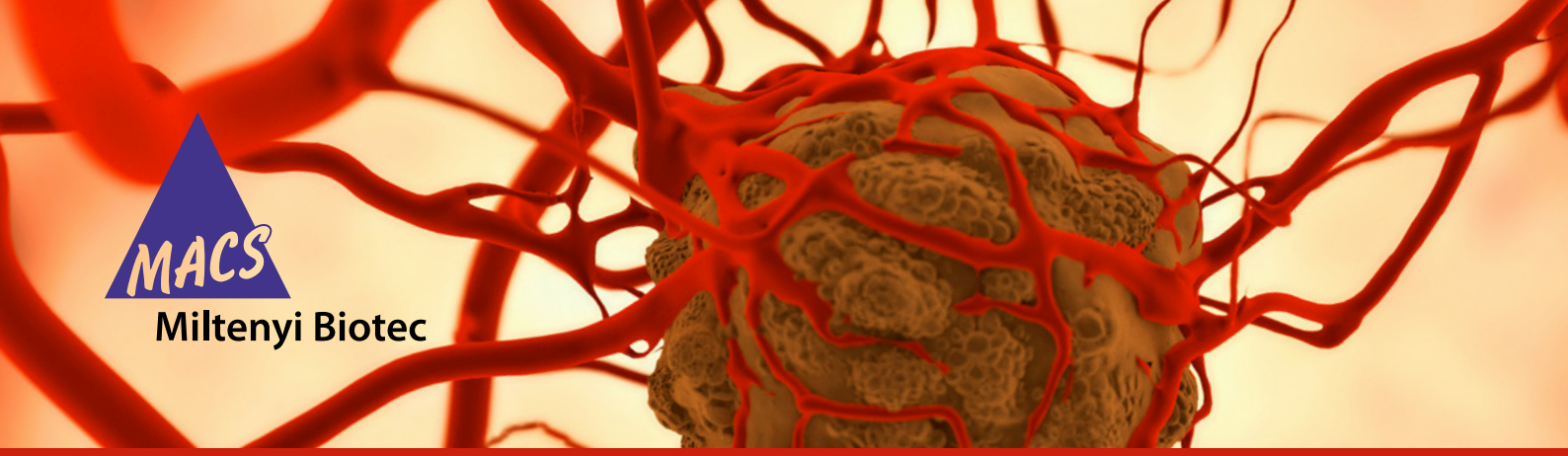

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Dissociation and isolation

Mouse cell depletion

Mouse cell depletion from patient-derived xenograft brain tumors and isolation of glial cancer stem cells

Jeremy Rich Lab, Cleveland Clinic, USA
Tyler E. Miller, Stephen C. Mack, and Jeremy N. Rich

Background

Glioblastomas are the most prevalent and lethal primary brain tumors and are comprised of cellular hierarchies with self-renewing cancer stem cells (CSCs) at the apex[1]. Like neural stem cells, CSCs reside in functional niches that provide essential cues to maintain the cellular hierarchy[2,3]. For this reason, it is essential when studying these cells that they are studied *in vivo* in a functional microenvironment. Access to these cells requires a reliable dissociation methodology. Traditional papain dissociation methods cleave the CD133 epitope that marks glial CSCs and therefore require post-dissociation incubation time in culture before isolation. This makes it difficult to capture the molecular profile of these CSCs in their microenvironment. Using the gentleMACS™ Octo Dissociator with Heaters in combination with the Tumor Dissociation Kit, human allows acute dissociation of primary brain tissue as well as primary brain tumor xenografts, immediately followed by sorting for CSC markers, such as CD133.

To maintain the tumor microenvironment, it is highly desirable to inject unlabeled tumor cells directly from the patient into a mouse intracranially to establish an orthotopic primograft. Historically, the field has been restricted to establishing primary xenografts subcutaneously in the flank of a mouse as it was technically restrictive to retrieve non-manipulated human cells – that did not express a marker such as GFP – out of the mouse brain due to the contaminating mouse cells and debris. Even if the human cells were fluorescently labeled, it would take several hours of flow cytometric analysis to obtain a sufficient amount of cells for experimentation. Using the Mouse Cell Depletion Kit allows to get a much larger pure population of human cells from the brain of a mouse, after dissociation, in approximately 30 minutes and at a fraction of the cost. $CD133^+$ and $CD133^-$ populations can then be separated for subsequent molecular analysis.

This note describes the dissociation of PDX brain tumors using the gentleMACS Octo Dissociator with Heaters and the Tumor Dissociation Kit, human. After dissociation, all mouse cells were depleted from the samples using the Mouse Cell Depletion Kit. Subsequently, glial cancer stem cells were isolated using the CD133 MicroBead Kit – Tumor Tissue, human.

Materials and methods

Materials

- gentleMACS Octo Dissociator with Heater
- Tumor Dissociation Kit, human
- MACS® SmartStrainers (70 µm)
- MACS Neuro Medium
- MACS NeuroBrew-21

Additional requirements for separation

- Mouse Cell Depletion Kit
- CD133 MicroBead Kit – Tumor Tissue, human

For a detailed protocol, please refer to the respective data sheet.

Methods

PDX engraftment

Glioblastoma tissues were obtained from excess surgical material from patients at the Cleveland Clinic after review from a neuropathologist in accordance with an approved protocol by the Institutional Review Board.

1. Dissociate the samples using the Tumor Dissociation Kit in combination with the gentleMACS Octo Dissociator with Heaters according to the protocol. Use the same setting as for PDX tumors.
2. Filter the single-cell suspension through a MACS SmartStrainer (70 µm).
3. Wash cells and perform a red blood cell lysis.
4. Wash cells again and inject in NSG mice intracranially.

   Note: To establish the xenograft, inject 50,000 cells intracranially or 2,000,000 subcutaneously.

5. (Optional) For initial experiments, e.g., to test the efficacy of the Mouse Cell Depletion Kit, utilize bulk tumor cells that are genetically modified to express GFP. Use unaltered bulk tumor cells to establish the PDX model.

Mouse cell depletion

1. After the PDX tumor is developed, harvest the brain of the mouse and macroscopically dissect the tumor, removing as much normal tissue as possible while leaving the tumor intact. Dissociate the tumor as well as the adjacent and infiltrating normal brain tissue by using the Tumor Dissociation Kit, human in combination with the gentleMACS Octo with Heaters applied with the following settings:
   A. Temp. ON
   B. Spin –25 RPM 5'0"
   C. Spin 25 RPM 5´0"
   D. Ramp 500 RPM 15"
   E. Loop × 15
   F. Spin 25 RPM 1'0"
   G. Spin –750 RPM 3"
   H. Spin –25 RPM 1'0"
   I. Spin 750 RPM 3"
   J. End Loop
   K. End
2. Filter the resulting single-cell suspension through a MACS SmartStrainer (70 µm) and wash thoroughly with MACS Neuro Medium.
3. Wash cells and perform a red blood cell lysis.
4. Spin cells at 750 rpm for 5 minutes. Aspirate supernatant to remove myelin and debris. Remaining myelin and debris will be removed with the Mouse Cell Depletion Kit.
5. Label mouse cells magnetically with the Mouse Cell Depletion Kit and separate according to the protocol. In general, 100 µL of the Mouse Cell Depletion Kit can be used for one mouse brain and the labeled cells can be passed over two LS Columns.

Isolation of glioblastoma stem cells

1. Label the human tumor cell population magnetically with the CD133 MicroBead Kit – Tumor Tissue, human and isolate according to the protocol.
2. Immediately harvest a fraction of the CD133$^+$ and CD133$^-$ cells for RNA next-generation sequencing, while culturing the remaining cells either in MACS Neuro Medium supplemented with MACS NeuroBrew-21, or re-inject into NSG mice to maintain the PDX model.

Results

Both intracranial and subcutaneous models were engrafted and grew up within two months after injection. To test the efficacy of the Mouse Cell Depletion Kit, GFP-labeled primary patient CSCs were utilized and injected into mice intracranially. Once mice showed neurological signs, tumors were removed and dissociated as described above. For the initial experiments with GFP-labeled cells, flow cytometry analysis showed that dissociated tumors contained much debris and only 8.75% of all events were gated as cells (figure 1A). Of events gated as cells, 25.4% were identified as infiltrating mouse cells (figure 1B). Dissociated tumors were treated with the Mouse Cell Depletion Kit and analyzed for purity by flow cytometry. Much of the debris was removed and 98.0% of all events fell into the area gated as cells (figure 1C) and human tumor cells were purified to 98.0% (figure 1D).

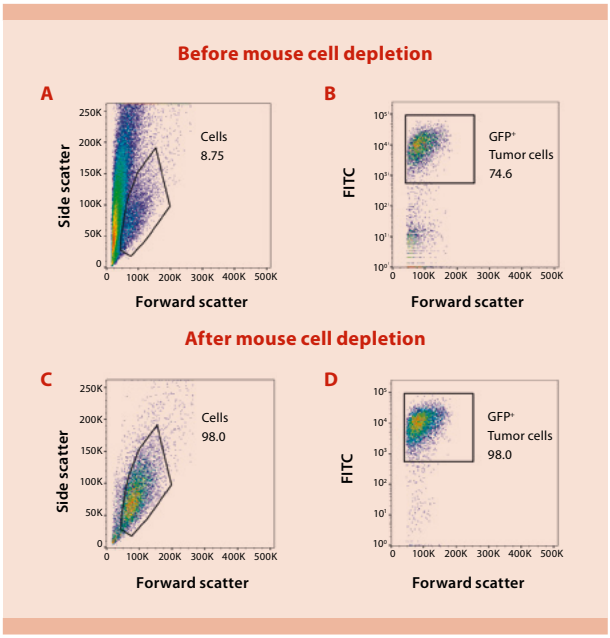

Figure 1: Flow analysis of dissociated PDX brain tumor samples before and after mouse cell depletion. (A) Before mouse cell depletion 9% of all events were gated as cells. (B) Of the events gated as cells, 25% were infiltrating mouse cells. (C) After mouse cell depletion, 98% of events were cells. (D) Human tumor cell were purified to 98%.

For the primograft intracranial tumors, 10 million live cells (at 76% viability) along with much debris were recovered, including mouse cells, post-dissociation for downstream processing. After using the Mouse Cell Depletion Kit, 2.7 million live cells were recovered at 81% viability. Cells were subsequently separated into CD133$^+$ and CD133$^-$ populations by using the CD133 MicroBead Kit, human. 235,000 CD133$^+$ cells, along with 2.2 million CD133$^-$ cells were obtained representing approximately 10% of total cells. RNA was isolated and RIN values greater than 9 were obtained. RNA was sequenced and over 60 million high-quality aligned reads were obtained for each population. CD133$^+$ cells were sequenced to a greater depth, obtaining greater than 120 million reads (table 1). CD133$^+$ cells formed spheres in culture which maintained the ability to be passaged sequentially (figure 2).

	Number of cells	Number of high quality aligned reads
Post dissociation	1.0×10^7	–
Post mouse cell depletion	2.7×10^6	–
CD133$^+$	2.4×10^5	1.2×10^8
CD133$^-$	2.2×10^6	6.0×10^7

Table 1: Cell numbers of dissociated PDX brain tumor samples prior and after mouse cell removal and CD133$^+$ and CD133$^-$ cells after CD133 cell isolation were determined. RNAs of CD133$^+$ and CD133$^-$ cells were applied for sequence analysis. The numbers of the resulting aligned high-quality reads are displayed.

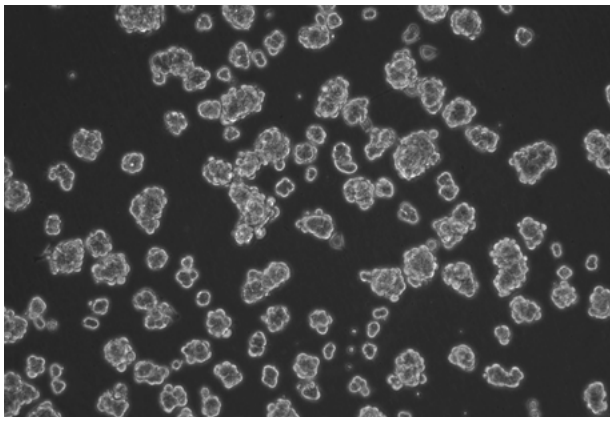

Figure 2: Cell cultures of CD133$^+$ cells isolated from PDX brain tumor samples after prior removal of mouse cells. Cells shown were imaged after 3 passages.

Conclusion

The presented data show that dissociation with the Tumor Dissociation Kit, human in combination with the gentleMACS Octo Dissociator with Heaters is suitable for obtaining viable single-cell suspensions from both primary GBM and PDX tissue. The data additionally show that the Mouse Cell Depletion Kit effectively removes post-dissociation debris and depletes infiltrating mouse cells from human GBM cells in PDX models. Furthermore, the CD133 MicroBead Kit – Tumor Tissue, human allows for subsequent separation of CD133$^-$ and CD133$^+$ CSCs for downstream experimentation.

References

1. Galli, R. *et al.* (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res. 64: 7011–7021.
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Visit **www.miltenyibiotec.com/mcd** for more information on the Mouse Cell Depletion Kit.

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Miltenyi Biotec GmbH | Friedrich-Ebert-Straße 68 | 51429 Bergisch Gladbach | Germany | Phone +49 2204 8306-0 | Fax +49 2204 85197
macs@miltenyibiotec.de | **www.miltenyibiotec.com**

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