Epigenetic regulation of leucine rich repeat containing G protein coupled receptor 5 *(LGR5)* in corneal endothelial cells

Miss Kulsiri Prakanrattana

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การควบคุมสภาวะเหนือพันธุกรรมของยีน *LGR5* ในเซลล์กระจกตาชั้นใน

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By	Miss Kulsiri Prakanrattana
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Thesis Advisor	Associate Professor NIPAN ISRASENA, M.D., Ph.D.

Accepted by the FACULTY OF MEDICINE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

.... Dean of the FACULTY OF MEDICINE
(Associate Professor CHANCHAI SITTIPUNT, M.D.)

THESIS COMMITTEE

.... Chairman
(Professor VILAI CHENTANEZ, M.D., Ph.D.)

.... Thesis Advisor
(Associate Professor NIPAN ISRASENA, M.D., Ph.D.)

.... Examiner
(Associate Professor WILAI ANOMASIRI, Ph.D.)

.... External Examiner
(Professor Pakpoom Kheolamai, M.D., Ph.D.)

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เซลล์กระจกตาชั้นในมีหน้าที่สำคัญในการควบคุมความใสของกระจกตา อย่างไรก็ตามเซลล์ดังกล่าวมีความสามารถในการแบ่งตัวที่จำกัดส่งผลให้มีความสามารถในการฟื้นฟูต่ำส่งผลให้ผู้ป่วยที่มีความเสียหายของเซลล์กระจกตาชั้นในสูญเสียความสามารถในการมองเห็น และจำเป็นต้องได้รับการผ่าตัดเปลี่ยนกระจกตา แต่จำนวนผู้บริจาคก็ยังไม่เพียงพอต่อผู้รับบริจาค ปัจจุบันจึงมีการพัฒนาการรักษาด้วยการฉีดเซลล์เพาะเลี้ยงกระจกตาชั้นในเข้าในตาเป็นอีกทางเลือกในการรักษา แต่ว่าการเพาะเลี้ยงเซลล์กระจกตาชั้นในยังมีข้อจำกัดหลายอย่างส่งผลให้จำเป็นต้องมีการพัฒนาการรักษาทางเลือกอื่นเพิ่มเติมและต้องมีการพัฒนาการเพาะเลี้ยงเซลล์กระจกตาชั้นในอีกด้วย ยีน *LGR5* มีสำคัญในการควบคุมเซลล์ต้นกำเนิดผ่านการกระตุ้นของ Wnt/β-catenin signaling pathway โดยพบว่า *LGR5* เป็นหนึ่งในยีนจำเพาะของเซลล์ต้นกำเนิดกระจกตาชั้นใน โดยพบว่ามีการแสดงออกลดลงเมื่อเซลล์เติบโตเต็มที่ซึ่งสอดคล้องกับความสามารถในการเพิ่มจำนวนที่ลดลง ในการศึกษานี้ผู้วิจัยได้นำแบบจำลองการกลับมาแสดงออกของยีน *LGR5* ผ่านการควบคุมสภาวะเหนือพันธุกรรมมาใช้ในเซลล์เพาะเลี้ยงกระจกตาชั้นใน จากการศึกษาพบว่า ระดับ DNA methylation และ DNA hydroxymethylation ที่ต่ำบริเวณตำแหน่ง promoter ของยีน *LGR5* ในเซลล์เพาะเลี้ยงกระจกตาชั้นใน เมื่อทำการใส่สารยับยั้ง HDAC ความเข้มข้นสูง ได้แก่ trichostatin A และ valproic acid สามารถเพิ่มการแสดงออกของยีน *LGR5* ในเซลล์เพาะเลี้ยงกระจกตาชั้นในได้ และการทดสอบสาร valproic acid ความเข้มข้นต่ำควบคู่กับสารดังต่อไปนี้ Wnt3A, R-Spondin1 และ BMP inhibitor สามารถกระตุ้นการแสดงออกของยีน *LGR5* ในเซลล์ดังกล่าวได้อีกด้วย อีกทั้งงานวิจัยนี้ยังมีการนำเทคนิค CRISPRa มากระตุ้นการแสดงออก *LGR5* พบว่าสามารถกระตุ้นการแสดงออก *LGR5* ใน HEK293 cells โดยเฉพาะอย่างยิ่งเมื่อใช้หลาย sgNRA และเมื่อนำเทคนิคดังกล่าวมาใช้ในเซลล์เพาะเลี้ยงกระจกตาชั้นในพบว่าสามารถกระตุ้นการแสดงออกของ *LGR5* ได้เช่นกัน จากผลการศึกษานี้คาดว่าการควบคุมสภาวะเหนือพันธุกรรมเป็นอีกหนึ่งแนวทางในการช่วยเพิ่มประสิทธิภาพในการพัฒนาการรักษาของโรคและการเพาะเลี้ยงเซลล์กระจกตาชั้นในต่อไปในอนาคต

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Human corneal endothelial cells (hCECs) are vital for maintaining corneal transparency. However, their limited capacity for proliferation can result in vision loss, requiring corneal transplantation. The *Leucine-rich repeat-containing G protein-coupled receptor 5* (*LGR5*) plays a critical role in maintaining various fetal and adult stem cell types by promoting the Wnt/β-catenin signaling. Although *LGR5* is present in corneal endothelial progenitors, its expression decreases as these cells mature, coinciding with the loss of replicative properties. In this study, we explore strategies to reactivate *LGR5* expression in cultured hCECs through epigenetic modulation. We found low levels of DNA methylation and hydroxymethylation at *LGR5* promoter in cultured hCECs. Our findings reveal that high-dose HDAC inhibitors, trichostatin A, and valproic acid enhanced *LGR5* expression in cultured hCECs. In addition, combining low-dose valproic acid with small molecules including Wnt3A, R-Spondin1, and BMP inhibitors also promoted *LGR5* expression in these cells. Furthermore, we generated CRISPRa targeting at LGR5 promoter which could activate *LGR5* expression in HEK293 cells, especially with multiple sgRNAs. Applying this method to cultured hCECs can also specifically enhance *LGR5* expression. Our results suggest that epigenome modification is a viable strategy for promoting corneal endothelium regeneration.

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CHAPTER 1

INTRODUCTION

The corneal endothelial layer is a single layer of hexagonal cells with Descemet's membrane at the posterior cornea which appears as a honeycomb pattern (1). The corneal endothelium has a critical role in maintaining corneal transparency through the pump-leak hypothesis (2). Adult human corneal endothelial cells (hCECs) have limited replication capacity in vivo and arrest in the G1 state of the cell cycle due to restrictions in the microenvironment (3). Consequently, following disease, aging, injury, and surgery lead to corneal endothelial dysfunction, corneal edema, and vision loss (4). 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 (5). 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) (6).

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 (7). It has been identified as a corneal endothelial progenitor marker, which has a role in the control of corneal endothelial proliferation, the preservation of endothelial characteristics, and the inhibition of EnMT through Hedgehog and Wnt signaling pathways (8, 9). *LGR5* is downregulated with endothelial maturation and especially absent in cultured hCECs (9, 10). Downregulation of *LGR5* could potentially lose the ability to proliferate 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 several tissues (7). Considering the properties of *LGR5*, we believe that the activation of *LGR5* in cultured hCECs has the

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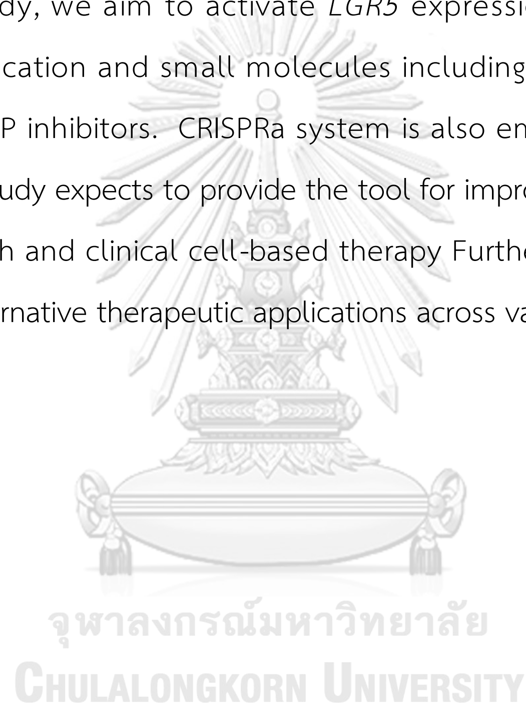
potential to enhance cell regeneration, thereby facilitating research investigation and advancements in therapeutics.

Epigenetic plays an important role in the regulation of gene expression during development and cellular differentiation, independent of changes to the DNA sequence (11). In normal cells, epigenetic changes are generally subtle, and each cell type possesses its unique epigenetic profile. However, it is noteworthy that epigenetic mechanisms play a pivotal role in facultative stem cells during tissue injury, especially in the liver (12). *LGR5* is known to be regulated by epigenetic modifications through various mechanisms, including DNA and histone modification in intestinal and liver stem cells (13-15). A previous study demonstrated that DNA methylation in the *LGR5* promoter region suppressed its expression in colon cancer cell lines (16). Additionally, DNA hydroxymethylation contributes to the regulation of *LGR5* expression in intestinal and liver stem cells through TET1 (14, 15). 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 (17). Qi and colleagues (2017) reveals that HDAC1 is recruited by Smad1/Smad4 to gene promoters, resulting in the suppression of *LGR5* gene expression in intestinal organoid (13). Consequently, the use of HDAC inhibitors including trichostatin A (TSA) and valproic acid (VPA) can enhance *LGR5* expression in intestinal organoids (13, 18, 19). Furthermore, several studies suggest that *LGR5* expression is under the control of both signaling pathway and epigenetic mechanism that might promote cell plasticity and reactivate quiescent after injury. Small molecules associated with signaling pathway such as Wnt ligand, R-spondin, and BMP inhibitors have been shown to promote and maintain *LGR5* expression in intestinal organoids (13, 20).

Additionally, clustered regularly interspaced short palindromic repeats transcriptional activation (CRISPRa) is a potential epigenetic modification tool for specifically activating *LGR5* expression. This CRISPR-based system enables the

activation of certain endogenous gene expressions without altering the DNA sequence. It involves the use of endonuclease deficient Cas9 (dCas9) fused with an effector protein or active domain, guiding a transcriptional activator complex to a targeted region of gene. This targeting is facilitated by an engineered specific guide RNA (sgRNA) (21). 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 combination of epigenetic modification and small molecules including TSA, VPA, Wnt ligand, R-spondin1 and BMP inhibitors. CRISPRa system is also employed to activate *LGR5* expression. 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 potential alternative therapeutic applications across various tissues.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CHAPTER 2
REVIEW LITERATURE

Corneal endothelial cells (CECs)

he cornea, a clear avascular tissue situated at the front of the eye, serves as both a physical barrier and a medium for refracting light into the eye. The human cornea is composed of five layers including epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium layer (22). The corneal endothelium layer is a single layer of hexagonal cells on Descemet's membrane at the most inner cornea which appears as a honeycomb pattern (1) (Figure 1).

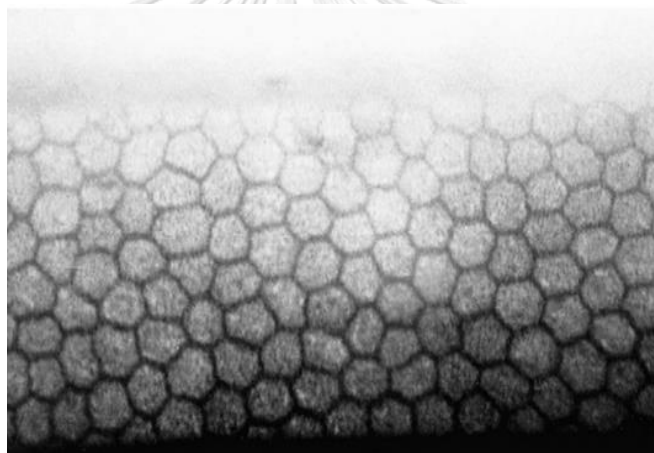

Figure 1 Endothelium layer on the specular microscopy (22)

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Corneal endothelial cells (CECs) are involved in preserving corneal hydration according to the pump-and-leak theory. The nutrition from aqueous humor can leak via the incomplete barrier of tight junction between CECs, this provides nutrition to stromal keratocyte. The CECs simultaneously pump fluid out of the stromal layer via active metabolic pump to counteract the leaking and maintain corneal homeostasis (2) (Figure 2).

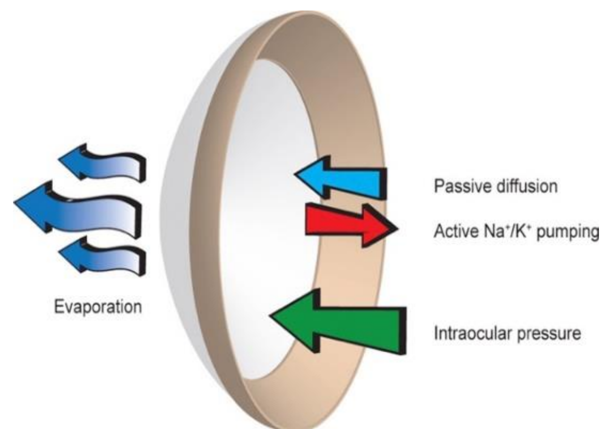

Figure 2 The pump-and-leak hypothesis in corneal endothelium layer (23)

After birth, the human corneal endothelial cells (hCECs) lack the ability to replicate in a living organism. Thus, this lack of cell division of hCECs is insufficient to replace cells from dead and injury. This consequences in a physiological loss of ECD about 0.3-0.6% per year (24). The reason for the restricted proliferation capacity of hCECs is G1-phase arrest of cell cycle by contract inhibition, insufficient of growth factor stimulation and mitogenic inhibitor in aqueous humor (Transforming growth factor-β; TGF-β) (3). Therefore, the wound healing process of hCECs are migration and spreading with enlargement to cover the wound after injury (25).

Dysfunction of hCECs from Fuchs' endothelial corneal dystrophy, aging, trauma, injury leads to a critical loss in number of hCECs, edematous cornea, bullous keratopathy, reducing of vision and eventually blindness. Therefore, the gold standard treatment for corneal endothelial dystrophy is corneal transplantation in worldwide (23). Nevertheless, corneal transplantation has several obstacles such as advanced surgical technique, postoperative complication, immune reaction, graft failure and deficiency of donor cornea. It is reported that the ratio of donor and patients is 1:70 and 33% of donor corneas is not suitable for transplantation due to low quality and contamination (26). Moreover, there is significant decreasing of tissue donor for corneal transplantation during COVID-19 pandemic (27). Therefore, the cell-base therapy is developed to be alternative treatment instead of corneal

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transplantation (28). Even though, the attempt to expansion of corneal endothelial cell in vitro is challenging. The challenges of culturing CECs are restricted by suitable donors, low proliferation rate, prone undergo senescence and spontaneously losing their morphology via endothelial-to-mesenchymal transition (EnMT) (6, 29).

Development of corneal endothelium and corneal endothelial progenitors

Human eye development begins at approximately the third week of gestation and continues through the tenth week, and the cornea is formed at about 5-6 weeks of human gestation (30). The corneal endothelium layer is developed form periocular neural crest cells (PNCCs) at periocular region which migrate and transform into periocular mesenchyme cells (POMs). Then, the POMs migrate into the space between anterior surface of lens and corneal epithelium and transdifferentiate into CEC (31). The exact mechanisms of NCCs migration and differentiation of CECs are incompletely determined. NCCs migrate from neural plate border into periocular region through epithelial-mesenchymal transition (EMT) (Figure 3). After lining into monolayer, the immature CECs may be differentiated into mature CECs with reversal step of mesenchymal to endothelial transition (MEndT). A combination of TGF-β, Wnt, Retinoic acid (RA) and Hedgehog signaling have been reported to involve these processes (32-36).

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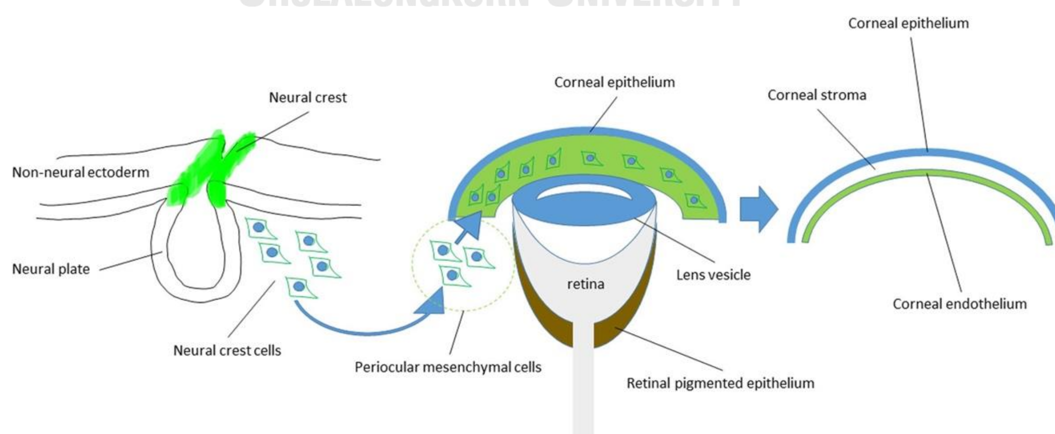

Figure 3 Scheme of corneal endothelium development (37)

Over the last few decades, several studies have attempted to discover corneal endothelial progenitors (CEPs). It is likely that CEPs are located at the peripheral cornea, Schwalbe’s line, transition zone and trabecular meshwork by tissue staining with progenitor or surface markers (8, 35, 38-41) (Table 1). The CEPs have also been demonstrated to form sphere in non-adherent culture, indicating that they have proliferative capacity (42, 43). The spheres express the stem cell-related gene including *AP2B1, LGR5, NES, OCT4, p75NTR, PAX3, SOX2, SOX9* and *TP63*. They also can be differentiated to corneal endothelial cell like which expressed corneal endothelial marker, phenotype, and function (39) (Table 2). Therefore, these results have suggested that CEPs are still present in adult cornea. However, there is no specific markers to identify CEPs and the location of the CEPs is still unclear.

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Table 1 The previous reports of locations, markers, and methods of identification in corneal endothelial progenitors

Method	Markers	Location	Reference
BrdU	Alkaline phosphatase, Telomerase	Peripheral endothelium	(38)
Sphere-forming assays	Nestin, Alpha-sma, beta3-tubulin, GFAP	ND	(42)
Sphere-forming assays	ND	Peripheral endothelium	(43)
Immunostaining	Nestin, Alkaline phosphatase, Telomerase, OCT-3/4, PAX6, WNT1, SOX2	Trabecular meshwork, transition zone, peripheral endothelium	(41)
FACs, Immunostaining	LGR5	Peripheral endothelium	(35)
FACs	Nestin, p75NTR, SOX9, FOXC2, TFAP2B	Transition zone	(40)
Sphere-forming assays	OCT4, p63, LGR5, SOX2, SSEA4, TRA-1-60	Subpopulation of neural crest-derived progenitor	(39)
Immunostaining	SOX2, LGR5, CD34, PITX2, Telomerase, Nestin	peripheral corneal endothelium , transition zone	(44)

* BrdU = Bromodeoxyuridine, ND = not determined, FACs = Fluorescence-activated cell sorting

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Table 2 The previous reports of locations, markers, and methods of identification in corneal endothelial cells

Markers	Location	Function	Method	Remark	Reference
Na^+/K^+ -ATPase	Basolateral membrane	Active transport pump	WB, ICC, IHC, qPCR	Found in epithelial cells, TM	(45-48)
ZO-1	Apical protein of the tight junction complex	Intercellular tight junction	WB, ICC, IHC, qPCR	Found in epithelial cells	(45-48)
COL8A1, COL8A2	Extracellular matrix	Produced by CECs	qPCR, WB	DM component	(49-51)
CDH2	Transmembrane	Adherents junctions	IHC, qPCR	Specific for CECs	(47, 50, 52)
CD166	Basolateral transmembrane protein	ND	ICC, qPCR, FCs, IHC, IP	Found in epithelial cells, stromal cells, TM	(47, 48)
SLC4A11	Transmembrane	Na^+/OH^- co-transport, Na^+-independent H^+ (OH^-) transport, NH_3 transport	qPCR	ND	(49, 53)

CEC: Corneal endothelial cell; DM: Descemet's membrane; FCs: Flow cytometry; ICC: Immunocytochemistry; IHC: Immunohistochemistry; IP: Immunoprecipitation; ND = not determined: Reverse transcription Quantitative PCR; TM: Trabecular meshwork; WB: Western blot

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Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)

LGR5 is a leucine-rich repeat-containing G protein-coupled receptor which is a coreceptor for R-spondin (RSPO) (54). In the absence of RSPO, transmembrane E3 ubiquitin ligase RNF43/ZNRF3 is an enzyme that degrades Wnt receptors including Frizzled (FZD) and lipoprotein receptor-related protein (LRP) 5/6. RSPO binding to LGR5 leads to neutralizing RNF43/ZNRF3 ligases which allows Wnt/FZD/LRP complex persisting at the plasma membrane. This enhances activation of the Wnt/β-catenin signaling (55) (Figure 4). LGR5 is additionally a transcriptional target gene of canonical Wnt signaling (56). It is commonly acknowledged to be a stem cell marker for homeostasis in several tissues including stomach, hair follicle, intestine, mammary gland, and ovaries (57-61). It also has been found on facultative stem cells in the liver, pancreas, and stomach after tissue injury (62-64).

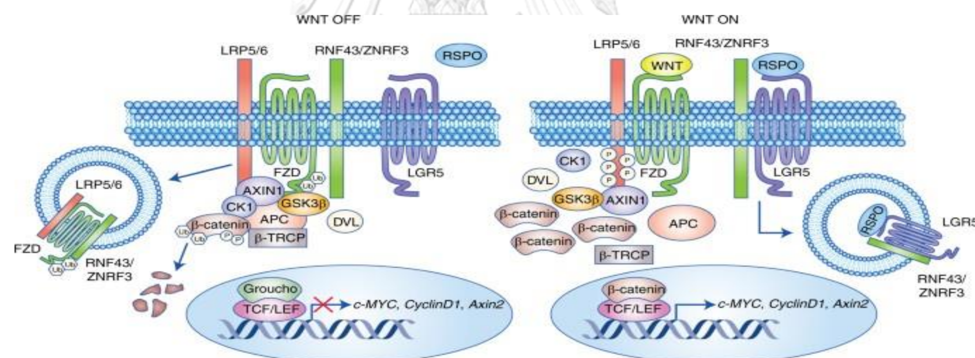

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Figure 4 LGR5 enhances canonical Wnt signaling via neutralizing RNF43/ZNF3 (65)

Wnt signaling pathway

The Wnt signaling pathways are a group of signal transduction pathways which involves in adult tissue homeostasis, embryogenesis, stem cell renewal and regeneration of tissue injury (66, 67). Wnt signaling pathways are divided into canonical Wnt/β-catenin pathway and non-canonical pathways, which is subdivided into the noncanonical planer cell polarity pathway and the noncanonical Wnt/calcium pathways (Figure 5). The canonical Wnt/β-catenin signaling is triggered by the binding of the Wnt proteins or Wnt ligands (WNTs) to the FZD and LRP 5/6

complex, leading to activating the Disheveled (DVL) protein to disrupt β-catenin destruction complex (68). Under unstimulated conditions, β-catenin is constantly low level in cytoplasm and controlled by the destruction complex which consisted of several protein including Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase (GSK3). This destruction complex phosphorylates β-catenin resulting in ubiquitination and proteasomal degradation of β-catenin (69). When the destruction complex is inhibited, β-catenin is accumulation in the cytoplasm and translocation into nucleus. Nuclear β-catenin interacts with transcription factor including T cell-specific transcription factor (TCF) and lymphoid enhancer-binding factor 1 (LEF) family and regulate Wnt target genes (70, 71). The planer cell polarity pathway, one of non-canonical or independent β-catenin pathways, activates the Rho family GTPase and c-Jun N-terminal Kinase (JNK) pathway which regulates cell adhesion and cell migration by controlled the cytoskeleton rearrangement and cell polarity (72). Another noncanonical Wnt pathway is noncanonical Wnt/calcium pathways. Its role is to help regulate cytoplasmic calcium level by regulating calcium release from the endoplasmic reticulum. Elevated cytoplasmic calcium can activate Nemo-like kinase (NLK) and nuclear factor of activated T cell (NFAT) signaling which important in neuronal growth during development (73).

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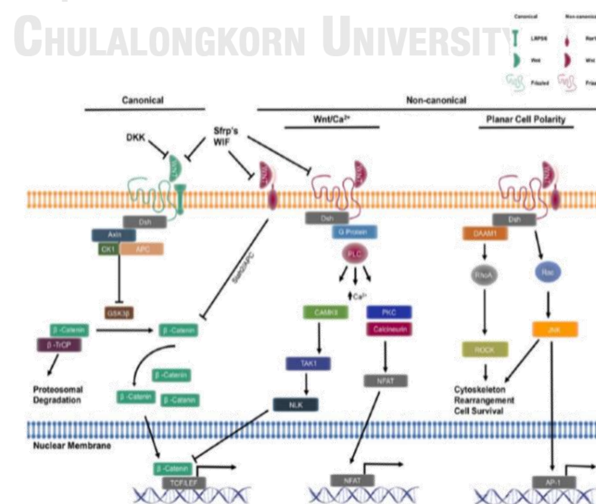

Figure 5 Wnt signaling pathways; canonical Wnt pathway, noncanonical Wnt/calcium pathways and noncanonical planer cell polarity pathway (74)

LGR5 and corneal endothelial cells

In cornea, LGR5 is likely to be the CEPs marker in corneal endothelium, which is high expression at the peripheral corneal endothelium and inner transition zone (8, 35, 39, 75). However, the expression of LGR5 was significantly reduced in cultured hCECs with similar to our preliminary experiment (35). Hirata-Tominaga and colleagues (2013) reported that $LGR5^{+}$ CECs were significantly smaller in size than LGR^{-} CECs. $LGR5^{+}$ CECs also have proliferative potential with some features of progenitor cells. In addition, they used the gain- and loss-of-function to investigate LGR5 role. They found that Hedgehog signaling pathway regulated LGR5 expression in peripheral CECs. The persistent LGR5 expression CECs have higher proliferation which exhibiting endothelial phenotypes via inhibiting EnMT through Hedgehog and Wnt signaling pathways (35). Although, in this study used monkey corneal endothelial cells (mCECs) to describe characteristics of $LGR5^{+}$ and LGR^{-} population and to study in LGR5 downregulation experiment. This might not fully represent the nature of hCECs. Furthermore, they did not evaluate the progenitor character and properties in corneal endothelium. It may be caused by difficult isolation of $LGR5^{+}$ cells. The challenging of purification LGR expressed cells might be from low expression $LGR5^{+}$ proteins, small population of $LGR5^{+}$ cells, low starter cell number, race $LGR5^{+}$ expression in cultured hCECs and ineffective LGR5-targeting antibodies (35, 76-78). Nonetheless, Katikireddy and colleagues (2016) can isolate a rapid proliferating subpopulation of hCECs which showed the character of neural crest-derived progenitor (NCDP) cells. This population showed high expression of LGR5 together with stem cell-related genes including SOX2, OCT4 and TP63 (39). Although, the isolation method of rapid proliferating CECs is not fully described and unable to be repeated by other groups. Furthermore, there are two studies investigating the role of RSPO which is ligand of LGR5. They found that CECs treated with RSPO1 have higher proliferation than non-treated CECs which maintaining CECs phenotype (35, 46). However, the association of RSPO1 and Wnt signaling in CECs is still unclear.

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Hirat-Tominaga and colleagues (2013) reported that RSPO1 accelerated pLRP degradation, which implies that RSPO1 might inhibit Wnt signaling. This contrast to Okamura and colleagues (2014), they found that RSPO1 significantly enhanced CECs proliferation especially at peripheral CECs via canonical Wnt signaling (46). The contrasting results might be forming different treatment times, different state, and different species of CECs. As aforementioned, activation of LGR5 might be the potential alternative treatment of corneal endothelial dystrophy. Though, the molecular mechanism and biology in LGR5^{+} CECs is still not fully elucidated.

Role of LGR5 in tissue homeostasis and injury

LGR5 has two main contexts in tissue homeostasis and injury. It is well known to be a stem cell marker for homeostasis in several tissues including antral stomach, hair follicle, intestine, mammary gland, ovaries (57-61). The most well-defined adult stem cell homeostasis is the intestinal crypt model. The expression of LGR5 cells are restricted at the base of the crypt which are actively cycling stem maintaining the stem cell pool and giving the rise differentiated cell lineages towards through the villus (56) (Figure 6). Additionally, single LGR5+ intestinal stem cells (ISCs) can establish intestinal organoids (three-dimensional self-organizing structure) in culture media containing RSPO-1, Wnt3a, EGF and Noggin (79).

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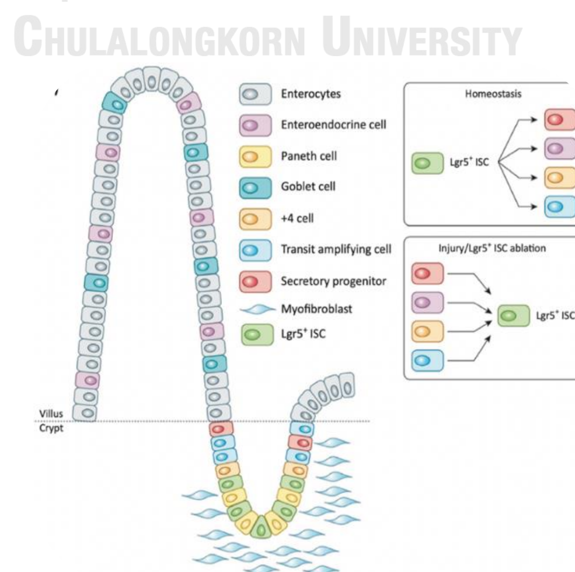

Figure 6 Intestinal homeostatic and injury (20)

The role of LGR5 in the context of injury has been studied and classified into two groups: injury-driven stem cell plasticity and $LGR5^{+}$ facultative stem cells system which can be further divided into LGR5 expressed before injury and LGR5 expression induced on injury. Injury-driven stem cell plasticity is found in intestinal epithelium regeneration after ablation of $LGR5^{+}$ ISCs in mouse model. They found that the +4crypt position, paneth cell precursors, enteroendocrine and secretory progenitors can dedifferentiate or reprogram to $LGR5^{+}$ ISCs (80) (Figure 6). While $LGR5^{+}$ facultative stem cells were found in the liver, pancreas, and stomach corpus after tissue injury (62-64). In stomach corpus gland, $LGR5^{+}$ cells are found in a subpopulation of quiescent, post-mitotic and differentiated chief cells at corpus gland base during homeostasis. The mature $LGR5^{+}$ chief cells are activated into proliferative stem cell to repopulation of the corpus epithelium after damage through activation of Wnt signaling (63) (Figure 7). In the liver regeneration. The early study suggested that $LGR5^{+}$ cells were not found during homeostasis, but they appeared near bile ducts of the portal triad area after liver injury in LGR5 knock-in reporter mouse model. A single $LGR5^{+}$ cells form previously liver injury or bile duct cell from healthy liver can generate organoids with hepatocyte-lineage and bile duct marker expression, which showed bipotent liver progenitor (81, 82) (Figure 7). These imply that bile duct cells are present in quiescent state in normal homeostatic, responding to tissue injury leading to turn into LGR5 stem cells to hepatic regeneration.

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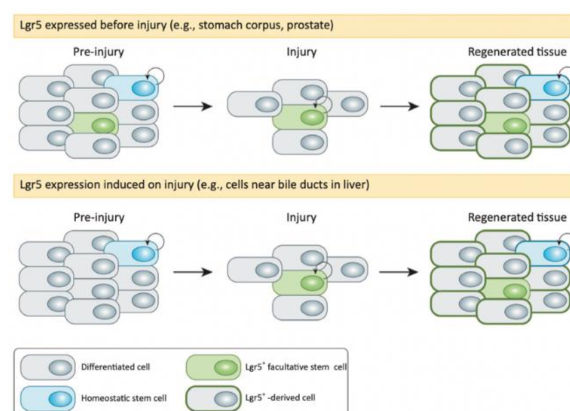

Figure 7 Lgr5+ facultative stem cell models (20)

Furthermore, LGR5 has been reported as a marker for cancer stem cells (CSCs). (83) The rapid renewal kinetic of intestinal epithelium prone to hyperproliferation and tumorigenesis, therefore the balancing niche homeostasis requires the both positive and negative signals (84). Wnt signaling plays a positive signal which promote the self-renewal and regeneration of ISCs. WNTs and RSPO are secreted form Paneth cells and stromal cells surrounding crypt bottom which also regulates intestinal crypt homeostasis (85). To maintain LGR5 expression, WNTs are required for initiation ISCs to response to RSPO after that RSPO regulates the ISC self-renewal and expansion (20). On the other hand, the stemness genes of LGR5+ stem cells were inhibited through directly Smad-mediated transcriptional repression, indicating that bone morphogenetic protein (BMP) plays a negative role. Consequently, BMP antagonist such as noggin and gremlin-1 is critical component of organoid culture to inhibit BMP signaling which might be promote LGR5 expression (86).

Epigenetics

Epigenetics is the study of changes in gene expression that do not involve changes to the DNA sequence, which plays a crucial role in gene expression regulation in development and cellular differentiation (11, 87). Epigenetic changes can occur through a variety of mechanisms, including DNA modification (DNA methylation and hydroxymethylation), histone modification, chromatin remodeling, and non-coding RNA (ncRNA) (88).

DNA methylation is an addition of a methyl group to the cytosine base of DNA (5-methylcytosine; 5mC) typically at the cytosine-guanine sequence (CpG). While CpG sites are found throughout the genome, they are commonly clustered in regions known as CpG islands, which are frequently located at promoter regions of genes (89). The enzymes responsible for DNA methylation are DNA methyltransferase (DNMTs) (90). Methylation of DNA can lead to silence gene expression by directly inhibiting the binding of transcription factors or by recruiting gene suppressor protein

(91). DNA hydroxymethylation is a DNA modification which involves adding a hydroxymethyl group to a cytosine nucleotide in DNA (5-hydroxymethylcytosine; 5-hmC) via Ten-eleven translocation (TET) family enzymes including TET1, TET2 and TET3 (92). Although the role of DNA hydroxymethylation in gene regulation is not well understood, several studies suggest that it may play a role in promoting gene expression by facilitating the recruitment of transcriptional activators or preventing the binding of transcriptional repressors (93) (Figure 9).

Histone modifications are a post translational modifications of amino-acid sequences of the histones tail via the addition or removal of chemical groups, such as acetyl, methyl, phosphate, or ubiquitin groups (94). Histone modifications affect their interaction with DNA in different ways. Some modifications can interfere with the way histones interact with DNA, leading to the unwinding of nucleosomes. When this happens, the chromatin structure becomes more open and is referred to as euchromatin, making it possible for transcriptional machinery to bind to DNA and activate gene expression. In contrast, some modifications can enhance histone-DNA interactions, leading to the formation of a compact chromatin structure called heterochromatin. In this state, DNA is less accessible to transcriptional machinery, resulting in the suppression of gene expression (95). Histone acetylation is a modification of histone protein that adding an acetyl group to lysine residues by histone acetyltransferase (HAT). This changes the positive charge of the lysine to a neutral one, which weakens the interaction between histones and DNA. As a result, transcription factors and other regulatory proteins can access DNA more easily, leading to increased gene expression. In contrast, histone deacetylase (HDAC) removing acetyl group cause hypoacetylation of histone, which plays a role in the suppression of gene expression (17). Histone methylation is adding methyl group on the residual lysine, arginine or histidine by histone methyltransferase (HMT). The effects on transcription differ depending on location of methylation and degree of methylation. For example, histone modification H3K4me3 is commonly related to

transcriptional activation. In contrast, H3K27me3 is related to repressed signals (96). Furthermore, DNA modification and histone modification has crosstalk. TET enzymes also play non-catalytic roles in histone modification by forming chromatin regulatory complexes with OGT, HDACs, and/or histone acetyltransferases (HATs) (97).

Non-coding RNA (ncRNA) is a type of RNA molecule that does not encode a protein, but instead plays a regulatory role in gene expression. There are several types of ncRNAs with varied functions, including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), small interfering RNAs (siRNAs), and ribosomal RNA (rRNAs). ncRNA has an ability to control gene expression at the level of the gene and chromosome regulation (98).

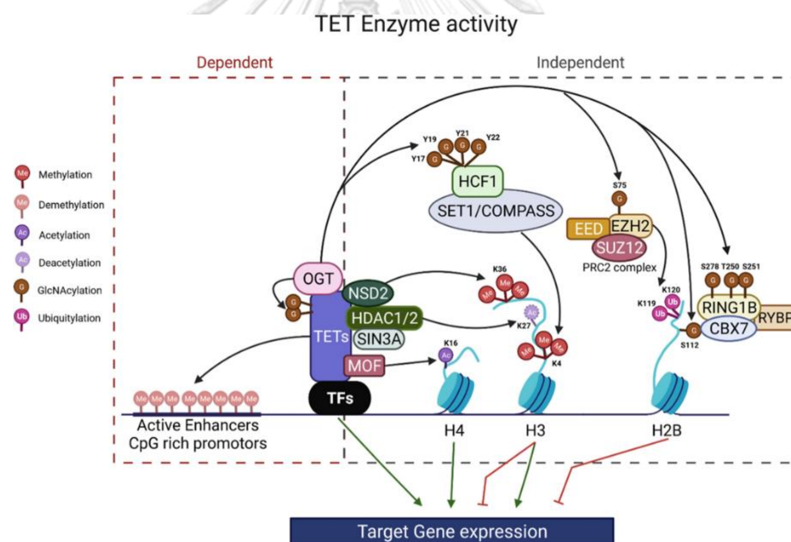

Figure 8 TETs regulate gene expression by both enzyme-dependent and -independent mechanisms (97)

Epigenetic regulation of LGR5

As previously stated, epigenetics play a crucial role in regulating gene expression during cellular differentiation, development, and homeostasis. Additionally, LGR5 is a marker for stem cells in several tissues. Therefore, we will concentrate on past research investigating the epigenetic mechanisms and profiles linked to *LGR5* gene. Previous genome-wide studies into DNA methylation dynamic

during differentiation of ISC in vivo and ex vivo. They found that DNA methylation is changed at specific location during differentiation process of ISC. However, DNA methylation is not gain at the promotor region of *LGR5* gene during differentiation, suggesting that *LGR5* expression does not regulate through DNA methylation at promotor of its gene (99-102). In the study of DNA hydroxymethylation, Kim and colleagues (2016) found that genes associated with Wnt signaling and developmental processes had high levels of hydroxymethylation correlated with abundant TET1. To investigate the role of 5hmC and TET1, TET1 deficient mice and their intestinal organoid were used in this study. They found that the decreased expression of Wnt target gene (*AXIN2* and *LGR5*) in the crypt of TET1 deficient mice is linked with reduced level of 5hmC at their promoter. Thus, TET1 plays as important epigenetic modulator of *LGR5* in ISC and progenitor cells during homeostasis and differentiation (14). This report corresponds with Aloia and colleagues' study, they found that elevated of 5hmC level at the regions surrounding promoters of *LGR5* gene as well as within gene body in cholangiocyte organoid. Thus, TET1-mediated hydroxymethylation controls expression of stem cell (*LGR5*), proliferation genes during liver injury to activate cell proliferation and dedifferentiation through ErbB/MAPK and YAP/Hippo signaling (15) (Figure 10).

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Furthermore, various research studies have shown that the expression of LGR5 is also regulated by modification of histone. Trimethylation on histone H3 lysine 27 (H3K28me3), which is a histone modification associated with gene silencing, is found to be increased on genes specific to intestinal stem cells, such as *LGR5, ASCL2*, and *MCY* in differentiated intestinal epithelial cells (100, 103). Additionally, Uchida and colleagues (2019) found that epigenetic modification in aging induces silencing of *LGR5* by H3K27me3 which reduces cell proliferation and suppresses Wnt signaling of intestinal epithelial organoids. Thus, the expression level of LGR5 was increased after treatment with DZnep (Histone methyltransferase EZH2 inhibitor) (102). Trimethylation on histone H3 at lysine 4 (H3K4me3), one type of histone

modification at the transcription start site of activate genes, is enriched at *LGR5* promoter in the crypts of intestine and in cholangiocytes organoids (Figure 10) (100, 104). Qi and colleagues found that histone deacetylase (HDAC) 1 has been recruited by Smad1/Smad4 to the promoters which represses gene expression such as *LGR5, SOX9* and *OLFM4* (13). After treated with trichostatin A (TSA), specific inhibitor of HDAC class I/II, *LGR5* expression is increased in intestinal organoid (13). In addition, other HDAC inhibitors, such as valproic acid (VPA), enhanced *LGR5* expression in mouse intestinal organoid but weakly increased colony-forming efficiency (18). The summary of epigenetic regulation of *LGR5* gene in mice and humans was shown in Figure 11 and 12.

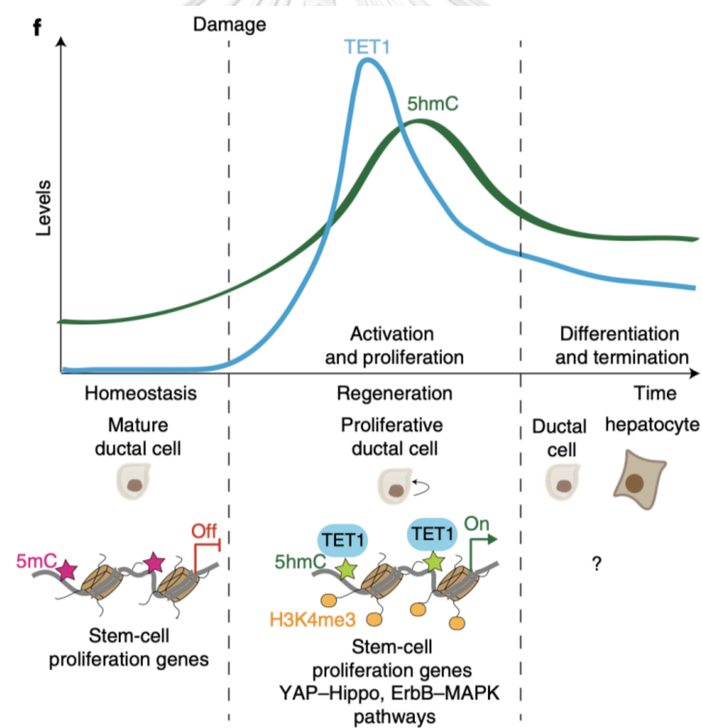

Figure 9 epigenetic change in liver injury model (15)

As mentioned above, a combination of intrinsic (involving transcription factor, signaling pathway and epigenetics mechanisms) and extrinsic mechanisms from niches environment can promote cell plasticity and reactivate quiescent, which

might regulate the repopulation of LGR5+ cells through induced-injury plasticity and LGR5+ facultative stem cells. Therefore, LGR5 might be activated by using small molecules and protein associated with LGR5+ stem cell homeostasis and LGR5+ facultative stem cell which is shown in table 3.

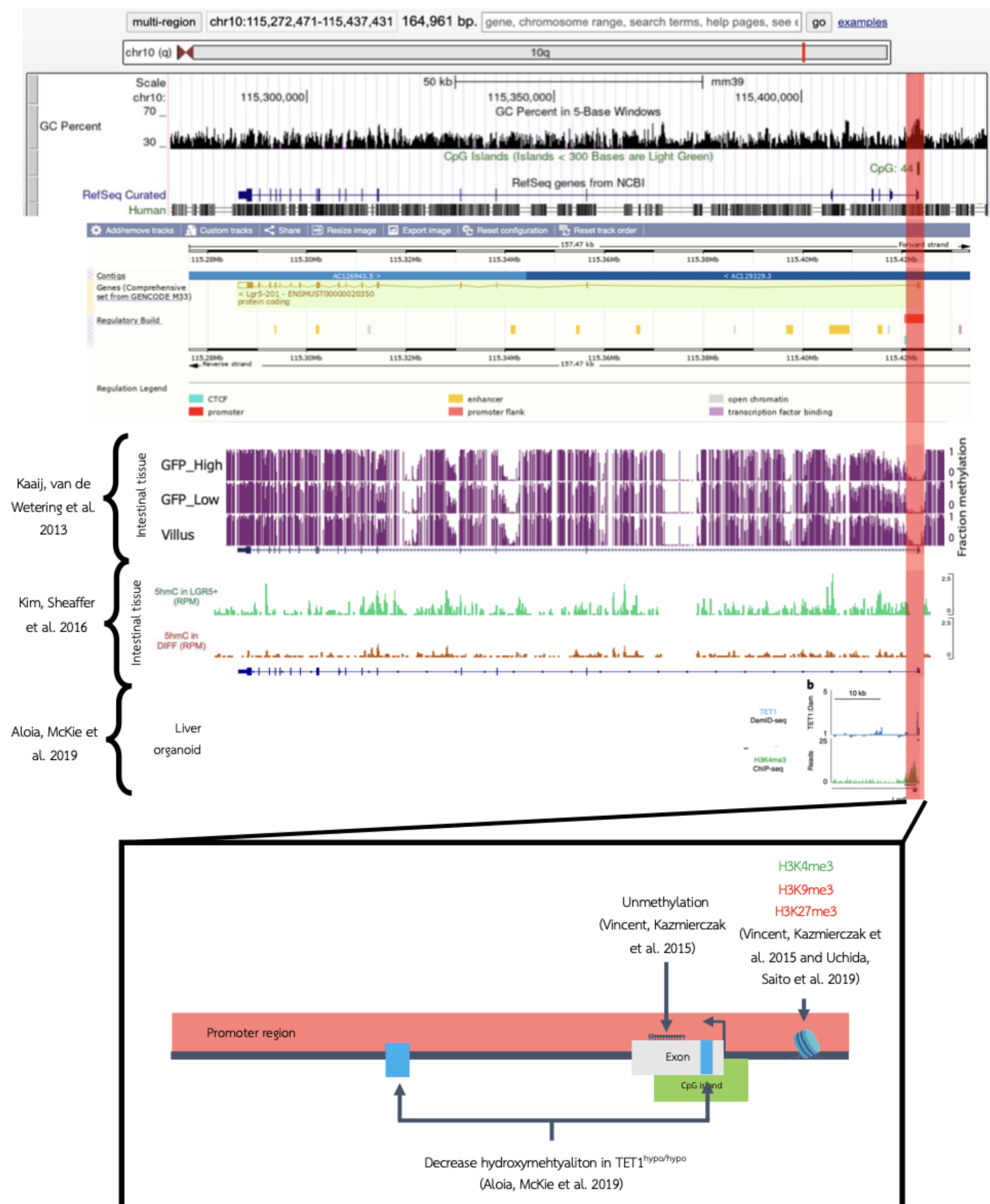

Figure 10 Schematic illustrates the epigenetic regulation at LGR5 gene in mouse by referencing from http://genome.ucsc.edu and https://www.ensemblgenomes.org.

Figure 11 Schematic illustrates the epigenetic regulation at LGR5 gene in human by referencing from http://genome.ucsc.edu and https://www.ensemblgenomes.org.

Table 3 Summary of small molecules effects LGR5 expression

Name	Function	References
SHH	Hedgehog ligand	(35)
Purmorphamine	Hedgehog agonist	(35)
R-spondin 1	- LGR5 ligand with coactivator of Wnt signaling - Essential for organoid culture	(86)
Wnt3a	- Wnt ligand activate Wnt signaling - Essential for organoid culture	(86)
Noggin, Gremlin-1	- BMP antagonist - Essential for organoid culture	(13, 86)
Valproic acid (VPA)	- HDAC inhibitor - Maintain self-renewal of mouse LGR5$^+$ intestinal organoid with/without CHIR99021	(18, 19)

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Trichostatin A (TSA)	- HDAC inhibitor - Increase expression of LGR5 in intestinal organoid	(13)
Dznep	- Histone methyltransferase EZH2 inhibitor	(102)
TET1	- DNA hydroxymethylation	(14, 15)

CRISPR-dCas9 system

Sequence-specific targeting of epigenetic modifier is used to reprogram and regulate target gene transcription via DNA-binding vehicle including Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and nuclease-deactivated Cas9 (dCas9). Recently, the dCas9 is an antiviral enzyme without nuclease activity which has been adopted as DNA-binding vehicle together with transcriptional activators (105). The Clustered regularly interspaced short palindromic repeats system for gene induction (CRISPR-on) or CRISPR-Cas transcriptional activation (CRISPRa) is one type of CRISPR systems which uses dCas9 fused with effector protein or active domain to guide a transcriptional activator complex at specific region of target gene via the engineered guide RNA (gRNA) (106) (Figure 12).

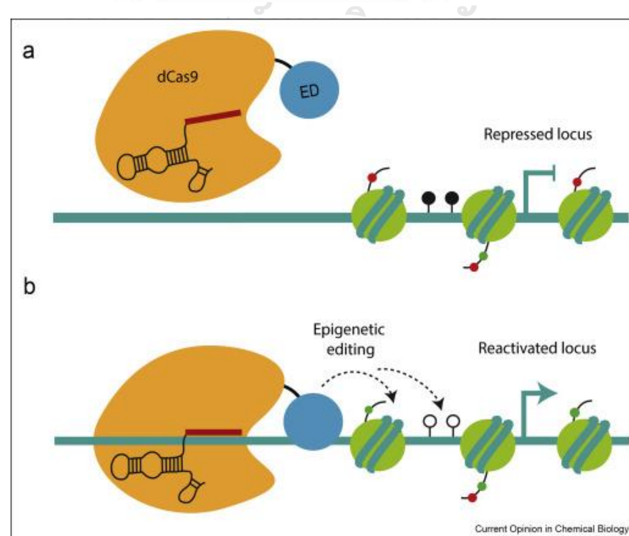

Figure 12 Epigenetic editing with the CRISPR/dCas9 platform (107)

The VP16-based CRISPR/dCas9 activators have been commonly used as transcriptional activator protein which consists of multiple repeats of the activation domain of herpe simplex viral protein 16, VP16. The first generation of CRISPRa is dCas9 fused to four copies of VP16 (dCas9-VP64). It can activate silent endogenous genes and upregulation of target genes (108). VP64 also fused with other activator domains such as VPR (VP64, p65 and Rta) and SunTag (repeated of VP64) to increase activation. Recently, the CRISPRa have been used to promotes regeneration of CECs via activated *SIRT1* and *SOX2* (109, 110). These used synergistic activation mediator (SAM) consisted of dCas9-VP64, engineered sgRNAs with MS2 RNA aptamers and activation fusion protein complex (MS2, HSF1 and p65) (111). Tammela and colleagues (2017) used SAM system to overexpress *LGR5* in *Kras*$^{G12D/+}$;*Trp53*$^{\Delta/\Delta}$ lung adenocarcinoma cell line which increased more than 100 fold (112). Therefore, the CRISPRa system could activate target gene expression such as *LGR5*.

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CHAPTER 3
MATERIALS AND METHODS

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 Helsinki Declaration. All human corneal tissues were collected form research-grade cornea tissue and remnant of clinical-grade tissue after corneal transplantation from the King Chulalongkorn Memorial Hospital and the Thai Red Cross Eye Bank, Bangkok, Thailand. The corneas were stored less than 14 days prior to use the hCECs in the culture.

Isolation and culture corneal endothelial cells

The Descemet's membrane-hCECs complexes were stripped and digested at 37 °C with 0.25% Trypsin-EDTA at 37 °C for 5 minutes followed by up-and-down pipetting. Then, they 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). The hCECs were cultured in expansion medium (basal medium, 5 ng/ml epidermal growth factor: EGF) and plated into a 6-well plate coated with collagen type I. The cultured hCECs were maintained in a at 37 °C in 5% CO_2 and replaced with fresh expansion medium every 3 days. When they reached confluency in 3 to 4 weeks, they were rinsed in PBS, trypsinized with 0.05% TrypLE™ Select for 10 minutes at 37 °C, and resuspended in expansion medium. The culture was passaged on a culture plate in a 1:2 to 1:3 ratio.

Culture of HEK293 cell line

The HEK293 cell line was obtained from the Excellence Center for Stem Cell and Cell Therapy, the King Chulalongkorn Memorial Hospital, Bangkok, Thailand. HEK293 cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, 1% GlutaMAX, and 1% antimycotic–antibiotic reagents (Gibco). They were maintained at 37 °C in a 5% CO_2 atmosphere.

Immunofluorescence

For fresh tissue, the cornea tissues were washed with 1XPBS three times, followed by fixation in cold methanol for 15 minutes at 4 °C. For cell culture, the hCECs were seeded on cover slip or chamber slide. The culture media was removed and rinsed with 1XPBS. The cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes and rinsed three times with 1XPBS for 10 minutes. The fresh tissue or cells were permeabilized with permeabilization solution (0.3% Triton X-100 in 1XPBS) for 30 minutes and blocked with blocking buffer (10% goat serum in 1XPBS) for 60 minutes. The cultured antibody was added and incubated overnight at 4 °C followed by washing three times with 1XPBST (1XPBS with 0.1%Tween®20) for 10 minutes each. After that, secondary antibody was added and incubated for 2 hours at room temperature in a dark room, followed by rinsing three time with 1XPBST for 10 minutes each. Nuclei were stained with DAPI at 1:1000 for 10 minutes, followed by washing with 1XPBST for 10 minutes. The samples were mounted with a gold antifade mountant. Finally, the fluorescence signals were observed and captured by florescence microscope (ZEISS).

Flow cytometry for LGR5 detection

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, they were washed with 1XPBS and centrifuged at 1000 rpm for 5 minutes at 4 °C. The stained cells were analyzed by flow cytometry using BD FACSAria II (Becton Dickinson, Franlin Lakes, NJ, USA)

Cytotoxicity Assay

The MTT assay was used to test the cytotoxicity of small molecules in hCECs. The hCECs were seeded in a 96-well plate at a concentration of 5,000 cells per well. Then, the cells were exposed to various concentrations of Trichostatin A (5, 10, 25, 50, and 100 nM) and Valproic acid (0.05, 0.1, 0.5, and 1 mM). After incubation for 7 days, the MTT solution (0.5 mg/ml) was added to each well, followed by incubation

at 37 °C for 3 hours. MTT-formazan crystals were solubilized by adding DMSO. Then, the plate was measured at a wavelength of 540 nm by using Varioskan Flash (Thermo).

DNA extraction and bisulfite sequencing PCR

Genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen). Bisulfite conversion was performed using MethylEdge® Bisulfite Conversion System (Promega) according to the manufacturer's instruction. For bisulfite sequencing, BSP primers from publish article were used to amplify the region from the promoter to exon 1 of LGR5 gene (428 bp). (16) Primers sequences were as follow: forward 5'-GGGTGTTTGGGAAGTTAGGTT -3' and reverse 5'-CAACTACAACAACACAAACAAAAAC-3'. The PCR products were cloned into pGEM®-T Easy Vector System, and DNA sequencing was performed on plasmid DNA obtained from five bacterial clones.

Hydroxymethylated DNA immunoprecipitation

Genomic DNA was sonicated for 8 cycles to obtain DNA fragment with sizes ranging from 200 to 600 base pairs (30 seconds on/ , 30 second off). The hydroxymethylated DNA Immunoprecipitation (hMeDIP) kit (Abcam) was employed for immunoprecipitation, following the manufacturer's instruction. hMeDIP-qPCR was performed to quantify the amount of specific hydroxymethylated DNA. The primers sequences are provided in table 6.

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Designed single guide RNA

The sgRNAs were designed following Zhang's protocol. Briefly, the sgRNAs were designed to target the -200 to +200 bp window of the transcription start site (TSS) using https://benchling.com/. Moreover, the four sgRNAs were designed as detailed in the previous article, covering a 300-base pair segment of LGR5 promoter that includes a TCF binding element. Primer sequences used are listed in Table 4.

Table 4 Primers used for sgRNA vector cloning.

	Forward primer	Reverse primer
sgRNA1	CACCGACTGGCGCGCAATTCGGGC	CTGACCCGCGCGTTAAGCCCGCAAA
sgRNA2	CACCGCGGGGGGTGCCTGGGAAGCC	CGCCCCCCACGGACCCTTCGGCAAA
sgRNA3	CACCGCGGGGGGTGCCTGGGAAGCC	CGCCCCCCACGGACCCTTCGGCAAA
sgRNA4	CACCGCCCAGCCCAGGACTTGGGAA	CGGGTCGGGTCCTGAACCCTTCAAA
sgRNA5	CACCGCTTACGTCTGCCGCACTGT	AAACGACAGTGCGGCAGACGTAAGC
sgRNA6	CACCGCGTCCCCGGCGAATGATAGG	AAACCCGATCATTCGCCGGGGACGC
sgRNA7	CACCGTTACGTTATCAGGGTAAGG	AAACCCTTACCCTGATAACGTAAC
sgRNA8	CACCGATTATTTGAAGCGGGCTCGG	AAACCCGAGCCCGCTTCAAATAATC

Plasmid construction of CRISPRa

The gRNA was cloned into pAC154-dual-dCas9VP160-sg expression (Addgene plasmid #48240). The specific oligos were phosphorylated and annealed with 10X T4 Ligation Buffer and T4 PNK by incubating them in thermocycler at 37 °C for 30 minutes, then at 95 °C for 5 minutes, followed by a gradual cooldown to 25 °C at a rate of 5 °C per minute. Then, the plasmids were digested with BbsI for 30 minutes at 37 °C, followed gel extraction by QIAquick Gel Extraction Kit. Then, the digested plasmid and annealed oligos were ligated at room temperature for 10 minutes. The ligated plasmids were transformed into Stellar competent bacteria using heat shock method. The mixture of competent bacteria and plasmid was incubated on ice again for 30 minutes, then heat shock at 42 °C for 45 seconds before placed back on ice for 3 minutes. After that, one milliliter of SOC media was added, and the mixture was incubated on shaker at 200 rpm at 37 °C for 45 minutes. The mixture was then centrifuged at 5,000 rpm for 5 minutes and the supernatant was removed. The pellet was plated onto 10 cm LB agar plate with 100 µg/ml of ampicillin and spread them using sterile spreader. The plate was incubated overnight at 37 °C. Colonies was picked into LB media with 100 µg/ml of ampicillin and then incubated at 37 °C overnight. The plasmids were extracted by using QIAGEN Plasmid Mini kit before

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doing cut-check to verify the correct insertion. The preferred plasmids were expanded in LB media with 100 µg/ml of ampicillin in large scale and isolated via QIAGEN Plasmid Midi kit.

Transfection in HEK293 cells

The electroporation technique, utilizing the SF cell line 4D-Nucleofector® X Kit S, was employed to introduce the plasmids into HEK293 cell line. The cells were cultured and allowed to reach passage before the Nucleofection process. A total of $5x10^5$ cells were employed. Subsequently, the cellular pellet was mixed with Nucleofector^{TM} solution, supplementary components, and plasmids. This mixture was then transferred into the Nucleocuvette^{TM} Strip. The strip was inserted into the 4D-Nucleofector^{TM} X Unit and initiated with the CM-130 program. Once the program concludes, the strip was left at room temperature for 10 minutes and then reconstituted with pre-warmed medium. The transfected cells were cultivated for 72 hours at 37°C in a 5% CO2 environment until they are ready for analysis.

Transfection in hCECs

The plasmids were transfected into hCECs using the electroporation method with the P3 cultured cell 4D-Nucleofector® X Kit S. The hCECs were passaged 2-4 days before Nucleofection. At least 10^5 cells were used for each transfection. The cell pellet was resuspended in NucleofectorTM solution, supplement, and plasmid, and subsequently transferred into the Nucleocuvette^{TM} Strip. The strip was placed into 4D-Nucleofector^{TM} X Unit and the CA-137 program was run. Upon completion of the program, the strip was incubated at room temperature for 10 minutes, followed by resuspension in pre-warmed medium. The transfected hCECs were incubated for 72 hours at 37°C in a 5% CO2 environment until analysis.

RNA extraction

RNA was extracted by TRIzol Reagent (Ambion). The hCECs were lysed with 1 ml TRIzol reagent per $1x10^7$ cells for 5 minutes. Next, Chloroform/Isoamyl alcohol was added and vigorously shaken for 10 seconds. The mixture was incubated for 10

minutes and then centrifuged for 15 minutes at 12,000 rpm at 4 °C. The supernatant was transferred to a new tube followed by precipitated with 0.5 ml isopropanol and incubated overnight at -80 °C. The mixture was centrifuged at 12,000 rpm for 20 minutes at 4 °C, then the supernatant was discarded. The RNA was washed with 75% Ethanol and centrifuged at 12,000 rpm for 10 minutes at 4 °C. The supernatant was removed without disturbing the pellet and air-dry for 10 minutes. The RNA pellet was dissolved in nuclease-free water and stored at -80 °C.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

The RT-qPCR was used to determine the expression level of the gene of interest. The Complementary DNA (cDNA) was generated from 1,000 ng of RNA template using the RevertAidH Minus Kit (Thermo Scientific). The RNA template was mixed with 1 µl of Oligo$(dT)_{18}$ primer and incubated in a thermocycler at 70 °C for 5 minutes, then immediately cooled on ice for 5 minutes. The RT PCR master mix (4 µl 5X Reaction buffer, 2 µl 10 mM dNTP, 0.5 µl Thermo Scientific™ RiboLock RNase inhibitor and 0.5 µl nuclease-free water) was added into the mixture, followed by incubation in a thermocycler at 37 °C for 5 minutes. Next, RevertAid H Minus Reverse Transcriptase (RT) was added to the mixture, and it was incubated in thermocycler at 42 °C for 60 minutes and 70 °C for 10 minutes. Subsequently, qPCR was performed using SYBR® Green PCR protocol. The 2 µl of cDNA was mixed with the qPCR master mix (12.5 µl 2X SYBR® Green PCR master mix, 1 µl forward primer, 1 µl reverse primer, 8.5 µl nuclease-free water). The thermocycling conditions were as follow: initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 45 seconds. Relative expression levels were obtained by comparing to the housekeeping gene GADPH and calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Primer sequences used for qPCR are listed in Table 5.

Table 5 Primers for qPCR

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')
GADPH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG
LGR5	GAGGATCTGGTGAGCCTGAGAA	CATAAGTGATGCTGGAGCTGGTAA
TET1	CAGAACCTAAACCACCCGTG	TGCTTCGTAGCGCCATTGTAA
TET2	GATAGAACCAACCATGTTGAGGG	TGGAGCTTTGTAGCCAGAGGT
TET3	TCCAGCAACTCCTAGAACTGAG	AGGCCGCTTGAATACTGACTG
TET1CD	CAGGACCAAGTGTTGCTGCTGT	GACACCCATGAGAGCTTTTCCC
hMeDIP	CTGAGTTGCAGAAGCCCA	GCACAGGCAAGGACAGGA

Data Analysis and Statistics

Statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software Inc., United States). All statistical tests were performed using triplicate experiments. Results are expressed as mean ± SEM. The unpaired two-tailed Student's t-test was used to analyze differences between 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.

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CHAPTER 4

RESULTS

Characterization of cultured human corneal endothelial cells

Several reports have emerged on protocols for the isolation and culture of cultured human corneal endothelial cells. The present study was conducted based on a previously published protocol with modification (5). The cells obtained from the Descemet membrane corneal endothelial complex were placed on culture plate as cultured cell cultures, followed by the observation of their morphology using phase-contrast microscopy. At the confluent, the cells exhibited corneal endothelium-like cellular morphology which displayed hexagonal pattern with dense packing in a continuous monolayer (Figure 13). After reaching confluency, the cells were sub-cultured and seeded at ratio of 1:2 or 1:3 from P0 to P4. The cultured cells at P1 and P3 displayed a compact monolayer of polygonal/hexagonal cellular morphology similar to that of the P0 cultured cells. While the P4 cultured cells have become heterogeneous in size and morphology. Some cells were slightly elongated, increased cell size and increased number of vacuoles in cytoplasm, which are the character of senescence (Figure 13). Thus, we used cultured cells in passage1 to 3 in our experiment.

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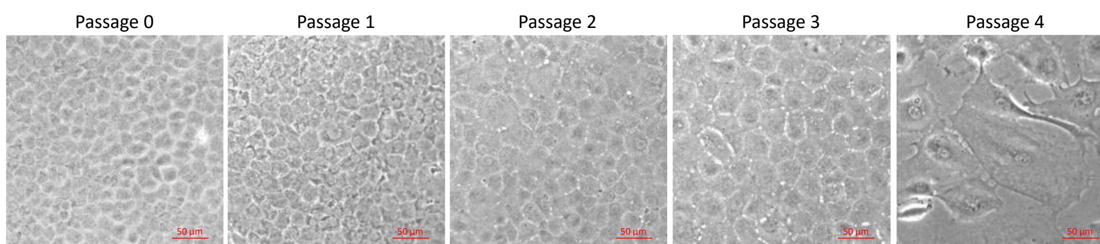

Figure 13 Brightfield morphological of cultured hCECs using phase-contrast microscopy by each passage.

To verify the identity of the cultured cells as corneal endothelial cells, immunostaining was performed on the human cornea tissue and cultured cells using the corneal endothelial cell markers which is the tight junction protein zona occludent 1 (ZO-1) (113). The results showed that ZO-1 was detected in almost all the cultured cells and in the cornea tissue, where it was primarily localized at the cell border, contributing to the formation of a hexagonal shape which is the characteristic of corneal endothelium morphology (Figure 14). Therefore, it can be concluded that the cultured cells were corneal endothelial cells, establishing the successful isolation and culturing of cultured human corneal endothelial cells.

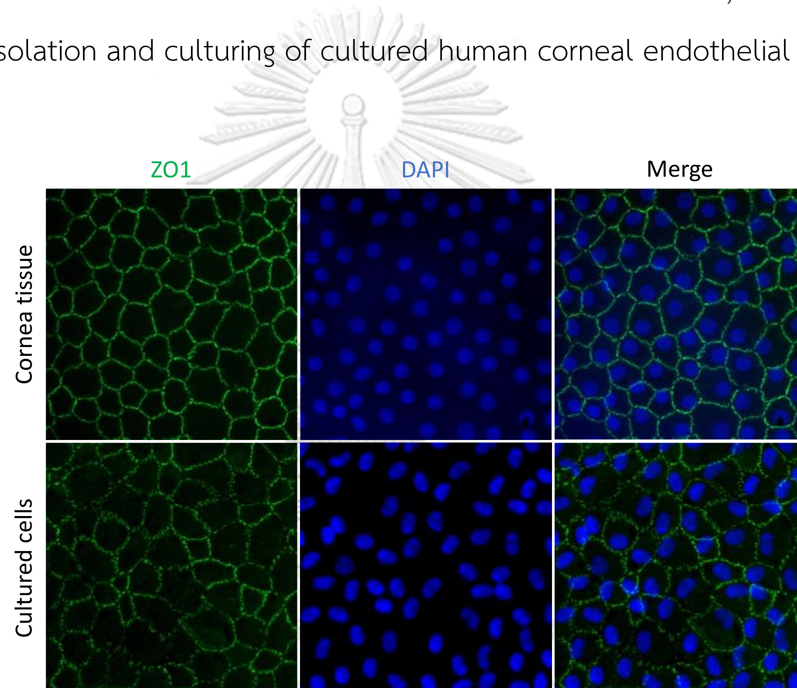

Figure 14 Characterization of hCECs in cornea tissue and culture. Immunofluorescence staining of cornea endothelial marker (ZO-1) was stained in green. Nuclei were stained in blue for DAPI.

Downregulation of LGR5 in cultured hCECs

The immunofluorescence technique was used to investigate LGR5 expression pattern in whole cornea tissue. We found that LGR5 was expressed both in central and peripheral tissue with increasing intensity at peripheral area (Figure 15).

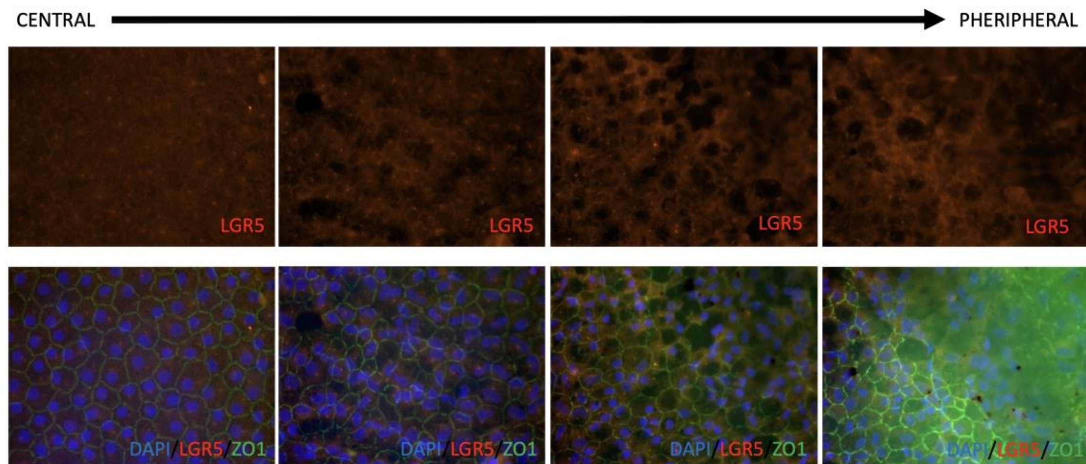

Figure 15 Characterization of LGR5 expression in cornea tissue. Immunofluorescence staining of ZO-1 and LGR5 in whole mount cornea tissue spanning from the central to peripheral regions. ZO-1 was visualized in green, LGR5 in red, and cell nuclei were stained in blue.

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To compare LGR5 expression in cornea tissue and cultured hCECs, we employed Real-time PCR and flow cytometry analyses. The real-time PCR showed a significant downregulation of *LGR5* mRNA expression in cultured hCECs compared to the expression observed in the donor tissue ($p \leq 0.05$) (Figure 16A). The flow cytometry analysis also demonstrated a downregulation of LGR5 protein levels in cultured hCECs compared to donor, with a notable gradual decrease across cell passages (Figure 16B). These findings indicate that LGR5 was downregulation in cultured hCECs.

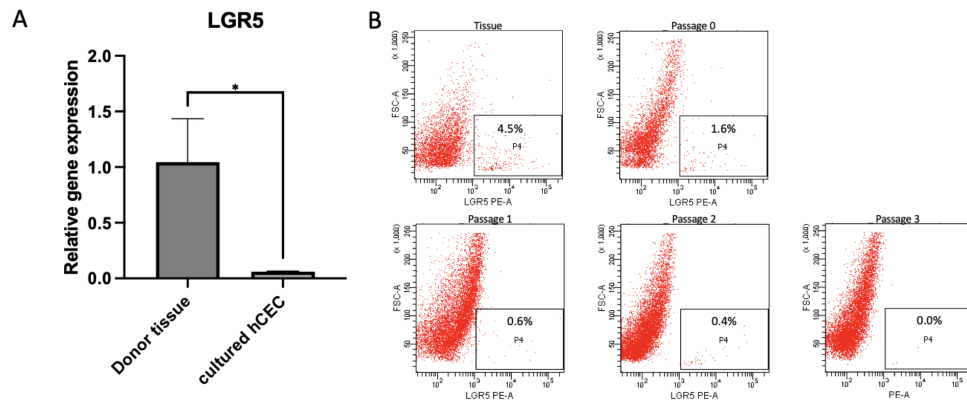

Figure 16 Comparison of LGR5 expression in donor tissue and cultured hCECs. LGR5 expression levels were quantitatively measured in donor and cultured hCECs using (A) qRT-PCR and (B) flow cytometry analysis.

Low level of DNA methylation at *LGR5* promotor in cultured hCECs

Epigenetic modifications regulate gene expression without altering the DNA sequence. These modifications can influence gene expression by either activating or silencing specific genes through DNA modification, histone modification and chromatin remodeling (88). DNA methylation is one of these DNA modifications, involving the addition of a methyl group to the cytosine base of DNA, typically at CpG sites (89). DNA methylation can lead to the silencing of gene expression by directly inhibiting the binding of transcription factors or by recruiting gene suppressor protein (91). A previous study suggested that DNA methylation in the promoter region of *LGR5* gene contributed to the silencing of LGR5 expression in colon cancer cell lines (16). To determine whether the suppression of *LGR5* expression in cultured hCECs might be due to DNA methylation at CpG island in the promoter region or not, we first examined the expression of *LGR5* gene after treatment with 5-aza-2'-deoxycytidine. We found that LGR5 expression did not significantly differ between different concentrations treatment with or without 5-aza-2'-doxycytidine, implying global demethylation could be insufficient to activate *LGR5* expression in cultured hCECs (Figure 17). We further investigated the methylation status at promoter region of LGR5 gene in cultured hCECs, we conducted bisulfite sequencing PCR (BSP) in

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cultured hCECs. The primer sequences for PCR were obtained from a previous article, designed to cover 50 CpG sites within the -91 to +337 region relative to the transcription start site (TSS) of *LGR5* gene (16) (Figure 18A). Our findings revealed that low levels of DNA methylation at promoter region of *LGR5* gene in cultured hCECs (Figure 18B). Therefore, the suppression of *LGR5* gene expression in cultured hCECs might not be due to DNA methylation at the promoter region of *LGR5* gene.

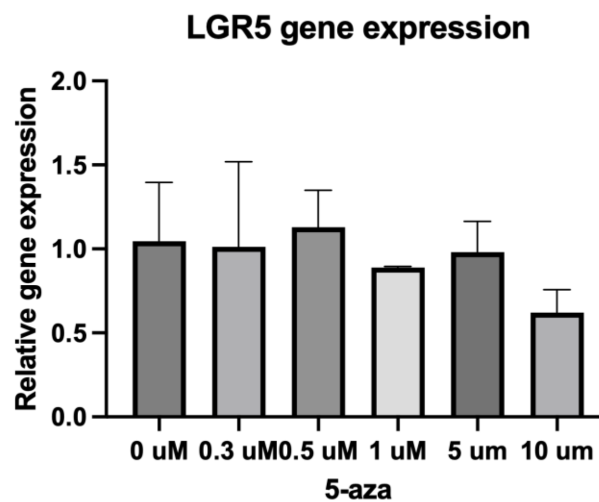

Figure 17 Comparison LGR5 gene expression in cultured hCECs after treatment with various concentration of 5-aza-2'-deoxycytidine using qRT-PCR

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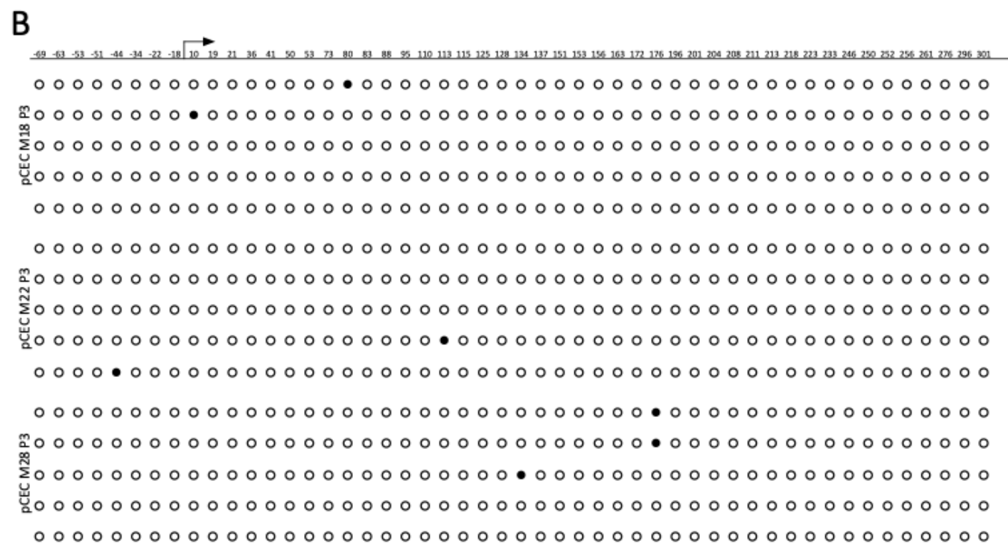

Figure 18 (A) Schematic illustrates the BSP region in CpG Island in the promoter region of LGR5 gene by referencing from http://genome.ucsc.edu and https://www.ensemblgenomes.org. (B) The DNA methylation status in the promoter region of LGR5 gene was assessed through BSP analysis in three cultured hCECs. In the upper panel, diagram of the distribution of CpG sites within the 5' UTR of LGR5 gene, with each vertical bar denoting a CpG site. In the lower panel. The BSP results depict the methylation status in three cultured hCECs. Each row represents a unique cloned allele that underwent sequencing after bisulfite DNA modification. Circles represent CpG site, a black circle signifies a methylated CpG site, while a white circle represents an unmethylated CpG site.

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Low levels of DNA hydroxymethylation at *LGR5* promoter in cultured hCECs.

DNA hydroxymethylation holds the potential to activate specific genes and influences over various processes such as development, tissue regeneration, and disease (93). Previous study has shown that a decrease in DNA hydroxymethylation at *LGR5* promoter region correlated with a reduction in *LGR5* expression in mouse cholangiocyte organoids (104). To investigate the DNA hydroxymethylation level at the promoter region of *LGR5* gene, we conducted a hydroxymethylated DNA immunoprecipitation (hMeDIP)-qPCR assay in HEK293 cell line and cultured hCECs. The HEK293 cell line served as positive control of LGR5, TET1 and DNA

hydroxymethylation. We designed a primer that is located in *LGR5* promoter region to measure the DNA hydromethylation levels (Figure 20A). Our results demonstrated a significant decrease in DNA hydroxymethylation levels at *LGR5* promoter region in cultured hCECs compared to HEK293 cell line ($p \leq 0.01$) (Figure 20B).

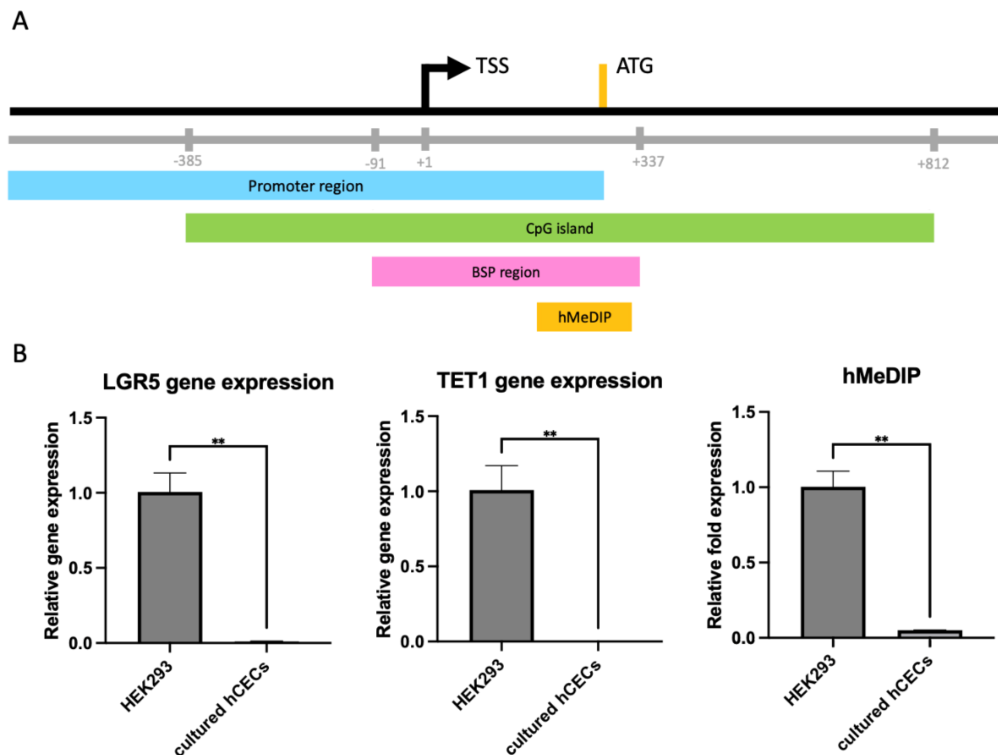

Figure 19 (A) A Schematic illustrates the hydroxymyethylation at the promoter region of LGR5 gene. (B) Gene expression levels of LGR5 and TET1 were measured using qRT-PCR and Hydroxymehtylation level at promoter region was measured using hMeDIP-qPCR in cultured hCECs and HEK239 cell line.

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DNA hydroxymethylation, catalyzed by enzymes known as Ten-Eleven Translocation (TETs) including TET1, TET2 and TET3, which involves the oxidation of methylated cytosine residues (5mC) within DNA (92). Previous articles have reported that TET1-mediated hydroxymethylation regulates the expression of LGR5 in intestinal stem cell and facultative stem cell during liver injury (14, 15). Together with previous results, we therefore hypothesized that low levels of DNA

hydroxymethylation might be due to low levels of TETs family in cultured hCECs. qRT-PCR was used to investigate gene expression levels of the TETs family (TET1, TET2 and TET3) in cultured hCECs and human cholangiocyte organoids (hCOs). The hCOs served as positive control for TET1 and LGR5 expression. We observed that *LGR5* and *TET1* gene expression in cultured hCECs were significantly lower than in hCOs ($p \leq 0.05$), whereas *TET2* and *TET3* showed no significant differences. (Figure 19) Collectively, these findings suggest that low levels of DNA hydroxymethylation at promoter region of *LGR5* gene might correlated with undetectable levels of *TET1*, *TET2* and *TET3*.

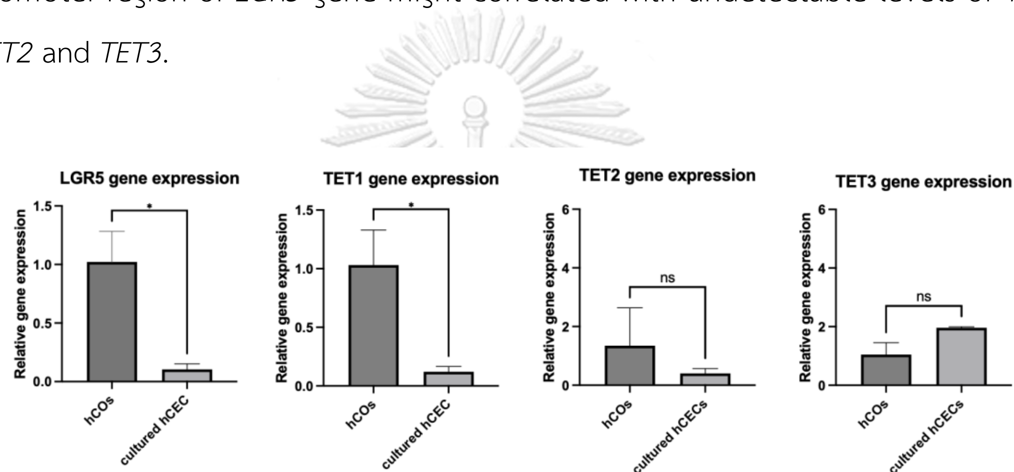

Figure 20 Comparison LGR5, TET1, TET2 and TET3 gene expression in cultured hCECs an hCOs using qRT-PCR.

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HDAC inhibitors can enhance *LGR5* expression in cultured hCECs.

Histone modifications are a post translational modification of amino-acid sequences of the histones tail through the addition or removal of chemical groups, including acetyl, methyl, phosphate, or ubiquitin groups (94). The previous studies reported that HDAC regulates the expression of LGR5 in intestinal organoids (13). To investigate the effect of HDAC inhibitor on *LGR5* expression in cultured hCECs. Cultured 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

$p \leq 0.001$ respectively) (Figure 21). This indicated that TSA and VPA can enhance *LGR5* gene expression in cultured hCECs.

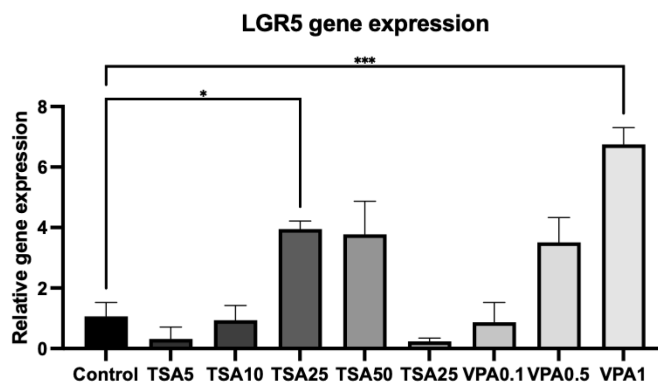

Figure 21 Comparison of LGR5 gene expression in cultured hCECs after treatment with virous concentrations of TSA (nM) and VPA (mM) for 7 days.

However, it has been reported that TSA and VPA have potential to induce cell death (114, 115). Therefore, we investigated to determine suitable concentration for use in cultured hCECs using MTT assay. As seen in Figure 22, three of out five TSA concentration (25, 50, 100 nM) exhibited significant impact on the percentage of cytotoxicity after 7-days incubation compared to untreated condition ($p < 0.05$, $p < 0.01$, and $p < 0.01$ respectively). Additionally, 1 mM VPA showed a significant increase in cytotoxicity percentages after a 7-day incubation, in contrast to the untreated condition ($p < 0.05$). Based on the experimental results of *LGR5* expression and cytotoxicity following treatment with TSA and VPA on cultured hCECs, we have opted to use TSA at a concentration of 25 nM and VPA at concentration of 1 mM for our subsequent experiments.

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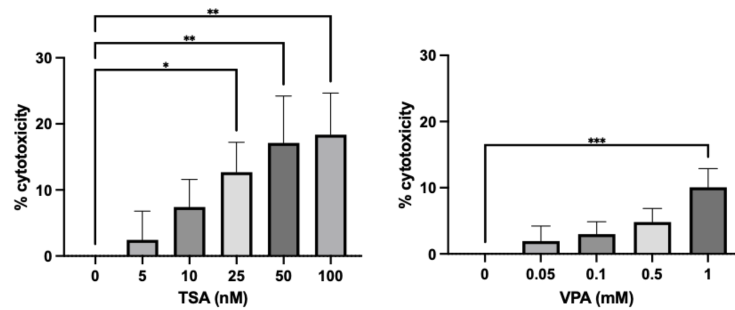

Figure 22 The assessment of cytotoxicity was performed on cultured hCECs using an MTT assay with varying concentrations of TSA (nM) and VPA (mM) for 7 days. The MTT transformed crystal was dissolved in DMSO and absorbance at 490 nm was measured. The absorbance values were normalized to the untreated control culture, which served as reference for 0% cytotoxicity.

Combination of small molecules and HDAC inhibitor can enhance *LGR5* gene expression in cultured hCECs.

Besides the regulation of LGR5 expression through epigenetic mechanism, small molecules associated with signaling pathways have also been crucial in maintaining and enhancing LGR5 expression in intestinal and cholangiocyte organoid. These include Wnt ligands, R-Spondin1, and BMP inhibitors (13, 86). To access whether small molecules, either individually or in combination with epigenetic modulators, can stimulate the expression of LGR5 gene in cultured hCECs. We utilized various small molecules, including Wnt3A (50 ng/ml), R-Spondin1 (100 ng/ml), BMP inhibitors (500 ng/ml Noggin and 10 mM SB431542). These were used with or without TSA (10 nM) and VPA (0.5 mM) for 7 days in cultured hCECs followed qRT-PCR analysis. Our results showed that the combination of small molecules, Wnt3A, R-spondin1 and Noggin/SB431542, can significantly stimulate *LGR5* gene expression, resulting in an approximate 4-fold compared to control ($p < 0.05$). Additionally, when VPA is combined with individual small molecules (Wnt3A, R-Spondin1 and Noggin/SB431542) were also significantly activates *LGR5* expression with an approximate 4-fold ($p < 0.05$) (Figure 23). These results indicated that the

combination of small molecules and HDAC inhibitors can enhance *LGR5* gene expression in cultured hCECs.

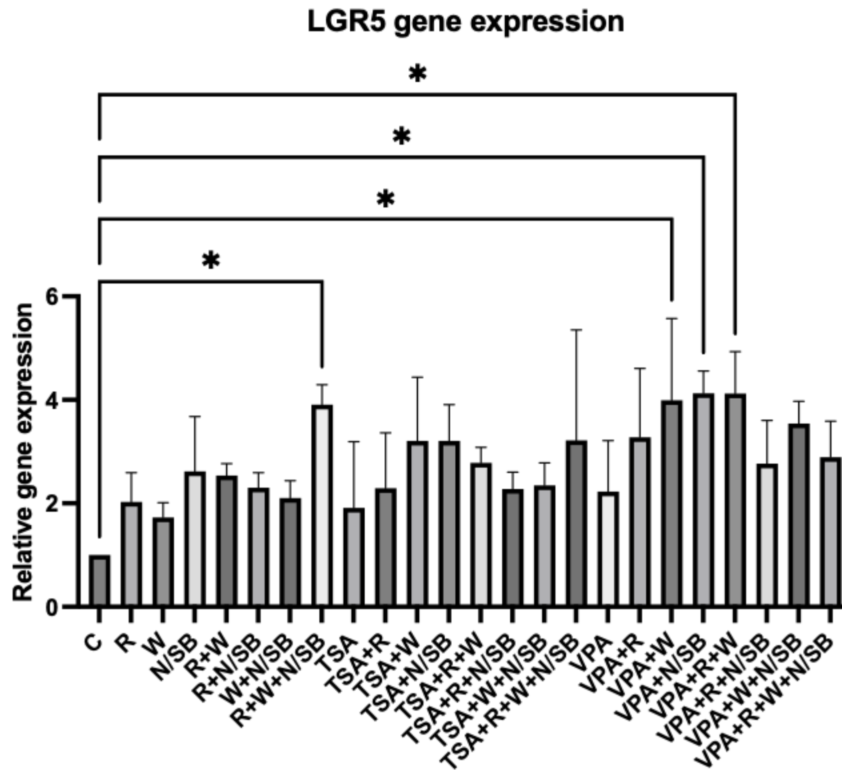

Figure 23 Comparison of LGR5 gene expression in cultured hCECs after individual small molecules treatment (W; Wnt3A, R; R-spondin1, N/SB; Noggin and SB23580) with or without epigenetic modulators (TSA and VPA) for 7 days using qRT-PCR.

CRISPRa targeted at -200 bp of TSS can activate *LGR5* expression in HEK293 cell line.

To achieve more specific activation of *LGR5*, we employed CRISPRa technique in HEK293 cell line through dCas9-VP160. Four sgRNAs were designed according to the recommendations in a previous article to target the 200 bp upstream region of the TSS of *LGR5* gene defined as (Figure 24) (116).

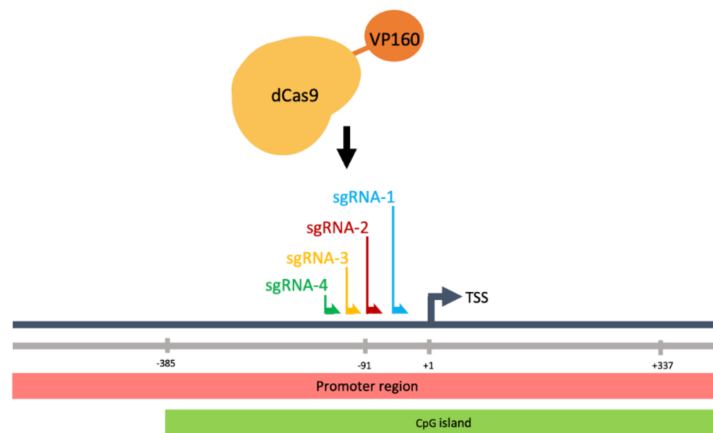

Figure 24 Diagram of CRISPRa system and the location of sgRNA on the 200 bp upstream of the transcription start site (TSS) of LGR5 gene.

To validate the most effective binding site, HEK293 cells were used and transfected with individual sgRNA by electroporation. The results of qRT-PCR indicated that each 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 25A). 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 25B).

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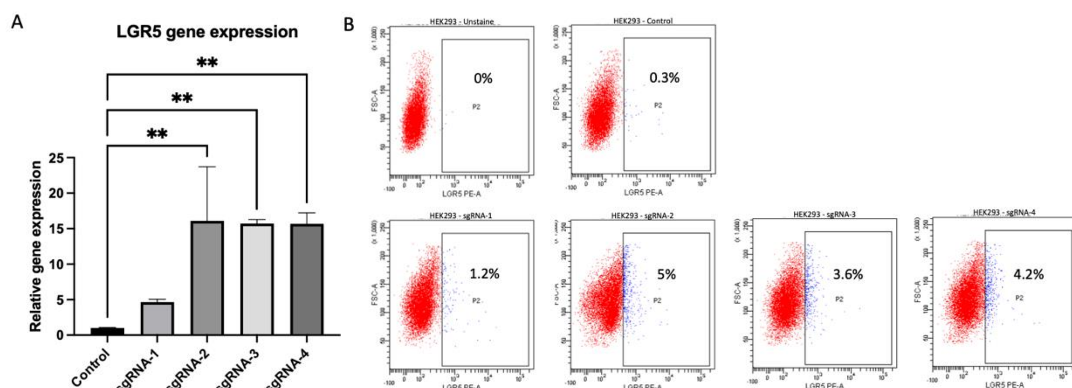

Figure 25 Comparison of LGR5 expression of individual sgRNA. LGR5 expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each single validation sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

According to Cheng et al., 2013, the use of a combination of sgRNAs can enhance endogenous gene expression more effectively than individual sgRNA (116). 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 *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 26A). Results from this experiment were consistent with the protein expression detected through flow cytometry analysis (Figure 26B).

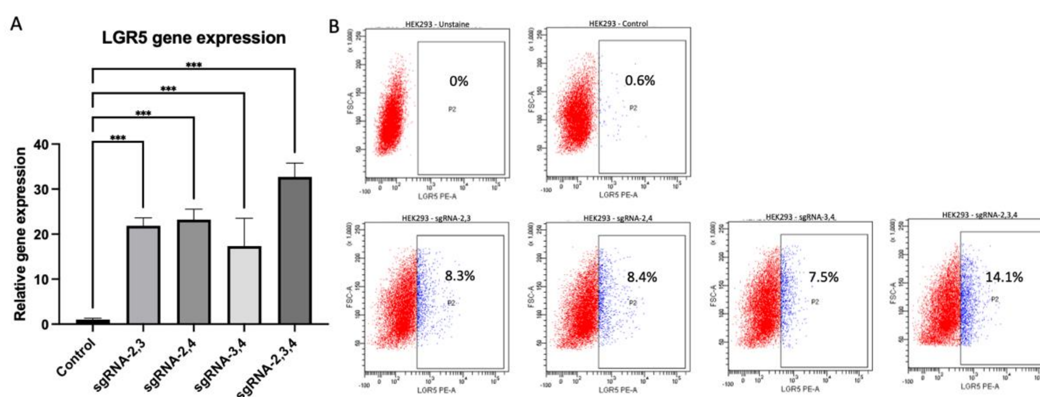

Figure 26 Comparison of LGR5 expression of each combination of sgRNA. LGR5 expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each combination of sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

CRISPRa targeted at WRE region at the promoter can promote *LGR5* expression in HEK293 cell line.

Recently article demonstrated that CRISPRa can epigenetically activate LGR5 expression in colorectal cell lines using dCas9-p300^{CORE} in a different region of the sgRNA target (117). They designed four guide sequences spanning a 300 bp segment of the promoter region defined as the Wnt-Responsive Element (WRE), with four guides named sgRNA5, sgRNA6, sgRNA7 and sgRNA8 (Figure 27).

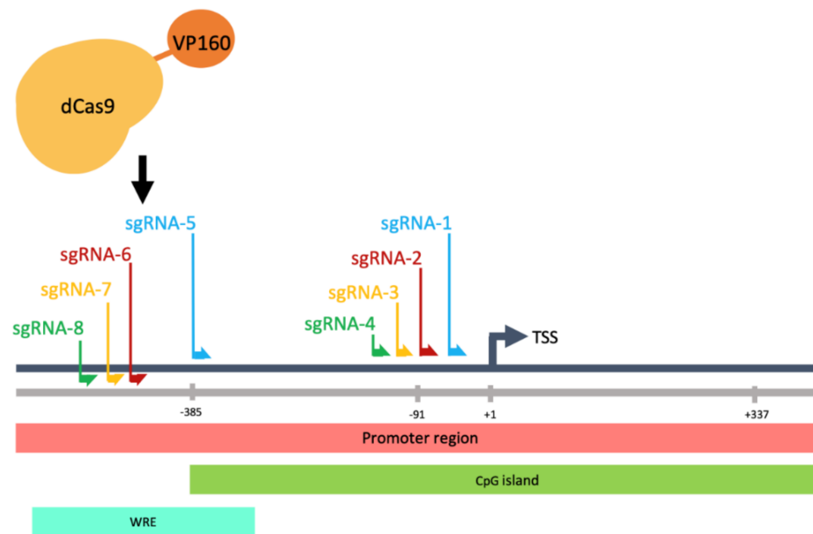

Figure 27 Diagram of CRISPRa system and the location of sgRNA on the 300 bp of the promoter called Wnt-Responsive Element (WRE) of LGR5 gene.

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To assess the efficiency of each sgRNA targeting WRE region in the promoter region of *LGR5* gene, we cloned these sgRNAs into dCas9-VP160 and transfected them via electroporation into HEK293 cell lines. Our finding revealed that a significant upregulation of *LGR5* gene expression by approximately 4-fold for sgRNA-5 and sgRNA-6 ($p \leq 0.05$). In contrast, sgRNA-7 exhibited a significantly increasing in *LGR5* expression, approximately 2-fold ($p \leq 0.01$). Meanwhile, sgRNA-8 showed the lowest level of *LGR5* gene expression activation, with no significant difference compared to the control group (Figure 28A). The findings from this experiment largely aligned with the protein expression observed via flow cytometry analysis (Figure 28B).

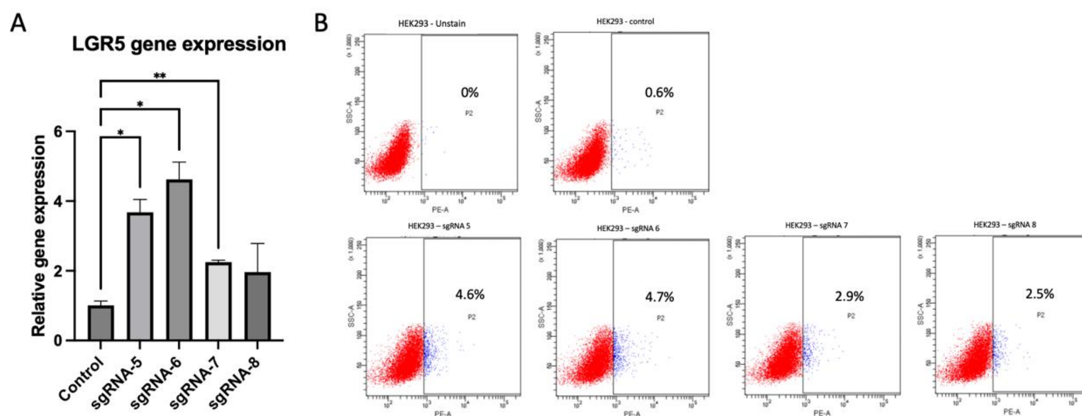

Figure 28 Comparison of LGR5 expression of individual sgRNA. LGR5 expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

Building upon the previous experiment, which demonstrated the synergistic activation of LGR5 expression through the combination use of sgRNAs. Our objective was to evaluate whether the specific targeting location of sgRNAs between the 200 bp upstream region of the TSS and WRE region, had a greater impact on the activation of LGR5 expression. We conducted an experiment using a combination of sgRNA2,3,4 and sgRNA5,6,7,8 transfected into HEK293 cell line. We observed a significant upregulation of *LGR5* gene expression in both combinations of sgRNAs ($p \le 0.05$ and $p \le 0.001$). sgRNAs targeting to the region 200 bp upstream of TSS resulted in significantly higher activation *LGR5* expression compared to sgRNAs targeting the 300 bp WRE region ($p \le 0.01$) (Figure 29A). However, the protein levels of LGR5 expression in both groups were nearly identical, at 27.6 and 23.6% (Figure 29B).

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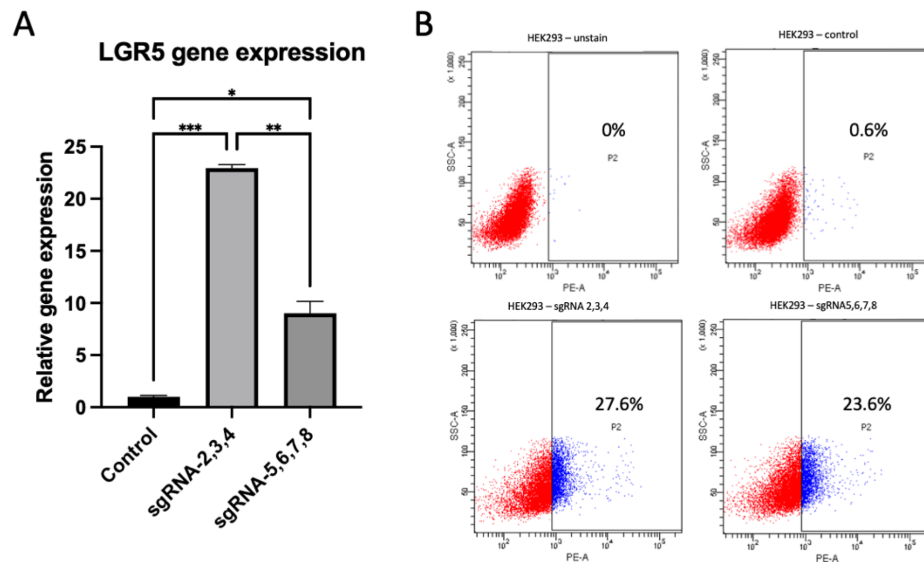

Figure 29 Comparison of LGR5 expression of combination of sgRNAs targeting between the 200 bp upstream region of TSS and WRE of LGR5 gene. LGR5 expression levels were quantitatively measured in HEK293 cells after 48 hours transfection of each sgRNA by using (A) qRT-PCR and (B) flow cytometry analysis.

CRISPRa can enhance *LGR5* expression in cultured hCECs.

It has been reported that *LGR5* expression was downregulated in mature corneal endothelium and especially in cultured hCECs. (9). 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 cultured hCECs by using CRISPRa. We proceeded to transfect the combination of sgRNA-2,3,4 into cultured hCECs via electroporation. We determined the transfection rate in cultured hCECs by using pmaxGFP, resulting in a rate of 25% (Figure 30A). This rate was considered sufficient for the CRISPRa experiment in cultured hCECs. The result of qRT-PCR analysis revealed a significant approximately 3-fold upregulation of *LGR5* expression ($p \leq 0.05$) (Figure 30B). The flow cytometry analysis showed a slight difference between the control group and the transfected group (Figure 30C).

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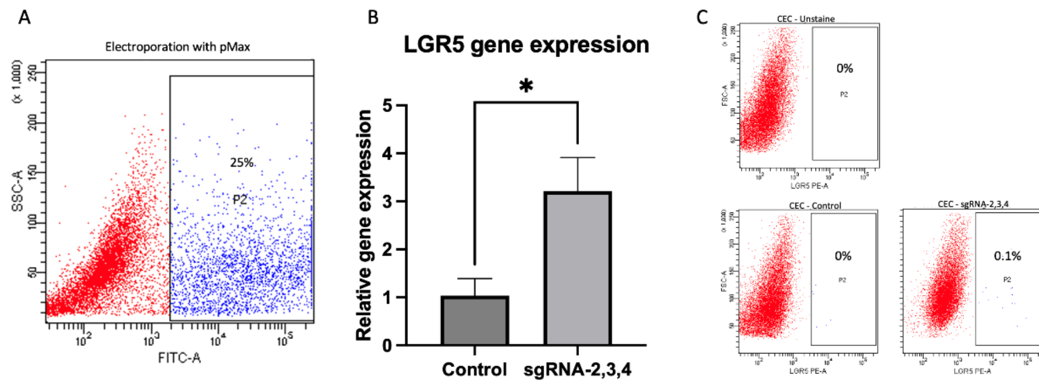

Figure 30 Combination of sgRNAs2,3,4 in cultured hCECs (A) Transfection rate by electroporation with pmaxGFP in cultured hCEC. LGR5 expression levels were quantitatively measured in cultured hCECs after 48 hours transfection of a combination sgRNAs using (B) qRT-PCR and (C) flow cytometry analysis.

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CHAPTER 5
DISCUSSION

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 (1, 4). *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 (9). However, it is downregulated during endothelial maturation and is absent in cultured hCECs (9, 10). 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, with epigenetic modification playing a role in regulating this process (7, 12, 118). According to strategy of reactivate LGR5 expression through epigenetic modification, we propose that the decrease in LGR5 expression in cultured hCECs might be due to low level of DNA hydroxymethylation mediated by TET1 rather than DNA methylation at promoter region of *LGR5* gene. We also enhance LGR5 expression in cultured hCECs through TSA, VPA, combination of VPA and small molecules and CRISPRa. These could provide insights into tissue maintenance, repair, and disease progression and offering potential therapeutic strategy through epigenetic modification.

LGR5 expression was detected in both the central and peripheral areas of corneal endothelial layer particularly in the peripheral region, which is also in line with those of a previous study (35, 44). Additionally, Hirata-Tominaga and colleagues (2013) also found a downregulation of LGR5 expression in cultured hCECs, consistent with our finding. Culturing hCECs is a complex process influenced by numerous factors, including the donor-specific characteristics, isolation methods, cell density, culture techniques, media preparation, EndMT and cellular senescence (78). In response to these challenges, we can successfully cultivate and maintain cultured

hCECs in this experiment. Our cultured hCECs exhibit compact monolayer of hexagonal cells along with the expression of ZO-1, a recognized marker of corneal endothelial cells (113). Nevertheless, they have limited capacity for expansion reaching four passages before exhibited changes in morphology, which might be from downregulation of LGR5 during cultivation (35).

Epigenetic refers to the study of changing in gene expression without altering the DNA sequence (88). Nowadays, there is increasing evidence indicating that the epigenetic landscape undergoes dynamic regulation, which facilitates in cell-fate changes and cellular plasticity in adult tissues during tissue damage (118). A previous articles study DNA methylation in normal intestinal tissue, they found that both LGR5-positive and LGR5-negative mouse intestinal stem cells exhibit the same unmethylation status at the *LGR5* promoter (99, 100). However, DNA methylation within the promoter region of the *LGR5* gene contributed to the suppression of LGR5 expression in colon cancer cell lines (16). Our findings demonstrated that there is low level of DNA methylation at the promotor region of *LGR5* gene in cultured hCECs, which agrees with previous articles (99, 100). This might imply that the reduction of LGR5 expression in cultured hCECs might not form DNA methylation at *LGR5* promoter region.

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Instead of DNA methylation, DNA hydroxymethylation also contributes to the regulation of *LGR5* expression in intestinal and liver stem cells (14, 15). Kim and colleagues (2016) found that a substantial amount of DNA hydroxymethylation not only near the promoter region but also throughout the gene body of the *LGR5* gene in mouse intestinal tissue. However, there is evidence of high TET1 binding at promoter region of LGR5 gene, corresponding to the reduction in DNA hydroxymethylation levels and LGR5 expression in Tet1-deficient and Tet1 hylomorphic allele mice (14, 15). Our results revealed a low level of DNA hydroxymethylation at the promoter region of *LGR5* gene in cultured hCECs,

coinciding with a decrease in TET1 level. These could be the possible causes for the reduction of *LGR5* expression in cultured hCECs. It should be noted that these findings do not fully represent of LGR5-positive and LGR5-negative hCECs due to the limited number of low cells available from donor tissue. Recently, a novel approach called joint single-nucleus (hydroxy) methylcytosine sequencing (Joint-snhmC-seq) has been developed to investigate 5hmC and true 5mC at the single-cells level (119). This technique could be employed for a more comprehensive evaluation of the DNA modification profile at the single-cell level, which is well-suited for studying hCECs. Furthermore, the role of TET1 in hCECs should be investigated in future studies.

DNA modification and histone modification has also crosstalk. TET enzymes play non-catalytic roles in histone modification by forming chromatin regulatory complexes with OGT, HDACs, and/or histone acetyltransferases (HATs) (97). Histone modification is one of the epigenetic mechanism that regulate *LGR5* expression (13). Our study revealed that high dose HDAC inhibitors can promote *LGR5* expression in cultured 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 (13). HDACs remove acetyl groups from histones, causing hypoacetylation and subsequent chromatin compaction. This compaction forms heterochromatin, making DNA less accessible to the transcriptional machinery and resulting in gene suppression (95). Therefore, the low expression of *LGR5* in cultured hCECs might be attributed to HDAC-induced heterochromatin formation. HDAC inhibitors like TSA and VPA are well-established drugs currently used in clinical treatments for seizures and bipolar disorder. These drugs might potentially be developed for the treatment for corneal endothelial cells in the future.

In addition to the epigenetic regulation of *LGR5* expression, small molecules targeting signaling pathways, such as Wnt ligands, R-Spondin1, and BMP inhibitors,

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have also been crucial in preserving and boosting LGR5 expression in intestinal and cholangiocyte organoid (13, 86). Combination of small molecules (Wnt ligand, R-spondin-1 and BMP inhibitors) can enhance *LGR5* expression in cultured hCECs. This suggests that the regulation of *LGR5* expression in these cells likely relies on the collaborative actions of multiple signaling pathways, including Wnt signaling and BMP signaling. This result agrees with previous reports in intestinal and liver organoids (13, 20). We did note that a combination of low dose VPA with individual small molecules can also enhance LGR5 expression in cultured hCECs. It is possible that VPA may inhibit HDAC, thereby allowing transcription factor associated with Wnt signaling and BMP signaling easier access to transcription site of DNA for activation. However, it is 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 genes.

CRISPRa is a genetic tool that enables activate the specific gene (120). In our experiment, we employed dCas9-VP160, which is the first generation of CRISPRa systems known to enhance gene activation moderately (116, 121). We were able to generate a combination of sgRNAs targeted at 200 bp upstream of TSS that efficiently activated *LGR5* mRNA and protein expression in a HEK293 cell line up to 30-fold. A recent study demonstrated that dCas9-p300 also can activate *LGR5* expression in colorectal cancer cell lines (117). A catalytic domain of p300 acetyltransferase promotes increase levels of H3K27ac histone modification at specific target region leading to gene activation (122). They designed sgRNAs to target the WRE at the *LGR5* promotor region, differing from the target location of our sgRNA. Therefore, we compared the effects of targeting sgRNAs to the 200 bp upstream TSS and the WRE in the promoter. Our finding revealed that targeting sgRNAs to the region 200 bp upstream TSS resulted in higher activation of LGR5 expression compared to sgRNAs directed at WRE region. These results is corresponded to previous study showed that high-throughput screens have identified high-activity windows for CRISPRa-VP64 in the 300 bp upstream region to TSS (123).

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Though, this combination of sgRNAs can slightly induce *LGR5* expression in cultured 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 (124, 125). Thus, several studies have explored method to enhance the transfection rate in hCECs, including magnetofection, calcium phosphate nanoparticle and recombinant adeno-associated virus (126-128). Further investigation should focus on utilizing different transfection methods to enhance transfection efficacy in cultured hCECs. Another possible reason is that the chromatin state in cultured hCECs could affect the efficacy of CRISPRa. As mentioned earlier, it has been suggested that the absence of *LGR5* expression in cultured 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 (129). 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.

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In conclusion, our study demonstrated the ability to activate endogenous *LGR5* expression in cultured 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 a promising therapeutic strategy of epigenetic modification for tissue repair and regeneration, particularly in the corneal endothelium. Further research should encompass the evaluation of alternative epigenetic profiles in various DNA modification regions, along with a concurrent exploration of histone modification. Furthermore, it is essential to delve into the characterization, proliferation, function, and quality assessment of the activated

LGR5-positive cells, as well as conducting animal model testing, before considering their clinical applications.

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CHULALONGKORN UNIVERSITY

VITA

NAME	กุลศิริ ปราการรัตน์
DATE OF BIRTH	11 พฤษภาคม 2537
PLACE OF BIRTH	กรุงเทพมหานคร
INSTITUTIONS ATTENDED	สัตวแพทยศาสตรบัณฑิต (สพ.บ.)
HOME ADDRESS	13/187 หมู่บ้านนนันทวัน ซอย 9 ถนนกาญจนาภิเษก แขวงบางไผ่ เขตบางแค กทม 10160

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