Novel methods for the isolation of tumor cells from human, mouse, and xenografted tumors

David Agorku, Anne Langhammer, Lena Willnow, Stefan Tomiuk, Jutta Kollet, Silvia Rüberg, Andreas Bosio, and Olaf Hardt
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Solid tumors are vascularized and infiltrated by stromal cells such as leukocytes, endothelial cells, and fibroblasts[1]. The amount and composition of those non-tumor cells depends on various factors including tumor entity and stage, treatment history, status of the host organism and site of tumor growth. The widely unpredictable and variable amount of non-tumor cells makes analyses of tumor samples difficult. Contaminating cells lead to hybridization of non-tumor cell–derived mRNA molecules to probes on microarrays, and a significant reduction of sensitivity caused by measurement of irrelevant signals during next-generation sequencing or proteome analysis can be expected. In addition, the culture of tumor cells is frequently hampered by fibroblasts overgrowing the target cells, which biases assays such as drug sensitivity tests.
To overcome these limitations, we have developed fast and easy methods to isolate 'untouched' tumor cells from tissue samples. The underlying procedure is based on the comprehensive depletion of cells of non-tumor origin by combining automated tissue dissociation and automated magnetic cell sorting. A negative selection strategy enables the isolation of the tumor cell population without specific knowledge of surface marker expression on these cells. Even from samples initially containing low numbers of tumor cells (<20%), the target cells could be isolated to purities of higher than 95% in less than 20 minutes. Here, we have applied these methods to isolate tumor cells from primary human breast carcinoma, three different syngeneic mouse tumor models, and three different patient-derived xenograft models. Bulk tumor and isolated tumor cells were cultivated for up to seven days. Additionally, we performed whole exome sequencing (WES) of bulk human tumor xenografts from lung, bladder, and kidney cancer, and compared the results to samples depleted of mouse cells.

Results

1 Rapid isolation of untouched tumor cells

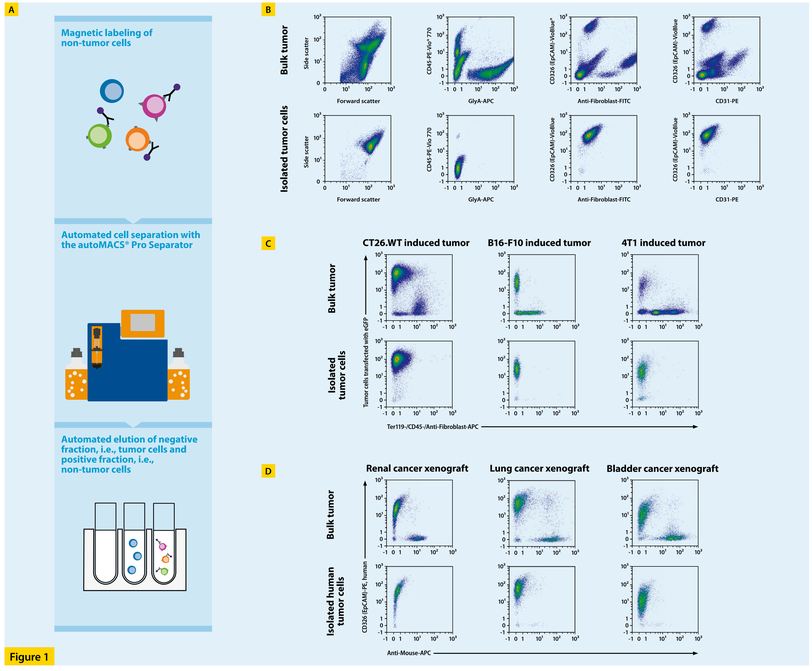


Figure 1

We have performed screenings on primary tumor material, cell lines, and healthy tissues to define combinations of antibodies recognizing all cells of the tumor microenvironment but not the tumor cells. Conjugates of these antibodies with superparamagnetic nanoparticles were used to develop optimized procedures for the depletion of non-tumor cells from mouse, human, and xenotransplanted tumor samples by magnetic separation (fig. 1A). The procedure allows for the elimination of >95% of the contaminating cells in less than 20 min, as shown for the isolation of tumor cells from a primary human tumor sample (fig. 1B). To evaluate the depletion efficiency by flow cytometry, cell fractions were labeled with human lineage markers (CD31, CD45, Gly-A, and anti-Fibroblast) and an antibody against human CD326 (EpCAM). Appropriately adapted antibody combinations allowed for the analysis of xenografted or syngeneic mouse tumors (fig. 1 C and D). As the antibody cocktails were developed to deplete the unwanted non-tumor cells, the isolation is independent of tumor cell–specific surface markers. Therefore, tumor cells can be isolated regardlessof the tumor entity, as shown for the isolation of tumor cells from different mouse tumors, which were induced by GFP-expressing cell lines (fig. 1C), and different entities of human tumor xenografts (fig. 1D). Additionally, the isolated cells stayed 'untouched' allowing for subsequent sorting of tumor subpopulations by MACS® Technology.

2 Depletion of non-tumor cells improves downstream culture of target cells

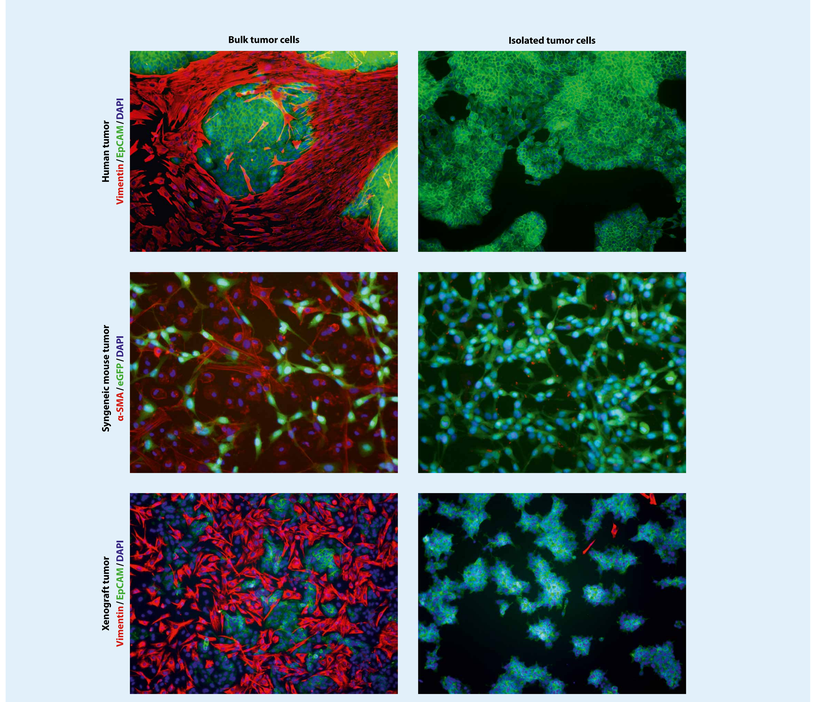


Figure 2

Cultivation of tumor cells from primary specimens is frequently hampered by the presence of fibroblasts, red blood cells, and debris. While debris and red blood cells impair efficient plating of tumor cells, fibroblasts attach and expand more efficiently, thereby overgrowing the target cells. Even when the target cells attach and grow well, *in vitro* cell culture assays (e.g. drug cytotoxicity testing) are problematic since mathematical correction for effects originating from contaminating cells is impossible in most cases. Upon magnetic separation, the original bulk and isolated tumor cell fractions were cultured for three to seven days, fixed, and stained. Syngeneic mouse tumor cells were detected by tumor cell–specific GFP expression and fibroblasts were stained with alpha-smooth muscle actin (α-SMA) (fig. 2, middle). Human tumors were stained for the human-specific epithelial tumor marker CD326 (EpCAM). As the human tumor cells were negative for vimentin, we were able to use this marker to unambiguously identify fibroblasts (fig. 2, top and bottom). Even after seven days, the cultures of isolated tumor cells were nearly pure.

3 Improved downstream analysis upon isolation of target cells

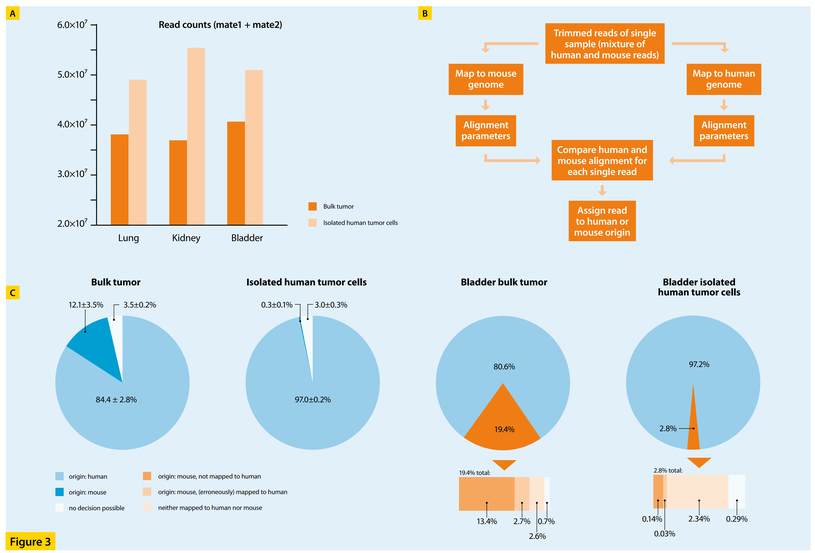


Figure 3

Samples of human tumor xenografts contain a significant amount of host-derived cells. To assess the impact of depletion of non-tumor cells on the quality of next-generation sequencing data, we conducted WES on three different xenograft models derived from human kidney, lung, and bladder cancer subsequent to mouse cell depletion. DNA from bulk tumor or isolated tumor cells was used to produce exome-captured sequencing libraries applying the Nextera® Rapid Capture Exome Kit (Illumina®). For sequencing on a MiSeq® instrument (Illumina) the MiSeq Reagent Kit v3 (150 cycles, Illumina) was utilized to generate 75-bp paired-end reads. As the capture oligonucleotides used for targeted enrichment of protein-coding sequences were designed based on the human genome, an initial pre-enrichment of DNA fragments of human origin from the mixture of mouse and human cells was expected. In order to assess the number of capture oligonucleotides that might cross-hybridize with mouse genomic DNA, we conducted BLAST searches of each single Nextera probe against mouse genome and used the resulting alignment parameters to determine possible cross-hybridization. Depending on the selection thresholds (alignment length, no. of mismatches, no. of gaps), we predicted a cross-reactivity of 5–10% of capture probes with mouse transcripts (data not shown). A significant increase ($p < 0.05$) in clusterdensity (not shown) as well as an average increase in read counts of 33% was observed for the samples depleted of mouse cells, indicating improved sample quality (fig. 3A). Correspondingly, we observed a strong reduction of debris and dead cells upon mouse cell depletion by flow cytometry analysis (data not shown).
After adapter clipping (trimmomatic v0.32[2]), we mapped the reads of all samples against human and mouse genomes (bwa v0.7.12[3]) and determined their putative origin based on the respective alignment parameters (LINUX shell, command-line Perl) (fig. 3B). An average of 12% of reads derived from bulk tumor samples was attributed to mouse cells. This amount could be reduced to 0.3% by prior depletion of mouse cells (fig. 3C). As on average 15% of the mouse-derived reads mapped erroneously to the human genome (1.9% of total reads) in the bulk tumor samples, a strong positive influence of mouse cell depletion (0.04% of total reads erroneously mapped to human genome) on downstream analyses can be expected. Figure 3C exemplifies the detailed read assignment for bulk tumor and isolated human tumor cells derived from the bladder cancer xenograft.

Conclusion

- Three novel methods have been established allowing for the untouched isolation of tumor cells from mouse, human, and xenotransplanted tumor tissue.
- The cell separation methods are easy and fast (<20 min) and allow for accurate downstream analysis of tumor cells, avoiding bias caused by contaminating cells of the tumor microenvironment.
- The contaminating non-tumor cells are specifically labeled prior to their depletion. Labeling of the tumor cells is not required. Therefore, the procedures can be used for the isolation of most tumor types without the need for knowledge of a positive marker expressed on the target cells.
- Isolation of pure populations of tumor cells improves downstream culture and molecular analysis by NGS.

References
1. DeRose, Y.S. *et al.* (2011) Nat. Med. 17: 1514–1520.
2. Bolger, A.M. *et al.* (2014) Bioinformatics 30: 2114–2120.
3. Li, H. and Durbin, R. (2009) Bioinformatics 25: 1754–1760.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. MACS and the MACS logo are registered trademarks or trademarks of Miltenyi Biotec GmbH. All other trademarks mentioned in this document are the property of their respective owners and are used for identification purposes only. Copyright © 2016 Miltenyi Biotec GmbH. All rights reserved.