

cells, remains to be identified. Until now, bone marrow has generally been perceived as a rich but relatively inaccessible source of vascular progenitors, and clinical studies have employed instead circulating EPCs for therapy [12]. Unfortunately, given the relative paucity and functional impairment of circulating progenitors in patients with vascular disease, this source of progenitors is far from ideal [13]. The presence of high-proliferative-potential colony-forming EPCs within the vessel wall suggests an additional, albeit mildly invasive, target site for harvesting progenitor cells. This source might be particularly auspicious in tissue engineering and non-acute therapeutic applications of these cells. However, further studies will be required to clarify if vessel-resident EPCs are subject to the same quantitative and qualitative deficiencies that circulating EPCs exhibit in the presence of intercurrent cardiovascular risk factors or disease. The mechanisms underlying mobilization of EPCs from the bone marrow stem cell niche have been quite well characterized [14], but might prove different to those governing mobilization of EPCs from within the vessel wall. Future therapies, either pharmacological or device based, might be able to exploit such local EPC populations to augment endothelial repair or, in the case of endovascular grafts or devices, speed-up luminal re-endothelialization through mobilization, recruitment and expansion of these cells. Alternatively, such devices might be configured to release EPCs from the vessel wall to target systemic or regional end-organ disease. Indeed, the existence of these cells within the broader systemic vascular tree provides numerous opportunities for local vessel wall and systemic targeting by a range of novel technologies.

This work by the Ingram group thus provides a robust benchmark to define EPCs *in vitro* akin to methodology already well established in the hematopoietic stem cell field. Such progenitor colony assays, if reproducible by other investigators, might be very useful in future efforts aimed at classifying the full hierarchy of progenitor cells supporting endopoiesis *in vivo*. Finally, the discovery of

such highly proliferative EPCs in the mature vasculature should prompt a re-appraisal of EPC origin and function, and suggests caution when using 'mature endothelial cells' as controls in studies of EPC biology and therapeutic efficacy.

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doi:10.1016/j.tibtech.2005.05.013

The best control for the specificity of RNAi

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RNA interference (RNAi) is revolutionizing functional genomics. However, there are several reasons to be concerned about the specificity and off-target effects of this technique. A recent paper by Kittler *et al.* describes a straightforward way to validate RNAi specificity, which exploits the increasing availability of bacterial artificial

chromosome (BAC) clone resources. Genetic rescue of the RNAi phenotype by BAC transgenesis is the best control yet described for specificity, and has further implications for reverse genetics.

The discovery of RNA interference (RNAi) uncovered an unexpected and fundamental aspect of eukaryotic molecular biology, the implications of which are still being

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Available online 23 June 2005

determined [1]. Currently, three basic mechanisms are acknowledged: mRNA degradation mediated by small interfering (si)RNA, translational inhibition mediated by microRNA (mi)RNA, and RNAi-provoked chromatin silencing mediated by methylation of nucleosomes. Despite the explosion of research activity in this area, none of these basic mechanisms is well understood. Similarly, the understanding of RNAi mechanisms in cell and developmental biology remains sketchy.

Besides its fundamental appeal, RNAi has attracted interest because of its usefulness as a tool in functional genomics. It can be used to provoke loss of function relatively easily compared with other approaches. The speed, ease and cost-effectiveness of RNAi gene knock-down has led to two main developments. First, large-scale functional screens, previously impractical with other methodologies, are now possible [2]. Second, targeted loss of function, previously limited to the few specialized systems that support efficient homologous recombination, is now widely applicable. These points are particularly relevant to studies with the model organisms *Drosophila* and *Caenorhabditis elegans*, as well as the vast untapped potential for reverse genetics with cultured cell lines. Now, both large-scale screens and testing for individual loss of function have entered routine practice.

Specificity issues

The rapidly spreading popularity of RNAi methodology emphasizes the need to understand its strengths and limitations as a tool. The issue of target specificity and off-target effects has been a source of concern since the first applications of RNAi to functional genomics [3]. Notably, work with vertebrate systems unveiled overlap with a general phenomena provoked by double-stranded (ds)RNA, mediated by protein kinase R (PKR), translational inhibition and the interferon response [4,5]. These widespread, deleterious responses are not related to the nucleotide sequence of the dsRNA and can significantly complicate RNAi loss-of-function studies. The recognition that RNAi is mediated by dsRNAs of less than 30 bps was key to defining the way to use RNAi for vertebrate analysis [6]. Hence, siRNAs (i.e. less than 30 bps) are now used in vertebrate systems. However, the dsRNA response remains a potential hazard for applications of RNAi. This is not the only reason for concluding that RNAi loss-of-function experiments need good controls.

To date, applications of RNAi aim to provoke loss of function by eliciting specific mRNA degradation. Often, mRNA levels are significantly depleted but not abolished. Because production of the target mRNA continues regardless of degradation, and a certain level of translation is expected, RNAi loss-of-function applications are termed 'knock-down' to distinguish them from 'knockouts' achievable by mutagenesis at the DNA level. An RNAi knock-down is an ongoing balance between the rate of production of the target mRNA and the efficiency of the RNAi-directed mechanism for target mRNA degradation. Because it is a balance, RNAi knock-down experiments are inherently variable. Therefore, they need exacting controls.

Furthermore, RNAi is not limited to targeted mRNA degradation. Two other short dsRNA mechanisms overlap

with siRNA-directed mRNA degradation. Of these, inhibition of translation by miRNAs has been the most worrisome for RNAi applications because miRNAs are not perfectly complementary to their targets. Consequently, it has proven difficult to determine the characteristics that define an miRNA, leading to the fear that siRNA designed for one target mRNA might inadvertently convey an miRNA effect on a different target. So little is known about the other known short dsRNA mechanism, which is involved in chromatin silencing, that no predictions as to specificities can safely be made. Because we do not yet understand RNAi well, we cannot depend upon its performance as a tool. Herein lie further reasons for exacting controls.

These issues have been recognized for some time. For example, an editorial in *Nature Cell Biology* [7] was dedicated to considerations of acceptable standards and controls. While discussing various sensible precautions and procedures, the editorial also noted that 'the ultimate control for any RNAi experiment remains rescue by expression of the target gene in a form refractory to siRNA (ideally within the physiological range)'. This restates a long-accepted gold standard for mutagenesis, namely genetic rescue.

Cross-species BAC rescue

The article by Kittler *et al.* [8] provides a straightforward way for genetic rescue of RNAi-induced phenotypes by use of bacterial artificial chromosome (BAC) transgenesis (Figure 1). The strategy exploits the increasing availability of annotated BACs that accompany genome databases. It is now possible to identify and obtain a BAC carrying a chosen gene from genomes representing most of the major model systems, including mouse, human, several *Drosophila* strains, and the nematode *Caenorhabditis briggsae*. Although the work by Kittler *et al.* uses HeLa cells, the strategy will be generally applicable not only to cells in culture but also to all systems that permit stable transgenesis. Aside from the virtue that BACs are online and readily obtainable,

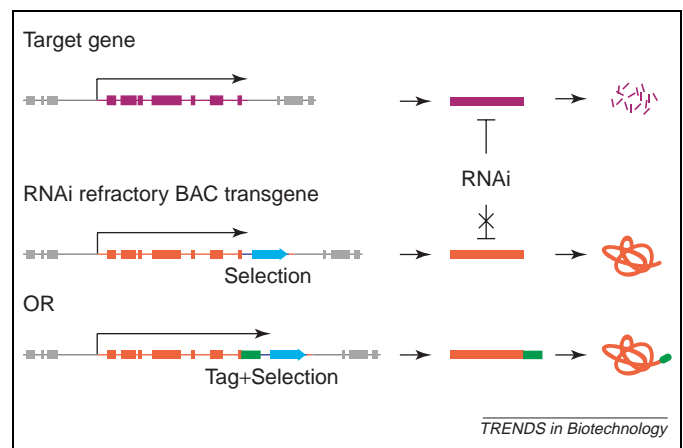


Figure 1. BAC rescue of knock-down by RNAi, according to Ref. [8]. A BAC transgene from a closely related species (orange) reconstitutes the expression pattern of the endogenous gene (purple) yet is divergent enough to avoid RNAi knock-down. In a simple elaboration of the same strategy, different modifications such as adding a protein tag (green) are introduced into the BAC transgene, thereby tagging (or mutating) the reconstituting protein.

expression of a gene from a BAC usually recapitulates splicing patterns and expression levels, including cell-cycle controls. Because BACs often contain all cis regulatory elements for gene expression, and are usually integrated at low transgene copy number, the artifacts associated with dysregulated cDNA overexpression from strong (usually viral) promoters are far less likely to be encountered.

The simplicity of the Kittler *et al.* strategy relies on two aspects. First, to facilitate selection of cells with BAC transgenes, Kittler *et al.* use 'recombineering' [9,10] to put a selectable gene onto the BAC. Because the selectable gene carries a dual *Escherichia coli*/mammalian cell promoter, the generation of a selectable transgenic construct takes only one recombineering step [11]. This simple application of recombineering greatly enhances the utility of BACs as reagents in functional genomics. For the above reasons, it is likely that BACs will become preferred to cDNA expression vectors.

Second, the rescuing transgenic mRNA must obviously differ from the mRNA targeted by RNAi. Therefore, Kittler *et al.* select BACs from a different species, in their case from the mouse. The great convenience of 'cross-species' rescue comes with a risk. At a frequency that will probably be very low, the cross-species gene/BAC will not rescue. Notably, the failure of cross-species BAC rescue will cast doubt on the RNAi specificity, rather than provoking a misleading functional conclusion. In these cases, recombineering-mediated point mutagenesis could be used to alter the RNAi target region in a BAC from the same species [12]. Although this is straightforward, it entails more work than simply choosing a cross-species BAC.

Further implications

Although it is a remarkable tool, RNAi can achieve only a loss of function. For example, it cannot achieve point mutagenesis or expression of fusion proteins. In a simple variation of the cross-species BAC rescue strategy, Kittler

et al. outline a way to achieve these goals using the combination of RNAi and BAC rescue. Rather than recombineering a BAC to carry a selectable gene, they recombineer the BAC to make a green fluorescence protein (GFP) fusion with the rescuing protein. Thereby, the endogenous protein, knocked-down by RNAi, is not merely replaced by its cross-species counterpart but by a GFP fusion protein. Further variations of the same theme are obvious. The paper by Kittler *et al.* therefore not only describes the best workable control for the specificity of RNAi knock-downs so far, but also suggests a new general strategy for reverse genetic approaches, which will be applicable to many experimental systems.

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doi:10.1016/j.tibtech.2005.06.007

Letters

Microalgae: the 'self-synchronized' eukaryotes

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Owing to the increase of sensitivity of high-throughput techniques in proteomics and genomics, 'truly' synchronized cultures should be a prerequisite for a reliable identification of key proteins and genes involved in the cell-division cycle (CDC), both in eukaryotes and prokaryotes. Recently, an interesting controversy regarding the accuracy and reliability of synchronization methods using

whole-culture versus Helmstetter's 'baby machine' has been raised in this journal [1,2]. This letter does not discuss the benefits and drawbacks of both methods but proposes a new tool to the scientific community requiring truly synchronized eukaryotic cells for CDC studies: the 'self-synchronized' cultures of microalgae.

Microalgae are photosynthetic eukaryotic microorganisms that exhibit a naturally 'phased' cell division, which occurs only during a particular time of the day, generally

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Available online 13 June 2005