Novel monoclonal antibodies for the analysis and isolation of Lgr5-positive cells

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Introduction

Cancer stem cells (CSCs), also known as tumor-initiating cells, have gained substantial interest over the past few years. CSCs have been isolated from multiple tumor entities and were shown to play a crucial role during tumor growth and metastasis. However, there is still a major debate about specific cell surface markers enabling the identification of CSCs in most tumor entities. The leucine-rich repeat–containing G-protein–coupled receptor (LGR) Lgr5 and its close homologs Lgr4 and Lgr6 associate with Wnt-receptors and act as R-spondin receptors, thereby playing a central role in the modulation of Wnt/β-catenin signaling in normal and neoplastic stem cells[1,2]. Initially described as a highly specific marker for stem cells in the small intestine, colon, hair follicle, stomach, and during kidney development[3–6], Lgr5-positive cells were also shown to be crucial factors during the development and progression of cancer. It was shown that Lgr5-positive crypt stem cells are the cells-of-origin of intestinal cancer and that CSCs in human colorectal cancer can be identified and isolated based on Lgr5 expression[7,8]. However, the analysis of Lgr5-expressing cells is hampered by the lack of highly specific monoclonal antibodies.

Results

1 Generation of cell lines stably expressing human Lgr4, Lgr5, or Lgr6

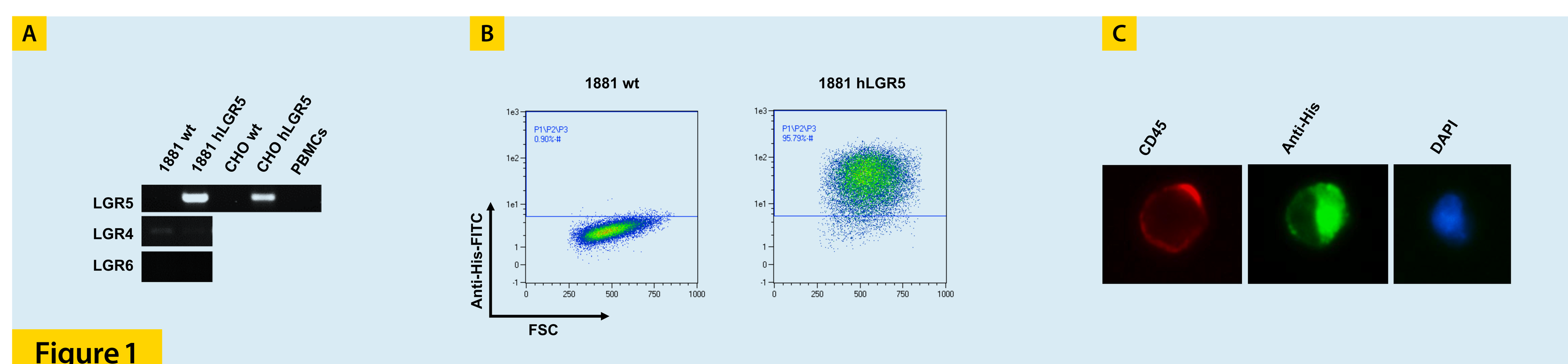

Figure 1

To establish a screening system for antibodies specifically recognizing human Lgr5, we transfected cell lines showing no intrinsic Lgr5 expression to generate cells stably expressing this protein (A). These cell lines allowed us to reliably assess the performance of anti-Lgr5 antibodies. To determine the level and validate the stability of Lgr5 expression by flow cytometry, a His-tag was fused to the intracellular C-terminus of the protein. This enabled us to intracellularly stain the Lgr5 antigen with Anti-His-FITC antibodies (B). Furthermore, cell surface localization of the protein was validated by co-staining of the cell surface marker CD45 (C). In addition, cell lines stably expressing human Lgr4 or Lgr6 were generated to prove that the Lgr5 antibodies do not cross-react with close homologs. Abbreviations: wt = wild type; PBMCs = peripheral blood mononuclear cells

2 Generation of highly specific anti-hLgr5 monoclonal antibodies

Rabbit monoclonal antibodies were generated against peptide sequences of Lgr5. Using the cell lines stably expressing Lgr5, we screened for high-affinity antibodies by flow cytometry identifying two suitable clones, 89.11 and 102.5. Using the cell lines stably expressing human Lgr4 or Lgr6, we demonstrated that the antibodies are specific for Lgr5 and do not cross-react with the close homologs. In a second approach, rat monoclonal antibodies were generated by immunization with cells expressing the full-length Lgr5 protein. By applying an equivalent screening strategy, we identified one clone, 22H2.8, which showed high affinity and specificity for Lgr5.

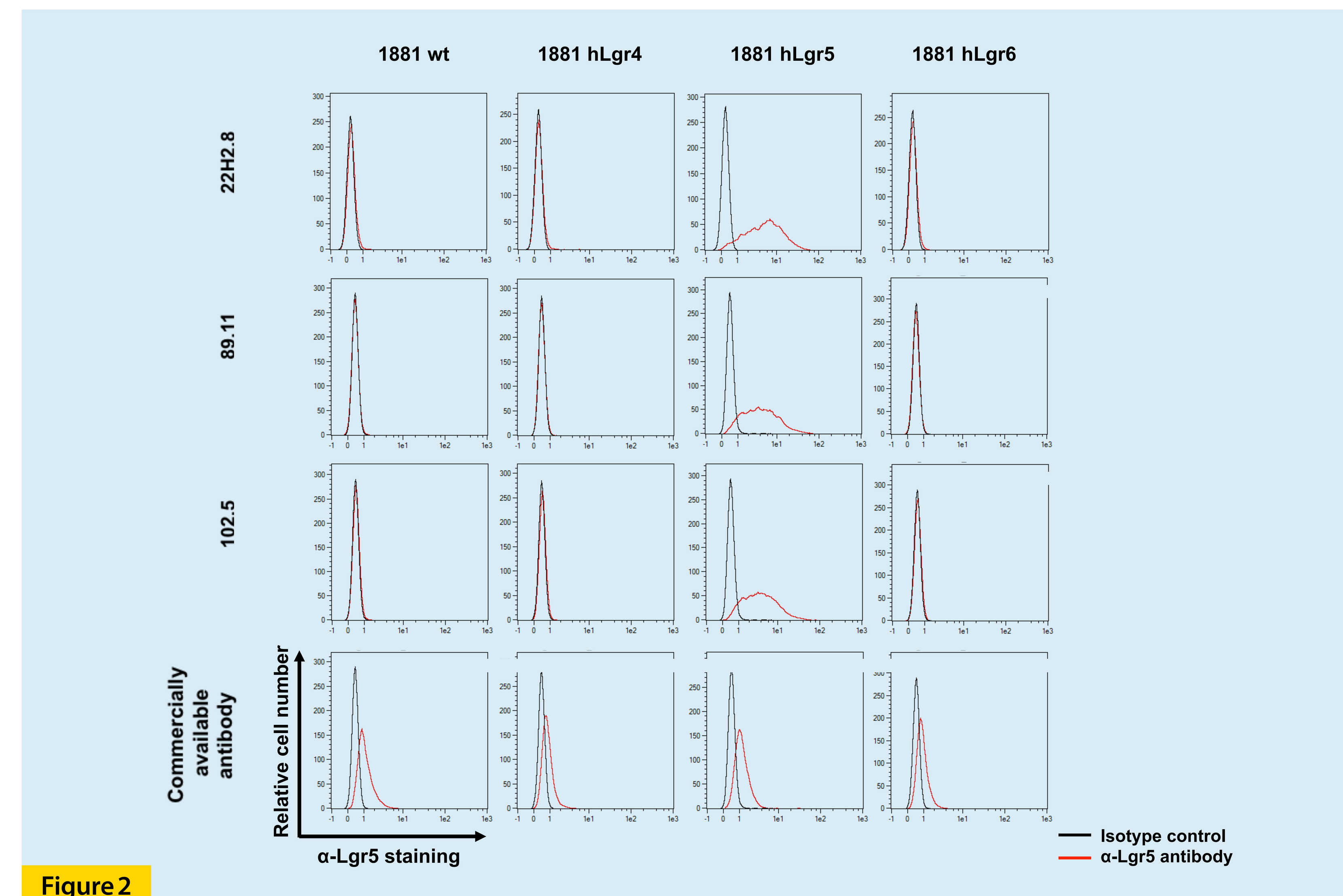

Figure 2

3 Identification of Lgr5-expressing cells in primary tissues

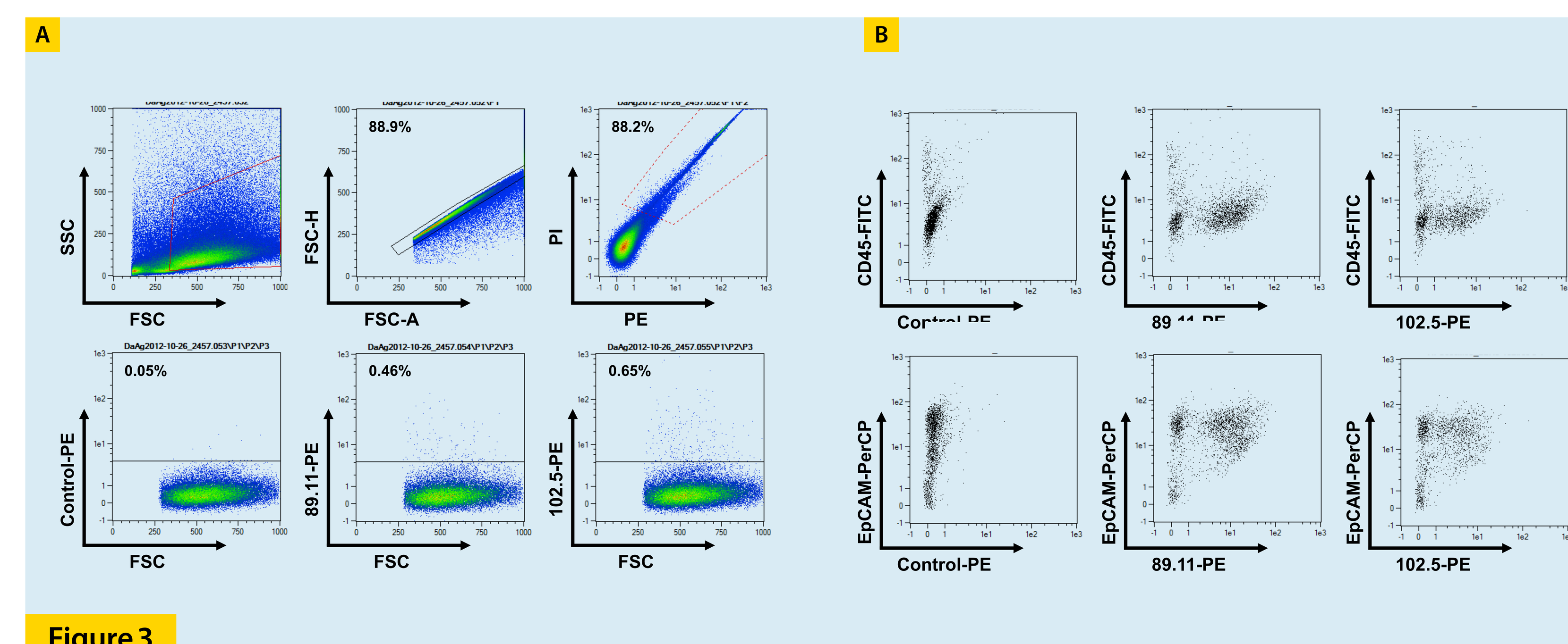

Figure 3

The cell surface expression level of Lgr5 in native tissues was reported to be very low. Therefore, we evaluated whether the generated antibodies were suitable for the identification of cells with intrinsic Lgr5 expression levels in healthy and neoplastic tissues. For the dissociation of solid tissues into single-cell suspensions with high cell viability, the gentleMACS™ Dissociator was used in combination with appropriate enzyme-based tissue dissociation kits, e.g., the Tumor Dissociation Kit in case of human tumor tissue. The highly pure enzymes contained in these kits allowed for optimal preservation of epitope integrity, which is a prerequisite for the reliable identification of target populations after tissue dissociation. Lgr5-expressing cells could be identified in single-cell suspensions of dissociated healthy human skin tissue (A). In addition, a significant amount of pancreatic neuroendocrine tumor cells were EpCAM⁺Lgr5⁺CD45⁻, representing a distinct sub-population in this tumor entity (B).

4 Isolation of Lgr5-expressing human skin cells

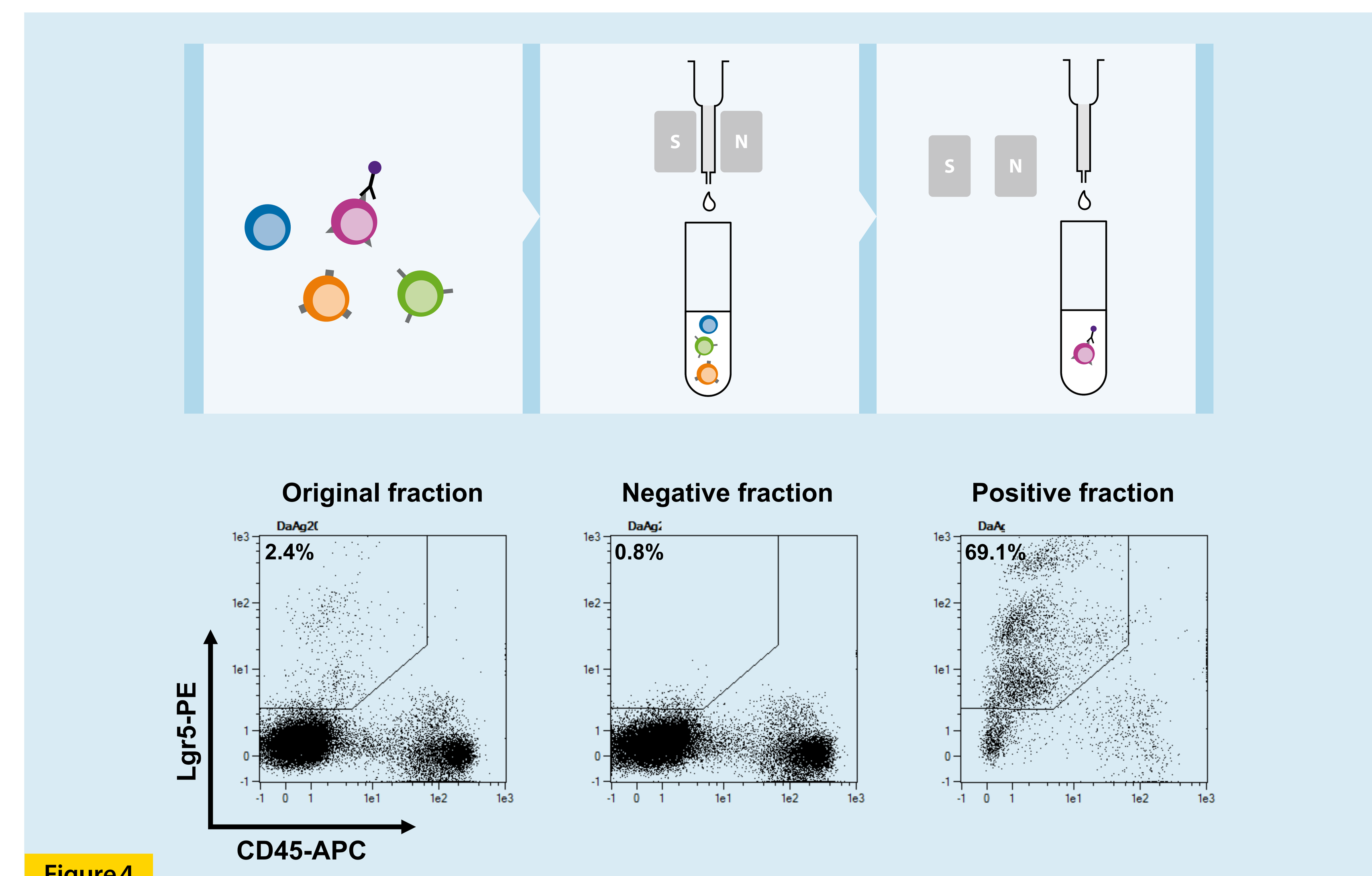

Figure 4

The purification of target cell populations is a prerequisite for accurate downstream analyses, e.g., next-generation sequencing or proteome profiling. Using non-purified cell populations for analysis, it is hard to differentiate between effects that are due to the cells of interest and effects resulting from contaminating cells. After dissociating skin tissue into single-cell suspensions with the gentleMACS™ Dissociator, we enriched cells expressing Lgr5. Utilizing the newly established antibodies in combination with MACS® Technology we were able to enrich the Lgr5-expressing cells from 2% to about 70%. This frequency already would allow for subsequent characterization of these cells. However, in order to further enhance the enrichment performance, additional experiments with varying titration parameters are currently in process.

Conclusion and Outlook

We have developed highly specific monoclonal antibodies allowing for the analysis and isolation of Lgr5-positive cells from cell lines and dissociated primary tissues.

Reliable methods for the dissociation, analysis, and isolation of Lgr5-expressing cells from healthy and neoplastic tissues in combination with molecular and functional downstream analysis will enable new insight into the biology of these highly interesting subpopulations.

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