ENHANCING LGR5 EXPRESSION IN PRIMARY CORNEAL ENDOTHELIAL CELLS USING EPIGENETIC MODIFICATION

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ABSTRACT

Human corneal endothelial cells (hCECs) are vital for maintaining corneal transparency, but their limited proliferation capacity can lead to vision loss requiring corneal transplantation. *Leucine-rich repeat-containing G protein-coupled receptor 5* (*LGR5*) is critical in maintaining many fetal and adult stem cell types by promoting Wnt/β-catenin signaling. *LGR5* is expressed in corneal endothelial progenitors but downregulated during corneal endothelial maturation simultaneously with the loss of replicative property and the transition to a quiescent state. In this study, we evaluate strategies to re-activate *LGR5* expression in primary hCECs through epigenetic modulation. Our finding demonstrates that HDAC inhibitors, trichostatin (TSA), and valproic acid (VPA) enhanced *LGR5* expression in primary hCECs by up to 6-fold. Targeting catalytically dead Cas9 (dCas9) fused to a transcriptional activator (VP160) to the promoter region of the *LGR5* gene with a single guide RNA increased *LGR5* mRNA by up to 15-fold in HEK293 cells. Multiplexing guide RNA further enhanced *LGR5* expression to more than 30 folds. Applying this method to primary hCECs can also specifically enhance *LGR5* expression. Our results suggest that epigenome modification is a viable strategy for promoting corneal endothelium regeneration.

Keywords: Corneal endothelial cells, Epigenetic modolutor, CRISPRa, *Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)*

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INTRODUCTION

The corneal endothelial layer is a single layer of hexagonal cells with Descemet's membrane at the posterior cornea, which plays a key role in maintaining corneal transparency through the pump-and-leak hypothesis (Sridhar 2018). Adult human corneal endothelial cells (hCECs) have limited replication capacity in vivo due to cell cycle arrest in G1-phase (Joyce 2012). Consequently, following disease, aging, injury, and surgery lead to corneal endothelial dysfunction, corneal edema, and vision loss (Hamill, Schmedt et al. 2013). The standard treatment of corneal endothelial dysfunction is corneal transplantation, but it is limited by the availability of donor tissues. Therefore, the cell-base therapy is developed to be alternative treatment (Kinoshita, Koizumi et al., 2018). Even though, culturing of corneal endothelium is challenging, including low proliferation capacity, easily undergo senescence and spontaneously losing their morphology via endothelial-to-mesenchymal transition (EnMT) (Smeringaiova, Utheim et al., 2021).

Leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*) is a marker for fetal and adult stem cells in several tissues including stomach, hair follicle, intestine, mammary gland, and ovaries (Leung, Tan et al., 2018). It has been identified as a corneal endothelial progenitor marker, which has a role in regulating corneal endothelial proliferation, maintaining endothelial phenotypes, and inhibiting EnMT through Hedgehog and Wnt signaling pathways (Hirata-Tominaga, Nakamura et al. 2013, Yam, Seah et al., 2019). *LGR5* is downregulated with endothelial maturation and especially absent in primary hCECs (Hirata-Tominaga, Nakamura et al. 2013, Espana, Sun et al., 2015). Downregulation of *LGR5* could be potentially loss ability to proliferation and enter a quiescent state, leading to low capacity of corneal endothelium regeneration. However, *LGR5* has the ability to reactive after injury in facultative stem cell population for tissue regeneration in the intestine, liver, pancreas, and stomach corpus (Leung, Tan et al., 2018). Considering the aforementioned properties of *LGR5*, we believe that the activation of *LGR5* in primary hCECs has the potentially to enhance cell regeneration, thereby facilitating research investigation and advancements in therapeutics.

Epigenetics plays a crucial role in gene expression regulation in development and cellular differentiation, which do not involve changes to the DNA sequence (Jaenisch & Bird 2003). It has been reported that *LGR5* expression is regulated by epigenetic modification through a variety of mechanisms, including DNA and histone modification (Kim, Sheaffer et al., 2016; Qi, Li et al., 2017; Aloia, McKie et al., 2019). Histone deacetylation is a modification of histone protein that removing an acetyl group by histone deacetylase (HDAC), which causes hypoacetylation resulting suppression of gene expression (Roth, Denu et al., 2001). Qi, et al. (2017) reveals that HDAC1 is recruited by Smad1/Smad4 to gene promoters, resulting in the suppression of *LGR5* gene expression in intestinal organoid (Qi, Li et al., 2017). Thus, the use of HDAC inhibitors including trichostatin A (TSA) and valproic acid (VPA) can enhance *LGR5* expression in intestinal organoids (Yin, Farin et al., 2014; Qi, Li et al., 2017; Park, Kwon et al., 2021). Furthermore, clustered regularly interspaced short palindromic repeats transcriptional activation (CRISPRa) is possible epigenetic modification tool for specifically activating *LGR5* expression. It is a CRISPR-based system that enables the activation of certain endogenous gene expression without altering the DNA sequence. It involves the use of endonuclease deficient Cas9 (dCas9) fused with an effector protein or active domain, which guilds a transcriptional activator complex to a targeted region of gene. This targeting is facilitated by a engineered specific guide RNA (sgRNA) (Gilbert, Horlbeck et al., 2014). Therefore, this system holds promise for activating the expression of specific genes both in vitro and in vivo, making it a valuable tool for research purposes.

In this study, we aim to activate *LGR5* expression using epigenetic modification including TSA, VPA and CRISPRa system. This study expects to provide the tool for improving corneal

endothelium culture in research and clinical cell-based therapy Furthermore, we expect that is also be potential alternative therapeutic applications across various tissues.

RESEARCH METHODOLOGY

Tissue Collection

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (IRB No. 0581/65), and was in accordance with the Helsinki Declaration. All human corneal tissues used in the study were collected from research-grade corneal tissue and remnants of clinical-grade tissue after corneal transplantation from the King Chulalongkorn Memorial Hospital and the Thai Red Cross Eye Bank, Bangkok, Thailand.

Culture of Primary Human Corneal Endothelial Cells (hCECs)

hCECs were cultured using published protocol (Kinoshita, Koizumi et al., 2018). Briefly, the Descemet's membrane-corneal endothelial complex was stripped and digested at 37 °C with 0.25% Trypsin-EDTA. The cells were washed with basal medium (modified Opti-MEM® I Reduced Serum media, 8% fetal bovine serum, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, and 1% antimycotic-antibiotic reagents), and cultured in expansion medium (basal medium, 5 ng/ml epidermal growth factor: EGF, 10 μM SB203580) on a collagen type I-coated plate. The cultured hCECs were maintained at 37 °C in 5% CO_2 and replaced with fresh expansion medium every 3 days.

Culture of Human Embryonic Kidney 293 cells (HEK293 cells)

The HEK293 cell line was obtained from Stem Cell and Cell Therapy Research Center, Chulalongkorn University, Thailand. HEK293 cells were cultured in high glucose Dulbecco's modifed Eagle's medium (DMEM) supplemented with 10% FBS, 1% GlutaMAX and 1% antimycotic–antibiotic reagents (Gibco) and maintained at 37 °C in 5% CO2 atmosphere.

Flow Cytometry Analysis

The cells were harvested and resuspended in sorting buffer (0.5% BSA, 2 mM EDTA in 1XPBS) followed by incubation with PE Anti-human *LGR5* at 4 °C for 30 minutes. Then, washing with 1XPBS and centrifugation at 1000 rpm for 5 minutes at 4 °C. The staining cells were analyzed by flow cytometry using BD FACSAria II. (Becton Dickinson, Franlin Lakes, NJ, USA)

CRISPRa sgRNA Generation and Transfection

The sgRNA was designed following Zhang's protocol. Briefly, the sgRNAs were be designed via using https://benchling.com/. The gRNA was cloned into pAC154-dual-dCas9VP160-sg expression (Addgene plasmid #48240) The plasmid was transfected into the cells by the electroporation method. The concentration 3 ug/ml per 10^6 cells. The transfected cells were incubated for 48 hours at 37 °C in 5% CO2 until analysis.

RNA Extraction and Quantitative Real-time RT-PCR (qRT-PCR)

RNA was extracted using TRIzol Reagent. Complementary DNA (cDNA) was generated from the mRNA template using the RevertAidH Minus Kit (Thermo Scientific). Subsequently, qPCR was performed using SYBR Green PCR Master Mix (Applied Biosystems). The relative expression was determined by comparing it to the housekeeping gene GADPH and calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method.

Data Analysis and Statistics

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., United States). All statistical tests were performed in triplicate. The unpaired two-tailed Student's t-test was used to analyze differences between the two groups. Comparison among three or more groups was performed using one-way analysis of variance (ANOVA) and post-hoc analysis with the Bonferroni test. P-values indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were considered statistically significant

RESEARCH RESULTS

Epigenetic Modulator Can Enhance *LGR5* Expression in Primary hCECs.

Epigenetic modifications regulate gene expression without changing the DNA sequence. This modification can influence gene expression by either activating or silencing specific genes by DNA modification, histone modification and chromatin remodeling (Portela & Esteller 2010). The previous studies reported that HDAC regulates the expression of *LGR5* in intestinal organoids (Qi, Li et al., 2017). To investigate the effect of HDAC inhibitor on *LGR5* expression in primary hCECs. Primary hCECs were treated with different concentration of TSA and VPA for 7 days. Our results revealed that 25 nM TSA and 1 mM VPA groups were observed significantly increase in *LGR5* expression compared to the control group ($p \leq 0.05$ and $p \leq 0.01$ respectively) (Figure 1). This indicated that TSA and VPA can enhance *LGR5* gene expression in primary hCECs.

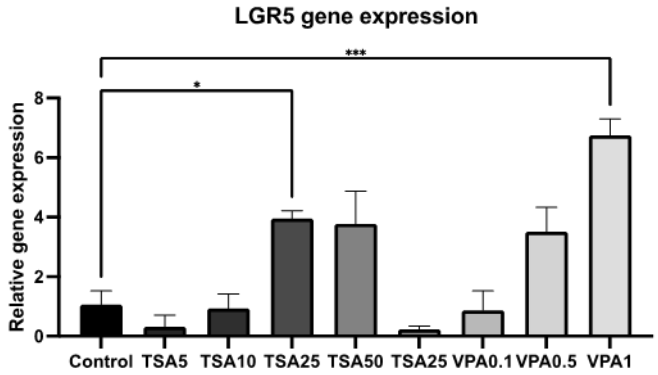

Figure 1 *LGR5* gene expression in primary hCECs after treatment with virous concentrations of TSA (nM) and VPA (mM) for 7 days.

CRISPRa Can Activate *LGR5* Expression in HEK293 Cells.

In order to achieve more specific activation of *LGR5*, we employed CRISPRa technique in HEK293 cell line. Four sgRNAs were designed according to the recommendations in a previous article to target the 200 bp upstream region of the transcriptional start site (TSS) of the *LGR5* gene defined as (Figure 2) (Cheng, Wang et al., 2013). To validate the most effective binding site, HEK293 cells were used and transfected with individual sgRNA by using electroporation. The results of qRT-PCR indicated that sgRNA-2, 3, and 4 significantly upregulated *LGR5* mRNA expression up to 15-fold ($p \leq 0.01$). While sgRNA-1 exhibited the lowest activation of *LGR5* gene expression with an approximately 5-fold (Figure 3A). These findings were corroborated by flow cytometry analysis, which showed the percentage of *LGR5*-positive cells increased to 1.2, 5, 3.6 and 4.2% in sgRNA1, sgRNA2, sgRNA3 and sgRNA4 respectively (Figure 3B).

According to Cheng et al., 2013, the use of a combination of sgRNAs can enhance endogenous gene expression more effectively than individual sgRNA (Cheng, Wang et al., 2013). Therefore, based on the outcomes of individual sgRNA in our previous experiment, we aimed to use different combinations of sgRNAs to induce a synergistic activation, as described in the following formats: sgRNA-2,3, sgRNA-2,4, sgRNA-3,4, and sgRNA-2,3,4. Our finding demonstrated that the combination of sgRNA-2,3,4 had the greatest efficiency in activating the *LGR5* gene in HEK293 cells up to approximately 30-fold, surpassing the upregulation seen with other combinations such as sgRNA-2,4, sgRNA-2,3, and sgRNA-3,4, which showed upregulation of approximately 20-fold ($p \leq 0.001$) (Figure 4A). Results from this experiment were consistent with the protein expression detected through flow cytometry analysis (Figure 4B).

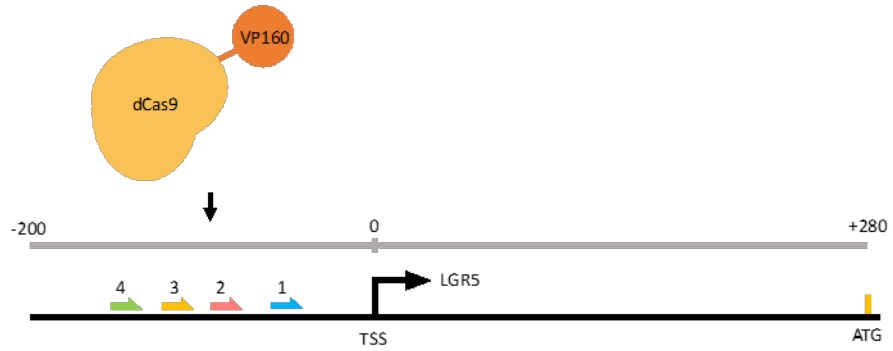

Figure 2 Diagram of CRISPRa system and the location of sgRNA on the 200 bp upstream of the transcription start site (TSS) of *LGR5* gene.

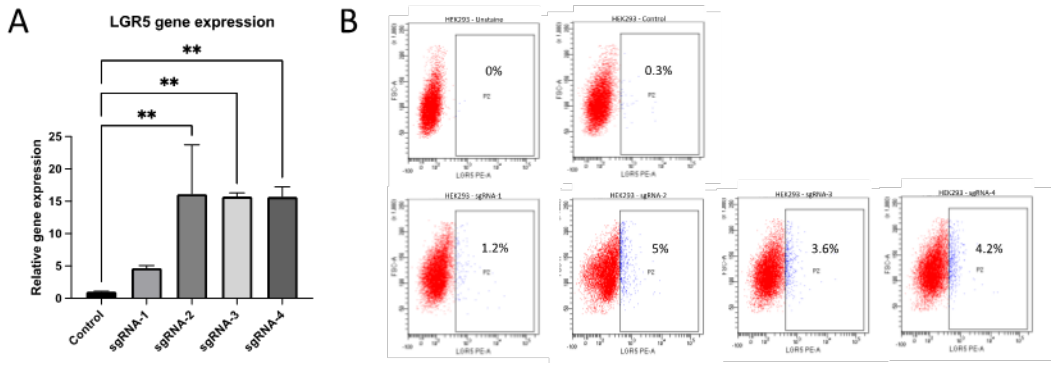

Figure 3 *LGR5* expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each single validation sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

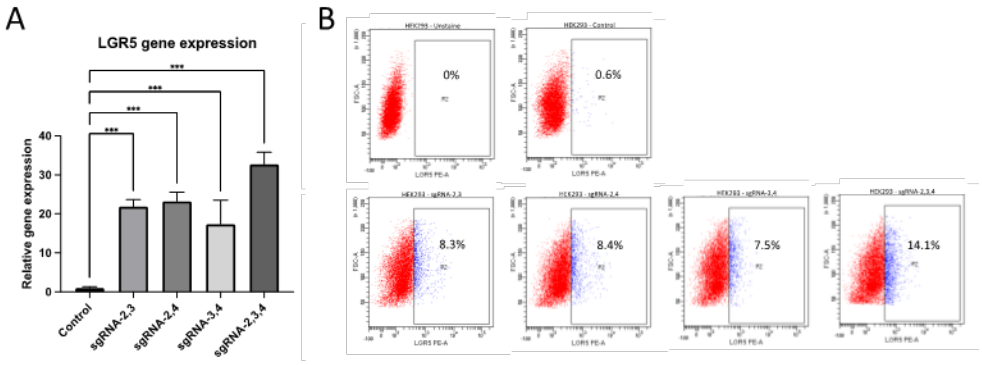

Figure 4 *LGR5* expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each combination of sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

CRISPRa in Primary hCECs

It has been reported that *LGR5* expression was downregulated in mature corneal endothelium and especially in primary hCECs (Hirata-Tominaga, Nakamura et al., 2013). This might be a reason for the inability to maintain cell growth and senescence. Therefore, it could be beneficial for activation of *LGR5* expression in primary hCECs by using CRISPRa. We proceeded to transfect the combination of sgRNA-2, 3, 4 into primary hCECs via electroporation. We determined the transfection rate in primary hCECs by using pmaxGFP, resulting in a rate of 25% (Figure 5A). This rate was considered sufficient for the CRISPRa experiment in primary

hCECs. The result of qRT-PCR analysis revealed a significant approximately 3-fold upregulation of *LGR5* expression ($p \leq 0.05$) (Figure 5B). The flow cytometry analysis showed a slight difference between the control group and the transfected group (Figure 5C).

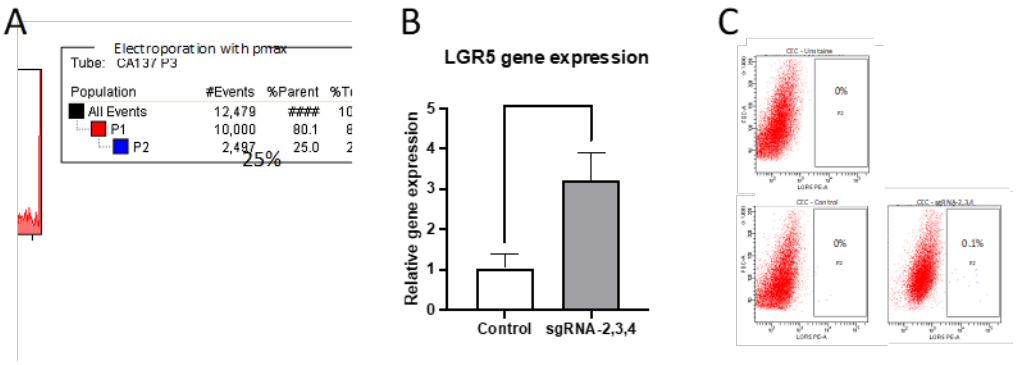

Figure 5 (A) Transfection rate by electroporation with pmaxGFP in cultured hCEC. *LGR5* expression levels were quantitatively measured in cultured hCECs after 48 hours transfection of a combination sgRNAs using (B) qRT-PCR and (C) flow cytometry analysis.

DISCUSSION & CONCLUSION

Maintaining the transparency of the cornea is crucial for vision by hCECs, but damaged corneal endothelium is unable to regenerate in vivo requiring corneal transplantation (Hamill, Schmedt et al., 2013; Sridhar, 2018). *LGR5* is known for its critical role in stem cell maintenance and tissue regeneration through Wnt/β-catenin signaling It also involves the regulation of proliferation, preservation of endothelial phenotypes, and prevention of EnMT (Hirata-Tominaga, Nakamura et al., 2013). However, it is downregulated during endothelial maturation and is absent in primary hCECs (Hirata-Tominaga, Nakamura et al., 2013; Espana, Sun et al., 2015). This might lead to a limited passage capacity and accelerated senescence. *LGR5* has been observed in facultative stem cells responsible for post-injury tissue regeneration in the liver, pancreas, and stomach corpus (Leung, Tan et al., 2018). According to strategy of reactivate *LGR5* expression, we can enhance *LGR5* expression in primary hCECs through TSA, VPA and CRISPRa. These could provide insights into the tissue maintenance, repair, and disease progression and offering potential therapeutic strategy through epigenetic modification. Epigenetic refers to the study of changing in gene expression without altering the DNA sequence (Portela & Esteller, 2010). Histone modification is one of the epigenetic mechanism that regulate *LGR5* expression (Qi, Li et al., 2017). Our study revealed that HDAC inhibitors can promote *LGR5* expression in primary hCECs, suggesting that HDAC directly or indirectly influences *LGR5* regulation in these cells. Previous research demonstrated that the recruitment of HDAC1 to *LGR5* gene promoters leads to its repression in intestinal organoids (Qi, Li et al., 2017). HDACs remove acetyl groups from histones, causeing hypoacetylation and subsequent chromatin compaction. This compaction forms heterochromatin, making DNA less accessible to the transcriptional machinery and resulting in gene suppression (Allis & Jenuwein, 2016). Therefore, the low expression of *LGR5* in primary hCECs might be attributed to HDAC-induced heterochromatin formation. However, it important to note that the use of epigenetic modulators exerts a broader impact on epigenetic modification and gene expression across the entire genome, thereby lacking specificity to interesting gene.

CRISPRa is a genetic tool that enables activate the specific gene (Di Maria, Moindrot et al., 2020). In our experiment, we employed dCas9-VP160, which is the first generation of CRISPRa systems known to enhance gene activation moderately (Cheng, Wang et al., 2013; Chavez, Tuttle et al., 2016). We were able to generate a combination of sgRNAs that efficiently activated *LGR5* mRNA and protein expression in a HEK293 cell line up to 30-fold. These

sgRNAs could be used with different dCas9 fusions, such as VPR, SunTag and p300. A recently study demonstrated that dCas9-p300 can activate *LGR5* expression in colorectal cancer cell lines (King, Marx et al., 2023). A catalytic domain of p300 acetyltransferase promotes increase levels of H3K27ac histone modification at specific target region leading to gene activation (Nakamura, Gao et al., 2021). They designed sgRNAs to target the Wnt-responsive DNA elements at the *LGR5* proximal promotor region, differing from the target location of our sgRNA.

Though, this combination of sgRNAs can slightly induce *LGR5* expression in primary hCECs. There could be several reasons why these cells exhibit slightly increasing expression after transfection with CRISPRa. One possible reason is the low transfection efficiency related with plasmid size. The dCas9-VP160 plasmid, with a size of 8859 bp, is a large size vector that reduces survival and transfection efficiency after electroporation (Lesueur, Mir et al., 2016; Søndergaard, Geng et al., 2020). Thus, several studies have explored method to enhance the transfection rate in hCECs, including magnetofection, calcium phosphate nanoparticle and recombinant adeno-associated virus (Hu, Kovtun et al., 2012; Czugala, Mykhaylyk et al., 2016; Gruenert, Czugala et al., 2016). Further investigation should focus on utilizing different transfection methods to enhance transfection efficacy in primary hCECs.

Another possible reason is that the chromatin state in primary hCECs could affect the efficacy of CRISPRa. As mentioned earlier, it has been suggested that the absence of *LGR5* expression in primary hCECs might be attributed to the heterochromatin formation caused by HDAC. This chromatin state might impede the accessibility of CRISPRa and the transcriptional machinery to the target region, consistent with recent study (Wu, Wu et al., 2023). Therefore, the next study should investigate the use of epigenetic modulators in conjunction with CRISPRa to modify the compacted DNA structure, thereby promoting accessibility for CRISPRa and transcriptional machinery to target DNA region.

In conclusion, we study demonstrated the ability to activate endogenous *LGR5* expression in primary hCECs through epigenetic modification. This provides a valuable tool for investigating the role of *LGR5* role in various biological processes such as cell fate determination, tissue homeostasis, and disease progression. Additionally, our findings provide promising therapeutic strategy of epigenetic modification for tissue repair and regeneration, particularly in the corneal endothelium. Further research should evaluate the characterization, proliferation, function, and quality of the activated *LGR5*-positive cells, as well as conducting animal model testing, before considering their clinical applications.

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REFERENCES

Allis, C. D. and T. Jenuwein (2016). The molecular hallmarks of epigenetic control. *Nature Reviews Genetics 17*(8), 487-500.

Aloia, L., M. McKie, G. Vernaz, L. Cordero-Espinoza, N. Aleksieva, J. van den Ameele, F. Antonica, B. Font-Cunill, A. Raven, R. Aiese Cigliano, G. Belenguer, R. Mort, A. Brand, M. Zernicka-Goetz, S. Forbes, E. Miska and M. Huch (2019). Epigenetic

remodelling licences adult cholangiocytes for organoid formation and liver regeneration. *Nature Cell Biology, 21*.

Chavez, A., M. Tuttle, B. W. Pruitt, B. Ewen-Campen, R. Chari, D. Ter-Ovanesyan, S. J. Haque, R. J. Cecchi, E. J. K. Kowal, J. Buchthal, B. E. Housden, N. Perrimon, J. J. Collins and G. Church (2016). Comparison of Cas9 activators in multiple species. *Nat Methods 13*(7), 563-567.

Cheng, A. W., H. Wang, H. Yang, L. Shi, Y. Katz, T. W. Theunissen, S. Rangarajan, C. S. Shivalila, D. B. Dadon and R. Jaenisch (2013). Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Research 23*(10), 1163-1171.

Czugala, M., O. Mykhaylyk, P. Böhler, J. Onderka, B. Stork, S. Wesselborg, F. E. Kruse, C. Plank, B. B. Singer and T. A. Fuchsluger (2016). Efficient and safe gene delivery to human corneal endothelium using magnetic nanoparticles. *Nanomedicine (Lond) 11*(14), 1787-1800.

Di Maria, V., M. Moindrot, M. Ryde, A. Bono, L. Quintino and M. Ledri (2020). *Development and Validation of CRISPR Activator Systems for Overexpression of CB1 Receptors in Neurons*. Frontiers in Molecular Neuroscience 13.

Espana, E. M., M. Sun and D. E. Birk (2015). Existence of Corneal Endothelial Slow-Cycling Cells. *Invest Ophthalmol Vis Sci 56*(6), 3827-3837.

Gilbert, Luke A., Max A. Horlbeck, B. Adamson, Jacqueline E. Villalta, Y. Chen, Evan H. Whitehead, C. Guimaraes, B. Panning, Hidde L. Ploegh, Michael C. Bassik, Lei S. Qi, M. Kampmann and Jonathan S. Weissman (2014). Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. *Cell 159*(3), 647-661.

Gruenert, A. K., M. Czugala, C. Mueller, M. Schmeer, M. Schleef, F. E. Kruse and T. A. Fuchsluger (2016). Self-Complementary Adeno-Associated Virus Vectors Improve Transduction Efficiency of Corneal Endothelial Cells. *PLOS ONE 11*(3), e0152589.

Hamill, C. E., T. Schmedt and U. Jurkunas (2013). Fuchs endothelial cornea dystrophy: a review of the genetics behind disease development. *Semin Ophthalmol 28*(5-6), 281-286.

Hirata-Tominaga, K., T. Nakamura, N. Okumura, S. Kawasaki, E. P. Kay, Y. Barrandon, N. Koizumi and S. Kinoshita (2013). Corneal Endothelial Cell Fate Is Maintained by LGR5 Through the Regulation of Hedgehog and Wnt Pathway. *Stem Cells 31*(7), 1396-1407.

Hu, J., A. Kovtun, A. Tomaszewski, B. B. Singer, B. Seitz, M. Epple, K.-P. Steuhl, S. Ergün and T. A. Fuchsluger (2012). A new tool for the transfection of corneal endothelial cells: Calcium phosphate nanoparticles. *Acta Biomaterialia, 8*(3), 1156-1163.

Jaenisch, R. and A. Bird (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature Genetics 33*(3), 245-254.

Joyce, N. C. (2012). Proliferative capacity of corneal endothelial cells. *Experimental Eye Research 95*(1), 16-23.

Kim, R., K. L. Sheaffer, I. Choi, K.-J. Won and K. H. Kaestner (2016). Epigenetic regulation of intestinal stem cells by Tet1-mediated DNA hydroxymethylation. *Genes & development 30*(21), 2433-2442.

King, C. M., O. M. Marx, W. Ding, W. A. Koltun and G. S. Yochum (2023). TCF7L1 Regulates LGR5 Expression in Colorectal Cancer Cells. *Genes (Basel) 14*(2).

Kinoshita, S., N. Koizumi, M. Ueno, N. Okumura, K. Imai, H. Tanaka, Y. Yamamoto, T. Nakamura, T. Inatomi, J. Bush, M. Toda, M. Hagiya, I. Yokota, S. Teramukai, C. Sotozono and J. Hamuro (2018). Injection of Cultured Cells with a ROCK Inhibitor for Bullous Keratopathy. *New England Journal of Medicine 378*(11), 995-1003.

Lesueur, L. L., L. M. Mir and F. M. André (2016). Overcoming the Specific Toxicity of Large Plasmids Electrotransfer in Primary Cells In Vitro. *Molecular Therapy - Nucleic Acids 5*, e291.

Leung, C., S. H. Tan and N. Barker (2018). Recent Advances in Lgr5(+) Stem Cell Research. *Trends Cell Biol 28*(5), 380-391.

Nakamura, M., Y. Gao, A. A. Dominguez and L. S. Qi (2021). CRISPR technologies for precise epigenome editing. *Nature Cell Biology 23*(1), 11-22.

Park, M., J. Kwon, H. Youk, U. S. Shin, Y. H. Han and Y. Kim (2021). Valproic acid protects intestinal organoids against radiation via NOTCH signaling. *Cell Biol Int 45*(7), 1523-1532.

Portela, A. and M. Esteller (2010). Epigenetic modifications and human disease. *Nature Biotechnology 28*(10), 1057-1068.

Qi, Z., Y. Li, B. Zhao, C. Xu, Y. Liu, H. Li, B. Zhang, X. Wang, X. Yang, W. Xie, B. Li, J.-D. J. Han and Y.-G. Chen (2017). BMP restricts stemness of intestinal Lgr5+ stem cells by directly suppressing their signature genes. *Nature Communications 8*(1), 13824.

Roth, S. Y., J. M. Denu and C. D. Allis (2001). Histone acetyltransferases. *Annu Rev Biochem 70*, 81-120.

Smeringaiova, I., T. P. Utheim and K. Jirsova (2021). Ex vivo expansion and characterization of human corneal endothelium for transplantation: a review. *Stem Cell Research & Therapy 12*(1), 554.

Søndergaard, J. N., K. Geng, C. Sommerauer, I. Atanasoai, X. Yin and C. Kutter (2020). Successful delivery of large-size CRISPR/Cas9 vectors in hard-to-transfect human cells using small plasmids. *Communications Biology 3*(1), 319.

Sridhar, M. S. (2018). Anatomy of cornea and ocular surface. *Indian journal of ophthalmology 66*(2), 190-194.

Wu, Q., J. Wu, K. Karim, X. Chen, T. Wang, S. Iwama, S. Carobbio, P. Keen, A. Vidal-Puig, M. R. Kotter and A. Bassett (2023). Massively parallel characterization of CRISPR activator efficacy in human induced pluripotent stem cells and neurons. *Mol Cell 83*(7), 1125-1139.e1128.

Yam, G. H., X. Seah, N. Z. Yusoff, M. Setiawan, S. Wahlig, H. M. Htoon, G. S. L. Peh, V. Kocaba and J. S. Mehta (2019). Characterization of Human Transition Zone Reveals a Putative Progenitor-Enriched Niche of Corneal Endothelium. *Cells 8*(10).

Yin, X., H. F. Farin, J. H. van Es, H. Clevers, R. Langer and J. M. Karp (2014). Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny. *Nature methods 11*(1), 106-112.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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