Efficient Gene Disruption in Cultured Primary Human Endothelial Cells by CRISPR/Cas9

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Rationale: The participation of endothelial cells (EC) in many physiological and pathological processes is widely modeled using human EC cultures, but genetic manipulation of these untransformed cells has been technically challenging. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) technology offers a promising new approach. However, mutagenized cultured cells require cloning to yield homogeneous populations, and the limited replicative lifespan of well-differentiated human EC presents a barrier for doing so.

Objective: To create a simple but highly efficient method using CRISPR/Cas9 to generate biallelic gene disruption in untransformed human EC.

Methods and Results: To demonstrate proof-of-principle, we used CRISPR/Cas9 to disrupt the gene for the class II transactivator. We used endothelial colony forming cell–derived EC and lentiviral vectors to deliver CRISPR/Cas9 elements to ablate EC expression of class II major histocompatibility complex molecules and with it, the capacity to activate allogenic CD4+ T cells. We show the observed loss-of-function arises from biallelic gene disruption in class II transactivator that leaves other essential properties of the cells intact, including self-assembly into blood vessels in vivo, and that the altered phenotype can be rescued by reintroduction of class II transactivator expression.

Conclusions: CRISPR/Cas9-modified human EC provides a powerful platform for vascular research and for regenerative medicine/tissue engineering. (Circ Res. 2015;117:121-128. DOI: 10.1161/CIRCRESAHA.117.306290.)

Key Words: clustered regularly interspaced short palindromic repeats • endothelial cells • genetic engineering • genetic techniques • immunologic techniques

Endothelial cells (EC) are critical participants in and regulators of numerous processes, including inflammation, immunity, wound healing, coagulation, fibrinolysis, macromolecular transport, permselectivity, and organ perfusion. Animal models have offered important insights into EC biology, but cultured EC are widely used to dissect processes that are difficult to analyze in whole-animal studies. The majority of these in vitro experiments use untransformed human EC cultures, the most common system being human umbilical vein endothelial cells (HUVEC), described in >20,000 publications listed in PubMed since the original reports of successful HUVEC culture in 1973 and in >1,400 publications in 2014 alone. Cultured human EC offer 2 clear advantages over using cultured mouse EC: (1) they can be serially passaged without transformation, thereby avoiding a process that frequently alters their phenotypes, and (b) their properties differ from those in mouse EC thereby making study results more applicable to human biology and disease. For example, human EC can activate alloantigen-reactive memory CD4+ T cells to elicit effector functions, a property requiring both expression of class II major histocompatibility complex (MHC) molecules and the expression of CD58 (also known as lymphocyte function–associated antigen-3), a major positive costimulatory molecule not found in mice. In contrast, mouse EC, lacking CD58, only activate CD4+ regulatory T cells, leading to different outcomes about the roles played by EC in transplantation. Specifically, the ability of human EC to activate effector memory CD4+ T cells in vivo can explain why cell-mediated rejection of vascularized human allografts can occur despite deletion of professional antigen presenting cells (passenger leukocytes), whereas mouse grafts are significantly protected by the same approach. These immunologic functions of human EC are also a concern for the immune response to tissue-engineered grafts constructed from allogeneic sources of cells.

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Despite the importance of evaluating EC functions with untransformed human EC cultures, they are typically hard-to-transfect, have a limited replicative lifespan, and are not amenable to cloning after stable genetic manipulation. Antisense oligonucleotides or RNAi have been applied to the study of EC, but knockdown is often incomplete and, in the case of small
CRISPR/Cas9 Mutagenesis

The tetracycline-inducible Cas9 lentiviral vector (pCW-Cas9, produced by Eric Lander and David Sabatini, and available through Addgene as plasmid: #50661) was used to transduce ECFC-derived EC to create stable inducible Cas9 expressing EC. Guide RNA targeting CIITA and CD58 genes were identified using the online optimized software http://crispr.mit.edu. These guides were cloned into pLX-single guide RNA (sgRNA) vector (also produced by Eric Lander and David Sabatini, and available through Addgene as plasmid: #50662) and transduced into TetOn-Cas9-EC. Loss-of-function was identified by fluorescence-minus-one staining of human leukocyte antigen (HLA)-DR (in the case of CIITA mutagenesis) and CD58 (in the case of CD58 mutagenesis), and cells were isolated by single-cell fluorescence-activated cell sorter (FACS) and seeded into microwell tier plates containing Y-27632 (Sigma) for clonal expansion and further analysis.

Confirmation of CIITA and Off-Target Mutagenesis

CIITA mutagenesis was confirmed by immunoblotting for CIITA as well as loss-of-function of transcriptional activity by FACS and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for HLA-DR. CIITA target locus and likely off-target site were sequenced to characterize mutations. Refer to expanded Methods available in Online Data Supplement.

Phenotypic Analysis of CIITA and EC

FACS-isolated CIITAmut EC were phenotypically compared with wild-type (WT) EC by flow cytometry for endothelial-specific surface markers (CD31 and blood group H antigen), viability, expression of endothelial nitric oxide synthase (eNOS), acetylated low-density lipoprotein uptake, and tumor necrosis factor-α and interferon-γ (IFN-γ)–induced expression of intercellular adhesion molecule–1, E-selectin and programmed cell death ligand 1 (PD-L1), barrier integrity measured by transendothelial electric resistance, and analyzed for vascular endothelial (VE)-cadherin expression and cord formation by epifluorescence microscopy. Refer to expanded Methods available in Online Data Supplement.

Protein Gel Implants

All animal protocols were approved by the Yale Institutional Animal Care and Use Committee. Collagen protein gels containing either WT or CIITAmut EC were implanted subcutaneously in the abdominal wall of female 6- to 8-week-old C.B-17/SCID-beige mice (Taconic Biosciences, Germantown, NY) and analyzed after 14 days for formation of perfused human microvessels. In some experiments, recombinant human IFN-γ was injected into mice for detection of HLA-DR+ EC-lined vessels. Refer to expanded Methods available in Online Data Supplement.

Mixed Lymphocyte Reactions and CIITA Rescue

WT or CIITAmut EC were cocultured with allogeneic memory CD4+ T cells labeled with carboxyfluorescein succinimidyl ester (CFSE) dye. At 24 hours, T cell elaborated interleukin-2 and INF-γ were measured by enzyme-linked immunosorbent assay (E Biosciences) as a measure of early activation. At 7 days, dilution of CFSE dye and expression of HLA-DR were analyzed for proliferation and late activation marker. In some experiments, HLA-DR expression in CIITAmut EC was restored by CIITA retrovirus before mixed-lymphocyte reaction.

Statistics

All data are expressed as mean±SD. Statistical comparisons were made using Student t test or 1-way ANOVA with Bonferroni post hoc test as appropriate. P values of 0.05 or less were considered statistically significant. All results were computed using Prism v6.0 (GraphPad Software, Inc, La Jolla, CA).

Results

EC cultures from ECFC have been extensively characterized and they are essentially indistinguishable from HUVEC with the single exception that they have a much greater replicative lifespan before displaying features of senescence. They are readily cultured from umbilical cord blood and, of particular
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Relevance for this study, ECFC-derived EC display the same immunologic properties as HUVEC isolated from the same umbilical cord.9 Second, we used lentiviral vectors to introduce tetracycline-inducible Cas9 and constitutively expressed RNA guide strands.17 Lentiviral transduction of EC is well tolerated, highly efficient (routinely exceeding 60% after a single round of infection), and the use of a tetracycline-inducible promoter to control Cas9 limits the exposure of the cells to possible accumulation of random off-target mutations by continuous overexpression of Cas9. Third, we optimized cloning conditions to routinely produce multiple different colonies with distinct biallelic deletions by addition of a rho-associated protein kinase selective inhibitor to microwells seeded with single EC.19,20

Transduction of Inducible Cas9 in ECFC-Derived EC

Early passage ECFC-derived human EC cultures were transduced with a tetracycline-inducible FLAG-tagged Cas9 lentiviral vector as described in the Methods (Online Figure IA). After a single round of transduction, >95% of the cells remained viable and were FLAG-Cas9 negative in the absence of doxycycline. However, about 50% of the cells had detectable levels of Cas9 after doxycycline treatment (Online Figure IB), and the level of FLAG-Cas9 expression increased as a result of increased doxycycline ≤10 μg/mL (Online Figure IB). The cultures were then transduced with a second lentivirus that constitutively expressed an sgRNA designed to target an exonic region shared by all known splice variants of CIITA (Online Figure IC).23 CIITA is an IFN-γ–inducible transactivator of class II MHC but not class I MHC molecule expression and identification of loss-of-function can simply be assessed through flow cytometric analysis of surface expression of class I MHC (HLA-A,B,C in humans) and class II MHC (HLA-DR being the most highly expressed locus) before and after IFN-γ stimulation. Although essentially 100% of unmodified EC upregulated both class I and II MHC molecules on IFN-γ stimulation, delivery of CIITA-specific sgRNA followed by doxycycline treatment resulted in 3 distinct subpopulations differing in levels of induced class II MHC: HLA-DRhi, which are indistinguishable from unmodified EC, HLA-DRmid, which express reduced levels of MHC II, and HLA-DRneg, which express no MHC II molecules (Figure 1A). Because all 3 populations expressed equivalent levels of MHC I in response to IFN-γ stimulation, the HLA-DRneg subpopulation likely represent EC biallelic loss-of-function gene disruption of CIITA. This result was reproducible with several different donor EC cultures and with different CIITA-specific sgRNA sequences (Figure 1A; Online Figure IC). CIITA mutagenesis was minimal in the absence of doxycycline, which is consistent with reduced levels of detectable Cas9 expression.

Isolation and Characterization of CIITAnull EC

Because the cells that likely bore loss-of-function mutations in CIITA were detectable by surface staining, we could use FACS sorting of viable cells to isolate CRISPR/Cas9-modified HLA-DRneg EC for further characterization. qRT-PCR analysis of FACS sorted IFN-γ–stimulated unmodified EC and the HLA-DRneg EC subpopulation revealed 99.2% reduction of HLA-DRA

Figure 1. High efficiency disruption of class II transactivator (CIITA) by clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) in human endothelial cells (EC). A, Unmodified EC stimulated with interferon-γ (IFN-γ) increase expression of major histocompatibility complex (MHC) class I (human leukocyte antigen [HLA]-A,B,C) and class II (HLA-DR), whereas a high proportion of EC transduced with TetOn-Cas9/sgCIITA vectors upregulate MHC class I but show either reduced (HLA-DRneg) or complete loss of MHC class II (HLA-DRneg) expression. B, qRT-PCR analysis of fluorescence-activated cell sorter (FACS) isolated wild-type (WT) and HLA-DRneg EC for CIITA, HLA-DR, and CXCL10 transcripts. C, HLA-DRneg EC clonally sorted by single-cell FACS, expanded, and sequenced across single-guide RNA (sgRNA) target site demonstrates biallelic indel mutations in CIITA but not in the likeliest off-target site (SLC6A9). Representative plots shown from multiple donors from 3 independent experiments.
transcript in the latter, consistent with CIITA loss-of-function, but equivalent levels of transcripts for CIITA, as well as CXCL10, another IFN-γ–stimulated gene (Figure 1B). The preservation of CIITA transcription is not surprising, given that Cas9-mediated mutagenesis would be expected to insert frameshift or structural mutations that interfere with protein translation and function but not signals to terminate RNA transcription. We then isolated individual clones to further characterize mutations of CIITA in HLA-DRneg EC. Previous reports have described the use of the rho-associated protein kinase inhibitor Y-27632 to enhance the recovery and cloning of sensitive primary cells.19,20 We used FACS sorting for single-cell isolation of ECFC-derived EC and observed improvement in the cloning efficiency of HLA-DRneg EC in cultures supplemented with Y-27632 (Online Figure ID). Notably, we were able to inhibit IFN-γ–induced expression of MHC II in a subset of primary HUVEC using the same approach (Online Figure II), but single-cell FACS isolation followed by culture in medium supplemented with Y-27632 yielded few colonies and these, in contrast to our experience with ECFC-derived EC, could not be further expanded. Clonally expanded HLA-DRneg clones had a normal karyotype and could be grown for at least 10 passages after sorting before showing morphological evidence of cell senescence, similar to unmodified ECFC-derived EC (data not shown). After expansion, genomic DNA isolated from HLA-DRneg EC clones was used to amplify a region of CIITA containing the CIITA-specific sgRNA target site. Consistent with loss-of-function, randomly selected HLA-DRneg EC clones derived from 3 distinct donors were all confirmed to have biallelic indels of between 1 and 23 bp at the predicted CIITA locus with a bias toward deletions as previously reported for CRISPR/Cas9 gene disruption (Figure 1C).17 In addition, sequencing of the highest scoring putative off-target coding site in SLC6A9 revealed no mutations in any of the isolated HLA-DRneg clones (Figure 1C). We also confirmed, by Western blot analysis, ablation of both CIITA and downstream HLA-DRα expression in expanded HLA-DRneg clones, which were otherwise preserved in WT clones (Online Figure ID). Assuming that all of the cells which no longer increased HLA-DR in response to IFN-γ had biallelic deletions, then the FACS data suggest that this approach produced biallelic gene disruption in >40% of the individual EC (Figure 1A). To ascertain the generalizability of this method, we also targeted another gene, CD58, and observed a similar efficiency in loss of expression with no detected off-target mutation at the highest scoring coding off-target site (Online Figure IVA and IVB). Importantly, we could readily disrupt both genes in the same EC by simultaneously transducing the cultures with lentiviral constructs encoding different sgRNAs (Online Figure IVC).

Figure 2. Class II transactivator (CIITA)null endothelial cells (EC) retain their characteristic endothelial identity. A, Fluorescence-activated cell sorter (FACS) isolated CIITAnull EC expanded for 2 weeks remain refractory to interferon-γ (IFN-γ)–induced upregulation of major histocompatibility complex II but are otherwise indistinguishable from unmodified (wild-type [WT]) EC with respect to surface marker expression (platelet endothelial cell adhesion molecule 1 [PECAM-1], blood group H antigen), (B) viability, (C) formation of vascular endothelial-cadherin positive cell–cell lateral borders, and (D) characteristic tumor necrosis factor-α (TNF-α) and IFN-γ responses pooled from 3 independent donors. Scale bars, 50 μm. HLA indicates human leukocyte antigen; and ICAM-1, intercellular adhesion molecule–1.
EC maintained high levels of expression of the EC markers PECAM-1 (platelet endothelial cell adhesion molecule 1) (CD31) and blood group H antigens, the latter detected with Ulex Europaeus Agglutinin-1, and remained refractory to IFN-γ-induced expression of MHC II (Figure 2A). Both WT and CIITA<sup>-null</sup> EC were viable in culture (Figure 2B), maintained equivalent levels of eNOS expression as well as the ability to take up acetylated low-density lipoprotein (Online Figure VA and VB). When grown to confluence, both WT and CIITA<sup>null</sup> EC formed VE-cadherin positive cell–cell lateral borders (Figure 2C) as well as formed equivalent barriers that reversibly respond to thrombin (Online Figure VC andVD), suggesting preservation of key functions of cultured EC. Although these cells were refractory to IFN-γ–induced expression of MHC II (Figure 2A), they responded with similar kinetics and magnitude to tumor necrosis factor-α and IFN-γ induction of E-selectin, intercellular adhesion molecule–1, and PD-L1 (Figure 2D) expected of cultured EC. The singlemost characteristic feature of EC is their ability to self-assemble into blood vessels. When suspended and cultured in a 3-dimensional collagen matrix, CIITA<sup>null</sup> EC spontaneously formed cords that underwent vacuolization, an early step of lumen formation (Figure 3A), again in a manner indistinguishable from unmodified WT EC. CIITA<sup>null</sup> EC suspended in collagen protein matrix and implanted subcutaneously into the abdominal wall of SCID/bg mice<sup>24</sup> formed stable human EC-lined vessels that inosculated with host vessels, recruited murine smooth muscle α-actin positive supporting mural cells, and became perfused with murine blood (Figure 3B). Comparison of the number of perfused vessels formed by CIITA<sup>null</sup> EC to those from unmodified EC in the same host revealed no significant differences, suggesting CIITA-ablation in EC by CRISPR/Cas9 does not affect the intrinsic in vivo vessel-forming capability of these cells. Finally, consistent with in vitro results suggesting CIITA<sup>null</sup> EC are refractory to IFN-γ–induced expression of MHC II, perfused conduits formed from unmodified EC expressed MHC II on challenge with IFN-γ, whereas conduits derived from CIITA<sup>null</sup> EC implanted in the same mouse but on the contralateral side did not (Figure 3C).

**Immunogenic Function of CIITA<sup>null</sup> EC**

To demonstrate the use of Cas9-mediated gene disruption in untransformed human EC, we analyzed the immunologic functions of the modified cells. Immunologic rejection of differentiated allogeneic cells is a major hurdle for therapeutic applications of ECFC-derived EC in regenerative medicine because human EC, unlike mouse EC, are capable of initiating allogeneic CD4<sup>+</sup> T-cell responses as a consequence of direct presentation of non–self forms of class II MHC molecules. In particular, coculturing of IFN-γ–treated human EC with allogeneic CD4<sup>+</sup> memory T lymphocytes results in T-cell activation as indicated by expression of activation markers, including MHC II, cytokine production, and

![Figure 3. Class II transactivator (CIITA)<sup>null</sup> endothelial cells (EC) retain ability to form vessels in vitro and in vivo. A. CIITA<sup>null</sup> EC, like unmodified (wild-type [WT]) EC, spontaneously assemble into cord-like structures (outlined) in three-dimensional culture in vitro at 24 hours. B. CIITA<sup>null</sup> EC form perfused vessels (arrows) as detected by hematoxylin and eosin (H&E) that are lined by human EC (identified by Ab reactive with human CD31 and human leukocyte antigen [HLA]-A,B,C with insets of murine vessels to show species specificity) and that recruit host (smooth muscle [SM] α-actin expressing) mural cells when implanted in SCID/bg mice like unmodified WT EC and (C) like EC in vitro, perfused vessels formed from CIITA<sup>null</sup> EC are refractory to interferon-γ (IFN-γ)–induced expression of major histocompatibility complex (MHC) II in vivo as detected by immunofluorescence. Representative figures and means±SD from (A) 4 gels, (B and C) 9 mice bearing WT and CIITA<sup>null</sup> implants. Scale bar (A) 25 μmol/L and (B and C) 50 μmol/L.](#)
Although CIITA-null EC are refractory to IFN-γ-induced expression of MHC II (Figure 2A), transduction with a retrovirus expressing a wild-type copy of CIITA restores MHC II expression (Figure 4A). Consistent with CD4+ restriction to MHC II on EC, we found that ablation of CIITA by CRISPR/Cas9 in primary EC results in concomitant loss of the ability to activate alloreactive CD4+ memory T cells as measured by secretion of interleukin 2 (IL-2) and interferon-γ (IFN-γ) produced in 24-hour supernatants, as well as carboxyfluorescein succinimidyl ester (CFSE) dilution and human leukocyte antigen (HLA)-DR activation markers at 7 days. Representative plots and mean±SD from n=4 replicates. Similar results were seen in 3 independent experiments. LZRS is empty retroviral expression vector. Asterisks in B and C indicate statistically significant difference by post hoc Bonferroni correction.

**Discussion**

Studies examining the role human EC play in regulating physiological and pathological processes have extensively used primary human EC cultures. The range and power of this approach can be greatly extended by the application of genetic alteration using CRISPR/Cas9, but this has been difficult because of the limited replicative lifespan of such cells, the difficulty of their transfection, and their inability to be cloned. In this report, we demonstrate an approach to achieve high-efficiency gene disruption in primary EC using the CRISPR/Cas9 system. First, instead of the widely used HUVEC cultures, we used umbilical cord blood ECFC-derived EC. These cells have greatly increased replicative capacity and are otherwise indistinguishable from HUVEC, including the capacity to spontaneous self-assemble into vessels in vivo. They are derived from the same source as HUVEC, that is, umbilical cords, and should thus be as readily accessible to academic laboratories that isolate their own primary cells. They are not yet available from commercial vendors, but we predict that if they were to become so, their enhanced replicative lifespan would make them a more attractive alternative to HUVEC for most applications. Second, we used lentiviral transductions to introduce Cas9 coding sequences and guide strands instead of inefficient plasmid transfection or excessive overexpression that is characteristic with adenoviral vectors. Although lentiviral vectors have the potential for insertional oncogenesis, their efficiency at gene transduction make them an extremely simple and useful vehicle for Cas9-based mutagenesis in EC. We favor lentiviral or retroviral gene transfer over that of adenovirus for immunologic studies because the latter produce viral proteins that can trigger unwanted immune responses which lead to elimination of the transduced cells. The availability of a single vector, tetracycline-inducible system enables temporally limiting the period of Cas9 expression, which may help minimize accumulation of random off-target mutations after clonal isolation. The extent of the human genome that is accessible to CRISPR/Cas9 mutagenesis is presently unclear, but we were able to easily achieve 40% biallelic gene disruptions of the 2 genes we targeted in cultured

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**Figure 4.** Loss of the ability of class II transactivator (CIITA)null endothelial cells (EC) to activate allogeneic CD4+ memory T cells is rescued by CIITA transduction. 

A. Expression of class II major histocompatibility complex (MHC) molecules in CIITA-null EC is rescued upon reintroduction of functional copy of CIITA by retroviral transduction. CIITA-null EC lose ability to activate alloreactive memory CD4+ T cells, which is rescued with CIITA retrovirus, as measured by (B) quantitative enzyme-linked immunosorbent assay for interleukin 2 (IL-2) and interferon-γ (IFN-γ) produced in 24-hour supernatants, as well as (C) carboxyfluorescein succinimidyl ester (CFSE) dilution and human leukocyte antigen (HLA)-DR activation markers at 7 days. Representative plots and mean±SD from n=4 replicates. Similar results were seen in 3 independent experiments. LZRS is empty retroviral expression vector. Asterisks in B and C indicate statistically significant difference by post hoc Bonferroni correction.
human EC. Our own experience with short hairpin RNA suggests that knockdown is often incomplete and thus less definitive than gene disruption as a research tool. Furthermore, CRISPR/Cas9 can readily be adapted for gene mutation or correction, which is not possible with short hairpin RNA. Third, we used transient exposure to a rho-associated protein kinase inhibitor to improve the efficiency of EC cloning. This step is necessary to obtain uniformly modified populations, especially when FACS sorting cannot be used for isolation of living cells, for example, when the cell surface is unaffected by the genetic change. Cloning will be particularly important if CRISPR/Cas9 is used to alter rather than simply disrupt an endogenous gene.

To demonstrate the use of our approach, we chose to study CITA, the master regulator of MHC II expression, and interrogate the activation of alloreactive CD4+ memory T lymphocytes, a biologically important capacity of human EC that is not observed in mice.4,5,25 The relative ease with which a specific gene, or multiple genes, can be targeted for Cas9-mediated genomic perturbation provides an opportunity to study the effect of loss-of-function mutations in other genes that may be relevant to EC regulated processes, including vasculogenesis, barrier maintenance, fibrinolysis, and leukocyte recruitment, in ways that were not technically feasible before. Although off-target effects remain a concern, in this report, we temporally limited expression of Cas9 through use of a tetracycline-inducible promoter, used judicious selection of CITA-specific sgRNA sequences, ascertained that other major EC functions were intact, and demonstrated phenotypic rescue by reintroduction of CITA. Although off-target mutations at loci containing ≥3 mismatches are rare2,26 and often may not have functional consequences, single-cell cloning of ECFC-derived EC also permits screening for appropriate clones through polymerase chain reaction amplification and direct sequencing of putative off-target sites. The use of other Cas9 variants, including nickases27 or catalytically inactive variants fused to repressors or activators28,29 may also provide useful tools for human EC biology.

The intrinsic ability of human EC to self-assemble into vessels has been used to promote vasculogenesis in several preclinical models of ischemic injury to tissue engineer vessel replacements as well as to promote vascularization in larger bioengineered grafts.34,30–34 Although these results are promising, allogeneic sources of EC may provoke cell-mediated immune rejection.9 As demonstrated in this report, genetic ablation of CITA eliminated surface class II MHC expression but did not compromise the ability of EC to self-assemble into vessels. The application of genome-editing technologies like CRISPR/Cas9 to modulate human cell behavior opens a range of exciting possibilities in regenerative medicine, including methods to reduce endothelial immunogenicity and allow the use of allogeneic EC as a cellular therapy or in tissue engineering.

In summary, we established a method for high efficiency gene disruption in untransformed human EC in a manner that can be repeated to ablate expression of additional genes in the same cells. We think that our approach to combine the use of readily cultured human EC from ECFC, lentiviral transduction with CRISPR/Cas9 vectors and enhanced cell cloning efficiencies will greatly expand the range of studies and applications that can be performed using human EC.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Human endothelial cells are an important tool for understanding many biological processes that are not well reproduced in animal models. However, these cells have been difficult to modify genetically. Recent development of CRISPR/Cas9 methodology offers an opportunity to apply genetic engineering to cultured human endothelial cells, but the approach is limited by technical barriers. We present a highly efficient method of CRISPR/Cas9 gene ablation in cultured human endothelial cells using 3 technical advances: efficient lentiviral vectors, cultures derived from cord blood endothelial colony forming cells that display extended replicative lifespan, and efficient cell cloning conditions. As proof-of-principle, we show how our approach can reduce the capacity of human endothelial cells to activate a T cell-mediated immune response to nonself-derived endothelial cells, the initiating event in human organ transplant rejection. Importantly, the modified cells retain all other endothelial characteristics, including the ability to form blood vessels, suggesting the possibility of generating engineered tissues that may evade immune recognition. This general approach can be used for many other purposes and represents a unique application of the CRISPR/Cas9 technology to vascular biology.

- Genetic modification of cultured cells is a powerful approach for dissecting mechanisms underlying biological processes or for modifying cell functions that until now has been difficult to apply to human endothelial cells.

- Genetic engineering using clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 nuclease (Cas9) is a new method for modifying cells that has not previously been applied to human endothelial cells.

What New Information Does This Article Contribute?

- We describe a simple and highly efficient method for using CRISPR/Cas9 technology to modify cultured human endothelial cells.

- As proof-of-principle, we show how this technique can be used to reduce a human immune response to nonself human endothelial cells by selectively deleting specific genes that encode proteins involved in T-cell activation without altering other properties of human endothelial cells.

- The specific changes we describe might be important for using these cells in therapeutic applications, such as organ repair or tissue engineering.
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Detailed Methods

Isolation of ECFC
Umbilical cord blood was obtained with informed consent under a protocol approved by the Yale Human Investigation Committee. ECFC were differentiated from cord blood mononuclear cells in vitro as “late outgrowth” cells, as previously described. Briefly, umbilical cord blood collected immediately following elective caesarean section was anti-coagulated with heparin and enriched for mononuclear cells by density centrifugation using Lymphocyte Separation Medium (MP Biomedicals, Santa Ana, CA) per manufacturers instructions. These cells were then plated onto gelatin-human-plasma fibronectin (0.1%, J.T. Baker, Phillipsburg, NJ and 20 ug/ml, Millipore, Temecula, CA, respectively) coated tissue culture plates with EGM-2 media supplemented with 10 ng/ml VEGF (Lonza, Walkersville, MD). Non-adherent cells were removed by washing after 4d. Colonies of proliferating, differentiated cells were typically identified at 7-10d, at which time the media was changed to EGM-2/15% FCS for expansion. ECFC-derived EC Cultures were serially propagated in gelatin (Sigma-Aldrich, St. Louis, MO) coated tissue culture flasks with EGM-2/5% FBS (Lonza, Walkersville, MD). After transduction with tetracycline-inducible Cas9 lentiviral vector, cultures were maintained under the same medium using tetracycline-free FBS (Clontech, Mountain View, CA) was used.

Isolation of memory T lymphocytes
PBMCs were collected with informed consent from anonymized healthy volunteer donors under a protocol approved by the Yale Human Investigation Committee. Mononuclear cells were further enriched by density gradient centrifugation of leukapheresis products using Lymphocyte Separation Medium according to the manufacturer's protocol. Purified PBMCs were cryopreserved in 10% DMSO-90% FBS in liquid nitrogen before use. CD4+ T cells were isolated from PBMCs using Dynabeads CD4+ Positive Isolation Kit (Invitrogen/Life Technologies, Carlsbad, CA) per manufacturer’s protocol. Naïve and activated T cells as well as monocytes were removed by negative selection using anti-CD45RA (Clone: H100, eBioscience, San Diego, CA) and anti-HLA-DR mAb (Clone: LB3.1, a gift from J. Strominger, Harvard University, Cambridge, MA) at a concentration of 5µg/ml for 20 min, washed twice, and magnetically depleted using pan-mouse IgG beads (Invitrogen). The population obtained by this procedure was routinely >98% HLA-DR- CD45RA CD4+ T lymphocytes by flow cytometry (data not shown).

Lentiviral vector construction
Guide RNA sites in CIITA exonic loci were identified using the online optimized design software at http://crispr.mit.edu. The highest scoring sgRNA, which had no off-target sequences with perfect matches in the human genome and the nearest coding off-target sites contained +3 mismatches, were used to create IDT gBlock segments composed of XhoI restriction site, U6 promoter, sgRNA target sequence, chimeric sgRNA scaffold, and Nhel restriction site (Integrated DNA Technologies, Coralville, IA). gBlocks were cloned into pLX-sgRNA (produced by Eric Lander & David Sabatini and available through Addgene as plasmid: #50662), which contained blasticidin resistance gene. The CIITA sgRNA targeting sequences used in this study include: GCTGAACCTGGTCGAGTTGA (sgCIITA1), GATATTGGCAATAAGCCTCCC (sgCIITA2), GTCAACTGGCGACCATGGC (sgCIITA3). Unless otherwise stated, sgCIITA2 was utilized for CIITA gene disruption. The CD58 targeting sequence: TGGTTGCTGGAGCAGC, which was prepped with a G nucleotide for efficient U6 transfection. A separate lentiviral vector encoding tetracycline-inducible FLAG-Cas9 (pCW-Cas9, also produced by Eric Lander & David Sabatini, and available through Addgene as plasmid: #50661) and puromycin resistance gene was used to create TetOn-Cas9-EC.

Lentiviral packaging and transduction
Lentiviral vector plasmids were co-transfected with psPAX2 (produced by Didier Trono, available as Addgene plasmid: #12260) and CMV VSV-G (produced by Robert Weinberg and available through Addgene as plasmid: #8454) packaging plasmids into human 293T (ATCC, Manassas, VA) cells using Lipofectamine 2000 (Invitrogen) per manufacturer’s protocol. Lentiviral supernatant was collected at 48 and 72h, filtered through 0.45 µM filter, and used to transduce EC in C-12 well plates at an MOI of 10 (Lenti-X p24 Lentiviral Titration Kit, Clontech) in the presence of 8 µg/ml polybrene (Sigma-Aldrich). TetOn-Cas9-EC were generated by transduction with pCW-Cas9 vector for 8h and after 24h, drug-selected with 1 µg/ml puromycin (Invitrogen) for 3d. Cas9 expression was confirmed by intracellular flow cytometric analysis using APC-conjugated antiFLAG mAb (Clone: L5, BioLegend, San Diego, CA) after doxycycline treatment. TetOn-Cas9-EC were then treated with lentiviral supernatant containing CIITA (sgCIITA2) or CD58 specific sgRNA vectors at an average MOI of 20 and 8 µg/ml polybrene for 8h. After 24h, cells were selected with 10 µg/ml blasticidin (Invitrogen) and 1 µg/ml doxycycline (Sigma-Aldrich) for 5d. When multiplexed, a 50/50 mixture of supernatants containing CIITA and CD58 sgRNA lentiviral vectors was used.

FACS Analysis and Isolation of CRISPR/Cas9-modified EC
After transduction and selection, EC were stimulated with IFN-γ (50 ng/ml, Invitrogen) to up regulate MHC II. EC were harvested with trypsin (TrypLE Express, Invitrogen) and subsequently stained with directly conjugated Pacific Blue anti-
CD31 (Clone: WM-59), FITC anti-HLA-A,B,C (Clone: W6/32), and APC anti-HLA-DR (Clone: LN3) mAb (all from eBioscience). CIITA loss-of-function was identified by CD31+ cells that increased expression of HLA-A,B,C and not HLA-DR, with the positive threshold defined by fluorescence minus one staining with CD31 and HLA-A,B,C labeled cells. This gate was then used to collect CIITA<sup>null</sup> EC using a 100 μm low-pressure nozzle on BD FACSaria II and then to deposit single cells into C-96 flat-bottom well plates containing either medium, medium supplemented with 10 μM ROCK-selective inhibitor Y-27632 (Sigma-Aldrich). After 24h, cells were re-fed with fresh EGM-2/5% FBS medium that was changed every other day. Colonies were scored after 14d and serially expanded into larger vessel sizes. To analyze phenotypic stability, HLA-DR<sup>neg</sup> EC or unmodified EC were FACS isolated and expanded in EGM-2/5% FBS for 2 weeks. These cells were then challenged with TNF-α (10 ng/ml, Invitrogen) or IFN-γ (50 ng/ml) and harvested at 0, 1, 6, 12, and 24h and stained with PE anti-E-selectin (CD62E, clone: 68-SH11, BD Pharrmingen, San Jose, CA), FITC ICAM-1 (CD54, clone: LB-2, BD Pharrmingen), PE PD-L1 (CD274, clone: MIH1, BD Pharrmingen), FITC PECA-1 (CD31, clone: WM-59, eBioscience) as well as APC HLA-DR (Clone: LN3, eBioscience), fluorescein labeled UAE-1 lectin (Vector Labs, Burlingame, CA), and eFluor 450 fixable viability dye (eBioscience) and analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, CA) with post-acquisition analysis using FlowJo software (FlowJo LLC, Ashland, OR). Unmodified and CIITA<sup>null</sup> EC were also stained for VE-cadherin (CD144, clone: F-8, Santa Cruz Biotechnology, with secondary stain: goat anti-mouse IgG Alexa-488, Life Technologies) and mounted on slides using mounting medium (Prolong Gold; Invitrogen), and examined by microscopy with an Axiovert 200M microscope (Carl Zeiss, Thornwood, NY).

q-RT-PCR
RNA from EC was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) and used to make cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) per manufacturer’s protocols. q-RT-PCR reactions were assembled with TaqMan 2x Gene Expression Master Mix (Applied Biosystems) and pre-developed Taqman gene expression probes and analyzed on a CFX96 Real-Time system using CFX Manager Software (Bio-Rad, Hercules, CA). Probes used in this study were purchased from Applied Biosystems: GAPDH (Hs99999905_m1), CIITA (Hs00172094_m1), HLA-DRA (Hs00219575_m1), and CXCL10 (Hs01124251_g1). Gene expression levels were normalized to GAPDH.

PCR and Sanger Sequencing
Genomic DNA was isolated from clonally expanded EC using QuickExtract DNA Extract Solution (Epicentre, Madison, WI) according to the manufacturer’s protocol. A 200bp segment containing the CIITA sgRNA (sgCIITA2) target site was amplified by PCR using Platinum SuperMix High Fidelity (Invitrogen) using primers CIITA<sub>forward</sub>: CACCAACCCTCTTTCCAGA, CIITA<sub>reverse</sub>: CCCCTTGCAATGATTTCTGT. The PCR amplicon was then column purified and subcloned into a TOPO TA vector using TOPO TA Cloning Kit (Invitrogen). Random colonies were picked and submitted for Sanger Sequencing using universal M13 forward and reverse sequencing primers at the W.M. Keck Sequencing Facility, Yale University. Same donor but unmodified EC were used as controls for comparison. The highest scoring off-target site by sgCIITA2 was GAAAAGTGCAATCGCTCCCCGAG (SLC6A9, 4 mismatches 3:5:7:12). To determine if this site was mutated, the following sequencing primers were used for sgCIITA2: SLC6A9<sub>forward</sub>: GCCCAGGTGGCCTTTCTAAA, SLC6A9<sub>right</sub>: TAGAAGGGAGGGATTTGCTA. For CD58 target site: CD58<sub>forward</sub>: GAACCTAGGGCTGGTGTG and CD58<sub>reverse</sub>: GTTCTCTGATCGGCAACC. To assess off-target cleavage the highest scoring off-target coding site TGACTGTGGGGGCACGGGGG (COL20A1, 3 mismatches in 3:4:12), the following sequencing primers were used: COL20A1<sub>left</sub>: CCTTCTTGCGCCTACTTC, COL20A1<sub>right</sub>: GAGGCTGGACTTCTCCTGAA.

Mixed lymphocyte-endothelial reactions
Unmodified or CIITA<sup>null</sup> ECs (1.5e5 cells/well) were plated into gelatin-coated wells of 24-well culture plates (Falcon; BD Biosciences) and treated with IFN-γ (50 ng/ml) (Invitrogen) where indicated. Purified memory CD4+ T lymphocytes were then added to each well (1.5e5 cells/well). All cultures were maintained in 5% CO2 at 37°C. The medium for co-culture consisted of RPMI 1640 supplemented with 10% FBS serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Supernatants collected from cocultures were assayed using an ELISA kit for human IL-2 or IFN-γ (Platinum ELISA kits, eBioscience), according to directions provided by the manufacturer. To measure proliferation by CFSE dilution, lymphocytes were stained with 250 nM CFSE (Molecular Probes/Life Technologies) for 20 min before coculture with EC and collected after 7d and stained with APC anti-CD4 (Clone: RPA-T4, eBioscience) mAbs and Pacific Blue anti-HLA-DR (Clone: LN3, eBioscience), and analyzed by flow cytometry. In some experiments, MHC II expression was restored by CIITA retrovirus as previously described. The retroviral supernatant was concentrated using 100KD Amicon Ultra Centrifugal Filters (EMD Millipore, Billerica, MA). The empty retroviral expression vector, LZRS, was used as a control (a gift from Garry Nolan, Stanford University, Stanford, CA).

Mice and Protein Gel Implants
All animal protocols were approved by the Yale Institutional Animal Care and Use Committee. Human microvessels were generated and implanted subcutaneously in the abdominal wall of female 6-8 week old C.B-17/SCID-beige mice (Taconic Biosciences, Germantown, NY) as previously described. Briefly, ECFC-derived EC were suspended in a rat tail type I collagen gel and 400ul of the cell suspension was gently poured into a single well of a 48-well tissue culture plate. The protein gel was polymerized at 37°C/5% CO2 and then implanted. Each mouse received bilateral gel implants: one
containing unmodified EC and the other containing CRISPR/Cas9 modified EC; mice were split into two cohorts defined by anatomic side of CIITA\textsuperscript{null} EC implant. Two weeks after implantation, animals were euthanized and the grafts harvested for analysis of human microvasculature. Gels and surrounding soft tissue were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5 \( \mu \)m thick sections were cut for H&E staining or immunostaining for pan-HLA-A,B,C (Clone: EM8R-5, Abcam, Cambridge, MA) human CD31 (BioGenex, Fremont, CA) and SM alpha-actin. Vessel number was quantified by number of perfused vessels, defined as containing murine erythrocytes, normalized to gel area and only vascularized implants were included for analysis. For challenge experiments, mice were given 400 ng recombinant human IFN-\( \gamma \) (Invitrogen) or PBS by subcutaneous injection every other day for an additional week. These gels were snap frozen in Tissue-Tek OCT (Sakura Finetek, Torrance, CA), cut in 5 \( \mu \)m thick cryosections, and subsequently fixed in acetone and stained with fluorescein labeled UAE-1 and HLA-DR (Clone: LB3.1, with secondary stain: goat anti-mouse IgG DyLight-649, Vector Labs) for detection of HLA-DR\textsuperscript{+} human EC-lined vessels within engrafted protein gels on a fluorescent microscope (Axiovert). Investigators were not blinded to allocation of implants either during the experiment or when accessing outcomes. Previous experience with the collagen implant model was used to determine the number of animals needed to obtain statistical significance\textsuperscript{1, 4}. From this experience, 9 mice were implanted as approximately 80% of implanted collagen/EC matrices inosculate with the murine vasculature and at minimum 5-6 matrices with functional vessels are needed in each group for statistical comparisons.

**Barrier Formation Assay**

Transendothelial electrical resistance (TEER) of EC monolayers was assessed by electrical cell-substrate impedance (ECIS; Applied Biophysics, Troy NY). Briefly, ECFC-derived EC were plated at two-thirds confluence on a fibronectin-coated 8-well array (#8W10E+, Applied BioPhysics). TEER measurements were obtained daily over 3 to 5 days to monitor increasing barrier integrity until EC monolayers reached a plateau. To initiate thrombin-induced changes in barrier function, thrombin (1 U/ml; GE Healthcare #27-0846-01, Pittsburgh PA) was introduced during real-time data acquisition without replacing the growth medium. Barrier function is presented as expressed in units of ohms per cm\textsuperscript{2} or normalized TEER (where 1.0 represents the basal TEER measurement immediately before adding thrombin).

**acLDL Uptake Assay**

EC were incubated with 10 \( \mu \)g/ml Dil-labeled acetylated low-density lipoprotein (Dil-acLDL, a gift from Carlos Fernandez-Hernando, Yale University, New Haven, CT) in complete EGM-2 for 4h at 37\( ^\circ \)C. Cells were washed 3x in PBS, briefly acid washed (1 minute, PBS/pH 2.5), detached with TrypLE Express and then analyzed by flow cytometry.

**Immunoblotting**

Cultured cells were washed in ice-cold PBS and lysed directly by adding 1x Laemmli buffer (Bio-Rad) containing protease inhibitor. Cell lysates were resolved by SDS-PAGE and proteins transferred to Trans-Blot Transfer Medium nitrocellulose (Bio-Rad), blocked in 5% BSA/TBST and stained with primary antibodies against CIITA (clone: 7-1H, Santa Cruz Biotechnology), HLA-DR\( \alpha \) (clone: DA6.147, Santa Cruz Biotechnology), or Hsp90 (clone: F-8, Santa Cruz Biotechnology). Membranes were stained with secondary goat anti-mouse Alexa-680 (Life Technologies) and visualized using Odyssey LI-COR system (LI-COR Biosciences, Lincoln, NE)

**References:**

Online Figures

Online Figure I. Doxycycline-induced mutagenesis, analysis and recovery of CIITA\textsuperscript{null} EC. (a) Schematic of isolation, transduction, selection and induction of TetOn-Cas9 ECFC-derived EC, (b) EC transduced with tetracycline-inducible Cas9 vector express high levels of Cas9 in the presence of doxycycline and remain viable, (c) comparison of three CIITA-specific sgRNA on inhibition of IFN-γ-induced expression of MHC class II in absence or presence of doxycycline, demonstrating reproducible Cas9-mediated ablation of CIITA that is minimal in the absence of doxycycline, and (d) transient culture with ROCK-selective inhibitor Y-27632 enhances recovery of FACS isolated CIITA\textsuperscript{null}.
Online Figure II. CRISPR/Cas9-mediated mutagenesis of CIITA in cultured HUVEC. Unmodified HUVEC stimulated with IFN-γ increase expression of MHC class I (HLA-A,B,C) and class II (HLA-DR), whereas a significant proportion of HUVEC transduced with TetOn-Cas9/sgCIITA vectors upregulate MHC class I but show either reduced (HLA-DR$_{\text{mid}}$) or complete loss of MHC class II (HLA-DR$_{\text{neg}}$) expression, suggestive of CIITA loss of function.
**Online Figure III.** CIITA gene disruption results in loss of both CIITA and HLA-DR protein expression. Individual clones of both WT and HLA-DR$^{\text{neg}}$ EC were isolated by single cell FACS, expanded and re-stimulated with 200 ng/ml IFN-γ for 24h and analyzed for HLA-DR by FACS (left) and CIITA as well as HLA-DRα by western blot (right).
Online Figure IV. High efficiency disruption of CD58 by CRISPR/Cas9. (a) CD58-specific sgRNA ablates surface expression of CD58 on EC whereas EC transduced with CIITA-specific sgRNA express levels equivalent to unmodified EC. (b) CD58<sup>neg</sup> EC clonally isolated, expanded and sequenced across sgCD58 target locus and likeliest off-target site (COL20A1) demonstrates bi-allelic indel mutations with no detected off-target mutagenesis in examined clones. (c) CIITA and CD58 sgRNA can be multiplexed together generate CD58<sup>neg</sup>HLA-DR<sup>neg</sup>CD31<sup>+</sup>. Results are representative from 2 independent experiments using distinct donors.
Online Figure V. CRISPR/Cas9-modified EC retain their characteristic endothelial identity. (a) WT and CIITA\textsuperscript{null} ECFC-derived EC express eNOS, (b) internalize acLDL, and (c) form equivalent barriers that are (d) responsive to thrombin-induced perturbation. * indicates statistical significance