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Short Communication

A novel shRNA vector that enables rapid selection and identification of knockdown cells

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Abstract

Small interference RNA (siRNA) is a powerful tool for disrupting expression of specific genes in a variety of cells. We have developed a vector, piMARK, which mediates expression of both small hairpin RNA (shRNA) and the blasticidin resistance (Bsr) protein fused with enhanced green fluorescent protein (EGFP), enabling rapid selection and identification of knockdown cells. Using this vector, we targeted Ect2, a gene encoding a guanine nucleotide exchange factor for several small GTPases, in human cell lines. Incubation in the presence of 10 μg/ml blasticidin S rapidly killed untransfected cells, so that after 24 h >90% of surviving HeLa S3 cells emitted green fluorescence and >70% were binucleate as a result of the frequent failure of cell division. The GFP-Bsr fluorescence enabled easy identification of individual knockdown cells under a fluorescence microscope, which in turn enabled unambiguous assessment of the morphological consequences of silencing Ect2. Moreover, because untransfected cells rapidly died and detached from the substrate, they were easily removed by simply rinsing the culture dishes. It thus should be possible to analyze the biochemical consequences of gene silencing *en masse* in the absence of a background of untransfected cells.

Keywords: RNA interference; Short hairpin RNA; Ect2; Enhanced green fluorescent protein; Blasticidin

RNA interference (RNAi) has proven to be a powerful tool with which to suppress expression of specifically targeted genes in a variety of cell types (Zhou et al., 2006). Today, there are a number of vectors available that are useful for this application (Wadhwa et al., 2004), but the transfection efficiency rarely reaches 100%, which creates practical problems. First, the presence of untransfected cells diminishes the apparent effect of gene silencing. Second, the sampling error may be problematical in cases of microscopic analysis of limited numbers of individual cells. To overcome these problems, we constructed a vector, piMARK, which mediates expression of both shRNA under the control of the human U6 promoter and the blasticidin S resistance gene fused to green fluorescent protein cDNA (GFP-Bsr) under the control of the CMV promoter. This enables easy identification and selection of transient knockdown cells, even when the transfection efficiency is suboptimal (Fig. 1b).

Blasticidin S is an antibiotic that inhibits protein synthesis in both prokaryotes (Takeuchi et al., 1958)

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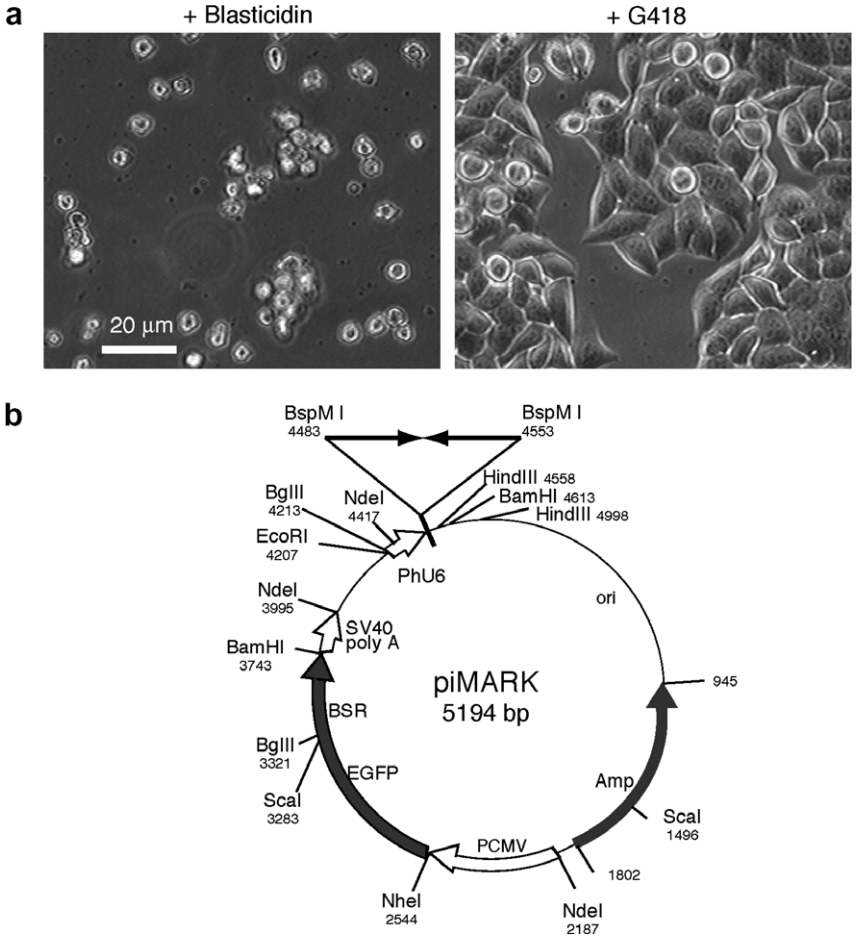

Fig. 1. (a) Blasticidin S killed HeLa S3 cells more rapidly than G418, which is commonly used for selecting cells expressing the neomycin resistance gene. (b) Construction of the piMARK vector. The expression of the EGFP-Bsr (gray arrow) and shRNA (black arrow) genes was driven by the CMV promoter and the human U6 promoter, respectively. The synthetic oligonucleotide coding shRNA was inserted at the *Bsp*MI site downstream of the U6 promoter.

and eukaryotes, and the blasticidin S resistance gene (Bsr) was originally isolated from *Bacillus cereus* (Itaya et al., 1990). We chose blasticidin S because it has been shown to kill sensitive cells more rapidly than G418 (Izumi et al., 1991) and has been used as a selection drug to express shRNA in mammalian cells (Hung et al., 2006). For example, the control HeLa S3 cells depicted in Fig. 1a exhibited no apparent changes after exposure to 800 µg/ml G418 for 24 h (right); this concentration of G418 was sufficient to kill all the cells within 5–6 days, however (not shown). By contrast, exposure to 10 µg/ml blasticidin S killed all the control HeLa S3 cells within 24 h, as judged by the fact that the cells appeared refractive and detached from the substrate (Fig. 1a, left). Fusion of EGFP to Bsr enables easy visual identification of individual transfected cells under a fluorescence microscope, as well as observation of the effects of gene silencing, even when untransfected cells are present. In addition, it is psychologically assuring to be certain that the cell one is analyzing contains the plasmid harboring the shRNA of interest.

DNA encoding the EGFP-Bsr fusion protein was generated by inserting Bsr cDNA into pEGFP-C3 (Clontech, Tokyo, Japan) between the *Bam*HI and *Kpn*I sites. The fragment of the EGFP-Bsr expression unit containing the CMV promoter and SV40 polyA was amplified by polymerase chain reaction (PCR) using a 3′ primer that added an *Nde*I restriction site. As a result, the PCR product had two *Nde*I sites, one near the 3′ end (derived from the primer) and another within the CMV promoter. The PCR product digested with *Nde*I, which contained a truncated CMV promoter-EGFP-Bsr-SV40 terminator, was inserted into the unique *Nde*I site in piGENE-