Magnetic isolation and rare cell gene expression analysis of breast cancer stem cells

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Olaf Hardt[1], Stefan Wild[1], Kay Hofmann[1], Shujun Luo[2], Yvonne Wiencek[1], Eva Kantelhardt[4], Geoffrey P. Smith[3], Gary P. Schroth[2], Andreas Bosio[1] and Jürgen Dittmer[4]
[1] Miltenyi Biotec GmbH, 51429 Bergisch Gladbach, Germany | [2] Illumina Hayward, 25861 Industrial Blvd., Hayward, CA 94545, USA | [3] Illumina Cambridge Ltd, Chesterford Research Park, Little Chesterford, Nr Saffron Walden, Essex CB10 1XL, UK | [4] Klinik für Gynäkologie, Universität Halle, Ernst-Grube-Str. 40, 06097 Halle (Saale), Germany

Introduction

Research over the last decade supports the model that tumors show an organization similar to normal tissues during development and maintenance, where stem cells maintain cellular hierarchies[1]. In analogy to normal stem cells, the first cells in the tumor hierarchy are called cancer stem cells (CSCs). It is thought that CSCs are critically involved in the development and metastatic spread of cancer. CSCs have also demonstrated resistance to a number of conventional therapies and this might explain why it is difficult to completely eradicate the disease and why recurrence is an ever-present threat[1].
The molecular characterization of CSCs may therefore be crucial for novel diagnostic and therapeutic approaches. However, signatures of cells with highly tumorigenic potential might escape detection during analysis of the bulk tumor mass due to the low frequency of CSCs. Similarly to conventional stem cells and somatic cells, gene expression profiles of CSCs are expected to significantly differ from the surrounding tumor tissue. To achieve a reliable gene expression analysis of CSCs in tumors we had to overcome two main challenges: Isolation of CSCs from the bulk tumor and amplification of tiny RNA amounts for transcriptome sequencing.

Methods

1 Isolation of cancer stem cells

One prerequisite for the isolation of cell subpopulations is to generate viable single-cell suspensions from solid tissue. We have developed a method for the semi-automated dissociation of human tumor tissue yielding high recoveries of viable single cells using the gentleMACS Dissociator[2].
Human mammary carcinoma stem cells are defined by the expression of CD44 and the absence of CD24[3]. In addition, analysis of further surface markers showed that CD45 has to be used as exclusion marker as the majority of CD44$^+$ cells are CD45$^+$CD44$^+$ tumor-infiltrating leukocytes (fig. 1A). Based on these results, a magnetic cell sorting strategy was established, allowing the isolation of CD45$^-$CD24$^-$CD44$^+$ breast CSCs (fig. B).
Due to the limited starting material, 500 CSCs as well as bulk tumor cells per replicate were used for global PCR-based RNA amplification (SuperAmp Technology, fig. 2). The amplified cDNA was sheared, size-selected, and processed for high-throughput gene expression analysis on the Illumina Genome Analyzer system. Samples were prepared in triplicate and over 10 million individual purity-filtered reads were analyzed per sample. In addition, Agilent Whole Genome Microarrays were used for validation of expression differences.

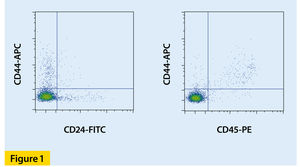


Figure 1

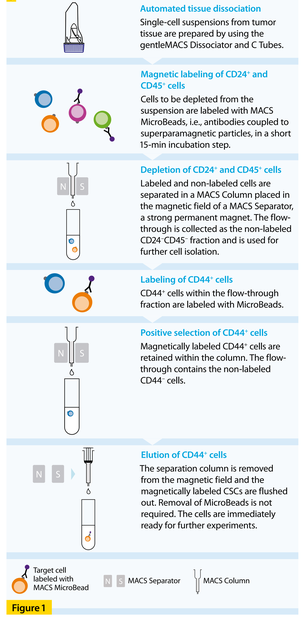


Figure 1

2 RNA amplification

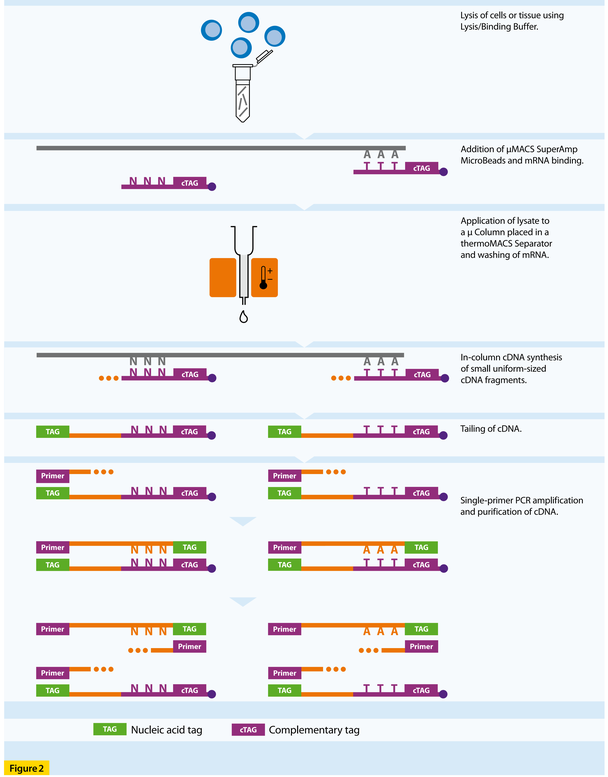


Figure 2

Results

3 Isolation of breast cancer stem cells

Human mammary carcinoma tissue was dissociated using a standardized, semi-automated procedure. Breast CSCs were isolated by depletion of CD24$^+$ and CD45$^+$ cells and subsequent enrichment of CD44$^+$ cells. All populations were checked for co-expression of CD24 and CD44 (A) as well as CD45 and CD44 (B). Using this method, CSCs were enriched from 5% in the original fraction to a purity of 94%.

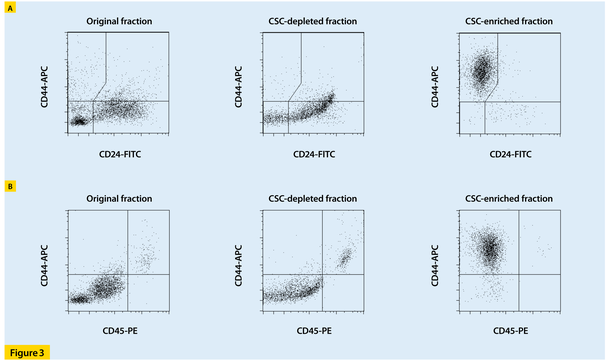


Figure 3

4 Gene ontology analysis of CSC-related pathways

Genes with higher expression in breast CSCs compared to the bulk tumor were grouped according to their gene ontology classification. Subsequently, significantly overrepresented functional classes were identified. A strong overrepresentation of genes connected to TGF-β, as well as Wnt/GSK3 signaling, supports the correlation of the CSC phenotype with epithelial-mesenchymal transition (EMT). Other differentially expressed pathways are known to be involved in pluripotency, cell migration, and regulation of apoptosis, further indicating a stem cell–like fate of CSCs. Interestingly, a strong enrichment of genes important for building and modification of extracellular matrix was observed, suggesting a participation of CSCs in the modulation of their surrounding environment.

Functional annotation	p-Value	Enrichment	Annotated genes
Extracellular matrix	2.53E-75	26.65	166
Tissue morphogenesis and development	4.48E-29	8.08	29
TGF-β pathway	6.52E-25	9.09	99
PTEN pathway	1.26E-17	9.32	29
Pluripotency	2.24E-15	8.48	29
Regulation of apoptosis	1.19E-14	1.95	38
Regulation of cell migration	1.41E-14	12.57	67
PI3K pathway	6.42E-14	4.61	38
Angiogenesis	1.10E-11	15.00	25
NF-κB family pathway	2.09E-11	7.55	24
Response to hormone stimulus	1.02E-7	3.30	31
Cancer and metastasis	1.01E-5	5.63	11
Wnt/GSK3 signaling pathway	1.27E-4	14.21	22

5 Regulation of stem cell– and epithelial-related genes

In purified CSCs, CD24 and CD45 were expressed at lower levels compared to the bulk tumor, whereas CD44 was expressed at a higher level in CSCs. This result matches the expression pattern of cell surface markers in CSCs used as a basis for CSC isolation. Known mammary CSC markers, such as PROCR and ITGB1/CD29 [4], but also other stem cell–related markers, such as CD34, CD133, and THY1, were highly enriched and may represent novel markers for breast CSCs. In addition, genes connected to a mesenchymal fate, such as VIM were overrepresented, while breast epithelial markers, such as GATA3 or keratins, were expressed at lower levels, again supporting a connection between a stem cell and a mesenchymal phenotype. Three replicates were analyzed for each marker.

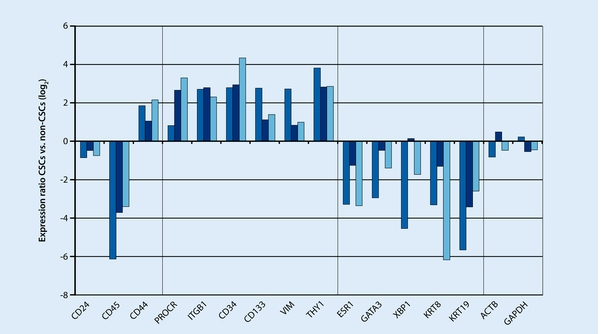


Figure 4

Conclusion

Our proof-of-principle experiment demonstrates the advantage of isolating rare cell populations for subsequent molecular analysis by high-throughput sequencing. The analysis of purified CSCs revealed new insights on regulation, maintenance, and biology that would not have been possible by assessing only the bulk tumor mass. Analyzing the genetic setup of defined tumor subpopulations by next-generation sequencing can improve our understanding of cancer and could pioneer targeted diagnostic and therapeutic approaches.

References

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