A Platform for Reverse Genetics in Endothelial Cells

Fatma O. Kok, Nathan D. Lawson

The recent development of programmable nucleases has the potential to revolutionize biological sciences. In particular, the Cas9 nuclease, which functions as a component of the clustered regularly interspaced short palindromic repeats (CRISPR) system in bacteria,1 has proven to be a highly efficient tool for genome editing in a wide range of model organisms, including mouse, zebrafish, Drosophila, and Caenorhabditis elegans.2–5 Application of Cas9 also allows straightforward genetic manipulations in cultured cells and is efficient enough to perform genome-wide screens in cell lines.6–7 However, applying genome editing tools in this manner in vascular biology is challenging because of the widespread use of primary cell cultures, which have a limited lifespan and are difficult to use for clonal analysis. Fortunately, studies by Abrahimi et al in this issue describe several solutions that facilitate the application of Cas9 in cultured endothelial cells. Together, these technical advances provide a valuable platform to enable straightforward and robust reverse genetic analysis in endothelial cells.

The discovery of RNA interference8 and its derived or related applications (eg, short hairpin RNAs and small interfering RNAs) revolutionized the analysis of gene function in both model organisms and cell culture.9 However, these approaches often only partially decrease gene function and can have widespread off-target effects leading to false-positives.10 Accordingly, Abrahimi et al chose to apply a more definitive genetic approach by directly introducing targeted deletions into the endothelial genome. In this case, they used the CRISPR/Cas9 system in which small RNA guides (referred to hereafter as sgRNA) target the Cas9 nuclease to a specific genomic locus.1 On binding of the sgRNA/Cas9 complex to target DNA, Cas9 nuclease introduces a double-stranded break. In general, this break is repaired by nonhomologous end joining leading to mutations at the target site. The work of Abrahimi et al11 is the first reported use of CRISPR/Cas9 technology in cultured human endothelial cells to introduce targeted mutations. To achieve this task, they used a previously established and efficient CRISPR/Cas9 lentiviral system to introduce Cas9 and guide RNA sequences instead of transient transfection. They also incorporated a tetracycline-inducible promoter to limit Cas9 expression to avoid the accumulation of possible off-target mutations in the genome at later passages. Together, these components clearly prove to be a robust system to introduce a desired deletion at a target of interest in endothelial cells.

Introduction of sgRNA and Cas9 into cells leads to a broad spectrum of distinct target site lesions within the cell population. This initial step usually requires selection for sgRNA and Cas9 expressing cells for highest lesion efficiencies. Subsequently, it is important to isolate clonal lines bearing a particular lesion of interest. Unfortunately, the most commonly used cell type in the vascular biology field—human umbilical vein endothelial cells—has numerous limitations that make application of Cas9 challenging. For example, they have limited replicative lifespan, are hard to transfect, and are not capable of forming stable clonal progeny derived from single cells.12,13 However, endothelial progenitor cells isolated from umbilical cord blood or adult peripheral blood display phenotypic, molecular, and functional characteristics that are similar to human umbilical vein endothelial cells, but display more robust proliferation potential and the possibility for clonal expansion.14–16 These last 2 characteristics solve a major hurdle in applying Cas9 for genome editing. Therefore, as a starting point in this study,11 Abrahimi et al used endothelial cell colony forming units grown from umbilical cord blood for their target cell type. They find that endothelial cell colony forming units can maintain general endothelial identity throughout the various manipulations to which they are subjected during genome editing, including introduction of Cas9 and sgRNA, induction of subsequent genetic lesions, and clonal isolation. In this latter step, the authors borrowed from the stem cell biologists’ toolbox to apply a small molecule inhibitor, specifically Rho-associated protein kinase inhibitor, to facilitate isolation of clonal lines. Despite all of these manipulations, endothelial cell colony forming units bearing a desired targeted lesion could be maintained up to 10 further passages after cloning. This is more than enough for a multitude of cellular, molecular, and in vivo analysis. Thus, endothelial cell colony forming units can be a valuable cellular platform for facilitating application of gene knockout technology in endothelial cells.

As a proof of concept, Abrahimi et al11 targeted CIITA (class II, major histocompatibility complex, transactivator), which regulates class II major histocompatibility complex expression in a interferon-γ–dependent manner. They monitored the efficiency of Cas9-induced mutations in bulk culture by performing fluorescent-activated cell sorting (FACS) for class I and class II major histocompatibility complex surface markers in the presence and the absence of interferon-γ. Based on
this straightforward assay (ie, loss of human leukocyte antigen [HLA]-DR expression), they estimated that biallelic CIITA disruption via CRISPR/Cas9 activity was ≈40%. Consistent with the loss of CIITA, these cells failed to activate allo-active CD4+ memory T cells. This immunogenic phenotype could be rescued by overexpression of CIITA in CIITAnull cells confirming that the observed phenotype is the result of loss of CIITA but not because of random off-target mutations. Just as importantly, CIITAnull endothelial cells retained viability, expressed endothelial markers, and exhibited normal endothelial biological and functional properties, such as acetylated low-density lipoprotein uptake, endothelial nitric oxide synthase expression, and ability to self-assemble into blood vessels both in vitro and in vivo.

It is clear that the CRISPR/Cas9 system has several advantages over existing molecular methods to alter various gene functions making it a powerful genomic engineering tool to study biological functions. For example, as shown in Abrahimi et al and others,1,17-19 Cas9 can be used to induce homozygous loss-of-function mutations in the genome while siRNAs can only reduce the translated protein levels by targeting RNA transcript. This feature can be especially beneficial where partial knockdown of the gene is not enough to interfere with its function. Moreover, different from RNA interference technology, Cas9 can be used to target noncoding regions of the genome, such as enhancers and microRNAs. Cas9 may also be rescued by overexpression of CIITA in CIITAnull cells with the loss of CIITA, these cells failed to activate allore sponsive in transplanted cells. Those problems can be partially resolved using inducible promoters or possibly transfection of protein/sgRNA complexes. At the same time, ex vivo edited cells can be sequenced for off-target mutations before therapeutic use. Despite these potential hurdles, the current studies clearly demonstrate the power of genome editing and its potential to facilitate robust genetic analysis in endothelial cells.

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**References**


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