Organotypic brain cultures for metastasis research

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Running Head: Organotypic brain cultures for metastasis research

i. Summary/Abstract

Despite the emergence of brain metastasis as an unmet clinical need, our knowledge of the underlying biology is scarce and current treatments provide limited positive responses to the majority of patients, which have generally poor prognosis upon diagnosis. Modelling of brain metastasis has been based on brain metastatic cell lines that are injected in mice. This has remained as the only relevant pre-clinical platform for the validation of mechanistic findings obtained *in vitro*. We describe the use of organotypic brain cultures for brain metastasis research, which recapitulate the *in vivo* phenotype in an *ex vivo* procedure, and discuss their multiple applications for basic and clinical research, thus providing a useful tool for improving the current landscape of brain metastasis preclinical models.

ii. Key Words

Organotypic brain cultures, metastasis, therapy, tumor microenvironment, patient derived organotypic culture (PDOC).

1. Introduction: experimental models for brain metastasis research

Brain metastasis constitutes a major clinical problem due to its increasing incidence, poor prognosis and limited available therapies that improve survival of most patients [1]. Modelling of this advanced progression of cancer has been

challenging limiting major advances on the knowledge of the underlying biology. Human and mouse brain metastatic (BrM) derivatives have been previously described as valuable resources for brain metastasis research [2-6]. However, *in vitro* cell lines do not fully recapitulate the complex multi-step process of the disease. Thus, different experimental mouse models are broadly used for mimicking the human course of the disease, including human and mouse BrM cell lines from most common sources of brain metastasis (lung and breast cancer and melanoma) that are inoculated into immunosuppressed or immunocompetent hosts, respectively, to induce metastasis [2-6]; genetically engineered mouse models (GEMMs) that generate spontaneous brain metastases [7-9]; and patient-derived xenografts (PDXs) that maintain pathological features of the human brain metastases of origin [10-13]. The study of brain metastasis using these preclinical models has allowed to dissect mechanistically several molecular processes involved at different stages of the metastatic disease. For instance, experimental models have helped to dissect interactions of cancer cells with the brain microenvironment at early stages of brain colonization that are necessary for survival of metastasis initiating cells in this hostile secondary organ and their subsequent growth and expansion [4, 14] as well as the role of the pro-metastatic tumor-associated microenvironment (i.e. reactive astrocytes) for the maintenance of established brain metastasis [5, 15]. Although mice are the experimental model of choice for brain metastasis research, they are costly and thus they impose obvious economical limitations for extensive validation of preliminary results obtained *in vitro*.

Organotypic brain cultures *ex vivo* constitute a novel experimental platform for brain metastasis research since they faithfully recapitulate phenotypically and

functionally metastatic cells *in vivo* [4, 14] and allow rapid evaluation of multiple experimental conditions at the same time at affordable economical costs. We describe here the use of organotypic brain cultures for brain metastasis research and discuss specific applications for interrogating different steps within the last part of the metastatic cascade corresponding to organ colonization.

2. Materials

2.1. Cell lines

Human and mouse BrM cell lines from melanoma, lung and breast adenocarcinoma have been previously described [2-5]. To establish a new BrM cell line a 100 µl cell suspension containing 10^5 cells of the respective parental cell line is injected intracardiacally in anesthetized 4-6-week-old athymic nu/nu (Envigo) or C57BL/6 mice (or the specific strain corresponding to the cancer cells), for human and mouse cell lines respectively. Parental cell lines are engineered with lentiviral constructs expressing firefly luciferase and the fluorescent protein GFP, which allow non-invasive *in vivo* imaging in mice and cancer cell tracking in histological sections, respectively. Tumor development is monitored by weekly bioluminescence imaging (BLI) using an IVIS Xenogen machine (Caliper Life Sciences). Brain lesions are localized by *ex vivo* BLI and resected under sterile conditions. Tissue is minced and placed in culture medium containing a 1:1 mixture of DMEM/Ham's F12 supplemented with 0.125% collagenase III and 0.1% hyaluronidase. Samples are incubated at 37°C for 1h, with gentle rocking. After collagenase treatment, cells are briefly centrifuged, resuspended in 0.25% trypsin and incubated at 37°C for 15 min. Cells are resuspended in culture media and allowed to grow to confluence on a 10-cm dish. Additional rounds of *in vivo*

selection, usually three, are performed until BrM derivatives are obtained, which are characterized by their superior ability to colonize the brain.

2.2. Vibratome

A vibratome (Leica VT1000 S) is used for slicing dissected brains with or without brain metastases (Fig1A-B) (see below) into 250 μm brain slices at speed = 2 and frequency = 5 (Fig2A).

2.3. Brains

Tumor-free brains from C57BL/6 or nude mice of 4-10 weeks of age are used for preparing organotypic brain cultures that mimic early stages of brain colonization or for toxicity assessment of inhibitors on different brain cell types in the presence or absence of metastatic cells.

Metastases-bearing brains obtained at the endpoint of the disease (5-7 weeks after intracardiac inoculation of human BrM cell lines or 2 weeks in syngeneic mouse BrM cell lines) are used for preparing organotypic brain cultures that interrogate established macrometastases as well as their associated reactive brain microenvironment.

Metastases-bearing brains could be also obtained from mice in which certain components of the microenvironment have been genetically engineered (Fig1C). Thus, the behavior of inoculated syngeneic mouse BrM cell lines could be studied in the context of a modified brain microenvironment.

2.4. Media

Brains are dissected on ice cold Hank's balanced salt solution (HBSS) supplemented with HEPES (pH 7.4, 2.5 mM), D-glucose (30 mM), $CaCl_2$ (1 mM), $MgCl_2$ (1 mM) and $NaHCO_3$ (4 mM). Brains/brain slices are maintained on ice cold supplemented HBSS during slicing at the vibratome and before culturing (Fig2A).

Brain slices are cultured in 12 or 24-well plates with 1 ml of slice culture media (DMEM, supplemented HBSS, fetal bovine serum 5%, L-glutamine [1 mM], 100 IU/ml penicillin and 100 mg/ml streptomycin) per well at 37ºC, 5% CO_2 (Fig2C).

2.5. Membranes

0.8 µm Whatman® Nuclepore™ Track-Etched Membranes (Sigma Aldrich) are used for culturing brain slices (Fig2B-C).

2.6. IVIS

An IVIS Xenogen machine (Caliper Life Sciences) is used for bioluminescence imaging (BLI) of metastases-bearing mice, brains and brain slices (Fig2B).

2.7. Immunofluorescence

BrdU (Sigma-Aldrich, ref. B9285) pulses consist on adding 20 µl/ml to the media. Primary antibodies for immunofluorescence: GFP (Aves Labs, ref. GFP-1020, 1:1,000), GFAP (Millipore, ref. MAB360, 1:1000), Iba1 (Wako, ref. 019-19741, 1:500), NeuN (Millipore, ref. MAB377, 1:500), cleaved caspase-3 (1:500, ref. 9661; Cell Signaling), BrdU (Abcam, ref.ab6326, 1:500), Collagen IV (Millipore, ref. AB756P, 1:500), Olig2 (Millipore, ref. AB9610, 1:500). Secondary antibodies:

Alexa-Fluor anti-chicken 488, anti-rabbit 555, anti-mouse 555, anti-rat 555, anti-rabbit 633, anti-mouse 647 (Invitrogen, dilution 1:300) (Fig2D-G).

3. Methods

3.1. General consideration of organotypic cultures

-Short term experiments

Organotypic brain cultures are short term assays that can be maintained up to 7 days with intact cellular architecture and functionality of different resident cell types present in the brain tissue as well as metastatic cells able to colonize the brain (Fig2D-G). Common assays performed for interrogating functional and mechanistic insights of brain metastasis using organotypic brain cultures include evaluation of the efficacy of monotherapies or combined therapies (targeted or immunotherapy, including blocking antibodies), cellular response to radiotherapy, physical interactions between cancer cells and the brain microenvironment (i.e. vascular co-option) [4] and the contribution of the tumor-associated microenvironment to the metastatic disease [5, 15] (Fig1A-C).

-Imaging with IVIS

Anesthetized mice (isofluorane) are injected retro-orbitally with D-luciferin (150 mg/kg) and imaged with an IVIS Xenogen machine for monitoring tumor development. Bioluminescence analysis is performed using Living Image software, version 4.5. Metastatic lesions from brains obtained from these animals at the endpoint of the disease are localized by *ex vivo* BLI. For brain slices imaging, D-luciferin (300 µg/ml) is added to supplemented HBSS or slice culture media.

-Immunofluorescence

Free-floating immunofluorescence of brain slices at the endpoint of the culture assays is performed for histological analysis (Fig2C6). Cellular death and proliferation of cancer cells are evaluated by staining against cleaved-caspase 3 and bromodeoxyuridine (BrdU) markers, respectively. Brain tissue architecture is visualized using respective markers for different cell types from the brain as described in the methodology (Fig2D-G).

-RNA extraction

Whole RNA isolation of brain slices using RNAeasy Mini Kit (Qiagen) is performed for qRT-PCR analysis at the endpoint of the culture assays. Brain slices are mechanically lysed in RLT buffer with β-mercaptoethanol (1:100) and centrifuged at full speed for 3 min in Qiashredder columns (Qiagen). Homogenates are processed following manufacturer's indications for whole RNA isolation. 3 brain slices in 500 µl of RLT buffer yield between 300-400 ng of RNA. Human versus mouse cells could be differentiated using specific primers.

-Western blotting

Protein lysates of brain slices are obtained by mechanical homogenization of brain tissue in commercial RIPA buffer 1x (Cell Signaling) + protease inhibitors (PMSF 1 mM, NaF 1 mM and Na_3VO_4 1 mM). Samples are maintained on ice for 30 min, vortexing every 10 min. Samples are centrifuged at 14.000g at 4ºC for 10 min and supernatants are collected for protein quantification. 3 brain slices in 500 µl of lysis buffer yield between 500-1000 µg of protein.

3.2. Plating cancer cells for mimicking early stages of colonization

Brain colonization by metastatic cells *in vivo* occurs after extravasation of circulating cancer cells through the blood-brain barrier (BBB) and their subsequent close interaction with several cell types present in the brain, mainly endothelial cells (i.e. vascular co-option) and reactive astrocytes (i.e. suppression of Fas-mediated cancer cell killing), which allow survival of infiltrated cancer cells in the hostile brain microenvironment followed by metastatic expansion [4]. Organotypic brain cultures *ex vivo* have been shown to recapitulate phenotypically and functionally these early events of brain colonization by metastatic cells [4] (Fig1A, Fig2D). Furthermore, translating ribosome affinity purification and mRNA sequencing (TRAP-seq) of the same brain metastatic cell line cultured under *in vitro* conditions and on *ex vivo* organotypic brain cultures shows differential enrichment of certain transcriptomic signatures in the *ex vivo* condition that have been further validated *in vivo* [14]. Thus, *ex vivo* mimicking of processes that occur at early stages of brain colonization by using organotypic brain cultures provides a fast, reliable and novel approach for studying brain metastasis initiating events.

Organotypic brain cultures can be used for evaluating the impact of different therapies on cancer cell growth by adapting previously reported methods [16]. For this purpose, tumor-free brains from C57BL/6 mice of 4-10 weeks of age are dissected in supplemented HBSS and embedded in 4% low-melting agarose (Lonza) preheated at 42°C. The embedded brains are cut into 250 µm slices using a vibratome. Slices are divided at the hemisphere into two symmetrical pieces (Fig2A). Brain slices are placed with flat spatulas on top of 0.8 µm pore membranes floating on slice culture media in the presence of inhibitors or vehicle. Brain slices are incubated at 37°C and 5% CO_2 for 1-2h, and then 3×10^4 cancer

cells suspended in 2 µl of slice culture media are placed on the surface of the slice with a micropipette and incubated for 16-24h (Fig2C4), during this time cells will infiltrate the brain tissue and interact with the capillaries in a process named vascular co-option [4] (Fig2D). At this moment, brain slices are imaged (Day 0) to confirm the presence of cancer cells using BLI with an IVIS Xenogen machine (Fig2B). Brain slices are incubated for up to 7 days with intact cellular architecture and reimaged by BLI (Fig2B). Cancer cell growth as measured by BLI is obtained by normalization of BLI at the end of the experiment over Day 0. For cultures over 3 days, slice culture media is replaced every 3 days with fresh inhibitors.

Organotypic brain cultures can be also utilized for functional studies by using cancer cells that carry genetic silencing of the gene of interest (i.e. shRNA, siRNA, CRISPR/Cas9 induced *knockout*, etc) [4, 5]. Both constitutive and conditional lentiviral vectors can be used. Doxycycline (1 µg/ml) is added to the slice culture media and replaced every 48h for induction of conditional vectors. Successful induction of *knockdown* or *knockout* in the cancer cells at the end of the experiment can be confirmed by qRT-PCR or western blotting of brain slices.

At the end of the experiment, brain slices are fixed in paraformaldehyde (4%) overnight at 4ºC and free-floating immunofluorescence is performed for histological analysis and for evaluation of cellular death and/or proliferation (Fig2C6). Brain slices are blocked in 10% NGS, 2% BSA and 0.25% Triton X-100 in PBS for 2h at room temperature (RT). Primary antibodies are incubated overnight at 4°C in the blocking solution and the following day for 30 min at RT. After extensive washing in PBS-Triton 0.25%, secondary antibodies are added in the blocking solution and incubated for 2h at RT. After extensive washing in PBS-Triton 0.25%, nuclei are stained with bis-benzamide (1 mg/ml; Sigma) for 7 min

at RT. To confirm cancer cell growth by histology as a complementary analysis to BLI, anti-GFP primary antibody is used and total number of GFP+ cells is quantified. Cancer cell death is evaluated by staining with anti-cleaved caspase 3.

Proliferation rate of cancer cells is measured by adding a BrdU pulse of 2-4h before fixation of brain slices followed by immunofluorescence staining against BrdU (proliferation rate = BrdU+/GFP+ cells [%]). Permeabilization of brain slices is performed by incubation with HCl 2N 30 min at 37ºC followed by 0.1M borate buffer (pH 8.5) 10 min at RT. After extensive washing with TBS, brain slices are blocked in 3% NGS in TBS-Triton 0.25% for 1h and primary antibody anti-BrdU is incubated for 72h at 4ºC. After extensive washing with TBS-Triton 0.25%, the secondary antibody is incubated in blocking solution for 2h at RT followed by extensive washing with TBS. Brain slices are mounted with Mowiol-Dabco anti-fade reagent (Fig2D-G).

Organotypic brain cultures also allow simultaneous evaluation of potential toxic effects of the compounds tested on tumor-associated and non-associated (wild type) brain components. This simple and preliminary score based on the analysis of specific cellular markers that identify various brain cell types (i.e. endothelial cells (Collagen IV), reactive astrocytes (GFAP), neurons (NeuN), microglia (Iba1) and oligodendrocytes (Olig2)) could be considered as a fast way to discard compounds with manifest effects on non-tumor cells (Fig2F). Eventually, if cellular components of the tumor-associated microenvironment have pro-metastatic behaviors, targeting them might be of interest [5] (see below).

Immunofluorescence images of brain slices are acquired with a Leica SP5 up-right confocal microscope x10, x20, x40 and x63 objectives and analyzed with ImageJ software (Fig2D-G).

3.3. Organotypic cultures with established metastasis

Organotypic brain cultures can also be prepared from brains obtained at the endpoint of the metastatic disease (5-7 weeks after intracardiac inoculation of human BrM cell lines or 2 weeks in syngeneic mouse BrM cell lines) (Fig1B). This kind of preparation allows evaluation of advanced stages of brain metastasis by obtaining brain slices that contain macrometastases that were previously established *in vivo* (Fig2E). This allows interrogating pharmacologically and biologically clinically relevant stages of brain metastasis.

Additionally, this approach allows evaluation of tumor-associated stromal cells that are only present when established metastases are present (i.e. pSTAT3+ reactive astrocytes) [5]. This tumor-associated microenvironment also includes non-brain resident recruited immune cells that contribute to the biology of the tumor-stroma entity (Fig1C). Consequently, organotypic brain cultures from established macrometastases allow simultaneous evaluation and targeting of the cancer cells and the pro-metastatic brain microenvironment as well as the interactions between them.

The methodology is similar to the one described on 3.2. with brains being dissected in supplemented HBSS and imaged by BLI for localizing the metastases. Brains are cut at the vibratome following the procedure previously described and then imaged by BLI (Day 0) for selection of luciferase+ brain slices, which are cultured in the presence of inhibitors or vehicle. Slices are incubated

up to 7 days replacing the media with fresh inhibitors every 3 days. BLI is performed at the endpoint of the experiment and brain slices are processed for histological analysis as previously described.

3.4. Use of inhibitors

The use of organotypic brain cultures for evaluating the efficacy of potential therapeutic agents for brain metastasis has been previously reported [4, 5]. We reported that silibinin, an inhibitor of activation of the transcription factor STAT3, efficiently reduces cancer cell growth in organotypic brain cultures with established macrometastases previously grown *in vivo* [5]. These findings were then validated *in vivo*, proving organotypic brain cultures as a reliable tool for evaluating efficacy of potential therapeutic agents and generating relevant preliminary results that can be later validated *in vivo*. This approach is not only restricted to the use of chemical compounds but can be also extended to other therapeutic agents such as blocking antibodies [4, 5]. In this regard, anti-CD8 blocking antibody in organotypic brain cultures has been used for the mechanistic dissection of the immunosuppressive and pro-metastatic role of pSTAT3+ reactive astrocytes in brain metastasis [5]. Another example would be the use of anti-FasL blocking antibody in brain slices for obtaining mechanistic insights of the role of neuroserpin in early stages of brain colonization by disseminated cancer cells [4]. Altogether, these evidences support the use of organotypic brain cultures as a valid approach for both drug efficacy assays and functional studies, providing a potent complementary approach to those methodologies already available for drug discovery and basic research.

3.5. Organotypic cultures to study the tumor associated microenvironment

Among many advantages of using organotypic brain cultures for brain metastasis research, the possibility of evaluating the interaction of the tumor-associated brain microenvironment with the metastatic cells represents the major difference compared to *in vitro* experimental models.

Organotypic brain cultures outperform *in vitro* models when used for drug efficacy assessment. The *ex vivo* approach permits simultaneous evaluation of the contribution of each entity (cancer cells or microenvironment) to the pharmacological phenotype of interest, allowing at the same time mechanistic dissection of the observed phenotype. This can be scored by combining genetic silencing of the targeted gene together with the effect of the inhibitor. In this regard, the study of Priego and colleagues has nicely shown that organotypic brain cultures allow evaluation of drugs that target the pro-metastatic microenvironment in brain metastasis, specifically pSTAT3+ reactive astrocytes, instead of only cancer cells [5]. For this purpose, a GEMM with a tamoxifen-inducible *knockout* of *Stat3* in reactive astrocytes and cancer cells that incorporate shRNA against the *STAT3* gene were used. Comparing silencing of the gene in cancer cells or the astrocytes versus the effect of the inhibitor (which targets both cancer cells and the microenvironment), authors were able to define a novel pro-metastatic role for pSTAT3+ reactive astrocytes in brain metastasis [5].

Increasing the knowledge about the mechanisms that metastatic cells use for interacting with and thus adapting to the brain microenvironment allows designing novel therapeutic options for patients with brain metastasis. In this regard, organotypic brain cultures can be also used to study physical interactions between metastatic cells and different components of the brain [4, 14, 17]. For

this purpose, brain slices are cultured either with tumor plugs [17] or seeding cancer cells on top of them [4, 14] in order to study the brain parenchyma-metastasis interface. In this sense, organotypic brain cultures have not only provided a useful tool to visualize physical interactions between brain metastatic cells and endothelial cells that occur at early stages of brain colonization (Fig2D) by cancer cells, a process named vascular co-option, but also allowed functional validation of key molecules required for this process, such as the axon pathfinding molecule L1CAM [4, 14]. Since studying early stages of brain metastasis at the single cell level is complex and time-consuming, the fact that organotypic brain cultures recapitulate faithfully both at the phenotypic and at the transcriptomic level these processes [14] strongly supports this *ex vivo* approach as a valid and reliable tool for basic research on brain metastasis able to provide results that could help to better design a subsequently necessary *in vivo* validation using mouse models.

3.6. Use of Patient Derived Organotypic Cultures (PDOC)

The use of organotypic cultures is not limited to research with experimental models, but they can be also utilized for performing patient-derived organotypic cultures (PDOCs) using surgically-resected fresh human samples from a variety of cancers such as breast, lung, prostate, colon, head and neck, gastric and esophageal [18-25] (Fig1D). PDOCs derived from brain tumors have previously been reported for exploring susceptibilities of glioblastoma tumors to different therapies [26]. PDOCs allow rapid drug efficacy assessment of multiple therapies directly on the human sample, which maintains intact its pathological features and intra-tumor heterogeneity. Real-time results obtained from these assays

might be used for clinical advice, which will validate PDOCs as valuable therapeutic tool for cancer precision medicine [27].

4. Conclusions

Experimental models extensively used for brain metastasis research include *in vitro* and *in vivo* models. However, *in vitro* models do not recapitulate entirely the complexity of the metastatic disease and *in vivo* models present economical limitations for medium and high-throughput based applications. We describe here the use of organotypic brain cultures *ex vivo* for brain metastasis research, as they overcome main limitations imposed by the two previous models. Applications of this approach include drug-screening, mechanistic dissection of different stages of brain colonization by metastatic cells, evaluation of different components of the tumor-associated brain microenvironment and patient-derived organotypic cultures for precision medicine. *Ex vivo* cultures allow studying relevant molecular mediators and signaling pathways by both pharmacological and genetic approaches, and results have been reported to be validated *in vivo* and in human samples.

However, organotypic brain cultures do present limitations as an experimental model, since they do not consider steps of the metastatic disease that occur before extravasation of cancer cells from the vessels. In this regard, they do not allow interrogating the ability of metastatic cells to intravasate into capillaries from the primary tumor site, to survive in circulation and more importantly, to cross the blood-brain barrier (BBB). In addition, these cultures cannot be maintained over long periods of time.

In conclusion, organotypic brain cultures constitute a useful, convenient and advantageous experimental model for brain metastasis research and provide a rapid, easy and cost-effective tool for basic and clinical applications.

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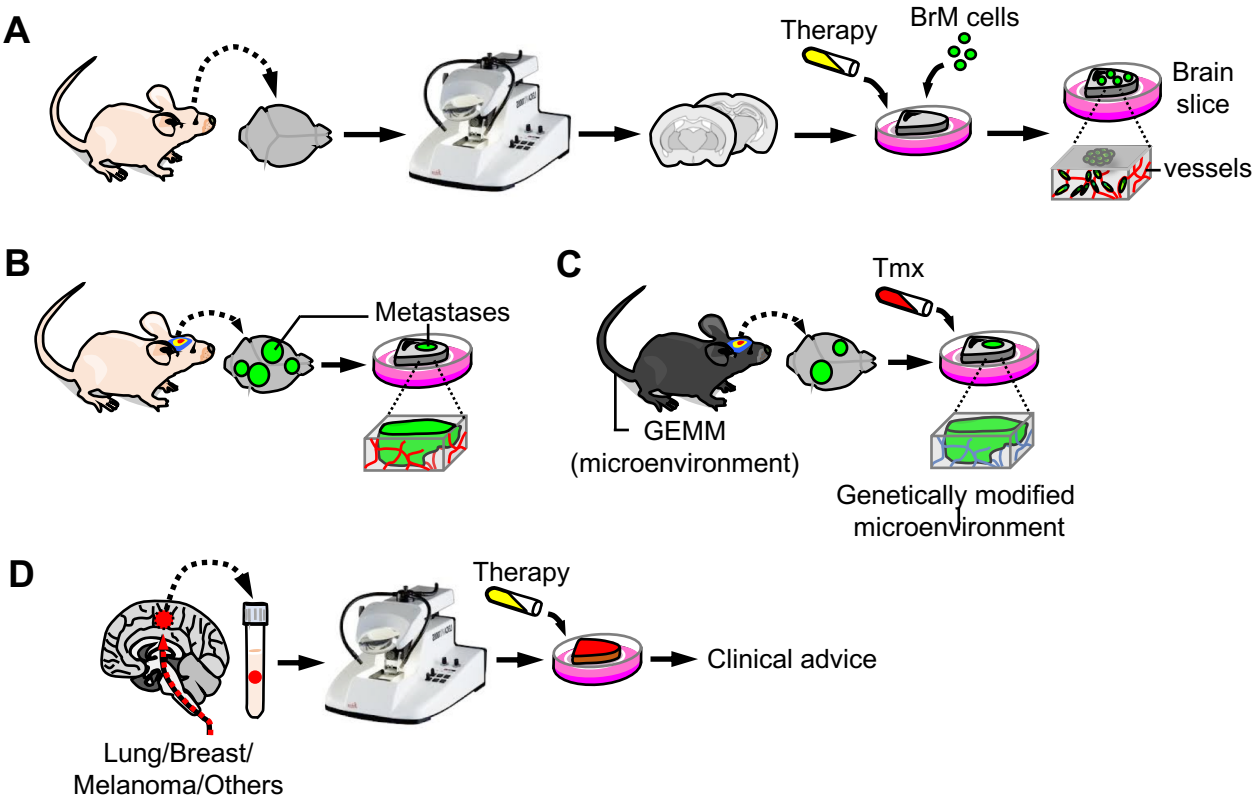
Figure legends

Figure 1. Organotypic cultures for brain metastasis research. (A) Schema representing organotypic brain cultures mimicking early stages of brain colonization. (B) Organotypic brain cultures from established brain metastases. (C) Organotypic brain cultures using genetically engineered mouse models (GEMM) to interrogate brain metastasis in the context of genetic modifications induced in specific components of the microenvironment in a conditional manner (i.e. Tamoxifen will activate Cre recombinase and delete floxed gene). (D) Patient derived organotypic culture (PDOC) as a personalized-medicine therapeutic strategy.

Figure 2. Preparation and processing of organotypic brain cultures. (A) Brain sectioning at the vibratome and collection of sectioned brain slices in 24-well plates with supplemented HBSS. (B) Bioluminescence imaging of brain slices with an IVIS Xenogen machine. This would be performed at this moment if brains have established metastasis or later, if cancer cells are plated on top (C4). (C) Preparation of organotypic brain cultures: 1. Placing of membranes; 2. Selection of brain slices; 3. Plating brain slices on membranes; 4. Seeding cancer cells on brain slices; 5. Organotypic brain cultures ready for culturing; 6. Immunofluorescence staining of fixed organotypic brain cultures. (D) Organotypic brain culture mimicking early stages of brain metastasis showing cancer cells (GFP) co-opting the vessels (Col.IV). Scale bar 50µm. (E) Organotypic brain culture with established brain metastasis showing proliferating (BrdU) cancer cells (GFP). Scale bar 75µm. (F) Capillaries (Col.IV) and neurons (NeuN) in an organotypic brain culture without cancer cells. Nuclei are stained with bis-benzamide (BB). Scale bar 20µm. (G) Patient derived organotypic culture (PDOC) with proliferating cancer cells (BrdU). Blue correspond to autofluorescence. Scale bar 100µm.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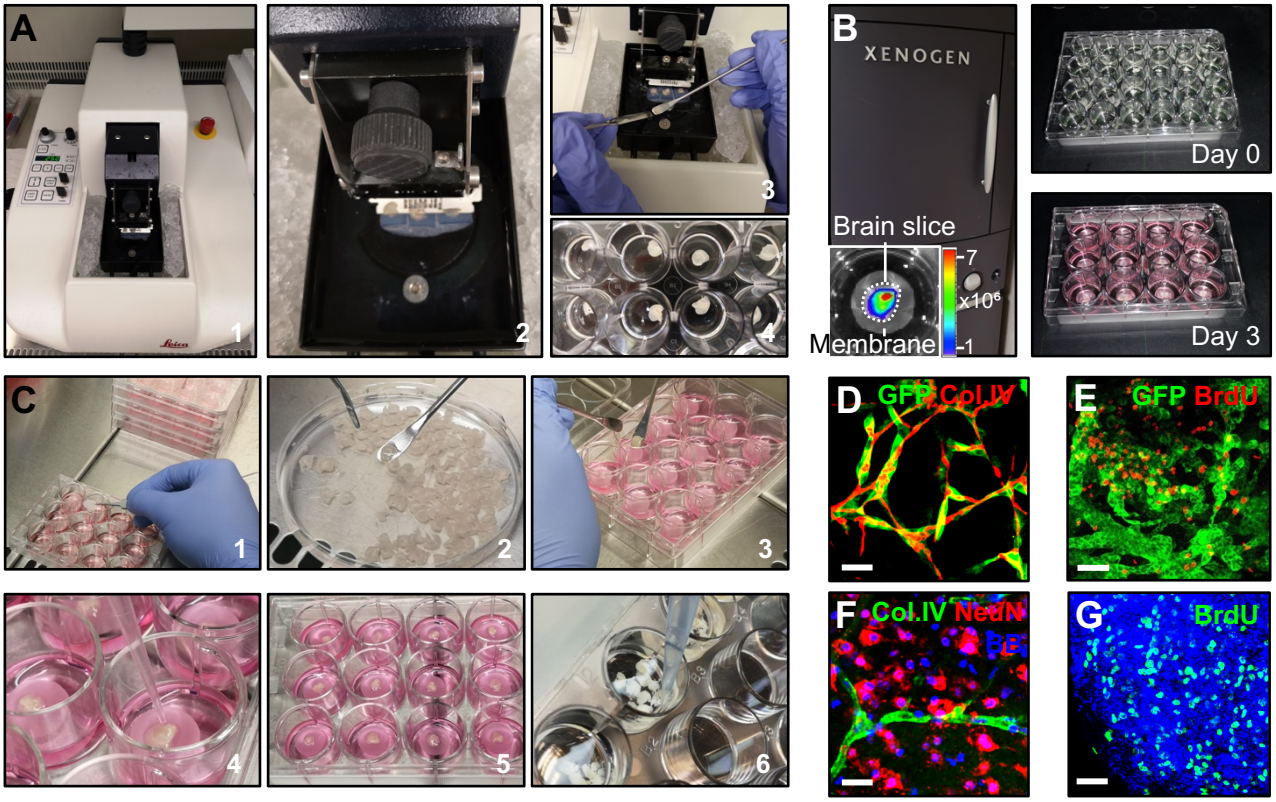
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