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

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Abstract

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)/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 bi-allelic 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 (CIITA). We used endothelial colony forming cell (ECFC)-derived EC and lentiviral vectors to deliver CRISPR/Cas9 elements to ablate EC expression of class II MHC molecules and with it, the capacity to activate allogeneic CD4⁺ T cells. We show the observed loss-of-function arises from bi-allelic gene disruption in CIITA 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 re-introduction of CIITA expression.

Conclusions—CRISPR/Cas9-modified human EC provides a powerful platform for vascular research and for regenerative medicine/tissue engineering.

Keywords

CRISPR; Cas9; genetic engineering; endothelial cell; immunogenicity; CIITA; endothelial function; genetic techniques; immunologic technique

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AUTHOR CONTRIBUTIONS

P.A. and J.S.P. conceived the study and wrote the manuscript. P.A., W.G.C. and M.S.K. conducted the experiments. P.A., W.G.C., M.S.K., Y.Q., G.T., W.M.S., J.S.P. helped design experiments.

INTRODUCTION

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.¹ 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 over 20,000 publications listed in PubMed since the initial reports of successful HUVEC culture in 1973² and in over 1,400 publications in 2014 alone. Cultured human EC offer two clear advantages over using cultured mouse EC: (a) they can be serially passaged without transformation³, 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 MHC molecules and the expression of CD58 (also known as LFA-3)⁴, a major positive co-stimulatory molecule not found in mice.⁵ In contrast, mouse EC, lacking CD58, only activate CD4⁺ regulatory T cells,⁶ leading to very different outcomes regarding the roles played by EC in transplantation. Specifically, the ability of human EC to activate effector memory CD4⁺ T cells *in vivo*⁴ 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.^{7, 8} These immunological functions of human EC are also a concern for the immune response to tissue-engineered grafts constructed from allogeneic sources of cells.⁹

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 siRNA, of limited duration. Permanent gene disruption by CRISPR/Cas9 is a transformative technology that utilizes the RNA-guided Cas9 endonuclease to introduce DNA double stranded breaks which trigger error-prone repair pathways that can result in frame shift mutations.¹⁰ This approach has been increasingly applied to ova for generating animals or to transformed cell lines to yield clonal progeny, but not previously to untransformed differentiated human cells.^{11–17} Given the importance of EC in human-focused biomedical research, the application of gene editing technologies to untransformed human EC would be of enormous value and in this study we describe a simple and efficient approach to leverage the CRISPR/Cas9 system for primary EC studies.

To produce stable genetic alterations of differentiated human EC, we combined three previously described technical advances. First, we produced untransformed human EC cultures from outgrowth of endothelial colony forming cells (ECFC), also known as late outgrowth EC or endothelial progenitor cells, isolated from cord blood.¹⁸ Secondly, we used lentiviral vectors to introduce tetracycline-inducible Cas9 and constitutively expressed RNA guide strands.¹⁷ Third we optimized cloning conditions to routinely produce multiple

different colonies with distinct bi-allelic deletions.^{19, 20} The combination of these advances allows highly efficient and simple gene disruption in human EC. As proof-of-principle, we generated EC lacking the transcriptional activator *CIITA*, the master regulator of MHC II expression²¹, and demonstrate that these cells lose the ability to express class II MHC molecules, thereby eliminating their ability to activate allogeneic CD4+ T cells without altering their basic properties, including the capacity to self-assemble into vascular structures in vivo.

METHODS

An expanded Methods section is available in Online Data Supplement.

Cell isolation and culture

Human ECFC-derived EC were isolated and cultured from umbilical cord blood obtained with informed consent under a protocol approved by the Yale Human Investigation Committee. Human memory CD4+ T cells were isolated from adult PBMCs that were collected with informed consent by leukapheresis from anonymized healthy volunteer donors under a protocol approved by the Yale Human Investigation Committee.

CRISPR/Cas9 mutagenesis

The tetracycline-inducible Cas9 lentiviral vector (pCW-Cas9, also produced by Eric Lander & 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-sgRNA vector (produced by Eric Lander & David Sabatini¹⁷ and available through Addgene as plasmid: #50662) and transduced into TetOn-Cas9-EC. Loss-of-function was identified by fluorescence-minus-one staining of HLA-DR (in the case of *CIITA* mutagenesis) and CD58 (in the case of *CD58* mutagenesis) and cells were isolated by single-cell FACS and seeded into microwell titer 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 qRT-PCR for HLA-DR. *CIITA* target locus and likeliest offtarget site were sequenced to characterize mutations. Refer to expanded Methods for details.

Phenotypic analysis of *CIITA*^{null} EC

FACS-isolated *CIITA*^{null} EC were phenotypically compared to WT EC by flow cytometry for endothelial-specific surface markers (CD31 and blood group H antigen), viability, expression of eNOS, acetylated-LDL uptake, and TNF- α and IFN- γ induced expression of ICAM-1, E-selectin and PD-L1, barrier integrity measured by transendothelial electrical resistance (TEER), and analyzed for VE-cadherin expression and cord formation by epifluorescence microscopy. Refer to expanded Methods for details.

Protein gel implants

All animal protocols were approved by the Yale Institutional Animal Care and Use Committee. Collagen protein gels containing either WT or CIITA^{null} EC were implanted subcutaneously in the abdominal wall of female 6–8 week old C.B-17/SCID-beige mice (Taconic Biosciences, Germantown, NY) and analyzed after 14d 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 for details.

Mixed lymphocyte reactions and CIITA rescue

WT or CIITA^{null} EC were co-cultured with allogeneic memory CD4+ T cells labeled with CFSE dye. At 24h, T-cell elaborated interleukin-2 and interferon- γ were measured by ELISA (eBiosciences) as a measure of early activation. At 7d, dilution of CFSE dye and expression of HLA-DR were analyzed for proliferation and late activation marker. In some experiments, HLA-DR expression in CIITA^{null} EC was restored by CIITA retrovirus prior to mixed-lymphocyte reaction.

Statistics

All data are expressed as mean $\pm$ SD. Statistical comparisons were made using Student's t test or one-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.²² They are readily cultured from umbilical cord blood and, of particular relevance for this study, ECFC-derived EC display the same immunological properties as HUVEC isolated from the same umbilical cord.⁹ Secondly, 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 bi-allelic deletions by addition of a Rho-associated protein kinase (ROCK) 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, over 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 following doxycycline treatment (Online Figure IB) and the level of FLAG-Cas9 expression increased as a result of increased doxycycline up to 10 μ g/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 form) before and after IFN- γ stimulation. While essentially 100% of unmodified EC upregulated both class I and II MHC molecules upon IFN- γ stimulation, delivery of *CIITA*-specific sgRNA followed by doxycycline treatment resulted in three distinct subpopulations differing in levels of induced class II MHC: HLA-DR^{hi}, which are indistinguishable from unmodified EC, HLA-DR^{mid}, which express reduced levels of MHC II, and HLA-DR^{neg}, which express no MHC II molecules (Figure 1A). Because all three populations expressed equivalent levels of MHC I in response to IFN- γ stimulation, the HLA-DR^{neg} subpopulation likely represent EC with 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 and 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 *CIITA*^{null} 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-DR^{neg} EC for further characterization. qRT-PCR analysis of FACS-sorted IFN- γ -stimulated unmodified EC and the HLA-DR^{neg} EC subpopulation revealed 99.2% reduction of *HLA-DRA* 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-DR^{neg} EC. Previous reports have described the use of the ROCK inhibitor Y-27632 to enhance the recovery and cloning of sensitive primary cells.^{19, 20} We employed FACS sorting for single-cell isolation of ECFC-derived EC and observed improvement in the cloning efficiency of HLA-DR^{neg} 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 cells using the same approach (Online Figure II), but single cell FACS isolation followed by culture in medium supplemented with Y-27632 yielded very few colonies and these, in contrast to our experience with ECFC-derived EC, could not be further expanded. Clonally expanded HLA-DR^{neg} ECFC-derived EC 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-DR^{neg} 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-DR^{neg} EC clones derived from three distinct donors were all confirmed to have bi-allelic indels of between 1 and 23bp at the predicted

CIITA locus with a bias towards deletions as previously reported for CRISPR/Cas9 gene disruption (Figure 1C).¹⁷ Additionally, sequencing of the highest scoring putative off-target coding site in *SLC6A9* revealed no mutations in any of the isolated HLA-DR^{neg} clones (Figure 1C). We also confirmed by western blot ablation of both *CIITA* and downstream HLA-DR α expression in clonally expanded HLA-DR^{neg} clones, which was otherwise preserved in WT clones (Online Figure III). 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 over 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 Figures 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).

Having established that we can efficiently produce EC with bi-allelic gene disruption, we next characterized the phenotypic functions of *CIITA*^{null} EC. Serially passaged *CIITA*^{null} EC maintained high levels of expression of the EC markers PECAM-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*^{null} EC were viable in culture (Figure 2B), maintained equivalent levels of eNOS expression as well as the ability to take up acetylated LDL (Online Figures VA and VB). When grown to confluence, both WT and *CIITA*^{null} EC formed VE-cadherin positive cell-cell lateral borders (Figure 2C) as well as formed equivalent barriers that reversibly respond to thrombin (Online Figures VC and VD), suggesting preservation of key functions of cultured EC. While these cells were refractory to IFN- γ -induced expression of MHC II (Figure 2A), they responded with similar kinetics and magnitude to TNF- α and IFN- γ induction of E-selectin, ICAM-1 and PD-L1 (Figure 2D) expected of cultured EC. The single most characteristic feature of EC is their ability to self-assemble into blood vessels. When suspended and cultured in a 3-dimensional collagen matrix, *CIITA*^{null} 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*^{null} EC suspended in collagen protein matrix and implanted subcutaneously into the abdominal wall of SCID/bg mice²⁴ 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*^{null} 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*^{null} EC are refractory to IFN- γ -induced expression of MHC II, perfused conduits formed from unmodified EC expressed MHC II upon challenge with IFN- γ , whereas conduits derived from *CIITA*^{null} EC implanted in the same mouse but on the contralateral side did not (Figure 3C).

Immunogenic function of CIITA^{null} EC

To demonstrate the utility of Cas9-mediated gene disruption in untransformed human EC, we analyzed the immunological functions of the modified cells. Immunological 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⁺ T cell responses as a consequence of direct presentation of non-self forms of class II MHC molecules. In particular, co-culturing of IFN- γ -treated human EC with allogeneic CD4⁺ memory T lymphocytes results in T cell activation as indicated by expression of activation markers, including MHC II, cytokine production, and proliferation by the alloreactive subset.⁴ While 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 IL-2 and IFN- γ (Figure 4B), proliferation and expression/acquisition of MHC II on the alloreactive subset (Figure 4C), and that this phenotype can be rescued upon reintroduction of CIITA to CIITA^{null} EC, ruling out off-target effects accounting for reduced EC immunogenicity.

DISCUSSION

Studies examining the role human ECs play in regulating physiological and pathologic processes have extensively utilized primary human EC cell 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 due to 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 utilized umbilical cord blood ECFC-derived EC. These cells have greatly increased replicative capacity and are otherwise indistinguishable from HUVEC, including the capacity to spontaneously self-assemble into vessels *in vivo*.^{9, 18, 22} They are derived from the same source as HUVEC, i.e. 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 over-expression that is characteristic with adenoviral vectors. While 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 immunological 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 system¹⁷ 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 two genes we targeted in cultured human EC. Our own experience with shRNA 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 shRNA. Third, we used transient exposure to a ROCK 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, e.g. when the cell surface is unaffected by the genetic change. Cloning will be particularly important if CRISPR/Cas9 is employed to alter rather than simply disrupt an endogenous gene.

To demonstrate the utility of our approach, we chose to study *CIITA*, 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, or leukocyte recruitment in ways that were not technically feasible before. While 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 *CIITA*-specific sgRNA sequences, ascertained that other major EC functions were intact, and demonstrated phenotypic rescue by reintroduction of *CIITA*. Though off-target mutations at loci containing three or more mismatches are rare^{23, 26} and often may not have functional consequences, single-cell cloning of ECFC-derived EC also permits screening for appropriate clones through PCR 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 activators^{28, 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 pre-clinical models of ischemic injury, to tissue engineer vessel replacements as well as to promote vascularization in larger bioengineered grafts.^{24, 30–34} While these results are promising, allogeneic sources of EC may provoke cell-mediated immunological rejection.⁹ As demonstrated in this report, genetic ablation of *CIITA* 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 believe 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

Cas9	CRISPR-associated protein 9 nuclease
CRISPR	clustered regularly interspaced short palindromic repeats
CIITA	class II transactivator
EC	endothelial cell
ECFC	endothelial colony forming cell
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cell
(IFN)-γ	interferon gamma
IL-2	interleukin 2
LFA-3	lymphocyte function-associated antigen-3
MHC	major histocompatibility complex
sgRNA	single guide RNA
TEER	transendothelial electrical resistance

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Novelty and Significance

What Is Known?

- Cultured human endothelial cells are a widely used model to study vascular cell biology and for potential use in organ repair or tissue engineering.
- 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 non-self 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

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 utilizing three technical advances: efficient lentiviral vectors, cultures derived from cord blood endothelial colony forming cells that display extended replicative lifespans 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 non-self-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.

Figure 1. High efficiency disruption of *CIITA* by CRISPR/Cas9 in human EC
(A) Unmodified EC stimulated with IFN- γ increase expression of MHC class I (HLA-A,B,C) and class II (HLA-DR), whereas a high proportion of EC transduced with TetOn-Cas9/s γ CIITA vectors upregulate MHC class I but show either reduced (HLA-DR^{mid}) or complete loss of MHC class II (HLA-DR^{neg}) expression, (B) qRT-PCR analysis of FACS isolated WT and HLA-DR^{neg} EC for *CIITA*, *HLA-DRA*, and *CXCL10* transcripts, (C) HLA-DR^{neg} EC clonally sorted by single-cell FACS, expanded, and sequenced across sgRNA target site demonstrates bi-allelic indel mutations in *CIITA* but not in the likeliest off-target site (*SLC6A9*). Representative plots shown from multiple donors from 3 independent experiments.

Figure 2. CIITA^{null} EC retain their characteristic endothelial identity
(A) FACS isolated CIITA^{null} EC expanded for two weeks remain refractory to IFN- γ -induced upregulation of MHC II but are otherwise indistinguishable from unmodified (WT) EC with respect to surface marker expression (PECAM-1, blood group H antigen), **(B)** viability, **(C)** formation of VE-cadherin positive cell-cell lateral borders, and **(D)** characteristic TNF- α and IFN- γ responses pooled from 3 independent donors. Scale bars 50 μ m.

Figure 3. CIITA^{null} EC retain ability to form vessels in vitro and in vivo
(A) CIITA^{null} EC, like unmodified (WT) EC, spontaneously assemble into cord-like structures (outlined) in 3-D culture in vitro at 24h, **(B)** CIITA^{null} EC form perfused vessels (arrows) as detected by H&E that are lined by human EC (identified by Ab reactive with human CD31 and HLA-A,B,C with insets of murine vessels to show species specificity) and that recruit host (smooth muscle 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^{null} EC are refractory to IFN- γ induced expression of MHC II in vivo as detected by immunofluorescence. Representative figures and mean $\pm$ SD from (a) 4 gels, (b and c) 9 mice bearing WT and CIITA^{null} implants. Scale bar (a) 25 μ M and (b and c) 50 μ M.

Figure 4. Loss of the ability of CIITA^{null} EC to activate allogeneic CD4⁺ memory T cells is rescued by CIITA transduction

(A) Expression of class II 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 ELISA for IL-2 and IFN- γ produced in 24h supernatants, as well as (C) CFSE dilution and HLA-DR activation markers at 7d. Representative plots and mean $\pm$ SD from n=4 replicates. Similar results were seen in 3 independent experiments. LZRS is empty retroviral expression vector. * in (B) and (C) indicates statistically significant difference by post-hoc Bonferroni correction.