AB} (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded onto the lower side of the insert were stained with Diff Quick and viewed with an Olympus inverted brightfield Microscope with a x20 objective and scale bars 20µm. The negative control (A) shows cells invaded after omitting treatment. Monoclonal Blocking Antibody for CD44 (B), CD155 (C) and (CD44+CD155) (D) result in a consequent decrease in invasion.](image-url)
Figure 36.2: Number of UPAB cells which invaded through the Transwell$^\text{TM}$ inserts after blocking with Monoclonal Antibody specific for either CD44, CD155 or (CD44+CD155). The graph is representative of the results obtained by repeating the study three times in triplicate.

There is very strong evidence that the treatment with Monoclonal Blocking Antibody significantly affects the potential of cells to invade through the Transwell$^\text{TM}$ insert (P<0.0001). When treatment with any monoclonal blocking antibody was omitted (control), approximately 100 cells invaded to the lower side of the filter. A complete ablation was reached upon treating with CD44 blocking antibody resulting in no cells invading through and only 2 cells on average invaded through when treated with CD155 blocking antibody. Treatment with both blocking antibodies (CD44+CD155) simultaneously resulted to an average of 1 cell invaded through. This shows that CD155 blocking promotes CD44 blocking to some extent.
3.7.2. Monoclonal Antibody blocking assay of CD44, CD155 and (CD44+CD155) on UPMC cells (P13)

Figure 37.1 below shows a marked decrease in invasion is reached when blocking with either CD44 or CD155 Mab alone or when double treatment is given.

![Figure 37.1: Invasive potential of UPMC cells after treatment with their specific Monoclonal Antibody Blocking regime via Transwell™ Boyden chambers. PDGF<sub>AB</sub> (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded onto the lower side of the insert were stained with Diff Quick and viewed with an Olympus inverted brightfield Microscope with a x20 objective and scale bars 20µm. The negative control (A) shows cells invaded after omitting treatment. Monoclonal Blocking Antibody for CD44 (B), CD155 (C) and (CD44+CD155) (D) results in a marked decrease in invasion.](image-url)
Figure 37.2: Number of UPMC cells which invaded through the Transwell™ inserts after blocking with Monoclonal Antibody specific for either CD44, CD155 or (CD44+CD155). The graph is representative of the results obtained by repeating the study three times in triplicate.

The treatment with Monoclonal Blocking Antibody significantly affects the invasive potential of cells to invade through the Transwell™ insert (P<0.0001). When treatment with any monoclonal blocking antibody was omitted (control), approximately 90 cells invaded to the lower side of the filter. A complete ablation was reached upon treating with CD44 blocking antibody resulting in no cells invading through the filter and only 4 cells on average invaded through when treated with CD155 blocking antibody. Treatment with both blocking antibodies (CD44+CD155) simultaneously resulted in an average of 5 cells which had invaded through the filter.
3.7.3. **Monoclonal Antibody Blocking assay of CD44, CD155 and (CD44+CD155) on SNB-19 cells (P52)**

Figure 38.1 below shows a marked decrease in invasion is reached when blocking with either CD44 or CD155 Mab alone or when double treatment is given.

![Image A](image1.png) ![Image B](image2.png) ![Image C](image3.png) ![Image D](image4.png)

**Figure 38.1: Invasive potential of SNB-19 cells after treatment with their specific Monoclonal Antibody Blocking regime via Transwell™ Boyden chambers.** PDGF<sub>AB</sub> (10ng/ml) used as a chemoattractant in the lower compartment. Cells which had invaded onto the lower side of the insert were stained with Diff Quick and viewed with an Olympus inverted brightfield Microscope with a x20 objective and scale bars 20µm. The negative control (A) shows cells invaded after omitting treatment. Monoclonal Blocking Antibody for CD44 (B), CD155 (C) and (CD44+CD155) (D) results in considerable inhibition of invasion.
Figure 38.2: Number of SNB-19 cells which invaded through the Transwell™ inserts after blocking with Monoclonal Antibody specific for either CD44, CD155 or (CD44+CD155). The graph is representative of the results obtained by repeating the study three times in triplicate.

The results show that treatment with Monoclonal Blocking Antibody significantly inhibits the invasive potential of cells to invade through the Transwell™ insert (P<0.0001). When treatment with any monoclonal blocking antibody was omitted (control), approximately 137 cells invaded to the lower side of the filter. A complete ablation was reached upon treating with CD44 blocking antibody resulting in no cells invading through the filter and only 3 cells on average invaded through when treated with CD155 blocking antibody. Treatment with both blocking antibodies (CD44+CD155) simultaneously resulted in an average of 1 cell which had invaded through the filter. This shows that CD155 blocking promotes CD44 blocking to some extent.
3.8 Transfection with siRNA

RNA interference (RNAi) can be used to suppress the function of a gene in cell lines by introducing siRNA into the cell which triggers an endogenous RNAi pathway inhibiting the target gene expression. To maximise knockdown, a target-gene-specific mixture (pooling of siRNA duplexes) of different siRNAs containing effective sequences was used. SNB-19 was grown to 40% confluency in 96 well plates and transfected with Accell CD44 and CD155 siRNA to knockdown (KD) gene expression. Western blotting was performed after transfection to assay for gene knock-down. The effects of CD44 knockdown, CD155 knockdown and knockdown of both proteins were also assessed through various experiments namely: ICC, flow cytometry invasion assay, adhesion assay, proliferation assay and through live cell imaging.

3.8.1 Transfection with Accell siRNA CD44 & siRNA CD155 and delivery media

Figure 39 below shows cell morphology of SNB-19 cells following a 96 hours post transfection with siRNA CD44 (A) and siRNA CD155 (B). Both figures demonstrate small and short cells with tiny slender processes at both ends. Both figures show many retractile cells indicating rapid cell proliferation. This transfection approach also shows minimal toxicity.
3.8.2 Western blotting

After transfection with siRNA for CD44 and CD155, gene knockdown was assessed by Western blotting. This method allowed us to determine if knockdown was successfully achieved. GAPD siRNA was used as a positive control for silencing and validation of experimental design. The house keeping marker β-actin was blotted to ensure all samples were loaded at 20µg/ml.

Figure 39: Post-transfection images of SNB-19 with Accell CD44 (A) and CD155 (B) siRNAs. (A) P52, 70% confluent small predominantly stellate cells with tiny slender processes. Many retractile cells indicating high rate of cell proliferation. (B) P53, 70% confluent short and small cells with tiny fat processes at both ends. Several retractile and dividing cells could be seen. Images obtained using the x4 objective under the phase contrast microscope with scale bar 10µm.
The images below are representative of blots obtained after carrying out the experiment at three independent times.

Figure 40: Western blots for the expression of GAPD, CD44 and CD155 on untreated cells and after knockdown with their specific siRNAs. Expression of β-actin is also shown.

Untreated cells show high expression of their particular protein determined by the sharp bands obtained. After transfection of their particular siRNAs, all cells show reduced amounts of their proteins as shown by the marked decreased band intensity for GAPD and CD155 however, CD44 show some protein was still present due probably to its high turnover as explained in chapter 4.

Equal levels of actin show that the differences in levels of proteins in untreated cells compared to cells treated with siRNA are not due to amount of protein loaded onto the gel.
3.8.3 Characterisation of silenced genes through ICC and flow cytometry

Levels of expression of antigens treated with siRNA for CD44 and CD155 were analysed using ICC and flow cytometry. Cells were harvested after 120 hours post transfection and labeled with their particular antigen. Each experiment was repeated three times in triplicate.

3.8.3.1 ICC results

Figure 41.1 (A) below shows CD44 is present on all cells and is evenly distributed on the cell surface. Particular intense expression was detected at the edges of the cells (arrows). The cells have well-defined long processes extending at both ends. Figure (B) shows a round cell with no processes. CD44 is uniformly expressed across the cell surface.

![Image of CD44 expression](image)

Figure 41.1: The images above show expression of CD44: no treatment given (A) and 120 hours post-transfection with siRNA CD44 (B) both stained by AlexaFluor 488 IgG (green) and counterstained with Hoechst blue (blue) on SNB-19 cells using x40 objective and scale bar 40µm. Figure A shows CD44 is uniformly distributed on the cell surface with a relatively high fluorescence fold as confirmed by flow cytometry in...
section 42.1. Particular intense staining was seen across the edges of the cells (arrows). The cells have a predominantly stellate shape or rather irregular, a characteristic of tumour cells with prominent long processes. siRNA-treated cell (B) shows a rather faint staining. The cell is round and devoid of any processes.

Figure 41.2 (A) below shows CD155 is evenly distributed on the cell surface. The cell has well-defined processes, some thin and long and some thick and short, extending at both ends. Figure (B) shows a round cell with no processes and CD155 is faintly and uniformly expressed across the cell surface with some CD155 “aggregates” at its periphery.

Figure 41.2: The images above show expression of CD155: no treatment given (A) and 120 hours post-transfection with siRNA CD155 (B) both stained by AlexaFluor 488 IgG (green) and counterstained with Hoechst blue (blue) on SNB-19 cells using x40 objective and scale bar 40µm. Figure A shows CD155 is universally distributed on the cell surface with a relatively high fluorescence fold at distinct zones on the cell (arrows). This result is consistent with that of flow cytometry described in section 42.1. The cell has well-defined processes, some of which are short and far while others are slender and long. siRNA-treated cell (B) shows that CD155 is rather faintly stained across the cell surface with some particular ‘clusters’ of CD155 expression at its periphery (arrows). The cell is round in morphology with no processes.
3.8.3.2 Flow cytometry results

Figure 42.1 below illustrates the percentage expression and mean fluorescence fold of CD44 and CD155 following 120 hours post-transfection with their siRNAs. A marked decrease in both parameters are seen in both (A) and (B) as explained in figure 42.2.

Figure 42.1: Effect of siRNA knock-down on percentage expression and mean fluorescence fold of CD44 (A) and CD155 (B) in SNB-19 cells by flow cytometry analysis. The solid black line is the control sample and the orange line which shows a shift to the right of the histograms demonstrates the positive expression of the antigens without any transfection treatment. The coloured lines (pink, blue and green) in the middle of the histograms demonstrate the positive expression of the antigens following 120 hours post-transfection with siRNA. Results are representative of three independent experiments carried out in triplicate (n=3).

Legend:

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<td>• Pink/Blue/Green-</td>
<td>Transfected sample with siRNA +primary antibody added</td>
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Figure 42.1 shows that after transfection with siRNA CD44 and siRNA CD155, less cells expressed CD44 and CD155 (% gated expression). The mean amount of CD44 and CD155 expressed by the cells (mean fluorescence fold) were also considerably low compared to non-transfected cells.

![Percentage positivity of cells expressing their particular antigen and mean fluorescence intensity after their respective siRNA knock-down treatment](chart.png)

**Figure 42.2: Percentage positivity and Mean fluorescence fold of cells expressing their particular antigens in untreated cells and post transfected cells with their respective siRNA by flow cytometry (unpaired t-test).**

There is very strong evidence that siRNA knock-down of CD44 and CD155 significantly affects the percentage of population of glioma cells that express CD44 and CD155 (P < 0.0001) as well as their mean fluorescence intensity (P < 0.0001). Figure 40 shows that
overall there is a marked decrease in both percentage expression and mean fluorescence intensity of CD44 and CD155 when the cells were silenced by their siRNAs.

Before transfection, 98.44% of the cell population expressed CD44 with a mean fluorescence intensity of 120.47. These values were drastically reduced to 74.17% of cells expressing CD44 with a mean fluorescence fold of 12 after siRNA ‘knock-down’.

Subsequent decrease was observed in the percentage of cells expressing CD155 and its mean fluorescence intensity by siRNA transfection of CD155 from 99.08% and 38.25 to 64.86% and 7.45 respectively.

### 3.8.4 Invasion assay

Diffuse local invasion of neoplastic cells into normal brain is one of the distinguishing features of primary brain tumours and affects the effectiveness of therapies. The modified Transwell™ Boyden chamber assay is an effective method to determine the invasive potential of cells and this technique was used to investigate the effect of siRNA-treatment of SNB-19 cells on their invasive potential. The images presented below are representative of the images obtained by capturing 5 fields of each Transwell™ unit in the study that was carried out three times in triplicate.
Figure 43.1: Effect of transfection with siRNA CD44 (B), siRNA CD155 (C) and siRNA (CD44+CD155) (D) on the invasive potential of SNB-19 cells as determined by the modified Transwell™ Boyden chamber assay. 8µm pores were used and scale bars 20µm. No treatment was given to the control (A). Results are representative of three independent experiments carried out in triplicate (n=3). The results show a marked decline in invasion when cells were treated with their specific siRNAs as compared to the control.

Figure 43.2 below shows the invasive potential of cells following 120 hours transfection with siRNA CD44, siRNA CD155 and siRNA (CD44+CD155). The results clearly show that silencing CD44, CD155 and both by their siRNA cause a marked decrease in invasion in SNB-19 cells. “knock-down” of both antigens does not seem to have any further effect on the invasive potential as seen in figure 43.2.
Figure 43.2: Number of SNB-19 cells invaded through the Transwell™ inserts after transfection with siRNA for either CD44, CD155 or (CD44+CD155). The graph is representative of the average number of cells captured per field and is representative of three independent experiments carried out in triplicate (n=3).

There is strong evidence to suggest that the treatment method significantly affects invasiveness of cells (P < 0.0001). There was a marked decline in invasive potential of cells from 188 cells invaded when no treatment was given compared to 63 cells invading after siRNA transfection of CD44. However, siRNA CD155-treated and siRNA (CD44+CD155) - treated cells both show 27 cells invading through.
3.8.5 Live cell imaging

Live cell imaging is a useful tool for monitoring the effects of a treatment on cell viability and motility or invasion in real time. Following transfection of SNB-19 cells with CD44, CD155 or both, they were monitored by live cell imaging for motility, proliferation and morphology for 72 hours post-transfection. Cells were imaged under a phase contrast microscope.

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Figure 44.1: Effect of transfection with siRNA [CD44, CD155 and (CD44+CD155)]. An overview of the proliferation, motility and morphology of the cells as shown by time-lapse microscopy over 72 hours.

The above figure shows that at 0 hours, for all treatment regimens and including the control, the cells were at approximately 10% confluent and viable. Progressively, it seems that wells treated with siRNAs tend to proliferate more eventually reaching at least 60% confluency for siRNA CD44 and siRNA (CD44+CD155) and 40% for siRNA CD155 after 72 hours. The lower confluency of siRNA CD155- treated cells was due to possibly of the controlled environment chamber used to image the cells live made it impossible to replenish media in any of the wells. A confluency of 30% was reached for control wells.

It has been observed that cells transfected with their siRNAs tend to acquire a rounder morphology (arrows) with less filopodias. A marked decline of the cells’ ability to migrate was also noted compared to control cells over 72 hours.

Time-lapse microscopy provided evidence that the treatments lead to a higher proliferative rate and a lower motility rate of the cells however, for quantitative comparisons; the cells were tracked with the ‘volocity’ software to permit evaluation of the motility rate and the speed by which the cells move. A BrdU proliferation assay was carried out to confirm the results obtained (figure 44.2).
**Figure 44.2:** Total distance moved or motility rate by cells (untreated + post transfection with siRNA) over a period of 72 hours under the live cell imaging microscope. The graph above is representative of repeating the study three times each in triplicate and averaging 30 cells per treatment regime. *** indicates statistically significant (P<0.001) difference compared to the control.

There is very strong evidence that siRNA knock-down of CD44 and CD155 significantly affects the distance which cells move over a period of 72 hours (P < 0.0001). The graph above (figure 43.2) shows a marked decrease in total distance moved by the cells in the well when the cells were treated with siRNAs over control (untreated) cells. The maximum distance travelled was achieved by untreated cells (4222.22 µm). Silenced-CD44 treated cells moved only 1036.31 µm over 72 hours whereas following treatment with siRNA CD155, the distance covered was 732.68 µm. The shortest distance (549.37 µm) travelled was obtained by siRNA (CD44+CD155).
Figure 44.3: Velocity of cells moved over a period of 72 hours under the live cell imaging microscope. The graph above is representative of repeating the study three times each in triplicate and averaging 30 cells per treatment regime.

The treatment method significantly impeded the speed at which cells moved over a period of 72 hours (P < 0.0001). There was substantial decrease in velocity of cell movement of post-transfected siRNA cells compared to control cells. Untreated cells (control) showed the highest speed by which cells moved (0.026 μ s⁻¹). A marked decrease in velocity was noticed when cells were silenced for CD44 (0.0092 μ s⁻¹). siRNA CD155 transfected cells moved at a rate of 0.0064 μ s⁻¹ and the minimum speed was reached when the cells were ‘knocked-down’ for both antigens (0.0058 μ s⁻¹).
3.9 Proliferation assay

Different approaches to measure the proliferation of cells have been defined intensively. In the scope of this thesis however, cellular incorporation of BrdU during the S phase have been employed to assess the proliferative rate of untreated cells versus cells treated with Monoclonal Blocking antibody (CD44, CD155 and CD44+CD155) and siRNA (CD44, CD155 and CD44+CD155) via detection by anti-BrdU-specific antibodies following membrane permeabilisation. Each experiment was done three times in triplicate and these are represented by the graphs below.
3.9.1 Monoclonal Antibody Blocking treatment on SNB-19 cells (P52) increases rate of cell proliferation

Figure 45.1: Proliferative rate of untreated cells and cells treated with their Monoclonal Blocking Antibody (CD44, CD155 and CD44+CD155). The results are representative of three independent experiments carried out in triplicate (n=3).

The BrdU proliferation assay showed significant differences (P<0.0001) when cells were blocked with their specific monoclonal antibody compared to untreated cells (control). The graph above showed a higher proliferative rate was achieved when cells were treated with MAb when compared to non-treated cells. The control gave a proliferative index of 0.168 which increased to 0.341 when cells were blocked with MAb CD44. Treatment with MAb CD155 showed that the amount of BrdU incorporated was 0.326 with a further increase when cells underwent simultaneous treatment with MAb (CD44+CD155) which gave a value of 0.363.
3.9.2 *siRNA treatment on SNB-19 cells (P52)* increases rate of cell proliferation

**Figure 45.2**: Proliferative rate of untreated cells and cells treated with their specific siRNA (CD44, CD155 and CD44+CD155). The results are representative of three independent experiments carried out in triplicate (n=3).

There was a substantial difference in the rate at which cells proliferated (P<0.0001) when they were transfected with their specific siRNAs compared to untreated cells. The graph above shows the highest proliferation rate was reached when cells were treated with siRNA ‘knockdown’ for both CD44 and CD155 and gave a proliferative index of 0.375. Untreated cells (control) showed that the cells were the least proliferative (0.175) of those studied. This value was increased to 0.314 when cells were transfected with siRNA CD44. A further increase to 0.333 was noticed when cells were silenced for CD155.
Overall, for both treatments (figures 44.1 and 44.2), there was significant evidence to suggest that treated cells under investigation had a higher proliferative rate when compared to non-treated cells. This further implies that these treated cells were less invasive since cells need to arrest from the cell cycle to migrate.

3.10 Adhesion assay

Cell adhesion is a fundamental process that gives valuable assessment of the adhesiveness of cells to a defined extracellular matrix substrate. The interaction of cells with an adhesive substrate, in this case laminin, fibronectin, vitronectin, Tenascin and Collagen I was quantified for each cell treatment (Monoclonal Blocking Antibody and siRNA) on SNB-19 cells. BSA was used as a control. Each experiment was carried out three times in triplicate to ensure statistical validity.
3.10.1 Monoclonal Antibody Blocking Treatment

Figure 46.1: Adhesive potential of untreated cells and cells treated with their specific Monoclonal Antibody Blocking (CD44, CD155 and CD44+CD155) on different Extracellular matrices (BSA, Fibronectin, Laminin, Vitronectin, Tenascin and Collagen I). The results are representative of three independent experiments carried out in triplicate (n=3). *** indicates statistically significant (P<0.001), ** (P<0.01) and * (P<0.05) difference compared to the control BSA.
The graph above shows cell adhesion potential on BSA (control), Fibronectin, Laminin, Vitronectin, Tenascin and Collagen I under different cell treatment. There was no significant differences shown under the different treatment regimes for BSA (P=0.442). Under no treatment condition, the rate by which the cell attached was 0.019 followed by 0.359 for MAb CD44. Blocking with MAb CD155 gave an adhesion potential of 0.012 whereas both treatment MAb (CD44+CD155) showed the cell adhesion rate was 0.018.

There is strong evidence to suggest that the treatment method significantly affected adhesiveness of cells when the plate was coated with fibronectin (P < 0.0001). There was a consequent decrease by which cells adhered to fibronectin from 0.181 when no treatment was given to 0.144 when MAb CD44 was applied to the cells. The lowest adhesion rate was achieved when cells were blocked for CD155 (0.103). A slight increase in adhesive potential was observed (0.123) with both treatment given-MAb (CD44+ CD155).

There was a marked decrease in cell adhesion for laminin when the different treatments were compared (P < 0.0001). The highest attachment rate at 0.256 was produced under no treatment condition followed by a significant decrease to 0.158, 0.131 and 0.145 under MAb CD44, MAb CD155 and MAb (CD44+CD155) respectively.

A significant difference was observed among treatment regimes when the plate was coated with vitronectin (P < 0.0001). Interestingly, the highest adhesive potential was induced when cells underwent treatment with MAb CD155 (0.161). Untreated cells gave a cell adhesion rate of 0.147 with a minor decrease observed under MAb CD44 (0.101). When both treatments were given simultaneously, the rate by which cells adhered to vitronectin was 0.143.
The rate by which cells adhered to tenascin differed under various treatment regimes (P=0.0008). Untreated cells gave the highest cell attachment rate (0.137) with a decrease under the other treatments such MAb CD44 (0.130), MAb CD155 (0.122) and MAb (CD44+CD155) to 0.126.

There was a substantial difference in the rate the cells adhered to Collagen I (P<0.0001) when they were treated with their specific MAb compared to untreated cells. There was a major decrease from adhesion potential from 0.178 under no treatment condition to 0.153 (MAb CD44) to 0.116 (MAb CD155). Blocking with both antibodies produced a cell attachment rate of 0.120.
3.10.2 siRNA Treatment

![Graph showing adhesion potential of SNB-19](image)

Figure 46.2: Adhesive potential of untreated cells and cells treated with their specific siRNAs (CD44, CD155 and CD44+CD155) on different Extracellular matrices (BSA, Fibronectin, Laminin, Vitronectin, Tenascin and Collagen I). The results are representative of three independent experiments carried out in triplicate (n=3). ** indicates statistically significant (P<0.01), *** (P<0.001) difference compared to the control BSA.
The graph above shows cell adhesion potential on BSA (control), Fibronectin, Laminin, Vitronectin, Tenascin and Collagen I under different cell treatments. There was a minor difference shown under the different treatment regimes for BSA (P=0.0046). Under no treatment condition, the rate by which cells attached was 0.019 followed by 0.0201 for siRNA CD44. Knock-down with siRNA CD155 gave an adhesion potential of 0.013, whereas both treatment siRNA (CD44+CD155) showed the cell adhesion rate was 0.029.

A significant difference was observed among treatment regimes when the plate was coated with fibronectin (P < 0.0001). A decrease in cell attachment was observed from untreated condition (0.1811) to 0.106 when cells were silenced for CD44. siRNA CD155 knockdown cells induced an adhesive potential of 0.131 with a small increase to 0.151 when cells were treated with both siRNAs.

Considerable changes were seen under different treatment regimens when the plate was coated with laminin (P < 0.0001). The rate of cell attachment was similar for siRNA CD155 and siRNA (CD44+CD155)-0.157. A significant increase in adhesive potential was however observed under no treatment condition (0.256) followed with a decrease with cells were silenced for CD44 (0.170).

There is strong evidence to suggest that the treatment method significantly affects adhesiveness of cells when the plate was coated with vitronectin (P < 0.0001). The highest adhesion rate was achieved when cells were treated with siRNA CD155 (0.168) followed by a decrease to 0.157 produced by siRNA (CD44+CD155). A further decrease in cell attachment rate was shown under no treatment condition (0.147). The lowest rate by which cells adhered to the ECM was induced by siRNA CD44 (0.076).
There was a marked decrease in rate of cell attachment to tenascin when cells were silenced for their specific antigen (P < 0.0001). Untreated cells produced the highest adhesive potential (0.137) value with a decrease observed when cells were siRNA “knockdown” for CD44 (0.077) and CD155 (0.090). Treatment with siRNA (CD44+CD155) showed the rate by which cells adhered to tenascin was 0.121.

There was significant evidence to suggest that different treatments affect the rate by which cells attach to Collagen I (P < 0.0001). The highest absorbance value was obtained when any treatment was omitted (0.178) followed by marked decrease in adhesion potential produced by knocking down CD155 (0.111). siRNA CD44 and siRNA (CD44+CD155) produced the same cell attachment rate (0.120).

3.11 Confocal Microscopy

Confocal microscopy permits individual planes of a specimen to be viewed. Cells (UPAB, UPMC and SNB-19) were co-labeled with β1, αvβ1 and αvβ3 integrins and F-Actin and CD44/CD155 together with cells silenced with siRNA CD44 and siRNA CD155. siRNA treatment was only applied to SNB-19 cells.
3.11.1 Expression of F-actin and integrins on UPAB cells (P13)

**Figure 47: UPAB confocal microscopy analysis**

**Figure 47.1: Single confocal microscopy staining**

The images above show expression of F-actin (A) and β₁ integrin (B) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in UPAB cells.

The green fluorescence shows both F-actin and β₁ integrin-positive cells. Clear strong filamentous F-actin staining of the cytoskeleton was observed. A uniform staining of β₁ integrin was seen throughout the cell with some dense clusters usually at the extremity of the cell.
The images above show expression of $\alpha_v\beta_1$ integrin (C) and $\alpha_v\beta_3$ integrin (D) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10$\mu$m and x40 objective (oil) in UPAB cells.

The green fluorescence shows both $\alpha_v\beta_1$ and $\alpha_v\beta_3$ integrin-positive cells. A uniform and rather faint granular staining of both integrins was seen throughout the cell. However, strong and crisp staining was seen to be concentrated generally at the edges of the cells.
Figure 47.2.1: Double confocal microscopy staining of CD44 and F-actin/integrins

The images above show expression of CD44 + F-actin (A) and CD44 + β₁ integrin (B). CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in UPAB cells.

Figure A shows strong filamentous F-actin staining throughout the cells with particular higher staining intensity at the periphery of the cells. CD44 seems to be well distributed across the cytoplasm of the cells. Prominent CD44 staining could also be seen in the filopodia of the cell.

Figure B shows good granular uniform staining of CD44 throughout the cell surface. β₁ integrin staining could be seen clearly at the perinuclear membranes and faintly in the cytoplasm of the cell. In addition, β₁ integrin seems to form dense aggregates usually near the vicinity of the cell edges.
The images above show expression of CD44 + αvβ1 integrin (C) and CD44 + αvβ3 integrin (D) in UPAB cells. CD44 (IgG2a) was stained with AlexaFluor 568 (Red). αvβ1 (IgG3) and αvβ3 (IgG1) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows an intense level of CD44 distributed all over the cells. αvβ1 integrin shows granular staining amassed around the nuclear region and sparsely spread in the cytoplasm. A relatively faint degree of co-localisation (yellow) of both antigens could be seen on the body mass of the cell (arrow).

Figure D shows positive staining of CD44 well-spread all over the cytoplasm of the cells. A clear granulated staining of αvβ3 integrin particularly congregated at the edges of the cells was observed (arrow).
Figure 47.2.2: Double confocal microscopy staining of CD155 and F-actin/integrins

The images above show expression of CD155 + F-actin (A) and CD155 + β₁ integrin (B) in UPAB cells. CD155 (IgG₁) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows bright, filamentous, thread-like staining of F-actin throughout the cell. CD155 was preferentially located to the leading edges of the cell (arrow).

Figure B shows CD155 to be present and evenly distributed on the cells. Granular dense staining of β₁ integrin was exceptionally expressed in the forming filopodia/invadopodia (arrows) of the cell with some sparsely staining across the cytoplasm. A very low degree of co-localisation was noticed.
The images above show expression of CD155 + α,β1 integrin (C) and CD155 + α,β3 integrin (D) in UPAB cells. CD155 (IgG₁) was stained with AlexaFluor 568 (Red). α,β₁ (IgG₃) and α,β₃ (IgG₁) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows high granular expression of both CD155 and α,β₁ integrin. Good staining of CD155 was seen to be uniformly expressed across the cells whereas α,β₁ integrin was prominently present around the nuclear region spreading to the periphery to the cells.

Figure D shows high degree of co-localisation of CD155 and α,β₃ integrin throughout the cells. CD155 is faintly expressed throughout the cell surface and α,β₃ integrin showed sprinkled staining across the cells with some exclusively intense expression at the leading edge of the cells.
3.11.2 Expression of F-actin and integrins on UPMC cells (P12)

Figure 48: UPMC confocal microscopy analysis

Figure 48.1: Single confocal microscopy staining

The images above show expression of F-actin (A) and $\beta_1$ integrin (B) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in UPMC cells.

Figure A shows well-defined, filamentous staining through the cytoskeleton with a relatively intense staining extending towards the ends of the cell.

$\beta_1$ integrin (figure B) was moderately expressed across the cells with some particular granular profiles (arrow) to be seen spread densely towards the edge of the cells.
The images above show expression of $\alpha_v\beta_1$ integrin (C) and $\alpha_v\beta_3$ integrin (D) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in UPMC cells.

$\alpha_v\beta_1$ integrin (Figure C) shows relatively good and granular staining, evenly distributed across the cell surface. A higher level of expression of the antigen was seen concentrated at the leading edges of the cells (arrow).

Figure D shows diffuse granular staining of $\alpha_v\beta_3$ integrin uniformly distributed throughout the cell surface with however, some particular zones of dense tiny, thread-like staining in the short, thick processes of the cells (arrows).
Figure 48.2.1: Double confocal microscopy staining of CD44 and F-actin/integrins

The images above show expression of CD44 + F-actin (A) and CD44 + β₁ integrin (B) in UPMC cells. CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10μm and x40 objective (oil).

Figure A shows a star-shaped cell with intense filamentous, thread-like F-actin staining throughout the cell. CD44 was present and showed consistent granular staining on the cell surface.

Figure B shows β₁ integrin granular staining to be more prominent in some zones especially around the perinuclear region of the cell with some expression extending to the periphery of the cell and the processes. CD44 was uniformly well-distributed across the cell. A slight co-localisation was observed slightly deeper into the cell (arrows).
The images above show expression of CD44 + αβ₁ integrin (C) and CD44 + αβ₃ integrin (D) in UPMC cells. CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). αβ₁ (IgG₃) and αβ₃ (IgG₁) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows αβ₁ integrin to be mostly expressed around the nuclear region of the cells. Faint diffuse staining was also seen throughout the cells. CD44 was evenly spread on the cell surface.

Figure D shows a scattered, rather faint expression of αβ₃ integrin all over the cells with some specific highly dense staining at the leading edge of cells and the invadopodia (arrows). Good granular expression of CD44 was seen distributed all over the cell surface.
Figure 48.2.2: Double confocal microscopy staining of CD155 and F-actin/integrins

The images above show expression of CD155 + F-actin (A) and CD155 + β₁ integrin (B) in UPMC cells. CD155 (IgG₁) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows a well-defined filamentous staining of F-actin expression throughout the cell but not extending into the long, fine processes. CD155 show consistent granular staining on the cell surface with zones of intense expression aggregation at the extremities and fine processes of the cells (arrows).

Figure B shows β₁ integrin granular staining to be amassed in some zones especially around the peripheral region of the cell (arrows). Some bright β₁ integrin expression was observed around the perinuclear region of the cell. CD155 was evenly distributed across the cell. A slight co-localisation was observed around the nuclear region of the cell.
The images above show expression of CD155 + αvβ1 integrin (C) and CD155 + αvβ3 integrin (D) in UPMC cells. CD155 (IgG1) was stained with AlexaFluor 568 (Red). αvβ1 (IgG3) and αvβ3 (IgG2a) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows αvβ1 integrin to be dispersed over the cell surface. Granular staining of the CD155 was evenly distributed on the cell surface but there was no evidence of co-expression.

Figure D shows a scattered, rather faint expression of αvβ3 integrin all over the cells with some specific highly dense staining at the leading edge of cells (white arrows). Dispersed and weak staining of CD155 was observed to be distributed throughout the cells with some particularly intense expression in processes and leading edges of migrating cells (blue arrows).
3.11.3 Expression of F-actin and integrins on SNB-19 cells (P49)

Figure 49: SNB-19 confocal microscopy analysis

Figure 49.1: Single confocal microscopy staining

The images above show expression of F-actin (A) and β₁ integrin (B) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in SNB-19 cells.

The green fluorescence shows both F-actin and β₁ integrin-positive cells. Clear thread-like F-actin staining of the cytoskeleton was observed with very slight aggregation at the peripheral adhesion points (arrow). A relatively faint staining of β₁ integrin was seen throughout the cell with dense clusters extending at the extremity of the cell.
The images above show expression of $\alpha_\text{v} \beta_1$ integrin (C) and $\alpha_\text{v} \beta_3$ integrin (D) both stained with AlexaFluor 488 IgG (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil) in SNB-19 cells.

$\alpha_\text{v} \beta_1$ integrin (Figure C) showed relatively crisp and granular staining, evenly distributed across the cell surface. A higher level of expression of the antigen was seen concentrated towards the perinuclear region of the cell.

Figure D shows a multinucleated cell with diffuse granulated staining of $\alpha_\text{v} \beta_3$ integrin uniformly distributed throughout the cell surface with however, some particular zones of dense staining all along the edge of the cell (arrows).
Figure 49.2.1: Double confocal microscopy staining of CD44 and F-actin/integrins

The images above show expression of CD44 + F-actin (A) and CD44 + β₁ integrin (B) in SNB-19 cells. CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows cells with intense filamentous, thread-like F-actin staining throughout the cell. CD44 granular expression was sparsely scattered over the cell surface.

Figure B shows β₁ integrin granular staining to be more prominent in some zones of the cell with some expression extending to the periphery of the cell. CD44 was uniformly well-distributed across the cell. A slight co-localisation was observed on the cell (yellow regions).
The images above show expression of CD44 + α,β1 integrin (C) and CD44 + α,β3 integrin (D) in SNB-19 cells. CD44 (IgG2a) was stained with AlexaFluor 568 (Red). α,β1 (IgG3) and α,β3 (IgG1) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows α,β1 integrin diffuse staining distributed throughout the cells. CD44 was evenly spread on the cell surface. Some co-localisation of both antigens could be seen at the edge of the cells (arrows).

Figure D shows a cluster of round cells with uniform expression of CD44 distributed all over the cells. α,β3 integrin shows specific intense staining concentrated along the edges of the cells (white arrows). Some co-localisation could also be seen across the cell surfaces, that is the interactive region with the substrate (yellow areas-blue arrows).
Figure 49.2.2: Double confocal microscopy staining of CD155 and F-actin/integrins

The images above show expression of CD155 + F-actin (A) and CD155 + β₁ integrin (B) in SNB-19 cells. CD155 (IgG₁) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows a cell with well-defined thread like staining of F-actin distributed across the cell. Sparse granular staining of CD155 was seen to be scattered all over the cell.

Figure B shows intense granular staining of CD155 evenly distributed all over the cells. β₁ integrin expression was particularly amassed in the peripheral edges of the cells (arrows) with some faint staining spread over the cell surface. Some vague areas of co-localisation could be seen along the edges of the cells.
The images above show expression of CD155 + α₃β₁ integrin (C) and CD155 + α₃β₃ integrin (D) in SNB-19 cells. CD155 (IgG₁) was stained with AlexaFluor 568 (Red). α₃β₁ (IgG₃) and α₃β₃ (IgG₂a) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows granulated staining of both CD155 and α₃β₁ integrin. CD155 was evenly distributed over the cell surface with some zones of concentration especially at the leading edges of the cell. α₃β₁ integrin was uniformly distributed across the cell with more intense expression around the perinuclear region of the cell. Considerable areas of co-localisation could be observed frequently around the peripheral edges of the cell (arrows).

Figure D shows good and granular staining of both CD155 and α₃β₃ integrin. Even distribution of CD155 was seen across the cell surface with higher expression at the leading edges of the cells. α₃β₃ integrin was sprinkled all over the surface of the cells with aggregates at the end of the cell (arrow). Some degree of random co-localisation could also be seen.
3.11.3.1 Expression of F-actin and integrins on siRNA-treated CD44 and CD155 on SNB-19 cells (P49)

After transfection with siRNA CD44 and CD155, level of integrins (β₁, β₃ and αᵥ) and F-actin were assessed by Western blotting. This method allowed us to determine if knockdown altered their expression level. GAPD siRNA was used as a positive control for silencing and validation of experimental design. The house keeping marker β-actin was blotted to ensure all samples were loaded at 20µg/ml. The images below are representative of blots obtained after carrying out the experiment three independent times.

<table>
<thead>
<tr>
<th>Wild type cells</th>
<th>GAPDH kd</th>
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**Figure 50.1:** Western blots for the expression of F-actin and integrins (β₁, β₃ and αᵥ) on untreated cells and after knockdown with siRNAs. Expression of β-actin is also shown.
Investigation of integrins ($\beta_1$, $\beta_3$ and $\alpha_v$) and F-actin protein levels by Western blots confirmed their expression levels obtained by confocal microscopy (figures 50.2 and 50.3).

Untreated cells and siRNA-transfected GAPD show high expression of the particular protein under investigation as determined by the sharp and thick bands obtained.

After transfection with siRNA CD44 and siRNA CD155, a substantial reduction in the amount of integrins ($\beta_1$, $\beta_3$ and $\alpha_v$) was noted as seen by the thin or rather fade bands (figure 50.1).

The level of F-actin was reduced following siRNA CD44 and siRNA CD155 as illustrated by their thinner bands in figure 50.1 with a further marked reduction in level of F-actin seen after co-transfection with CD44/CD155 as illustrated by the very faint band in figure 50.1 above.

Equal levels of $\beta$-actin show that the differences in levels of proteins in untreated cells compared to cells treated with siRNA are not due to amount of protein loaded onto the gel.
Figure 50.2: Confocal microscopy staining of siRNA CD44 transfected SNB-19 cells: Double staining for presence of CD44 + F-actin and CD44 + integrins

The images above show expression of siRNA CD44 + F-actin (A) and siRNA CD44 + β₁ integrin (B). CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows a cell with bright filamentous, spider web-like F-actin staining throughout the cell. Some of the filaments seem to have coalesced. CD44 was weak and sparsely scattered over the cell surface.

Figure B shows a faint β₁ integrin staining distributed across the cell surface. A relatively low level of CD44 was seen to be uniformly spread across the cell. A slight co-localisation was observed at the edge of the cell (arrow).
The images above show expression of siRNA CD44 + α,β₁ integrin (C) and siRNA CD44 + α,β₃ integrin (D). CD44 (IgG₂a) was stained with AlexaFluor 568 (Red). α,β₁ (IgG₃) and α,β₃ (IgG₁) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows a granular, fairly good expression level of α,β₁ integrin located at the perinuclear region with a weaker staining extending towards the cell surface. CD44 was faintly spread on the cell surface.

Figure D shows a cluster of rather rounded cells and no defined processes with a very weak, uniform expression of CD44 distributed all over the cells. Clustered α,β₃ integrin staining around the peri-nuclear cytoplasm was very weak.
Figure 50.3: Confocal microscopy staining of siRNA CD155 transfected SNB-19 cells:
Double staining for presence of CD155 + F-actin and CD155 + integrins

The images above show expression of siRNA CD155 + F-actin (A) and siRNA CD155 + β₁ integrin (B). CD155 (IgG₁) was stained with AlexaFluor 568 (Red). F-actin and β₁ integrin both IgG were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure A shows thread-like intense staining of F-actin distributed across the cell. Very faint staining of CD155 was seen to be sprinkled all over the cell.

Figure B shows weak granular staining of CD155 evenly distributed all over the cells. β₁ integrin expression was predominantly concentrated in the peripheral edges of the cells (arrows) with some faint staining spread over the cell surface. Some vague areas of faded co-localisation could be seen along the edges of the cells.
The images above show expression of siRNA CD155 + αvβ1 integrin (C) and siRNA CD155 + αvβ3 integrin (D). CD155 (IgG1) was stained with AlexaFluor 568 (Red). αvβ1 (IgG3) and αvβ3 (IgG2a) were stained with AlexaFluor 488 (Green) and counterstained with Hoechst blue (nuclei) at scale bar 10µm and x40 objective (oil).

Figure C shows a weaker, granulated staining of αvβ1 integrin compared to CD155. CD155 was evenly distributed over the cell surface. αvβ1 integrin was very faintly distributed across the cells. A relatively high degree of co-localisation was observed, frequently at the periphery of the cell (arrows).

Figure D shows granular staining of both CD155 and αvβ3 integrin. Even distribution of a weak level of CD155 was seen across the cell surface with higher expression at the leading edges of the cells. αvβ3 integrin was spread all over the surface of the cell.
3.12 Signal transduction pathways (Rho GTPases assay)

To further understand the role of CD44 and CD155 in invasion of brain tumours or the other pathways they may trigger or switch off intracellularly, some key players of the RHO proteins family were investigated.

After transfection with siRNA for CD44 and CD155, the level of RHO proteins (cdc42, Rac 1/1/3, RhoA, RhoB, RhoC, phospho-Rac1/cdc42) was assessed by Western blotting. This method allowed us to determine if knockdown altered expression levels of these proteins. Similarly, the level of CD44 and CD155 was also analysed to show whether by knocking down one protein, the expression level of the other was changed.

GAPD siRNA was used as a positive control for silencing and validation of experimental design. The house keeping marker β-actin was blotted to ensure all samples were loaded at 20μg/ml. The images below are representative of blots obtained after carrying out the experiment three independent times.
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Figure 51: Western blots for the expression of Rho GTPases proteins (cdc42, Rac 1/2/3, RhoA, RhoB, RhoC and phosphor-Rac1/cdc42), CD44 and CD155 on untreated cells and after knockdown with siRNAs. Expression of β-actin is also shown.
Equal levels of β-actin show that the differences in levels of proteins in untreated cells compared to cells treated with siRNA are not due to amount of protein loaded onto the gel.

Untreated cells and siRNA-transfected GAPD show high expression of the particular protein under investigation as determined by the sharp and consistent bands obtained.

Knock-down of CD44 by siRNA shows partial expression of CD44 with the same level of CD155 expressed as the untreated cells. A reduction in protein level was seen when both CD44 and CD155 were silenced as determined by the blurred band.

siRNA CD155 knockdown shows very slight reduction in the level of protein of CD44 and substantial decrease in CD155 as determined by the weak band. This shows that siRNA CD155 transfection was successful. Level of CD155 was further decreased when both proteins were silenced.

Interesting data was obtained involving the level of expression of Rho GTPases when CD44 and CD155 were subjected to siRNA transfection showing that silencing these proteins play a significant role in the intracellular signaling pathways of cells.

Figure 51 shows weak consistent bands of cdc42 when CD44 and CD155 were ‘knock-down’ singly and doubly.

The level of expression of Rac 1/2/3 and RhoA were greatly reduced further CD44 and CD155 transfection compared to non-treated cells. Weak bands could be seen.

Level of RhoB shows inconsistency further transfection of CD44 and CD155 by siRNA as determined by the bands. Relatively thick bands were obtained probably showing no effect was achieved.
Level of RhoC showed marked reduction when cells were knockdown for CD44 and (CD44 and CD155). No effect was observed on siRNA CD155 silenced cells as determined by the thick band.

The level of phosphor-Rac 1/cdc42 was inconclusive throughout. Very weak and fade bands were obtained for all treatment regimes and in untreated cells.