

Glioblastoma Invades by Co-opting Healthy Astrocytes via Gap Junctional Communication

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ABSTRACT

Glioblastoma multiforme (GBM) is the most aggressive and deadly CNS cancer. Its dismal prognosis is attributed to difficulty of resection, resistance to traditional chemotherapeutics, and invasion along the brain vasculature. Finding mechanisms by which GBM invades is critical for development of more effective surgical protocols and adjuvant therapies. Invasion of GBM involves alteration of non-cancerous cells, such as endothelial cells and astrocytes, in the tumor microenvironment toward a cancer-friendly phenotype. Evidence suggests post-transcriptional regulation by miRNAs may be the culprit. Astrocytes express gap junction proteins Connexin 30 and Connexin 43. Here we present evidence that functional Cx43 homotypic gap junctions form between GBM and astrocytes *in vitro*, and presence or absence of Cx43 causes a shift in miRNA profile for cells cocultured with the GBM cell line U87-MG. Further, presence/absence and functionality of astrocyte-GBM Cx43 channels alter the invasion potential of GBM *in vitro* and *ex vivo*. Our data indicate that GBM-GBM Cx43 junctions limit invasion, while astrocyte-GBM junctions promote invasion, consistent with literature. Previous studies have highlighted the potential transfer of miRNAs from GBM to astrocytes by means of gap junction channels, however it is widely accepted that gap junctions have a size exclusion of approximately 1 kDa, and miRNAs are on the order of 14 kDa. From here we seek to address the question of how gap junctions are implicated in the expression or transfer of miRNAs whether by direct passage through Cx43 junctions, exosomal release and uptake, or cell-cell endocytosis of Cx43 itself.

BACKGROUND

Glioblastoma and Glioblastoma Invasion

- Glioblastoma Multiforme (GBM), a stage 4 astrocytoma, is the most common, aggressive, and deadly CNS cancer.
- Median survival post diagnosis is 11-15 months with 5-year survival under 5%¹.
- GBM invasion is a driver of poor GBM prognosis and contributes to near certain recurrence
- GBM invasion involves co-opting of surrounding tissue, disruption of astrocyte/matrix interactions, and modification of astrocyte phenotype.

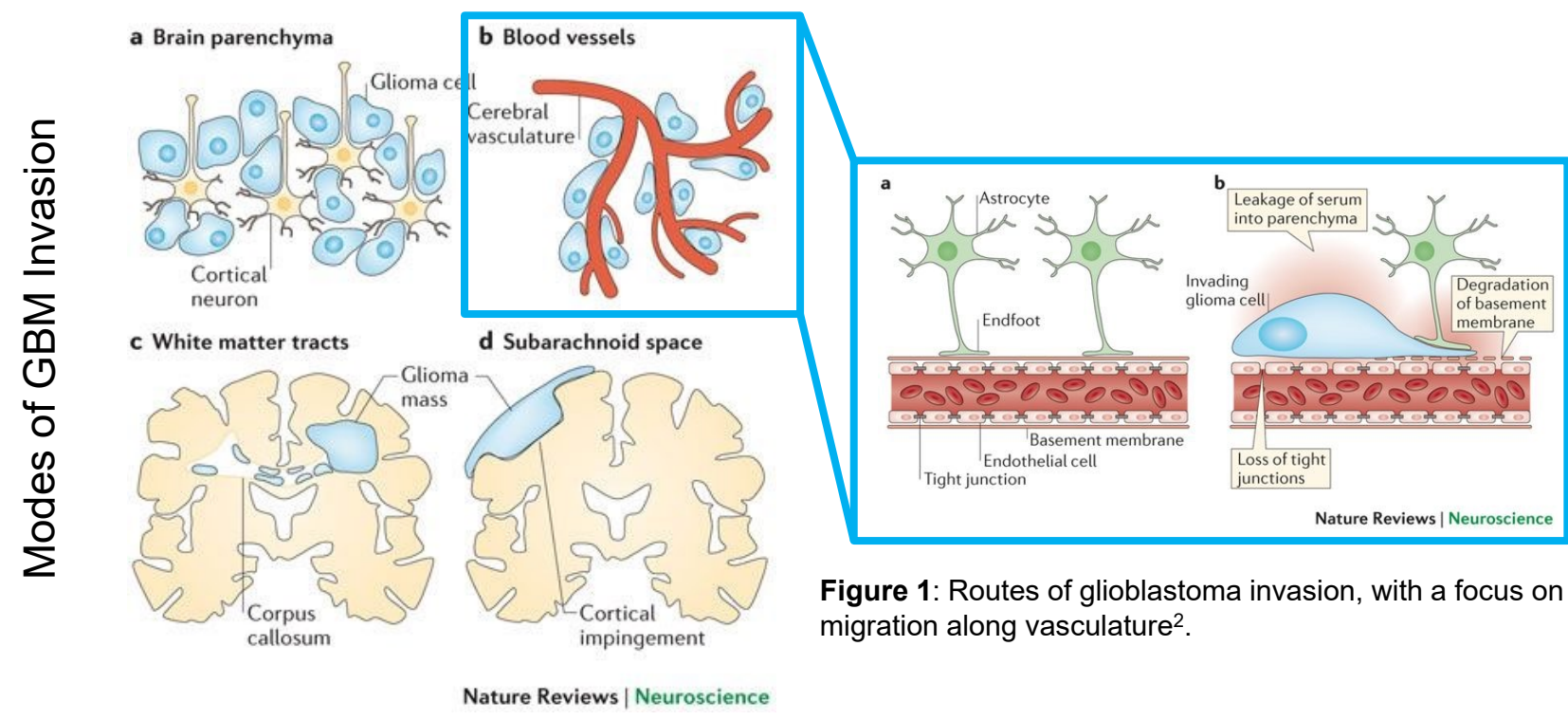
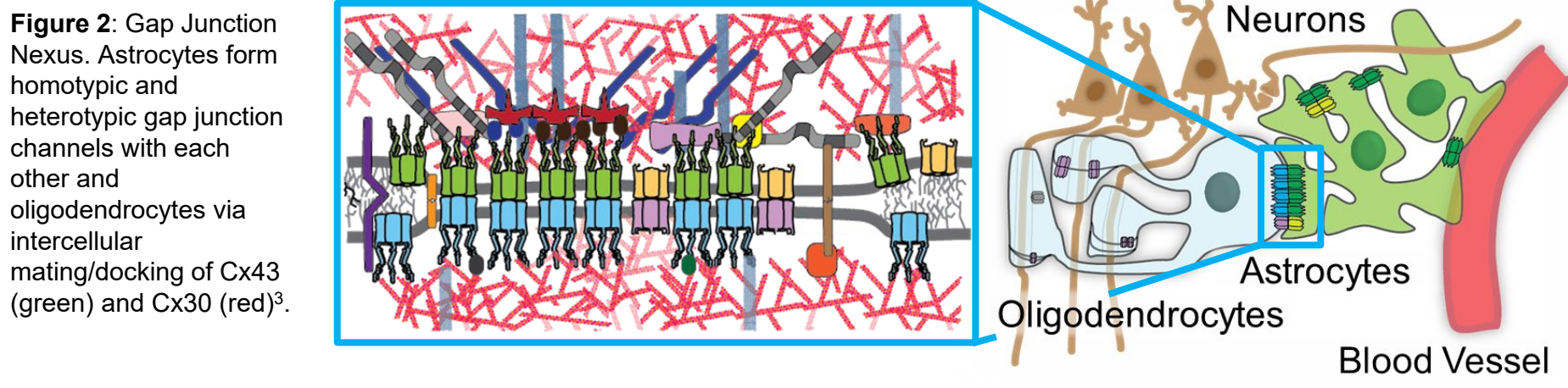


Figure 1: Routes of glioblastoma invasion, with a focus on migration along vasculature².

Astrocytic Gap Junctions

- Gap junction (GJ) channels permit small molecules, i.e. ions, metabolites/nutrients and second messengers, to transfer between cells.
- Astrocytes express Connexin 30 and 43 (Cx30, 43), gap junctions also expressed in GBM.
- Evidence suggests GBM-GBM and astrocyte-GBM Cx43 channel stoichiometry has a role in tumor invasiveness.



miRNAs and Cancer

- miRNAs are post-transcriptional regulators which, in their mature form, bind to specific mRNAs and prevent translation
- miRNAs regulate ~30% of the proteome
- Changes miRNAs are implicated in cancer diagnosis and prognosis
- miRNAs may act as tumor suppressors or oncogenes depending on which mRNAs they target.

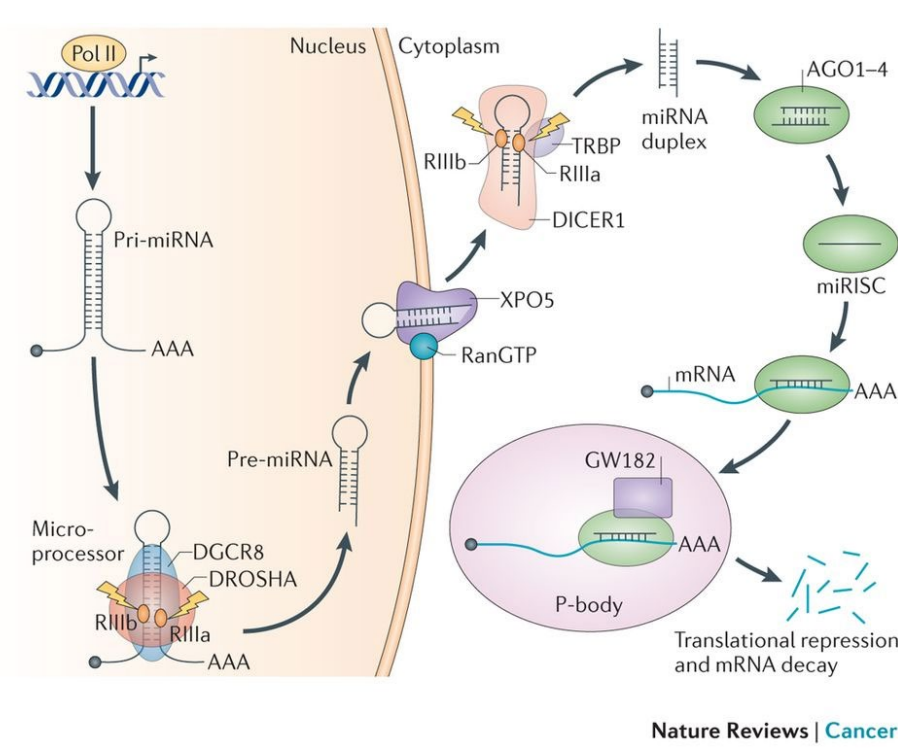


Figure 3: miRNA processing pathway⁴.

METHODS

In vitro studies - cell lines and cell culture

- Human GBM cell line U87 was used for all experiments. Parallel experiments were performed using murine GBM cell line GL261 (data not shown).
- Cell lines used for *in vitro* coculture experiments include: Immortalized primary astrocytes (IWCA), primary murine cortical astrocytes isolated from newborn pups (P1), and immortalized Cx43 knockout astrocytes (IKOCA).
- All cells were maintained in DMEM supplemented with 10% vol/vol fetal bovine serum and 1% vol/vol Antibiotic-Antimycotic.

Confirming Astrocyte-GBM Gap Junction Formation

- Formation of functional astrocyte-GBM GJs channels was assessed by parachute dye transfer assay. Donor cells were loaded with Calcein-AM (GJ permeable) and Dil (GJ impermeable) and "parachuted" at a concentration of 1:1000 onto a confluent monolayer of recipient cells. GJ transfer of Calcein was blocked by 50µM of GJ inhibitor 18α-glyzherretinic acid (18α-GA) or 100µM carbenoxolone (CBX).
- Formation of homotypic Cx43 plaques (both U87-U87 and U87-astrocyte) was visualized by fluorophore tagged Cx43 constructs. C-terminal GFP and BFP tagged Cx43 were transiently transfected into distinct cell populations. Transfected cells were cultured for 2 days and mixed at a 1:1 ratio, culturing for an additional 1-3 days before imaging. All imaging was performed on Zeiss LSM510 confocal or LSM880, at 10x or 63x magnification.

Exosome Isolation

- Cytosolic GFP-tagged U87 cells were cultured under standard conditions for 7 days, after which cell culture medium was collected, and centrifuged at 4,000 x g to remove any floating cells and debris.
- Exosomes were isolated by incubating cell culture medium with total exosome isolation reagent at 4°C overnight and centrifuging at 10,000 x g for 1 hour, followed by resuspension of the exosome pellet in 1x PBS.

METHODS

In vitro co-culture invasion model

- Transwell invasion assays were performed to assess the invasive potential of U87 cells.
- U87 cells were plated at 10⁴ cells/well on matrigel coated inserts with 8µm pores.
- FBS was used as a chemoattractant.
- U87 invasion was quantified alone, with GJ blockade, and in 1:1 astrocyte coculture.

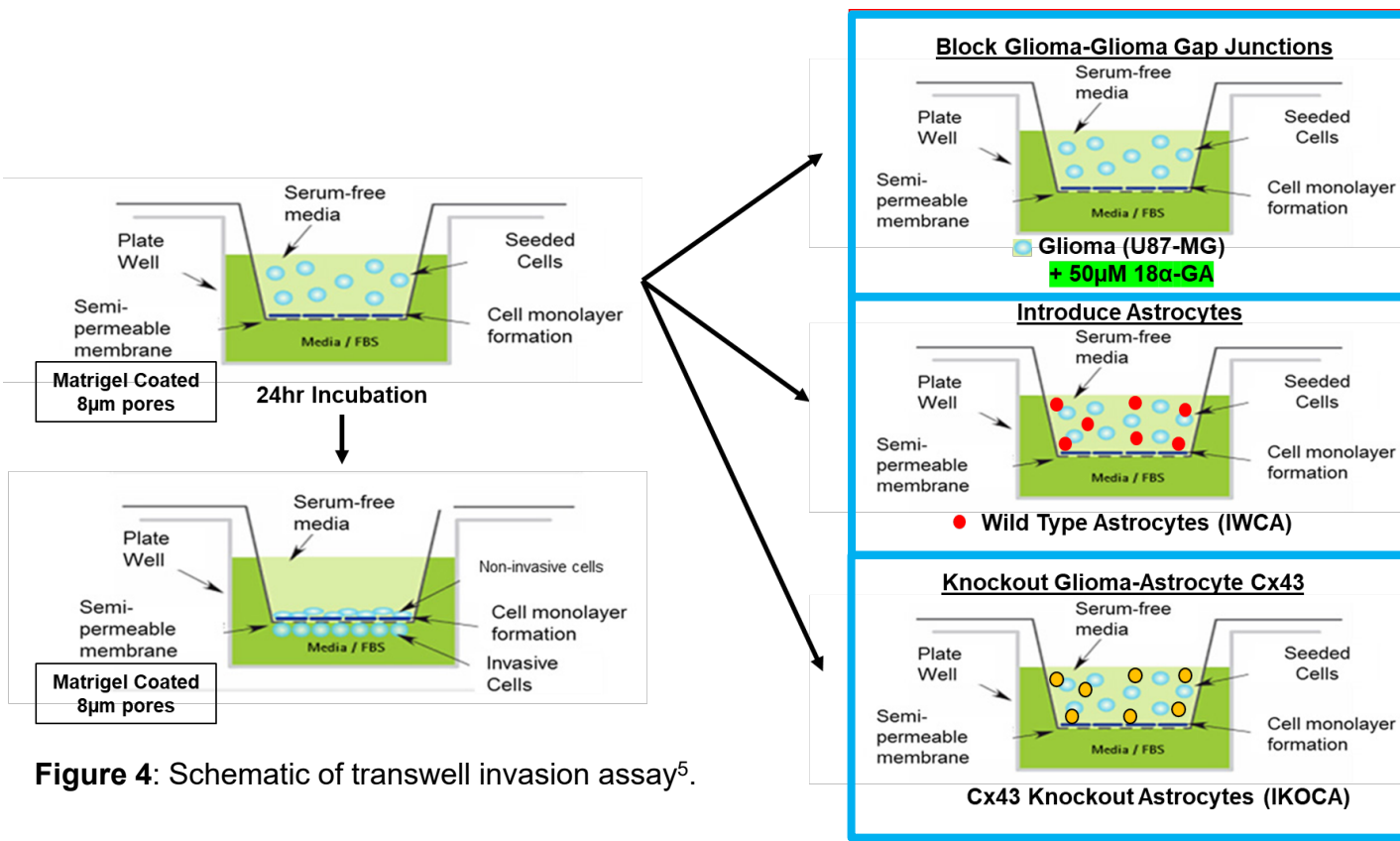


Figure 4: Schematic of transwell invasion assay⁵.

Ex vivo invasion model

- Cytosolic GFP tagged U87 were grown into 250µm diameter spheroids by standard culture.
- 400µm thick whole brain coronal slices were obtained from 6-8 week old wildtype C57BL/6J mice and maintained in culture for up to 7 days.
- U87 spheroids were harvested and injected by Hamilton syringe.
- Invasion was quantified at 1-3 days untreated or continuously dosed with 100µM CBX.
- Tissue samples were cleared by SeeDB⁷ protocol and imaged by confocal microscopy, at 10x magnification.

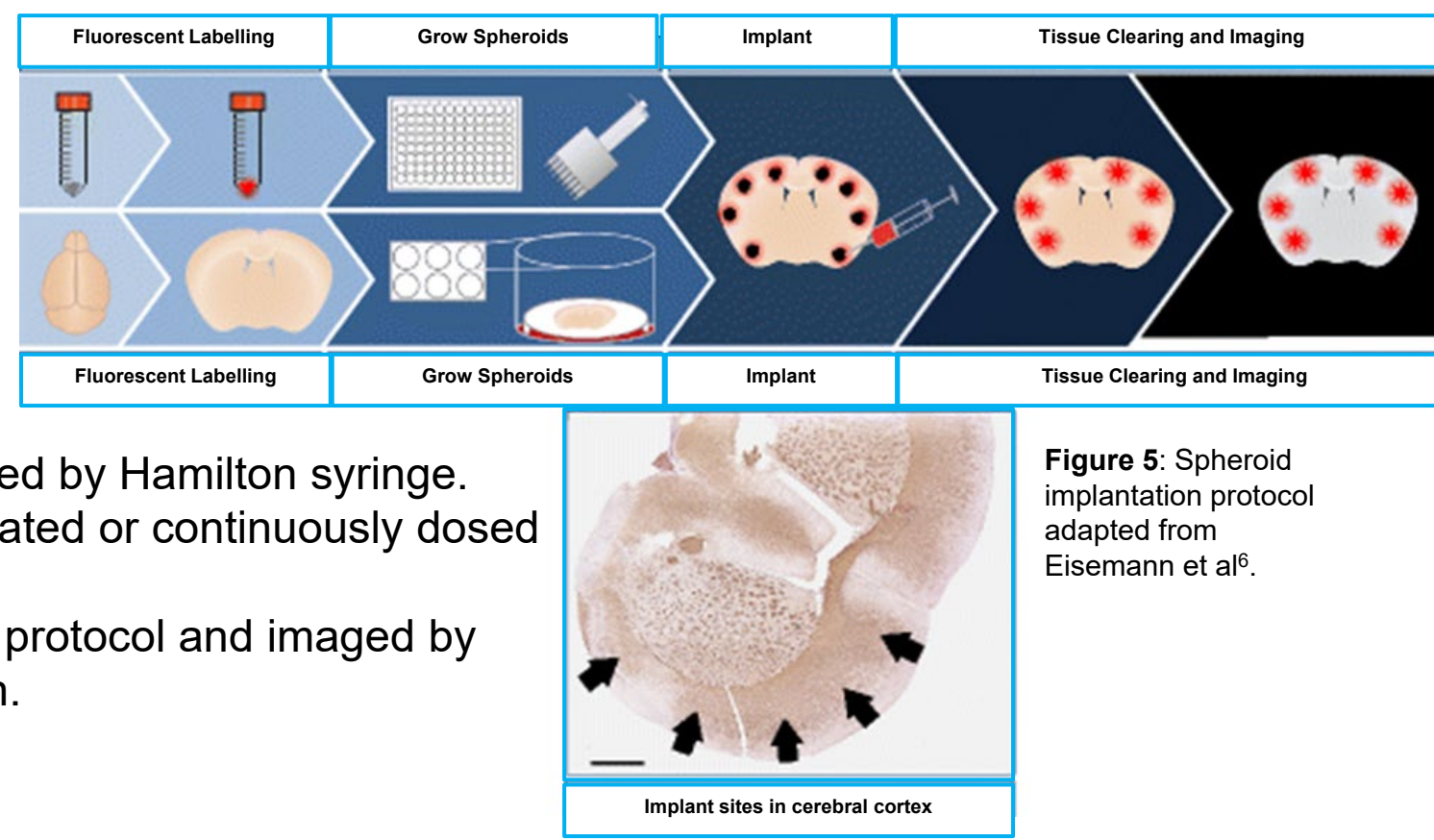


Figure 5: Spheroid implantation protocol adapted from Eisemann et al⁶.

miRNA sequencing

- IWCA or IKOCA were cocultured with Cytosolic GFP tagged U87 at a 1:1 ratio for 48 hours in standard tissue culture treated plates.
- Cells were harvested and isolated by FACs.
- Small RNAs were isolated from FACs sorted samples and sequenced for miRNA expression/content (LC Sciences).

RESULTS

U87 form functional Cx43 channels with astrocytes *in vitro*

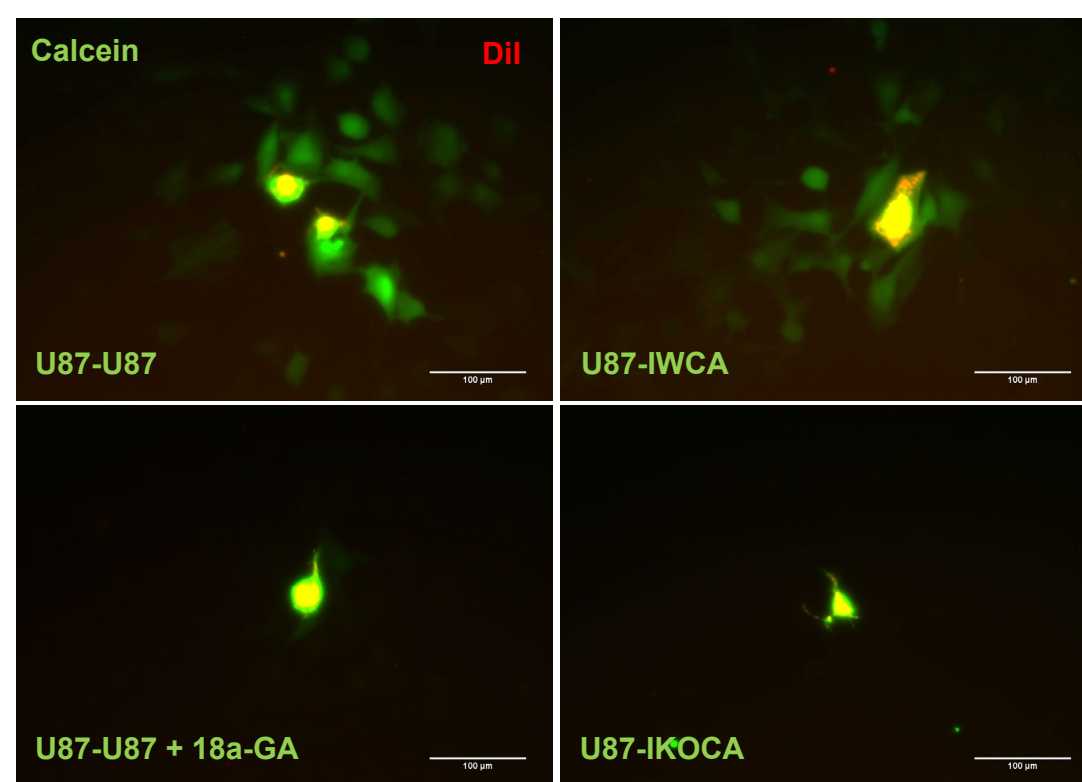


Figure 6: Formation of functional GJs between U87 GBM cells and astrocytes. Left panel shows transfer of GJ permeable dye from U87 to U87, and U87 to IWCA, blocked by GJ blockade and Cx43 knockout. Right chart shows degree of coupling per condition. Scale bars 100µm. Data shown as mean ± standard deviation.

- U87 formed functional gap junctions with other U87 cells and IWCA cells, and gap junctional transfer of Calcein was successfully blocked by gap junction blockade and culture with Cx43 null cells.
- U87 form distinct junctional plaques with IWCA cells and the plaques are internalized over time.

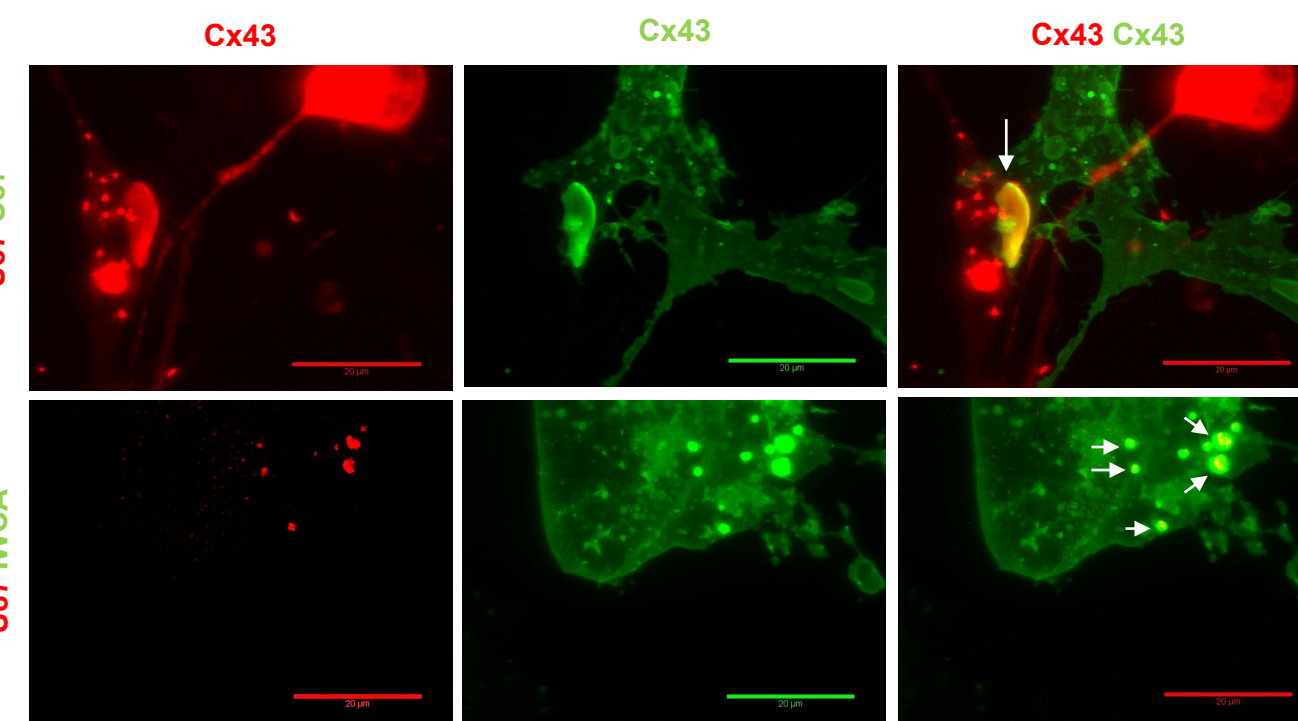


Figure 7: Formation of GJ plaques between U87 cells and astrocytes. C-terminus tagged Cx43 transfected into U87 and IWCA. BFP-Cx43 wildtype (red), GFP-Cx43 wildtype (green). Scale bars 20µm. Arrows show membrane or internalized plaques.

Cx43 presence and function alters the invasion potential of GBM *in vitro*

- Blockade of Cx43 increases invasion of U87 cells in monoculture.
- Culture of U87 with IWCA increases invasion of U87 cells.
- Blockade of Cx43 in U87-astrocyte coculture dramatically reduces U87 invasion.
- Culture of U87 with Cx43 knockout astrocytes dramatically reduces U87 invasion.

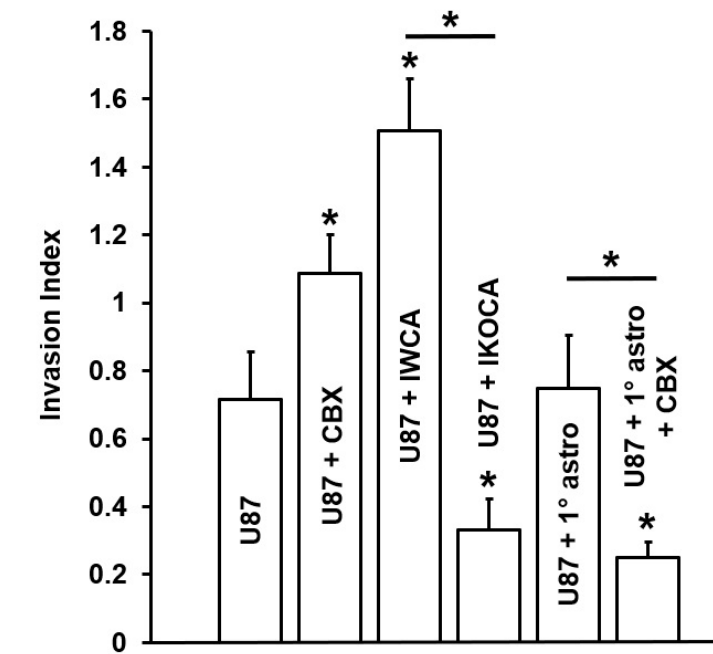


Figure 8: Invasion index relative to MDA-MB-231. Data shown as mean ± standard deviation. *p<0.05 vs U87 alone unless otherwise specified.

Cx43 blockade stunts GBM invasion in *ex vivo* slice model

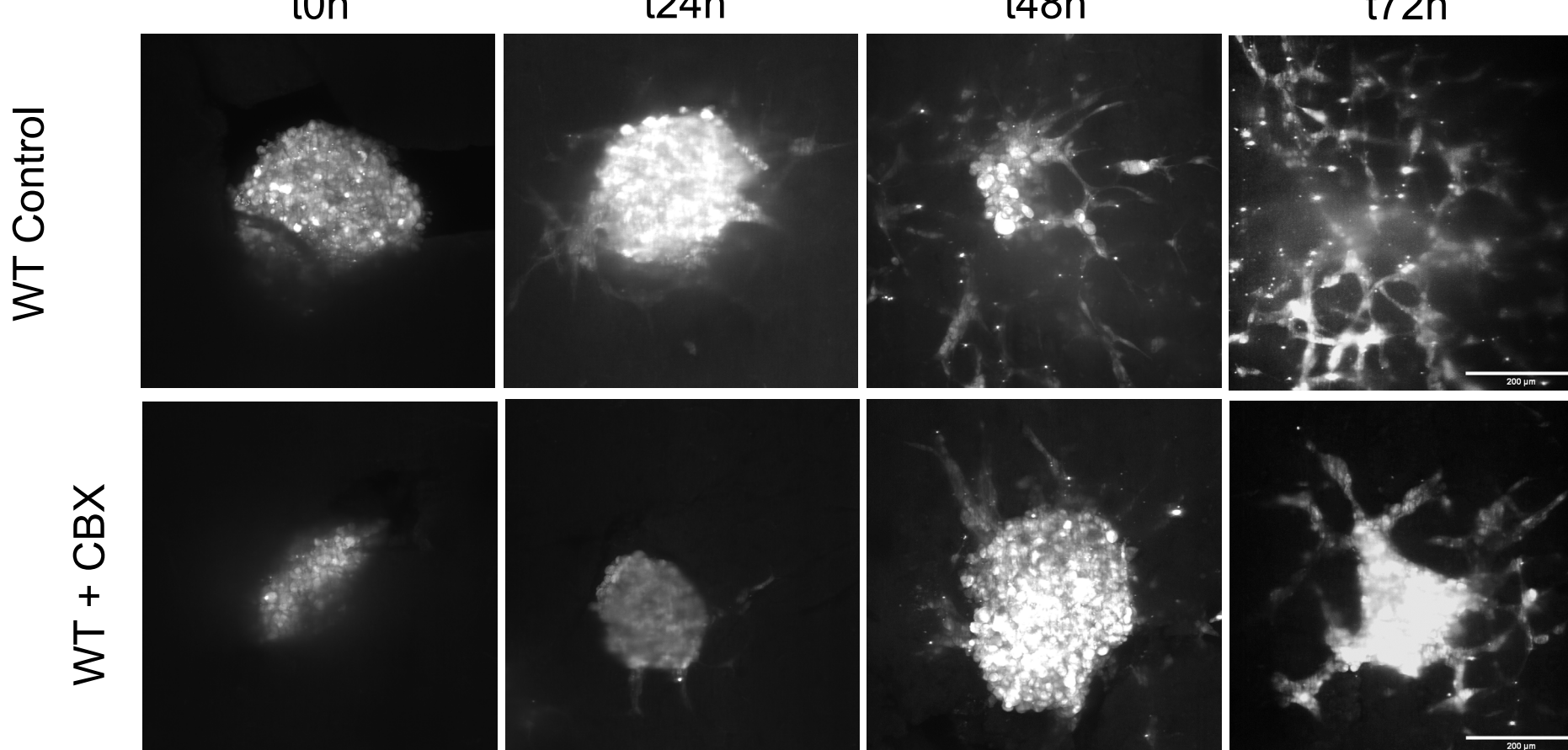


Figure 9: 250µm U87 spheroids implanted in coronal cortex of 400µm whole mouse brain coronal slices. Slices untreated or continuously treated with 100µM CBX for 1-3 days. Scale bars 200µm.

- Our *ex vivo* GBM invasion model successfully demonstrated tumor outgrowth and invasion in a network-like structure in the cerebral cortex within 48-72 hours.
- GJ blockade significantly reduced the invasion of tumor cells over the time course of the experiment.

RESULTS

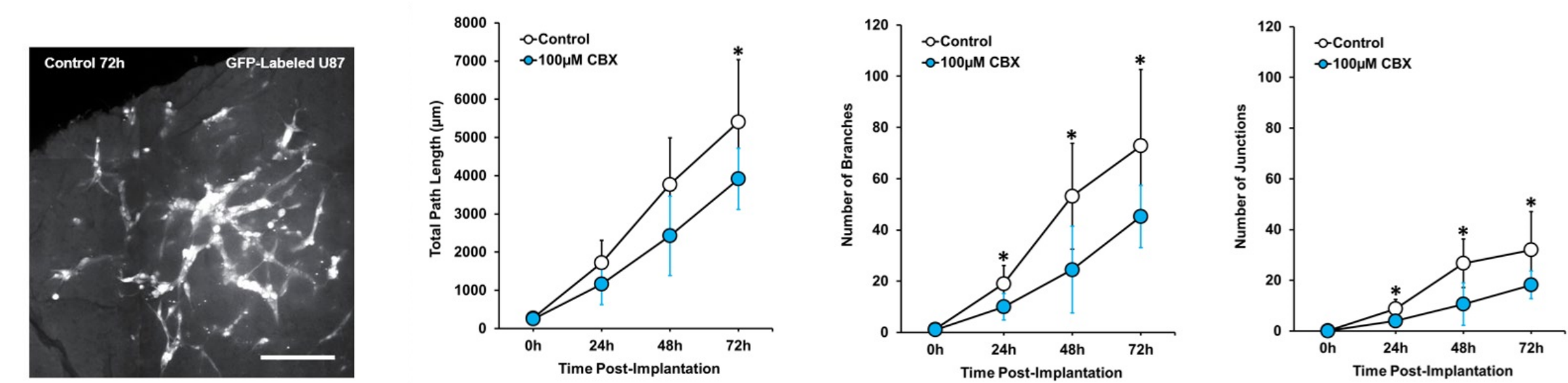


Figure 10: Invasion parameters of ex vivo slice culture U87 spheroid implantation. Left shows tile scan of U87-implanted cortical region at 72 hours without treatment. Right panels shown degree of invasion with and without GJ blockade by CBX. Scale bar 200µm. Data shown as mean ± standard deviation. *p<0.05.

miRNA is differentially expressed in astrocytes when cultured with Glioblastoma

- miRNAseq provided 11 miRNAs of interest for our studies, these miRNAs had 3 criteria:
 - Upregulated in IWCA cocultured with U87 vs IWCA monoculture
 - Lower expression levels in IKOCA cocultures vs IWCA cocultures
 - Expressed at much higher levels in U87 than IWCA or IKOCA
- From these 11 miRNAs, subsequent experiments have identified 2 main miRNAs of interest, has-miR-339-3p and has-miR-378a-5p

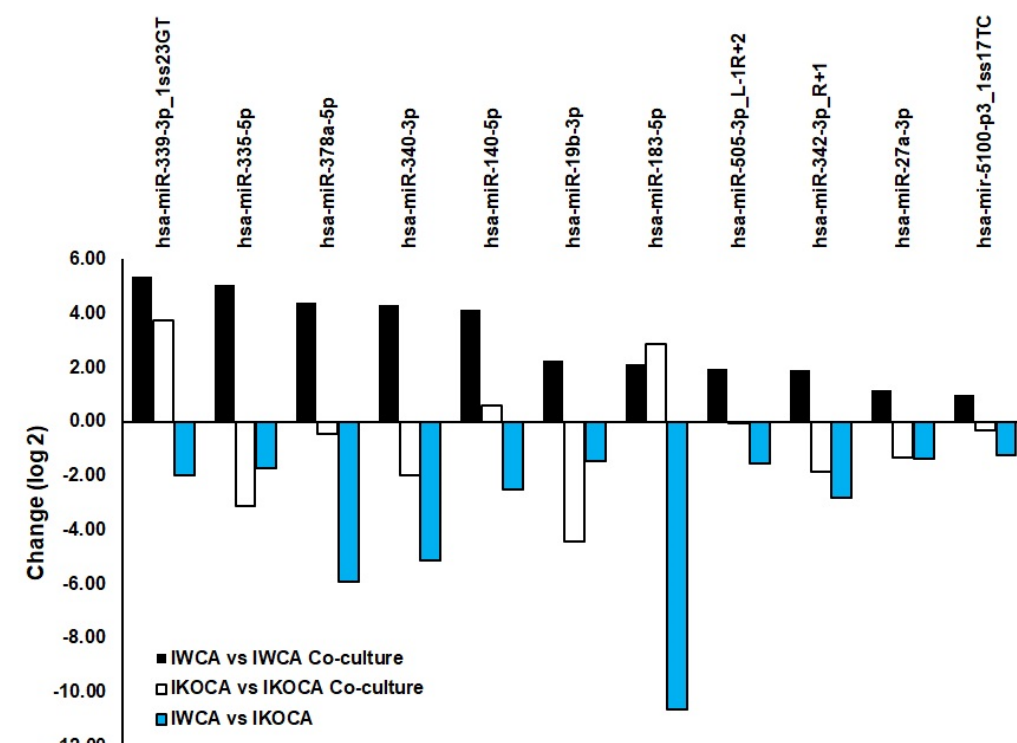


Figure 11: miRNAs of interest. All changes in IWCA monoculture vs coculture with U87 have p<0.01.

Glioblastoma transfers vesicles to astrocytes via biased internalization of Cx43 and transfers exosomes

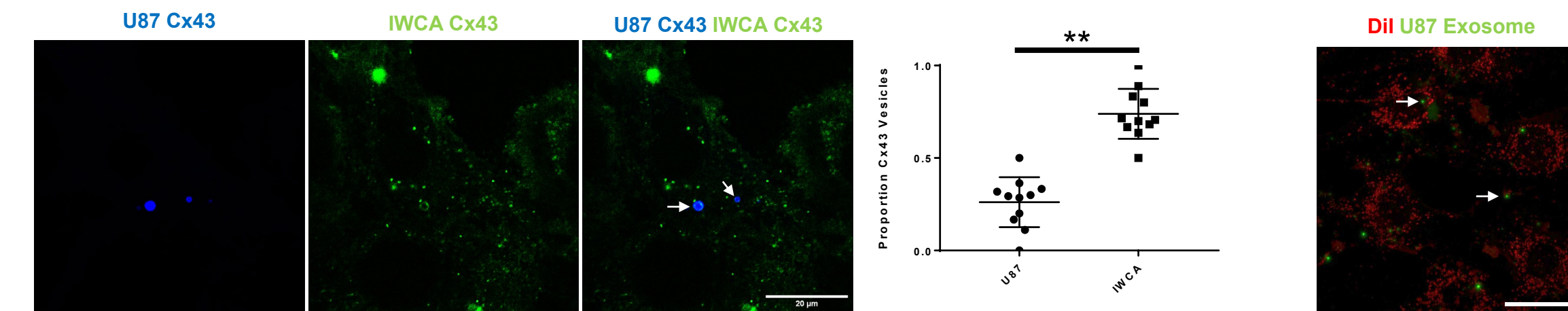


Figure 12: BFP-tagged Cx43 in U87 cocultured with EGFP-tagged Cx43 in IWCA, demonstrating biased internalization of Cx43 toward astrocytes of any cancer. Chart depicts proportion of endocytosed vesicles present in either cell of the U87-IWCA pair. Arrows show internalized Cx43. Data shown as mean ± standard deviation. **p<0.001. Scale bar 20µm.

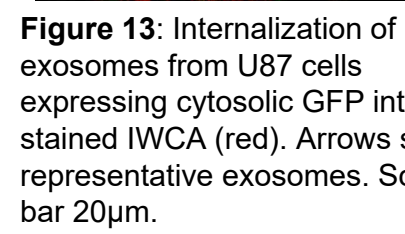


Figure 13: Internalization of exosomes from U87 cells expressing cytosolic GFP into Dil stained IWCA (red). Arrows show representative exosomes. Scale bar 20µm.

DISCUSSION

- Glioblastoma Multiforme (GBM) is the most aggressive and deadly CNS cancer and has one of the worst prognoses of any cancer. The invasive nature of GBM, characterized by growth along tracts within the brain, makes resection and adjuvant therapy less effective in the long-term, leading to a high incidence of tumor recurrence.
- Cell-cell communication mediates GBM invasion along vasculature, and disruption of the perivascular niche contributes to a permissive environment for GBM invasion.
- We show that GBM readily form functional Cx43 gap junctions with each other and with wildtype astrocytes, consistent with previous studies. We have also visualized the presence of junctional plaques between GBM and astrocytes, and confirmed they have similar properties to non-cancer wildtype Cx43 plaques.
- Our data indicate that Cx43 plays opposing roles in GBM invasion. Cx43 inhibits invasion by maintaining cohesion of the primary GBM tumor, with blockade of Cx43 increasing invasion when a GBM cell line is cultured alone. Cx43 also drives invasion at the edges of the tumor when GBM forms junctions with surrounding astrocytes, as demonstrated by increase invasion in the presence of astrocytes, and markedly decreased invasion when GBM-astrocyte Cx43 is blocked or knocked out.
- Our data recapitulate a decrease in GBM invasion with GBM-astrocyte Cx43 blockade in a standard *in vitro* invasion system and in an adapted *ex vivo* 3D slice culture model.
- Preliminary data indicate that there are significant shifts in miRNA content of astrocytes cocultured with GBM cells, and that these shifts can be abrogated by knocking out of Cx43.

FUTURE WORK

- Finding the mechanism(s) of miRNA transfer. Direct gap junctional transfer of single stranded linear mature miRNA molecules, exosomal transfer, or biased endocytosis of gap junction plaques.
- Tracking miRNA using fluorescently-labeled miRNA mimics and post-hoc single molecule fluorescence *in situ* hybridization (smFISH).
- In vivo* modeling of GBM invasion, using astrocyte specific GFAP-Cre Cx43 knockouts and non-functional Cx43 constructs.
- Microfluidics approach to miRNA transfer. Development of novel pseudo-3D microfluidics system using photolithographic techniques and tissue engineering for high throughput assessment of GBM invasion and miRNA localization.

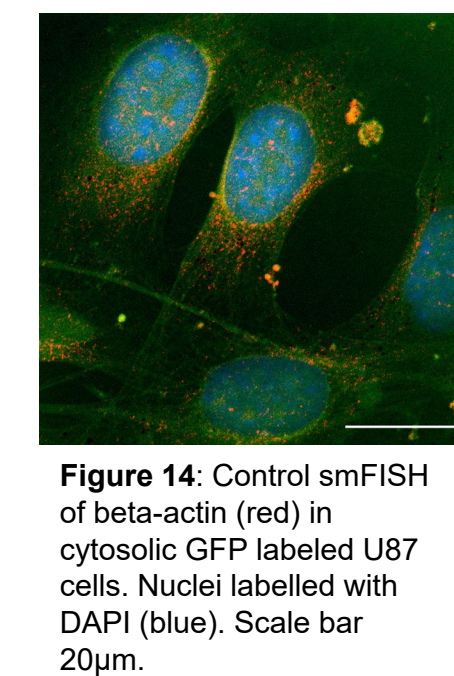


Figure 14: Control smFISH of beta-actin (red) in cytosolic GFP labeled U87 cells. Nuclei labelled with DAPI (blue). Scale bar 20µm.

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