LETTER TO THE EDITOR

Development of a novel method for rapid cloning of shRNA vectors, which successfully knocked down CD44 in mesenchymal triple-negative breast cancer cells

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Dear Editor,

Since the discovery of short hairpin RNA (shRNA) vector-mediated RNA interference (RNAi), this technology has been widely used in cancer research for its specificity, potency, and convenience. However, researchers may find it costly to purchase commercial vectors from biocompanies or time- and labor-consuming to construct their own shRNA vectors using traditional method by inserting annealed duplex into digested vectors. Despite intensive efforts to accelerate shRNA vector cloning in laboratories, the development of a reliable, rapid, convenient, and cost-effective method is still in great demand. To this end, we developed a novel method named SuperSH (Super rapid cloning of shRNA vector) for the effective and rapid construction of shRNA-expressing vectors based on high-performance DNA polymerase and seamless cloning technique [1] (Additional file 1: Figure S1a; the detailed methods can be found in Additional file 1). In our SuperSH method, the shRNA sequences are introduced into the vector via a pair of polymerase chain reaction (PCR) primers rather than via annealed duplex. In detail, the 3' ends of the primers are designed to bind the template to initiate a PCR to amplify the vector backbone, and the 5' portions are designed to introduce the sequences of interest as well as to form a short homologous arm for subsequent recombination via seamless cloning [1]. After the seamless recombination reaction, the seamed vector is transformed into competent E. coli using a quick transformation protocol that takes only 5 min [2] (Additional file 1: Figure S1). It's important to note that the SuperSH method requires a linearized vector template to achieve effective and rapid cloning, skipping the need for purification steps throughout the cloning procedure. For this aim, we created an intermediate vector pSuperSH-MX by introducing restriction enzyme sites for two non-isocaudomers, MluI and XbaI, to allow complete linearization of the template plasmid and to avoid self-ligation in subsequent cloning procedures as well.

Our SuperSH method outperforms traditional shRNA cloning method based on annealed complementary oligonucleotide duplex in the following aspects (Additional file 1: Figure S1b). Firstly, the SuperSH method requires only three steps, namely a low-cycle number PCR to amplify the vector backbone, a recombination reaction to seam the vector, and a quick transformation to replicate the vectors in E. coli, which altogether greatly saves researchers’ hands-on work. Secondly, the above procedures also allow users to complete shRNA vector cloning in as short as 30 min. Thirdly, SuperSH uses much shorter oligonucleotides in the application of shRNA cloning, as seamless cloning requires only a short homologous arm (10–15 nucleotides) to cyclize the PCR product, and thus there is no need to synthesize the full length of shRNA sequence (Additional file 1: Figure S2). In addition, we observed this novel method to be much more reliable and...
accurate than traditional cloning methods, by achieving an overall success rate higher than 95% in our work. Furthermore, SuperSH is a versatile method which allows researchers to construct various shRNA vector formats, such as 21-mer-, 29-mer-, and miR-N-shRNA vectors [3] (Additional file 1: Figure S2).

To further decipher the role of CD44 in mesenchymal TNBC cell lines, we stably knocked down CD44 in three mesenchymal TNBC cell lines (SUM159, MDA-MB-436, and MDA-MB-231) using shRNA vectors constructed by the above described SuperSH method. After selection for stable cell lines, we determined the knockdown efficiency of the shRNA vectors in the TNBC cell lines. Western blotting showed that the shRNA clones sh4 and sh5 achieved the best knockdown efficiency in all three cell lines (Fig. 1a–c), which was also confirmed by flow cytometry with fluorescence-conjugated antibody for CD44 (Additional file 1: Figure S4a). Therefore, we used these two shRNAs in subsequent studies to assess the role of CD44 in cell proliferation, colony formation, and invasion. Our results showed that deletion of CD44 significantly suppressed cell proliferation (Fig. 1d–f), colony formation (Fig. 1g, h) and invasion ability (Fig. 1i, j) of mesenchymal TNBC cell lines SUM159, MDA-MB-436, and MDA-MB-231, indicating a vital role of CD44 in promoting the proliferation and invasion of mesenchymal...

(Supplementary figure on next page.)

**Fig. 1** The knocking down of CD44 markedly suppresses the proliferation and invasion of mesenchymal TNBC cell lines. a–c The efficiency of shRNA vectors in knocking down CD44 was determined in stable cell lines at the protein level. d–f MTT assay was performed in stable cell lines to determine the effect of CD44 knockdown on cell proliferation. The OD490 value was read at three indicated time points and the values were normalized to that of day 3. The results are shown as mean ± SEM. ****P < 0.0001; this experiment was independently performed 3 times. g–h Colony formation assay was performed to assess the effect of CD44 knockdown on colony formation ability. i–j Transwell assay was carried out to decipher the impact of CD44 knockdown on invasion. A representative picture of three independent experiments is shown for each cell line and the related statistic results are shown as mean ± SEM. **P < 0.01; ***P < 0.001; this experiment was independently performed 3 times. Scale: The black rectangular box at the right bottom corners of figure i represents the 100 μm scale. k–l Western blotting was carried out in SUM159 cells with CD44 stably interfered with 29-mer shRNA and miR-N shRNA, respectively. TNBC triple-negative breast cancer, shRNA short hairpin RNA, MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, CD490 optical density at 490 nm, SEM standard error of the mean, NT non-targeting, GAPDH glyceraldehyde-3-phosphate dehydrogenase
TNBC cell lines, consistent with previous reports in other cancers [9].

To demonstrate the versatility of the SuperSH method in cloning various forms of shRNA vectors, we also constructed shRNA vectors targeting CD44 in two other forms, namely 29-mer shRNA and miR-N shRNA [3] (Additional file 1: Table S1) and determined their knockdown efficiency by using both Western blotting (Fig. 1k,
l) and flow cytometry (Additional file 1: Figure S4b, c). In our results, both shRNA formats achieved excellent knockdown efficiency with their best-performing clones, which were comparable to that of the best 21-mer shRNA vectors described above. To provide additional support to this novel shRNA vector construction technique, we also knocked down several other genes, such as vimentin (VIM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and achieved robust knocking-down effects in breast cancer cell line SUM159 (Additional file 1: Figure S5).

In summary, we developed a rapid, reproducible, and cost-effective method for shRNA vector cloning, which would evidently facilitate the shRNA cloning work for researchers, especially if they are not able to access commercial shRNA libraries. Using this method, we have successfully constructed several forms of shRNA vectors targeting one of the widely accepted breast cancer stem cell marker CD44 whose expression has been previously implied to promote metastasis in breast cancer. To better understand the roles of CD44 in breast cancer, we characterized the CD44 expression pattern in non–TNBC and TNBC and found that CD44 was significantly over-expressed in the mesenchymal subcategory of TNBC cell lines at both mRNA and protein level (Additional file 1: Figure S3). Epithelial-mesenchymal transition (EMT) state has been demonstrated to be involved in breast cancer invasion and subsequent metastasis [10]. Hence, our analysis of the CD44 expression pattern implies a vital role in these processes. Consistent with this notion, when we stably knocked down CD44 in mesenchymal TNBC cell lines, the invasion ability of all three tested mesenchymal breast cancer cell lines, SUM159, MDA-MB-436, and MDA-MB-231, were markedly inhibited, suggesting an essential function of CD44 in mesenchymal TNBC invasion. Our results may, to some extent, put forward the hope of targeting CD44 in mesenchymal TNBC to inhibit its proliferation, invasion, and metastasis and to obtain a better prognosis for breast cancer.

Additional file

Additional file 1. Additional materials.

Abbreviations
BL1: basal-like 1; BL2: basal-like 2; CCLE: Broad Institute Cancer Cell Line Encyclopedia; EMT: epithelial–mesenchymal transition; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; iM: immunomodulatory; LAR: luminal androgen receptor; M: mesenchymal-like; MSL: mesenchymal stem-like; MTT assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NT: non-targeting; PCR: polymerase chain reaction; RNAi: RNA interference; shRNA: short hairpin RNA; SuperSH: super rapid cloning of shRNA vector.

Authors’ contributions
SL and LZ designed the experiments and drafted the manuscript. LZ and DS performed the experiments, analyzed the data, and drafted the manuscript. QG and DW performed flow cytometry experiments. All authors read and approved the final manuscript.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
Additional data and methods are available online as an additional file at Cancer Communications. All materials mentioned in the manuscript and additional file are available upon reasonable request from the corresponding author.

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Not applicable.

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Not applicable.

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