In vitro functional characterization of genes mediating breast cancer metastasis to the brain

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1 – Summary
The aggressive behavior of tumor metastasis and the lack of effective treatments are conditions that warrant the study of basic mechanisms of metastasis. The brain is one of the sites where breast cancer often forms metastasis. A model of spontaneous breast cancer brain metastasis has been previously generated in the laboratory and a gene expression profile has been determined. From the microarray analysis a list of genes with potential clinical relevance for breast cancer metastasis to the brain has been identified, based on their correlation with reduced BMFS (Brain Metastasis-Free Survival) parameter as determined by bioinformatic analysis of breast cancer gene expression data bases.
The aim of this project was to functionally characterize a subset of these up-regulated genes previously identified in genome-wide screening to mediate spontaneous experimental breast cancer metastasis to the brain, in order to understand the biological implications of these selected genes. Here we provide evidence that the Cx31 protein promotes cell proliferation, resistance to apoptosis and stem cell self-renewal potential. In addition, the EDN1 peptide provides a pro-migratory pattern, promoting effects of resistance to apoptosis and stem cell self-renewal potential. Further findings are a pro-migratory activity of CTGF and a promoting stem cell self-renewal potential of IL1R2.
In short, results obtained from this project are expected to contribute to a better understanding of the mechanism of breast cancer metastasis to the brain and to identify novel therapeutic approaches.

2 – Introduction
Breast cancer is the most common type of cancer occurring in women, representing around 10% of all cancer incidences worldwide. Advanced therapeutic approaches as hormonal therapy, chemotherapy and targeted therapies continuously improved progression-free and overall survival of breast cancer patients over the past 20 years. Currently, the limiting factor in breast cancer survival is, as a matter of fact, the formation of distant metastasis to vital organs, and the lack of effective treatments to prevent or cure them.

Preferred primary metastatic sites for breast cancer are lung, bone and liver. Traditionally breast cancer metastasis to the brain occurred in less than 20% of the patients and was often associated with an advanced stage of the disease. Nowadays, however, the number of breast cancer patients experiencing brain metastasis is increasing due to their extended survival thanks the improved therapeutic management of the primary lesions. In fact it appears that adjuvant therapy effectively controls disease progression in the periphery (i.e. metastasis to lung, bone and liver), but fail to impinge on metastasis formation in the brain. This is in part due to the limited ability of chemotherapy and targeted drugs to pass the blood brain barrier.

Today brain metastasis occurs in approximately 30% of breast cancer patients and causes death within 6 months from the diagnosis. Autopsy studies suggest that its incidence is likely to be underestimated due to asymptomatic lesions in many cases. It is likely that the incidence of breast cancer brain metastasis will rise continuously, because on improved overall survival and lack of effective therapy against brain metastasis (Ref. 1). Thus there is an urgent need to better understand mechanisms governing breast cancer brain metastasis formation.

Metastasis, is defined as the spread and growth of cancer cells from a primary tumor site to distant secondary organs as a multi-step process. At a phenomenological level, cancer cells have to perform several discrete steps in order to metastasize. They include: (1) loss of cell-cell adhesion and gain of cell-matrix adhesion and local invasion, (2) intravasation, (3) intravasation and survival in the circulation, (4) extravasation, (5) formation of micro-metastasis, and (6) growth at the distant site. The current paradigm defines metastasis as a process driving the selection of cells with advantageous traits that allow them to overcome the diverse environmental defense mechanisms preventing growth of cells in tissues different from the ones of origin. Metastasis is associated with important changes in gene expression (Ref. 2). Our current understanding of metastasis formation indicates that the emergence of metastatic cells might be driven by the selection of a limited set of stochastically accumulated combinatorial functions necessary for the growth and survival of the tumor cells at the primary site, rather than the de novo selection of rare cells overexpressing a limited number of 'metastasis-specific' genes (Ref. 3). This situation is reminiscent of infection organisms becoming highly pathogenic through the acquisition of only a few 'virulence' genes. Malignant tumors often metastasize following an organ-specific pattern, defined by metastasis organ tropism. For example, bone metastases preferentially originate from lung, prostate or breast carcinoma. This relation between a certain type of cancer and a particular organ location for its metastatic colonization suggests the need for a tumor cell to interact with, adapt to and survive within a specific "foreign" tissue microenvironment. Paget first evoked this notion on 1889 in his "seed and soil" theory.
In case of breast carcinoma, metastases occur mainly in lung, bone, liver and brain. Breast cancer is the third most common tumor metastasizing to brain after lung carcinoma and melanoma. However, it is the most common one due to the higher incidence of breast cancer. The mechanisms mediating breast cancer brain metastases remain still largely unknown.
The group of Dr. Massagué recently reported the identification of genes mediating breast cancer metastasis to the bone, lung and brain, which include the epidermal growth factor receptor (EGFR) ligand epiregulin, COX2, MMP 1 and MMP2 (Ref. 4).
In order to identify additional genes mediating breast cancer metastasis to the brain, the host laboratory has developed the first orthotopic model of breast cancer metastasis to the brain in immunocompetent mice using the 4T1 breast cancer model in BALB/c mice. Through two cycles of in vivo selection this lab has generated 4T1-derivative lines (4T1-BM2) that form brain metastasis with 100% penetrance. Thereupon a list of genes associated with brain metastasis was compiled through gene expression profiling of brain metastatic and non-brain metastatic cells and bioinformatic analysis of gene expression profiles in human breast cancer patients datasets. 30% of the identified genes were identical or related to those reported by Massagué's group. Many genes related to inflammation and cell-cell communication were identified. A small set of up-regulated genes was selected based on the association of their human othologues with reduced brain metastasis-free survival in human patients, for further detailed in vitro and vivo characterization. In this project, we investigated five genes, which have been implicated in mediating brain metastasis in the 4T1 model, but their mechanism of action has not been elucidated yet.

One of them is the gap junction protein beta 3 (GJB3 also known as Connexin 31 - Cx31), a member of the connexin gene family. As specialized cell-cell contact molecules, their role is to mediate the passive diffusion of molecules such as nutrients, small metabolites (e.g. glucose), ions (K+, Ca2+) and second messengers (IP3, cAMP and cGMP) from one cell to another (Ref. 5). To date, little is known on the role of connexins in cancer. Using connexin-transfected HeLa cells, Graeber and Hülser reported that forced expression of connexins Cx31, Cx40, or Cx43 promoted invasion of tumor cells in an in vitro chicken heart model (Ref. 6).

Another analyzed molecule is the endothelin 1 peptide (EDN1), a member of the endothelin family, consisting of potent vasoconstrictor peptides with three endogenous isoforms and two distinct receptors (Ref. 7). EDN1 has been proposed to function as an auto- and paracrine

growth factor for certain tumor cells, especially for ovarian cancer cells (Ref. 8-9). Targeting endothelin A receptor and HER2 in HER2-overexpressing breast cancer cells reduced proliferation and invasion (Ref. 10).

A third gene of interest is connective tissue growth factor (CTGF). CTGF belongs to the CCN family of matricellular proteins (the CCN stands for CTGF, Cystein rich protein (Cyr61), and Nephroblastoma overexpressed gene, the first three identified members of the family) (Ref. 11), which biological properties that include the stimulation of cellular proliferation, migration, adhesion, extracellular matrix formation, as well as the regulation of angiogenesis and tumorgenesis (Ref. 12). Hence, CTGF plays multiple roles in various cancer types. In breast cancer cells CTGF overexpression has been linked to increased tumor size and lymph node metastasis (Ref. 13, 14), as well as to usual resistance to apoptotic stress (Ref. 12) and critical involvement in the formation of osteolytic bone metastasis (Ref. 15, 16).

A forth gene of interest encodes for the laminin gamma 2 chain (LAMC2), part of the laminin family of extracellular matrix glycoproteins, implicated in a wide variety of biological processes including cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis (Ref. 17). Today's findings on the LAMC2 expression show a correlation with hematogenous spread of cancer cells in contrast to lymphatic metastasis in bladder cancer (Ref. 18) and a coordinated promigratory activity with Membrane type 1 metalloprotease at the invasive front of colorectal carcinomas (Ref. 19).

A fifth gene of interest is the interleukin 1 receptor type II (IL1R2) that belongs to the interleukin 1 receptor family. IL1R2 has been proposed to act as a decoy receptor, inhibiting the activity of IL1A, IL1B, thereby reducing IL1R1 signaling (Ref. 20). IL1R2 plays a physiological role in inflammation, proliferation and apoptosis (Ref. 20). Rückert et al, reported for the first time an upregulation of IL1R2 in pancreatic cancer (Ref. 21). They proposed that IL1R2 protects pancreatic cancer cells from apoptosis, whereas the microenvironment might benefit from the angiogenic and proliferative properties of IL1 secreted by pancreatic cancer cells as well (Ref. 22).

3 – Aim of the study

Our objective was to test, whether the genes GJB3, EDN1, CTGF, LAMC2, IL1R2, among those identified in a gene expression analysis screen in non-brain metastatic (4T1-T2) vs. brain metastatic (4T1-BM2) cells, were functionally implicated in influencing the aggressiveness of breast cancer cells toward the formation of brain metastasis.

4 – Experimental approach

The proposed experiments are performed in vitro using 4T1-derived cell lines.

Part 1: Suppression of expression of selected genes by RNAi approach (gene silencing)

To knock-down genes of interest we used a gene silencing approach based on RNA interference (RNAi). Short hairpin RNA's are transduced into target cells (i.e metastatic 4T1 variants). Among genes of interest correlating with the metastatic phenotype five were previously silenced in the host laboratory (Cx31, CTGF, EDN1, IL1R2, LAMC2), by lentivirus-mediate gene transfer of short hairpins RNA (shRNA).

Plasmid containing the shRNA of interest were purchased commercially (Sigma) and used to generate a lenti virus by cotransfection in HEK 293 cells. Supernatants were then used to infect 4T1-BM2 cells. The validity of gene silencing was then determined by measuring mRNA level by quantitative real time qRT-PCR and by detecting the protein expression by Western blot analysis. 3-5 different shRNA constructs were used for each gene to be silenced. This part of the study was performed before my arrival in the lab.

Part 2: Analysis of the biological effect of gene silencing

The effect of gene silencing on 4T1-BM2 cell activities was monitored in cell culture and functional experiments. We analyzed 4T1 parental cells, 4T1-BM2 cells and 4T1-BM2 cells silenced for the specific genes (4T1-Bm2 Cx31^{kd}, T1-BM2 CTGF^{kd}, 4T1-Bm2 EDN1^{kd}, 4T1-BM2 IL1R2^{kd}, 4T1-BM2 LAMC2^{kd}) in assays testing properties potentially associated with metastasis: proliferation, survival, migration/invasion and "stem cell" activity. The following in vitro assays were used:

4.1. Proliferation assay

Cells were seeded in 96 well micro-titer plates at low density (1000 cells/well) and their growth was monitored daily for up to 5 days by crystal violet staining followed by elution and densitometry (O.D.) analysis. We used one 96 well plates for each time point (t=0 hours, t=24 hours, t=48 hours, t=72 hours, t=96 hours). The cells were allowed to attach for 18 hours before the experiment was started. Each cell line was seeded in 8 wells per plate in 200 µl of medium (500 ml DMEM, 10% FCS, 1% penicillin/streptomycin solution, 1% non-essential amino acids, NEAA) per well. Once the experiment started, plates were placed into a humidified cell culture incubator at 37°C and 5% CO_2. The procedure in terms of the reading points was to control microscopically the aspect of the cell attachment and the exclusion of contamination before the medium was aspirated. Afterwards the cells were washed once with phosphate-buffered saline (PBS), fixed and stained for 20 minutes at room temperature with a 160 µl of a solution containing 4 % Paraformaldehyde and 2 % crystal violet per well. The plates were rinsed and air-dried overnight. Finally, crystal violet was resolubilized using 100 µl destainer solution (1% acetic acid, 50% ethanol, 50% deionized water) per well to quantify absorbance at 595nm wavelenght.

4.2. Resistance to hypoxia assay

Cells were cultivated in 12 well plates at high density (100'000 cells/well) under standard (i.e. 20% oxygen) and hypoxic (0.1% oxygen) conditions. We used 4 wells per plate for each cell line in each oxygen condition with 1 ml of DMEM medium per well. The cells were allowed to attach for 18 hours before the experiment was started. Once the experiment started all plates were placed into the incubator at 37°C, 5% CO2 and the appropriated oxygenic condition during 24 hours. Finally, the cells were controlled microscopically before the medium was aspirated. After washing once with PBS, splitting the cell adhesion with trypsin during 4 minutes in the incubator, neutralizing the protease activity by adding DMEM medium and centrifuging, the number of cells per unit volume of a suspension was determined by using a counting chamber (Neubauer hemocytometer). Dead cells and debris were excluded by trypan blue exclusion staining.

4.3. Migration and invasion assays.

To test migration capability, cells were seeded in 6 well plates at high density (500'000 cells in 2 ml DMEM medium/well) to grow till confluence. A sharp a wound was applied in the middle of the well with a 1000 µl micropipette tip. Wound healing was monitored till its closure during 24 hours by time lapse imaging using a Leica DMI6000B microscope equipped with a CO2/Temp- conditioned incubator as documented by a 24 hours time-lapse movie. Duplicate wells were used per condition.

4.4. Mammosphere formation assay

Cells were seeded on a non-adhesive surface in a 96 well micro-titer plate at low density (5'000 cells/well). We used MEGM (mammary epithelial growth medium) to assure anchorage independent growth. Hence, each cell line obtained 10 wells per plate and 100 µl of MEGM. Every third day, the cells received 100 µl of culture medium MEGM over a period of 10 days. Finally, the number of formed spheroids and their sizes was assessed on microscopically collected/documented images.

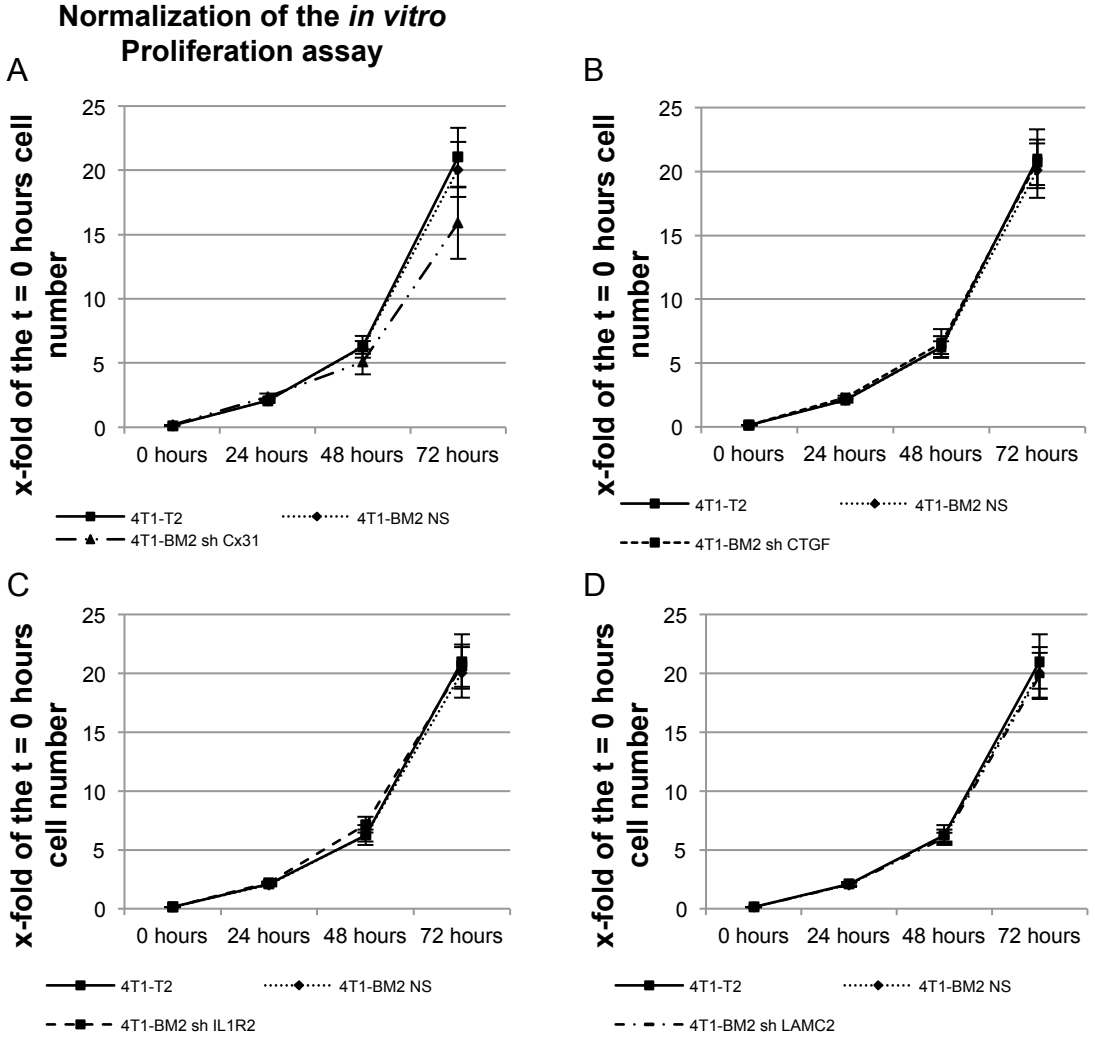
4.5. Statistical analysis

Statistical validation of the collected data was determined by using the bilateral Student's t-test. Results were then ranked as * for a t test value <0,05, ** for a t test value <0,005, *** for a t test value <0,001, or NS, non-significant, for a t test value >0,05.

5 – Results

5.1. – 4T1-BM2 Cx31^{kd} cells have a reduced proliferative capacity in an *in vitro* proliferation assay compared to the parental and 4T1-BM2 cell lines, as well as the other cell lines with selected gene-silenced.

To determine whether one of the selected genes (Cx31, CTGF, EDN1, IL1R2, LAMC2) silenced in the 4T1-BM2 cell line play a significant role in breast cancer cell proliferation, we examined the proliferation rate of each cell line with the silenced genes and compared it with two control cell lines (4T1-T2 parental and 4T1-BM2 cells) in an *in vitro* proliferation assay. We did a time course experiment and we first analyzed the raw values obtained at the time of 72 hours. Later time points were avoided due to cell confluence. We performed a normalization of the data obtained (**Figure 1**), by dividing the values of the different time points by the value at 24 hours, to illustrate the cell growth rate. We observed a significant reduction in cell growth rate of the 4T1-BM2 Cx31^{kd} cell line related to all the other gene-silenced cell lines (19.7 ± 14.0 %, $p < 0.006$; at 72 hours), as well as the controls (20.9 ± 13.8 %, $p < 0.005$; at 72 hours).



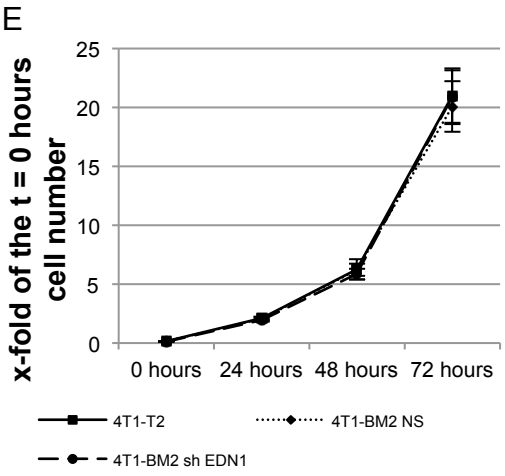


Figure 1. Cell proliferation. Proliferation rate of each cell line with the silenced genes, compared to the two control cell lines (4T1-T2 parental and 4T1-BM2 cells) at 72 hours. (A) reduced proliferation in 4T1-BM2 Cx31^{kd} cells (to 4T1-T2, **, P<0.0015, to 4T1-BM2, **, P<0.005, students t test). Unaltered proliferation in (B, C, D, E) 4T1-BM2 CTGF^{kd} cells (to 4T1-T2, NS, P=0.79, to 4T1-BM2, NS, P=0.52, students t test), in 4T1-BM2 IL1R2^{kd} cells (to 4T1-T2, NS, P=0.72, to 4T1-BM2, NS, P=0.58, students t test), in 4T1-BM2 LAMC2^{kd} cells (to 4T1-T2, NS, P=0.27, to 4T1-BM2, NS, P=0.78, students t test), in 4T1-BM2 EDN1^{kd} cells (to 4T1-T2, NS, P=0.90, to 4T1-BM2, NS, P=0.48, students t test). Unaltered proliferation rate from 4T1-T2 parental compared to 4T1-BM2 cells (NS, P= 0.41, students t test). All values represent mean ± SEM.

5.2. – The 4T1-BM2 Cx31^{kd} cell line have reduced survival under hypoxic conditions.
To determine the impact on 4T1-BM2 survival under hypoxia of one of the selected genes, we compared the growth of parental 4T1 and 4T1-BM2 cells as well as the silenced 4T1-BM2 lines under hypoxic vs. normoxic conditions.
Considering the number of cells and using the normalization of the data by calculating the hypoxia-normoxia ratio (**Figure 2-3**), the cell lines 4T1-BM2 Cx31^{kd}, 4T1-BM2 EDN1^{kd} showed a 49.4% and 12.1% decreased in survival, respectively, compared to the 4T1-BM2 cell line at 24 hours. In addition, the 4T1-BM2 Cx31^{kd} presents a decreased survival rate relative to the paternal cell line by 11.2% at 24 hours.
The numerous cell lines used made it difficult to quantify the assays. In order to ease the effort, we reduced the number of lines tested at once. We added all four wells along a cell line and counted them. Nevertheless, there is the need to perform additional assays to perform statistical analysis of the results.

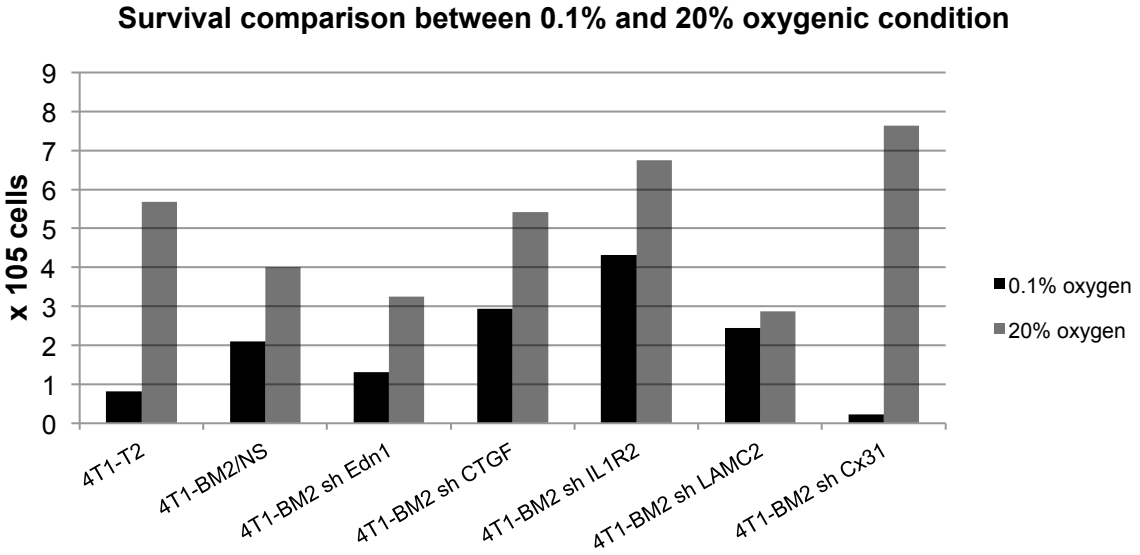


Figure 2. Cell survival under hypoxia vs normoxia.

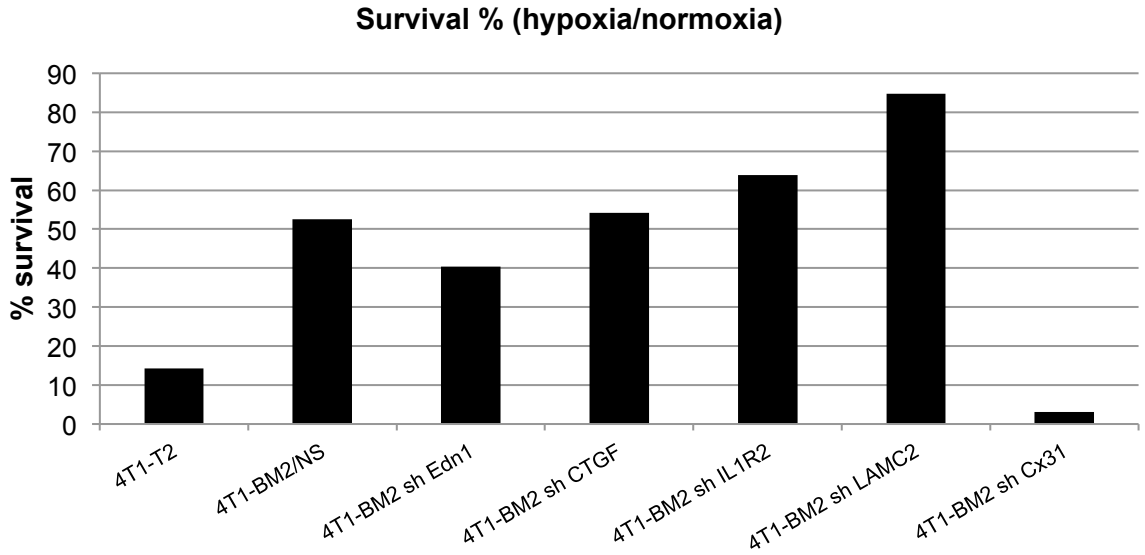


Figure 3. Relative cell survival under hypoxia vs normoxia.

5.3. – 4T1-BM2 CTGF^{kd} and 4T1-BM2 EDN1^{kd} cell lines show a collective pattern of migration and reduced velocity compared to controls and other gene-silenced cell lines.

To investigate the potential involvement of the selected genes in the migration process, we monitored the migration of the 4T1-T2, 4T1-BM2, 4T1-BM2 NS, 4T1-BM2 CTGF^{kd}, 4T1-BM2 Cx31^{kd} and 4T1-BM2 EDN1^{kd} cell lines during 24 hours. Microscopic analysis revealed a collective migration behavior of the 4T1-BM2 CTGF^{kd} and 4T1-BM2 Cx31^{kd} cell lines, particularly of the former (**Figure 4**). Conversely, a rather individual cell migration pattern was observed in other cell lines (**Figure 5**). The quantification of the migration velocity (μm / sec) revealed a clearly reduced speed of migration of the 4T1-BM2 CTGF^{kd} and 4T1-BM2 EDN1^{kd} cell lines, whereas the 4T1-BM2, 4T1-BM2 NS and 4T1-BM2 Cx31^{kd} cell lines has equal migration speed (**Figure 6**).

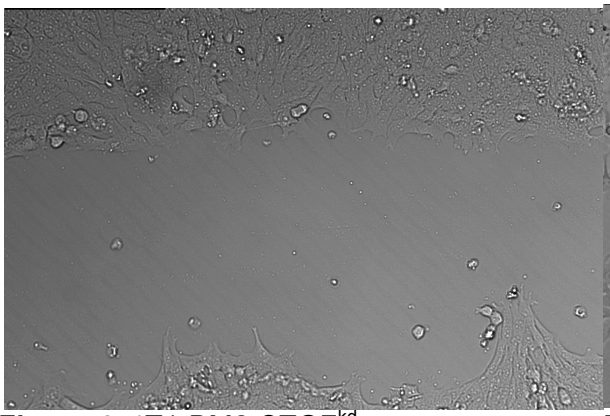


Figure 4. 4T1-BM2 CTGF^{kd} .
Example of collective cell migration

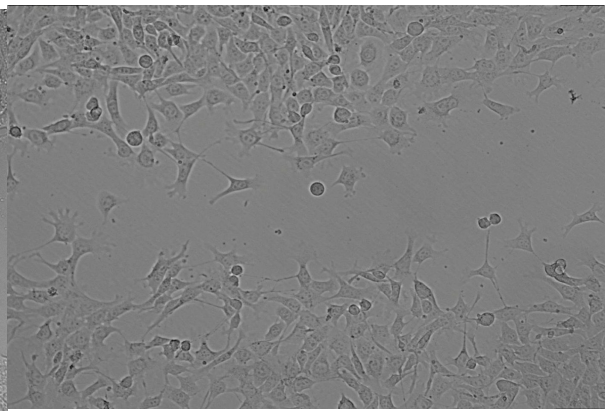


Figure 5. 4T1-T2
Example of individual cell migration

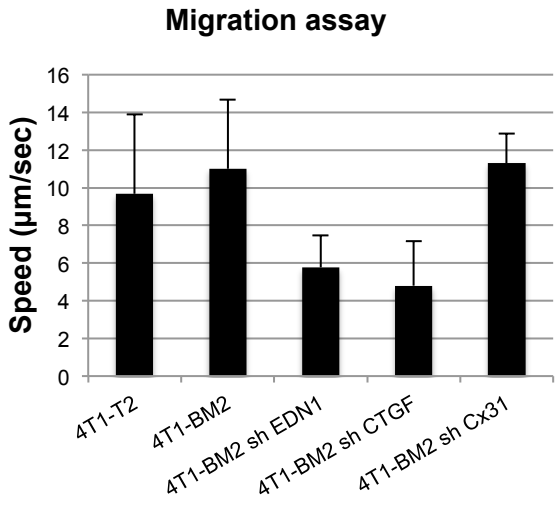


Figure 6. Cell migration. Migration during 24 hours of each cell line with the silenced genes, compared with the two control cell lines (4T1-T2 parental and 4T1-BM2 cells) is reduced in 4T1-BM2 CTGF^{kd} cells (to 4T1-T2, *, P<0.04, to 4T1-BM2, **, P<0.0085, students t test) and 4T1-BM2 EDN1^{kd} (to 4T1-T2, NS, P=0.076 to 4T1-BM2, *, P<0.02, students t test), unaltered in 4T1-BM2 Cx31^{kd} (to 4T1-T2, NS, P=0.53, to 4T1-BM2, NS, P=0.99, students t test). No difference in migration between 4T1-T2 parental and 4T1-BM2 cells (NS, P=0.60, students t test). All values represent mean ± SEM.

5.4. – Reduced capacity of 4T1-BM2 Cx31^{kd}, 4T1-BM2 IL1R2^{kd} and 4T1-BM2 EDN1^{kd} to form mammospheres.

To asses for stem cell self-renewal potential, we monitored for the capacity of the different lines to spontaneously assemble and grow as spheroids, using a mammosphere formation assay (**Figure 7**). We found a significant reduced number of spheroids formed by 4T1-BM2 Cx31^{kd}, 4T1-BM2 IL1R2^{kd} and 4T1-BM2 EDN1^{kd} lines compared to the 4T1-BM2 cell line by at least 18.4 ± 13.2 percent ($p < 0.002$). The 4T1-BM2 Cx31$^{\mathbf{kd}}$ also presents a significant reduced number of formed spheroids compared to the paternal cell line 4T1-T2 by 26.1 ± 17.1 percent ($p < 0.001$). In contrast, there were no significant differences in the number of formed spheroids by 4T1-BM2 CTGF^{kd} and 4T1-BM2 LAMC2^{kd} lines compared to the 4T1-BM2 cell line ($p > 0.1$). Nevertheless, the 4T1-BM2 LAMC2^{kd} cell line shows an increasing trend compared to all the other cell lines. According to experience, the parental 4T1-T2 cell line has a reduced ability to form spheroids compared with the 4T1-BM2 cell line (22.9 ± 8.4 percent, $p < 0.001$).

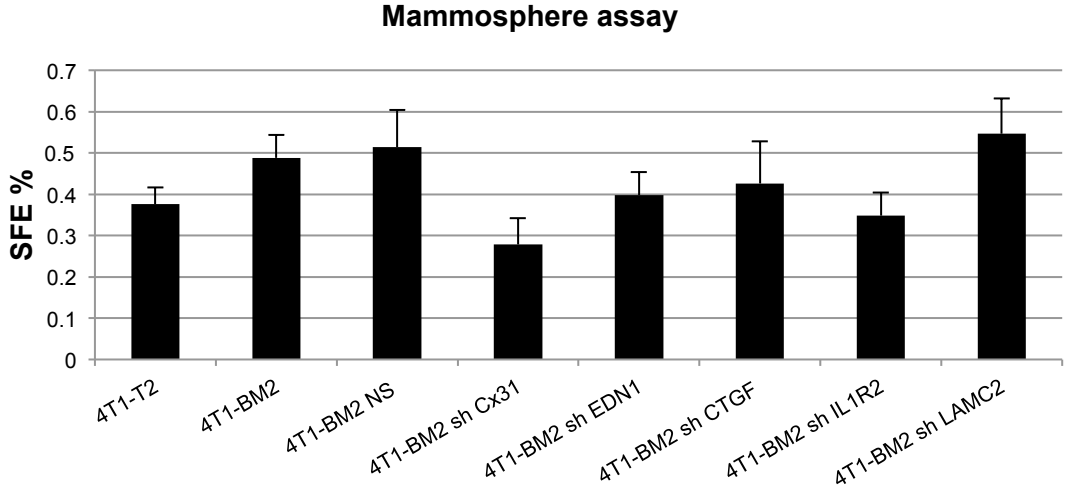


Figure 7. Mammosphere formation. Monitored spheroids formation of each cell line with the silenced genes, compared with the two control cell lines (4T1-T2 parental and 4T1-BM2 cells) at 10 days is reduced in 4T1-BM2 Cx31^{kd} cells (to 4T1-T2, ***, P<0.001, to 4T1-BM2, ***, P<0.0001, students t test), 4T1-BM2 IL1R2^{kd} (to 4T1-T2, NS, P=0.22, to 4T1-BM2, ***, P<0.0001, students t test) and 4T1-BM2 EDN1^{kd} (to 4T1-T2, NS, P=0.33, to 4T1-BM2, **, P<0.002, students t test), unaltered in 4T1-BM2 CTGF^{kd} cells (to 4T1-T2, NS, P=0.17, to

4T1-BM2, NS, P=0.11, students t test), in 4T1-BM2 LAMC2^{kd} cells (to 4T1-T2, ***, P<0.0001, to 4T1-BM2, NS, P=0.09, students t test). Reduced spheroid formation from 4T1-T2 parental compared to 4T1-BM2 cells (***, P=0.0001, students t test). All values represent mean ± SEM.

6 – Discussion

The mechanisms governing breast cancer brain metastasis are largely unknown at the molecular level. Models to identify and study genes involved in this process have just been developed. A model of spontaneous breast cancer brain metastasis has been previously generated in the laboratory. Gene expression profile of 4T1 brain metastasis has been studied by microarray analysis. The aim of my work was to characterize the biological effects of some of the gene identified in these screens as associated with brain metastatic capacity. We expected to obtain results allowing us to point to the role of the targeted genes in promoting one of these activities potentially involved in metastasis formation. The results should allow us to demonstrate or dismiss a potential correlation between one or more of the selected genes and the aggressive, brain metastatic behavior of the 4T1-BM2 cells.

We provide evidence that the Cx31 protein is relevant for the metastatic spread in breast cancer brain metastatic (4T1-BM2) cells. Increased Cx31 expression is a characteristic of 4T1-BM2 cells. While a previous study has identified members of the connexin family to promote HeLa cell invasion (Ref. 6) our findings show for the first time that a knockdown proceeding on Cx31 in brain metastatic cells decreases the growth rate. In comparing the proliferation rate of the gene-silenced cells with parental tumor and brain metastatic cells, we have been able to demonstrate a reduced proliferative capacity of 4T1-BM2 Cx31^{kd} compared to the 4T1-BM2 cells. But also the main difference from the parental cells is in particular to consider the role according to the growth capacity in general. This probably speaks for a reduced cell interaction with respect to growth-promoting factors and cell architecture.

Furthermore, our finding that Cx31 knockdown is correlated to greatly reduced survival under hypoxic conditions, compared the growth of the cell lines under hypoxic vs. normoxic conditions during 24 hours, provides compelling evidence that disruption of intercellular communication by connexins in breast cancer brain metastatic cells has a particular importance on overcoming stress conditions. Incidentally, even the knockdown of EDN1 peptide shows a reduced capacity for survival under hypoxia compared with the 4T1-BM2 but not with the parental tumor cells. Also, the EDN1 expression is particularly increased in 4T1-BM2 cells compared to the parental tumor cells. The involvement of auto- and paracrine growth factor activity (Ref. 8-9) in stress conditions is still open for discussion, but the presumption of the lack of increased growth factor secretion during hypoxia seems to prove as right.

Our results from the wound closure assay show a collective pattern of migration and reduced speed of the 4T1-BM2 CTGF^{kd} and 4T1-BM2 EDN1^{kd} cell lines. Concerning CTGF, this results complement the observations from the Wang, Xie and Chen reports (Ref. 12-14) as to the link to increased tumor size and lymph node metastasis, as well as to resistance to apoptotic stress by having an important impact on migration speed and character. EDN1 is regarding the same complemented properties.

Given the established role for the gene-silenced cell lines in proliferation, migration and resistance to hypoxia, it was important to demonstrate stem cell self-renewal potential of giving cell populations as well. We found greatly reduced capacity in 4T1-BM2 Cx31^{kd}, and slightly in 4T1-BM2 IL1R2^{kd} and 4T1-BM2 EDN1^{kd} to form mammospheres. Again, we found a large deficit in the Cx31 gene knock down compared to the two controls suggesting, that the absence of Cx31 decreases the capacity to form spheroids. The EDN1 peptide silencing shows only a models reduction of spheroid formation in comparison to 4T1-BM2 cells. With regard to IL1R2 the difference achieved in the ability to form spheroids is almost 30 percent lower than the 4T1-BM2 cells. This finding supports the report by Matsuo et al's (Ref. 22), proposing that the microenvironment might benefit from the angiogenic and proliferative

properties of IL1 secreted by pancreatic cancer cells. Consistent with this hypothesis, IL1R2 is overexpressed in 4T1-BM2 cells and findings of several studies regarding breast cancer cells in the past shown that IL-1 alpha enhances the invasiveness of tumor cells (Ref. 23-25), as well as IL-1 beta, including tumor-induced angiogenesis and immunosuppression (Ref. 26).

The obtained results suggest a possibly important role of Cx31 in brain metastasis, although EDN1, CTGF, as well as IL1R2 consistent with metastasis promotion, the next step would be to test their function in mice in vivo (i.e. to test the effect of gene silencing on brain metastasis formation). While this part of the work will be out of the scope of my Master thesis, it will be a necessary step toward the further understanding of the mechanisms of brain cancer metastasis. This will be essential, especially in order to confirm our findings from recent study reporting reduced cell proliferation, inhibited anchorage-independent growth and suppressed cell migration and invasion, in the non-small cell lung cancer (NSCLC) –derived cell line H1299 cells overexpressing Cx31.1 (Ref. 27).

In conclusion, though the *in vitro* functional characterization of proliferation, migration, survival in hypoxia and mammosphere formation, five different genes of interest selected for their potential clinical relevance for brain metastasis formation, we demonstrate that the Cx31 protein promotes cell proliferation, survival under hypoxia and stem cell self-renewal potential. These findings are consistent with a prometastatic role of Cx31. Our study also provides evidence for a pro-migratory, anti-apoptotic and stem cell self-renewal potential for the EDN1 peptide. CTGF seems also to promote migration, and IL1R2 to promote stem cell self-renewal potential.

Metastasis formation and lack of effective therapeutic options are the main causes of cancer patient's death, in contrast to localized cancers, which can be effectively treated by surgery. The Identification of genes mediating breast cancer metastasis in general (e.g. to bones, lung, liver) and to brain in particular is highly relevant to the understanding of the biology of breast cancer metastasis formation and might open novel therapeutic options to their prevention and treatment in patients (Ref. 28).

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