GENERATION OF CRISPR KNOCKOUT MODELS OF HETEROZYGOUS *HEPATOCYTE NUCLEAR FACTOR-1B* (*HNF1B*) TOWARDS UNDERSTANDING KIDNEY DISEASES

BY

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ABSTRACT

Hepatocyte Nuclear Factor 1 Beta (HNF1B) is a DNA-binding transcription factor that is essential for normal kidney development and is expressed in all tubular epithelial cells composing the nephrons and collecting ducts where it controls the expression of genes involved in membrane transport, cell differentiation, and metabolism. In this study, CRISPR/Cas9 technology was utilised to generate *HNF1B* gene knockout in HEK 293T cells, to investigate the effects of *HNF1B* gene loss. To achieve this, HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin (10,000 IU). Two guide RNAs (sgRNAs) targeting *HNF1B* gene were designed using the CRISPick web tool, and DNA breaks were repaired through non-homologous end-joining (NHEJ) pathway. The sg*HNF1B* was cloned into the pKLV-U6gRNA(BbsI)-PGKhygro2ABFP vector, transformed into *E. coli* and successful cloning was then validated using Sanger sequencing. Post-transduction of Cas9 lentiviral construct, cells were selected with 30 µg/mL Blasticidin antibiotic, and the edited cells were isolated. Subsequently, these cells (containing Cas9) were transduced with sg*HNF1B* lentivirus and further selected using 700 µg/mL Hygromycin selection for 6 days and continue with western blot to validate the protein expression. The western blot band intensity analysis indicates that HNF1B protein expression is reduced in both knockout cell lines, KO1 (60%) and KO2 (34%), compared to untransduced control (100%). The observed reduction in protein expression levels suggests that the knockout is heterozygous and not full knock out. This is likely due to a mixed cell population, where some cells are fully edited (bi-allelic KO), others are partially edited (monoallelic) and some may remain unedited resulting in intermediate levels of protein expression when analysed collectively. The *HNF1B* gene knockout cells were successfully compared with untransduced control cells and further validated using western blot analysis confirming the *HNF1B* gene knockout at the protein level. In conclusion, this study had successfully generated heterozygous HNF1B CRISPR-knockout models of kidney dysfunction as a proof-of-concept that gene function can be altered to treat kidney diseases. In addition, this study also highlights the utility of genome editing as a powerful tool for elucidating gene function and effectively modelling human pathophysiological conditions. Through this CRISPR/Cas9 technology, valuable insights are offered to drive advancements in personalized medicine and sustainable healthcare solution. Such innovations address key biomedical challenges and advance scientific progress to improve the well-being of both individuals and the planetary health.

Keywords: CRISPR/Cas9, Gene editing, HEK 293T, *HNF1B*, sgRNA cloning, kidney

ملخص البحث

،(DNA) هو عامل نسخ يرتبط بالحمض النووي (HNF1B) عامل الخلايا النووية الكبدية 1 بيتا وهو ضروري لنمو الكلى الطبيعي، ويُعبَّر عنه في جميع الخلايا الظهارية الأنبوبية التي تُشكِّل النيفرونات ،والقنوات الجامعة، حيث يتحكم في التعبير عن الجينات المشاركة في نقل الأغشية، وتمايز الخلايا في HNF1B لتعطيل جين CRISPR/Cas9 والاستقلاب .في هذه الدراسة، استُخدمت تقنية ولتحقيق ذلك، زُرعت خلايا .HNF1B وذلك لدراسة آثار فقدان جين ،HEK 293T خلايا HEK 293T في وسط Dulbecco's Modified Eagle Medium (DMEM) .و1 %من البنسلين)10,000 وحدة دولية((FBS) المضاف إليه 10 %من مصل الجنين البقري باستخدام HNF1B يستهدفان جين (sgRNAs) صُمِّم نوعان من الحمض النووي الريبوزي الدليل (DNA) وأُصلِحت كسور الحمض النووي الريبوزي منقوص الأكسجين ،CRISPick web أداة pKLV- في ناقل sgHNF1B استُنسخ .(NHEJ) من خلال مسار الربط النهائي غير المتماثل وحُوِّل إلى بكتيريا الإشريكية القولونية، ثم تم ،U6gRNA(BbsI)-PGKhygro2ABFP اختيرت ،Cas9 التحقق من نجاح الاستنساخ باستخدام تسلسل سانجر .بعد نقل بنية الفيروس العكوس الخلايا باستخدام مضاد حيوي بلاستيسيدين بتركيز 30 ميكروغرام/مل، وعُزلت الخلايا المحرَّرة .بعد ،sgHNF1B باستخدام الفيروس العكوس (Cas9 التي تحتوي على) ذلك، استُنسخت هذه الخلايا ثم اختيرت باستخدام هيغروميسين بتركيز 700 ميكروغرام/مل لمدة 6 أيام، واستمرت العملية باستخدام لطخة ويسترن للتحقق من صحة التعبير البروتيني .يشير تحليل شدة نطاق لطخة ويسترن إلى انخفاض ،(34%) KO2و (60%) KO1 ،في كلا خطي الخلايا المعطَّلة HNF1B التعبير البروتيني لـ مقارنةً بالمجموعة الضابطة غير المحوَّلة)100 .(%يشير الانخفاض الملحوظ في مستويات التعبير البروتيني بنجاح مع خلايا HNF1B إلى أن التعطيل متغاير الزيجوت وليس كاملاً .قورنت الخلايا المعطوبة جين التحكم غير المتحولة، وتم التحقق من صحتها باستخدام تحليل لطخة ويسترن، مؤكدةً تعطيل جين على مستوى البروتين .في الختام، نجحت هذه الدراسة في توليد نماذج متغايرة الزيجوت معطلة HNF1B بتقنية كريسبر لخلل وظائف الكلى، كدليل على إمكانية تعديل وظيفة الجينات لعلاج HNF1B جين أمراض الكلى .بالإضافة إلى ذلك، تُسلط هذه الدراسة الضوء على فائدة تحرير الجينوم كأداة فعّالة .لتوضيح وظيفة الجينات ونمذجة الحالات المرضية البشرية بفعالية

APPROVAL PAGE (MASTER'S DEGREE)

I certify that I have supervised and read this study and that in my opinion, it conforms toacceptable standards of scholarly presentation and is fully adequate, in scope and quality,as a dissertation for the degree of Master of Health Sciences (Planetary Health)

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF ABBREVIATIONS

CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
NHEJ	Non-Homologous End Joining
HDR	Homology Directed Repair
KO	Knockout
WT	Wild-type
HNF1B	Hepatocyte Nuclear Factor 1 Beta
SNP	Single nucleotide polymorysm
sgRNA	Single guide RNA
HEK 293T	Human Embryonic Kidney cell
CAKUT	Congenital anomalies of the kidney and urinary tract
PKD	Polycystic kidney disease
CKD	Chronic kidney disease
TALEN	Transcription Activator-Like Effector Nuclease
ZFN	Zinc Finger Nuclease
UMBI	UKM Medical Molecular Biology Institute
PAM	Protospacer Adjacent Motif
PTM	Post-Translational Modifications
DBD	DNA-binding domain
WD	Wolffian Duct
UB	Ureteric Bud
MM	Metanephric Mesenchyme
CM	Cap Mesenchyme
MET	Mesenchymal-Epithelial Transition
FBS	Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffered Saline
Pen-Strep	Penicillin-Streptomycin
sgRNA	Single guide RNA

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF RESEARCH

The kidneys play a crucial role in the body by being responsible for the filtration of waste products, elimination of excess fluids, and regulation of electrolyte levels in the bloodstream, ultimately leading to the production of urine (Röck, Rizzo, & Lienkamp, 2022). There are several kidney diseases that are related to the kidneys structure and function, including congenital anomalies of the kidney and urinary tract (CAKUT), polycystic kidney disease (PKD) and chronic kidney disease (CKD). The CAKUT encompass a range of diseases that show up either at birth or during early childhood (Webster et al., 2017), and it refer to a range of developmental defects in the kidneys and urinary tract that arise due to disruptions during nephrogenesis (Walawender et al., 2023). Conditions like renal agenesis occur when the ureteric bud (UB) fails to connect with the metanephric mesenchyme (MM), while renal hypodysplasia arises from defective UB-MM interactions. Genetic factors, particularly mutations in HNF1B and PAX2, are reported to be strongly associated with CAKUT phenotypes such as renal agenesis, hypoplasia, and cyst formation (Walawender et al., 2023).

CKD is a condition that occurs due to various disease pathways, which cause permanent changes in the function and structure of the kidney over an extended period of time, typically months or years. The primary risk factors for CKD includes advanced age, hypertension, diabetes, obesity, proteinuria, and dyslipidaemia. Additionally, environmental factors, including excessive dietary salt intake and, more recently, pollution, have emerged as significant contributors to CKD development (Mallamaci & Tripepi, 2024). These environmental factors often interact with traditional CKD risk factors, such as age, genetic predisposition, and diabetes, thereby exacerbating the overall burden of kidney disease. Addressing both traditional and environmental determinants is essential for mitigating the prevalence and progression of CKD on a global scale (Mallamaci & Tripepi, 2024).

In this study, *HNF1B*, a gene encoding the protein hepatocyte nuclear factor-1 Beta and located on the 17q12 locus of the human chromosome, was knocked out. The HNF1B protein is classified as a homeodomain protein, possessing a specific structural domain that facilitates its binding to DNA molecules (Weber et al., 2006). This protein is essential for regulating gene expression by binding to specific DNA sequences and influencing the activation of various target genes. This protein can be found in a variety of organs and tissues, including the lungs, liver, intestines, pancreas, kidneys, genital system, and urinary tract. This particular gene also plays a crucial role in both the development and proper functioning of the kidneys, as well as the pancreatic beta cells (Bockenhauer & Jaureguiberry, 2016).

Heterozygous mutations in *HNF1B* gene are responsible for a dominantly inherited disorder in humans, presenting with both renal and extrarenal phenotypes (Bellanné-Chantelot et al., 2004). Approximately 50% of affected individuals carry whole-gene deletions, while the majority of the remaining cases involve point mutations (Bellanné-Chantelot et al., 2005). Heterozygous mutations in *HNF1B* gene cause a multisystem disorder in humans (Clissold et al., 2015). One prominent manifestation of this disorder known as renal cysts and diabetes (RCAD) syndrome, characterized by renal cysts as the most commonly observed clinical feature, alongside early-onset diabetes mellitus. Additional clinical manifestations include pancreatic hypoplasia (Haldorsen et al., 2008), abnormal liver function (Montoli et al., 2002), hypomagnesemia (Adalat et al., 2009), hyperuricemia, and early-onset gout (Bingham et al., 2003). These studies highlight the role of HNF1B protein in multiple organ systems, including the kidneys, pancreas, liver, and genital tract. Furthermore, *HNF1B* is expressed in various fetal tissues and plays a crucial role in nephrogenesis, particularly in ureteric bud branching and tubular development (Clissold et al., 2015). This broad expression pattern explains the diverse developmental abnormalities and organ dysfunctions observed in individuals with HNF1B protein mutations.

This study focuses on human embryonic kidney (HEK 293T) cells as an in vitro model, which are widely utilised almost 20 years in scientific research. It is reported that these cells were derived from embryonic kidney cells using adenoviral DNA in the 1970s (Kavsan et al., 2011). The HEK 293T cell line has primarily been employed for the transient production of research grade proteins in scientific and pre-clinical studies, and widely employed in the field of gene therapy for the purposes of protein expression and viral production (Abaandou et al., 2021); Zhang et al., 2017). DuBridge et al. (1987) reported that HEK 293T cells are often used to produce retroviral vectors because these cells produce a protein called SV40 large T-antigen. This protein helps certain plasmids to replicate more efficiently when they are introduced into the cells, making the process more effective.

The clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system is a prominent gene-editing technology comprising of two crucial elements: a guide RNA that specifically complements a target gene of interest, and CRISPR-associated protein 9 (Cas9), an endonuclease responsible for inducing a double-stranded DNA break. This breakage facilitates alterations to the genome, enabling precise modifications, insertions and deletions of DNA in living cells (Charpentier & Doudna, 2014). The Cas9 and single guide RNA (sgRNA) work together to ensure accuracy and selectivity in genome editing (Redman et al., 2016). CRISPR knockout can occur when the genetic material undergoes self-repair, leading to the missing bases.

There are two main approaches to repair double-strand breaks (DSBs) in DNA: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Charpentier & Doudna, 2014) . NHEJ is an error-prone pathway that directly repair the broken DNA ends without requiring a homologous template. This process frequently introduces insertions or deletions (indels), which can result in premature stop codons, frameshift mutations or nonsense-mediated mRNA degradation. Consequently, this repair mechanism can lead to the loss of gene function and is often utilised in genome editing to disrupt target genes. The second approach is HDR. This is a high-fidelity repair pathway requires homologous DNA template, such as a donor DNA sequence, to precisely repair the damaged site (Mitra et al., 2022). This mechanism allows for the accurate introduction of specific genetic alterations, making it a valuable tool for studying gene function and developing therapeutic interventions. By utilising HDR, genetic defects can be corrected or desired mutations be inserted, thereby advancing the understanding of gene roles and improving the potential for targeted gene therapies. Figure 1.1 illustrates the CRISPR/Cas9 mechanism and its role in gene knockout (Liu et al. 2019).

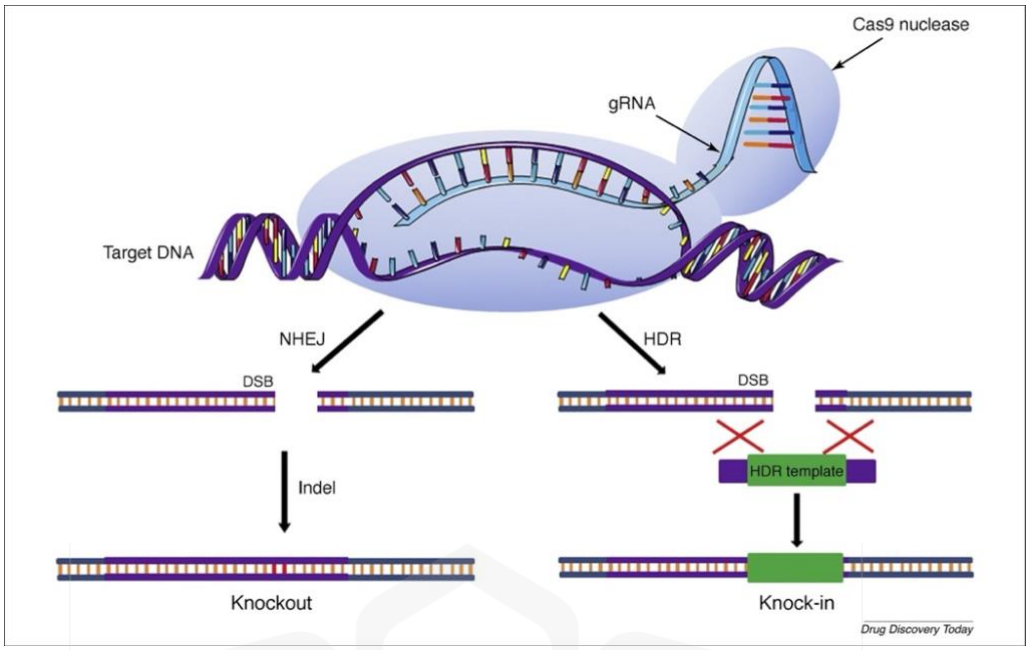

Figure 1.1 CRISPR/Cas9 mechanism and its relation to gene knockout (Liu et al., 2019)

1.2 STATEMENT OF PROBLEM

The prevalence of kidney disease is increasing worldwide, with more than 10% of the population reported having Chronic Kidney Disease (Przepiorski et al., 2020). According to Cockwell & Fisher (2020), dysfunction of the nephron resulted in CKD, which contribute to global prevalence of 9.1%. Webster et al. (2017) have highlighted that CKD is predominantly caused by diabetes and hypertension in various countries, including countries from high, middle, and low-income groups. Moreover, 30 to 50% of all CKD cases is caused by diabetes, which affects 285 million individuals worldwide. This number is expected to increase by 69% in high income countries and by 20% in low and middle-income countries by 2030 (Webster et al., 2017). According to a recent study conducted in Malaysia by Daim (2022), there has been a notable rise in the occurrence of CKD among the younger population, leading to increase demand for dialysis treatment. Malaysia has over 40,000 kidney patients in 2022, with over 8,000 additional patients diagnosed per year since 2018. According to Health Minister, if this increased tendency continues, it was predicted that around 106,000 kidney patients will require dialysis by the year 2040, of which 30% will be under 45 years old (Daim, 2022).

Although HNF1B mutations do not directly cause typical polycystic kidney disease (PKD), there is a link between HNF1B and both autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD) (Shao et al., 2020). HNF1B protein regulates PKHD1 gene, encodes fibrocystin, which is a protein crucial for kidney tubule formation. Hence, HNF1B mutations can disrupt the PKDH1 expression. Its dysregulation will result in cystic kidney disease, which look similar to ARPKD. Because of both have similar cystic features, misdiagnose is common. Exploration of HNF1B-regulated signalling pathways has highlighted potential therapeutic targets for human cystic kidney diseases (Ferrè et al., 2019). This research aims to bridge these gaps, contributing to more accurate diagnosis. Nishigori et al. (1998) were the first to describe the connection between Maturity-Onset Diabetes of the Young type 5 (MODY5) and non-diabetic kidney disease, which manifests as renal dysfunction, hypertension, low-grade or absent proteinuria, and bilateral cystic kidneys. Mutations in HNF1B can cause MODY5, a monogenic form of diabetes that arises due to defects in pancreatic beta-cell function and reduced insulin secretion (Mateus et al., 2020). These genetic changes interfere with normal glucose homeostasis, contributing to the onset of the disease.

The transcription factor *HNF1B* plays a pivotal role in kidney development and function, with mutations or disruptions in this gene being strongly associated with developmental kidney disorders, such as cystic dysplasia and renal hypoplasia. Despite its critical importance, the underlying mechanisms through which *HNF1B* influences kidney physiology and pathology remain poorly understood. This gap in knowledge hampers efforts to develop targeted therapeutic interventions for *HNF1B*-related kidney diseases. Current researches are also lacking in addressing this issue especially to dissect the functional roles of *HNF1B*, particularly in the context of cellular and molecular processes underlying kidney disorders. Therefore, this study focuses on generating an *HNF1B* knockout line in HEK 293T cells by utilising the CRISPR/Cas9 system. This research aims to lay the groundwork for future gene-editing approaches that could potentially repair defective *HNF1B* and offer therapeutic solutions for patients with *HNF1B*-associated kidney diseases.

1.3 JUSTIFICATION OF THE RESEARCH

This study focuses on the *HNF1B* gene, a critical regulator of nephrogenesis and kidney function, utilizing CRISPR/Cas9 genome-editing technology to create a knockout model in HEK 293T cells. Additionally, this study aims in addressing the substantial gaps in the current understanding of the molecular mechanisms underlying kidney pathophysiology, particularly the role of *HNF1B* mutations in disease progression. Although many genes linked to kidney disease have been identified, functional studies are essential to confirm candidate genes identified through genetic research and to uncover the mechanisms by which mutations contribute to disease at the cellular and tissue levels. Moreover, numerous genes implicated in kidney disease remain unidentified. With kidney disease increasingly becoming a global health crisis and Malaysia experiencing a rapid escalation in dialysis demand projected to reach alarming levels by 2040, the absence of innovative genetic models would impede the development of precision medicine approaches (New Straits Times, 2024). Inadequate research would severely hinder advancements in focused treatment approaches and better clinical results for individuals afflicted by kidney disease, thereby intensifying the strain on healthcare systems and impacted communities globally.

The CRISPR/Cas9 system represents a groundbreaking advancement in the fields of genetic research and medicine, providing remarkable possibilities for tackling genetic disorders and enhancing scientific comprehension. By identifying and rectifying defective genes, CRISPR presents the possibility of addressing inherited conditions, setting the stage for groundbreaking treatments. Clinical trials have commenced utilizing this technology to address conditions like specific types of cancer, polycystic kidney disease (PKD), Alport syndrome (Zhao et al., 2024) and glomerulonephritis, highlighting its potential for therapeutic application. In previous research conducted by Cruz et al. (2018), CRISPR/Cas9 technology was used to study the mechanism of *PKD* by introducing loss-of-function mutations in the *PKD1* and *PKD2* genes in human pluripotent stem cells (hPSCs). These cells were then differentiated into kidney organoids, which developed cysts, replicating the key features of PKD. The findings from this research revealed that cyst formation in PKD is a cell-intrinsic process, as mutant organoids exhibited disease-specific characteristics compared to controls (Cruz et al, 2018). Thus, this study highlights the potential of CRISPR in modeling kidney diseases and advancing understanding of PKD mechanisms.

In kidney research, particularly in the context of Alport Syndrome, Zhao et al. (2024) recently utilised CRISPR technology to investigate gene correction strategies targeting mutations in the COL4A3 and COL4A5 genes. Using CRISPR/Cas9, patient-derived podocytes were edited to correct these pathogenic mutations, achieving correction rates of 44.2% for *COL4A3* and 58.8% for *COL4A5* in vitro. Additionally, a dead Cas9-based gene activation system was employed to upregulate COL4A6, which compensated for the defective COL4α3α4α5 collagen network by promoting the formation of an alternative COL4α5α5α6 network (Zhao et al., 2024). This approach successfully restored the structural and functional integrity of the glomerular basement membrane (GBM). Therefore, these findings highlight the potential of CRISPR-based technologies for advancing gene therapies in Alport Syndrome. In this research, the capacity of CRISPR to generate specific gene knockouts proves to be especially significant for investigating the function of the *HNF1B* gene in the pathophysiology of kidney disease.

1.4 RESEARCH OBJECTIVES

General objectives: To develop and validate a CRISPR/Cas9-mediated gene editing approach for the targeted knockout of the *HNF1B* gene in HEK 293T cells.

Specific objectives:

i. To clone the sg*HNF1B* into PKLV-U6gRNA (Bbs1)-PGK hygro2BFP vector.
ii. To generate the viable HNF1B-knocked out HEK 293T cell using CRISPR/Cas 9.
iii. To validate the successful knockout of the *HNF1B* gene via CRISPR experiment using western blot.

1.5 RESEARCH QUESTIONS

i. How *HNF1B* gene being cloned into PKLV-U6gRNA (Bbs1)-PGK hygro2BFP vector?
ii. How *HNF1B* gene knockout be generate in HEK 293T cells via CRISPR/Cas9 technology?

iii. Does the HNF1B-knocked out HEK 293T cells is successful using CRISPR/Cas9 method?

1.6 RESEARCH HYPOTHESES

Null Hypothesis

a) The *HNF1B* gene cannot be cloned into the PKLV-U6gRNA (Bbs1)-PGK hygro2BFP vector.

b) The viable *HNF1B* gene knockout cannot be generated in HEK 293T via CRISPR/Cas9.

c) The knockout of HNF1B gene is not successful using CRISPR/Cas9 method.

Alternative Hypothesis

a) The *HNF1B* gene can be cloned into the PKLV-U6gRNA (Bbs1)-PGK hygro2BFP vector.

b) The viable *HNF1B* gene knockout can be generated in HEK 293T cells via CRISPR/Cas9.

c) The knockout of HNF1B gene is successful using CRISPR/Cas9 method.

CHAPTER TWO

REVIEW OF LITERATURE

2.1 ANATOMY AND PHYSIOLOGY OF THE KIDNEYS

The kidneys play a key role in maintaining the body's fluid and acid-base balance, osmolarity, pH, blood filtration, and excretion of waste materials. According to Mahadevan (2019), the adult kidneys measure approximately 12 cm in length, 6 cm in width, and 3 cm in anteroposterior thickness, with the left kidney typically being slightly longer and narrower than the right. For infants, kidney length begins at a median of 60.1 mm in boys and 57.3 mm in girls, gradually increasing with age to 114.2 mm in 18-year-old boys and 105.2 mm in 18-year-old girls (Obrycki et al., 2022). The kidneys are made up of nephrons, which are comprised of a glomerulus and a renal tubule and these components work together to filter blood and ultimately produce urine (Yoshimura et al., 2023).

In contrast to the mature mouse kidney, which has about 13,000 nephrons, each human kidney has between 0.2 and 1.8 million nephrons (Bertram et al. (2011). According to Yoshimura et al. (2023), the mammalian kidney is composed of over 20 distinct cell types that work together to maintain homeostasis. These cells are responsible for regulating various functions such as water and electrolyte balance, blood pressure, and the elimination of waste materials. The collecting duct (CD), which also controls pH, reclaims free water, and drains urine into the renal pelvis, is part of the nephron. The nephron consists of segments of proximal tubule, loop of Henle, and distal tubule, that perform specialized functions in transporting glucose and solutes as well as controlling acid-base balance (Breshears & Confer, 2017). Figure 2.1 shows nephrons part of kidney and its specific functions performed by each part of the nephron are described in Table 2.1.

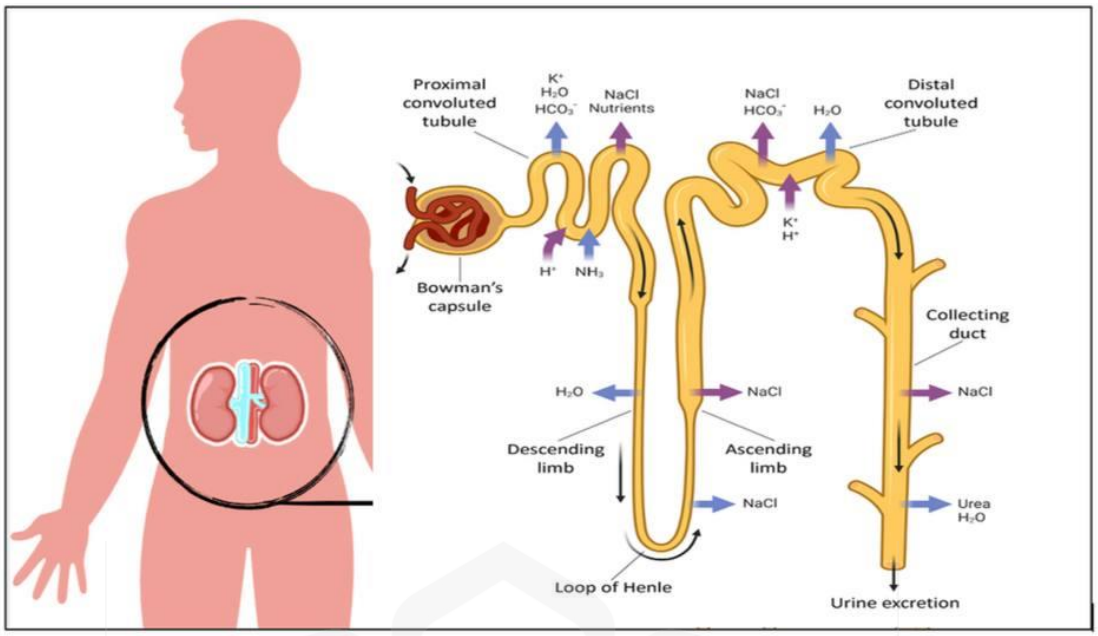

Figure 2.1 Nephrons as the functional unit of the kidney

Table 2.1 An overview of the specific functions performed by each part of the nephron

Nephron part	Function
Glomerulus	Filters small solutes from the blood
Proximal Convoluted Tubule	Reabsorbs ions, water, and nutrients; removes toxins and adjusts filtrate pH
Descending loop of Henle	Aquaporins allow water to pass from the filtrate into the interstitial fluid
Ascending loop of Henle	Reabsorbs Sodium (Na^+) and chloride (Cl^-) from the filtrate into the interstitial fluid
Distal tubule	Selectively secretes and absorbs different ions to maintain blood pH and electrolyte balance
Collecting duct	Reabsorbs solutes and water from the filtrate

Before the metanephros develops, embryos have two temporary kidneys, the pronephros and mesonephros, in a spatial and temporal sequence (Krause et al., 2015). Metanephros, a permanent secretory organ, will replace the early two types. Kidney organogenesis is regulated by cell-tissue interactions. Most of these interactions occur between the epithelial Wolffian duct (WD) derived, ureteric bud (UB) and metanephric mesenchyme (MM) (Krause et al., 2015). At its distal caudal end, epithelial WD forms UB. After invading the kidney mesenchyme, the UB starts to branch and induces nephrogenesis.

Metanephric kidney occurs in two stages: the initial phase involves inductive signaling between the UB and the MM, followed by nephron formation within the nephric cap (Ludwig & Landmann, 2005). In general, kidney development can be classified into five stages: primary ureteric bud (UB), cap mesenchyme (CM), mesenchymal-epithelial transition (MET), glomerulogenesis, tubulogenesis, and interstitial cells (Faa et al., 2012). Human kidney formation starts in the third week of embryogenesis with the emergence of the pronephros. This is followed by the development of the mesonephros around the fourth week and the metanephros by the fifth week. Renal development is initiated through the mesenchymal-to-epithelial transition. During this transition, the intermediate mesoderm (IM) grows into the WD, which then develops and specifies the MM (Davidson et al., 2019). The definitive kidney develops when the WD forms a single bud that grows into the near MM, forming the UB (Monica et al., 2012; Dressler G. R., 2011). Figure 2.2 illustrates a schematic overview of the three stages of kidney development: the pronephros, mesonephros, and metanephros (Krause et al., 2015). Figure 2.3 shows the development of the metanephros (Veikkolainen, 2014).

This is followed by elongation, where the UB tip enlarges into an ampulla. Eventually, the ampulla bifurcates into T-shaped branches (Li et al., 2021). Following the initial bifurcation of the UB and the subsequent formation of MM, the process of branching continues for a total of 12 cycles (Sampogna et al., 2015; Short et al., 2014). The formation of nephron occurs on embryonic day 16.5 (E16.5), which is a critical phase in the process of kidney development called embryogenesis (McMahon, 2016). At E16.5, nearly 85% of the branching process has been finalized, resulting in the formation of a complex network consisting of approximately 2,580 branch and tip segments, with an average of 1,300 tips positioned at the periphery (Krause et al., 2015). After this, the phase of elongation of the UB trunk begins, and the final generations of branches are formed before birth (Sampogna et al., 2015; Short et al., 2014). According to Li et al. (2021), progenitor cells found in the embryonic kidney have the ability to form the whole collecting duct, the stroma's connective tissue, and the functional segments of the nephron.

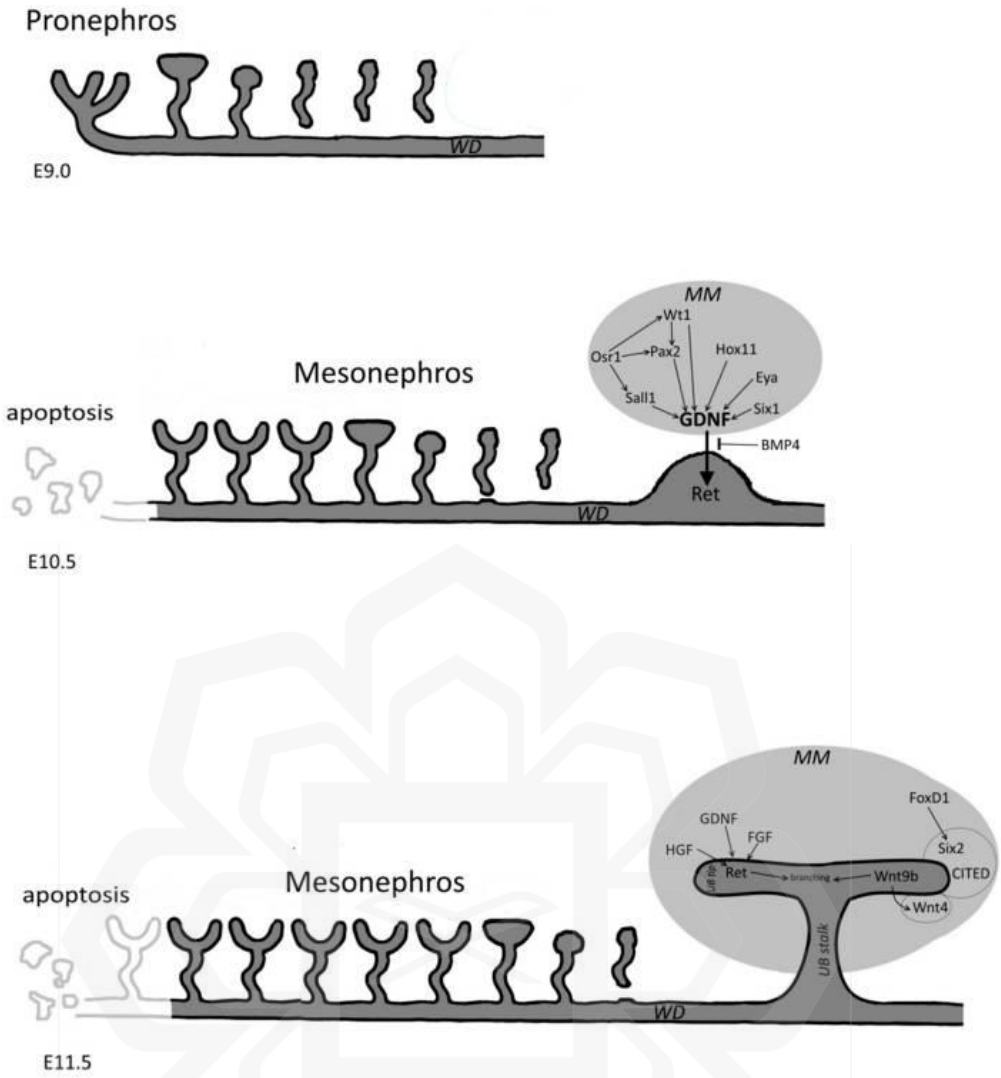

Figure 2.2 A Schematic overview of the three stages of kidney development (Krauseet al., 2015)

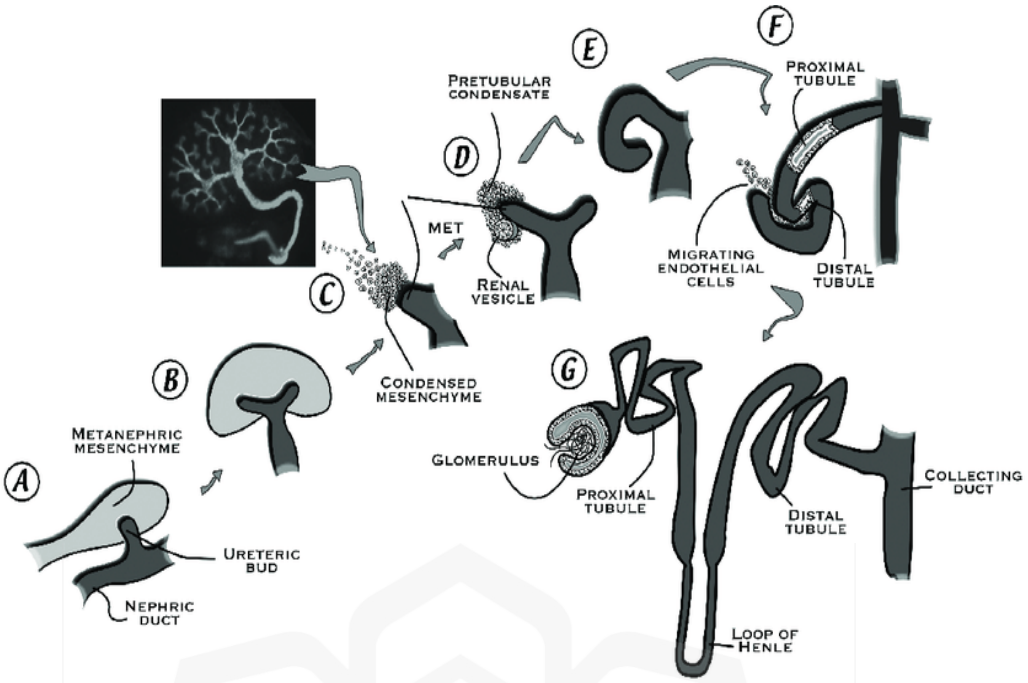

Figure 2.3 Development of metanephros into mature kidney structures (Veikkolainen, 2014)

2.2 GENETICS KIDNEY DISEASE

In the early stages of renal disease, specific structures are often targeted by particular type of stressors or damage. Immune-mediated mechanisms predominantly impact the glomeruli, whereas exposure to toxins commonly results in tubular necrosis. (Breshears et al., 2017). As disease advances into chronic stages, the kidney undergoes structural changes driven by nephron loss. Most kidney disease occurs due to genetic factors, where approximately 15% of all cases can be traced back to a Mendelian mutation (McKnight et al., 2010). For example, progressive reduction in kidney function due to genetic abnormalities such as polycystic kidney disease (PKD) can be caused by nephrotoxic medicines leading to acute kidney damage (AKI), or underlying illnesses such as diabetes and hypertension, which are the primary causes of CKD (Przepiorski et al., 2020). For instance, PKD primarily resulted from hereditary mutations, constitutes the principal diagnosis in roughly 10% of individuals requiring renal replacement therapy (McKnight et al., 2010).

In CKD, the occurrence of this disease is attributed to the malfunctioning of nephrons, which are the functional units of the kidneys. It is noteworthy that CKD has a global prevalence of 9.1% among the population, as reported by Cockwell & Fisher in 2020. According to the Global Health Observatory in 2024, the mortality rate associated with CKD expected to rise steadily, reaching 14 deaths per 100,000 individuals by the year 2030 worldwide. Additionally, Lanktree et al. (2018) reported that autosomal dominant polycystic kidney disease (ADPKD) is a common genetic renal disorder. It is estimated to affect approximately 1 in 500-2,500 individuals, with over 10 million people worldwide expected to be affected (Igarashi et al., 2007).

HNF1B protein regulates *PKHD1*, a gene encoding fibrocystin which is essential for maintaining tubular architecture. Mutations in HNF1B can result in the downregulation of *PKHD1*, thereby disrupting normal tubule structure and contributing to cyst formation, resembling the pathogenesis observed in ARPKD (Shao et al., 2020). According to Toronto Genetic Epidemiology of Polycystic Kidney Disease performed between 1999 and 2003 it was reported that 55.5% of patients who did not have a family history of ADPKD, did not have any detectable variants in the *PKD1* or *PKD2* genes (Iliuta et al., 2017). Mutations in the PKD1 gene are responsible for approximately 80% of ADPKD cases and are associated with a more severe disease phenotype, with kidney failure (KF) typically occurring between the ages of 55 and 65. In comparison, *PKD2* mutations account for roughly 15% of cases, with KF manifesting later, typically in the mid-70s (Yang et al., 2023). ADPKD, in contrast, is marked by progressive cyst formation and contributes to 5-10% of KF cases (Yang et al., 2023).

In addition, The *HNF1B* gene is essential for kidney development, and its mutations have been closely linked to different manifestations of CAKUT, such as renal cyst formation, structural kidney abnormalities, and defects in the urinary tract. CAKUT can result from various environmental and genetic factors. Mahmoud et al., (2024) reported that environmental contributors include maternal diabetes, obesity, malnutrition, alcohol consumption, and exposure to medications that impair kidney development. Genetically, CAKUT arises from disruptions in the interaction between the metanephros and the ureteric bud. Mutations in genes such as *PAX2, TBX18, NRIP1, REX, SIX2, BMP4*, and regions of chromosome 17 have been implicated. Additionally, more than 50 genes have been identified as underlying causes, including the *HNF1B* gene (Capone et al., 2017; Mahmoud et al., 2024). Nevertheless, it is important to note that the precise genetic factors responsible for the majority of CAKUT cases are still unidentified, with one of the gene involved is *HNF1B*. The *HNF1B* is expressed in the embryonic kidney and is essential for several stages of kidney development, such as branching morphogenesis, nephrogenesis, nephron patterning, and tubulogenesis (De Stefani et al., 2015).

2.3 THE ROLE OF *HNF1B* IN KIDNEY DEVELOPMENT

Over the last two decades, there are about 40 genes involved in nephrogenesis and more than 95 genes are associated with cystic kidney disease (Gimpel et al., 2018; Goodyer et al., 2016: Sanna. C et al., 2018). Additional genes regulated by signaling, including *Lfng, DII1, Jag1,* and the *Irx1/2* factors, also induce renal tubular segmentation and glomerular development (Cheng et al., 2007; Heliot et al., 2013). According to Vivante et al. (2016) and Uy et al. (2016), *HNF1B* is the most common gene associated with cystic kidney disease that occur as either deletion or intragenic mutations. The *HNF1B* gene is located on the 17q12 locus of the human chromosome, and responsible for encoding a distinctive homeobox HNF1B transcription factor. Kettunen et al. (2017) reported that *HNF1B* is a transcription factor that plays a vital role in the development of various organs derived from ventral endoderm. It is crucial for regulating gene expression in multiple tissues, including the liver, kidneys, intestines, and pancreatic islets. Additionally, it controls the embryonic development of these organs (Ulinski et al., 2006). Figure 2.4 shows *HNF1B* gene (or *TCF2*) locus on chromosome.

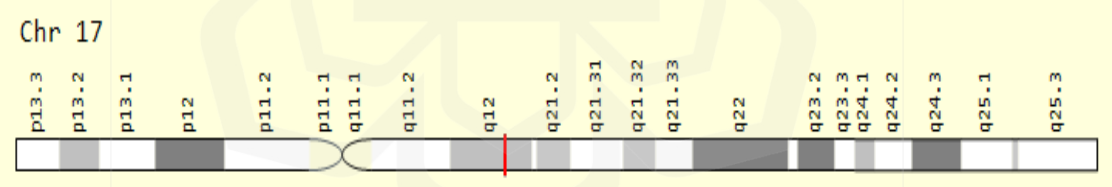

Figure 2.4 *HNF1B* gene (or *TCF2*) locus on chromosome

HNF1B is a transcription factor that regulates the expression of several genes involved in kidney, genitalia, pancreas, liver, thymus, and gut morphogenesis (Vivante et al., 2016; Verhave et al., 2016; Bockenhauer et al., 2016). In a study conducted by Nie et al. (2020), it is reported that the expression of *HNF1B* was higher in uterine corpus endometrial carcinoma and bladder urothelial carcinoma. Similarly, this transcription factor exhibits developmental role during the initial induction of kidney formation, particularly within the collecting duct of metanephros. However, minimal expression levels of *HNF1B* were observed in liver hepatocellular carcinoma, colon carcinoma, glioblastoma multiforme, kidney chromophobe, and kidney renal clear cell carcinoma (Bártů et al., 2020).

Human HNF1B protein is made up of 557 amino acids (De Stefani et al., 2015). Most of current understanding on *HNF1B* is derived from animal studies, particularly mouse models. However, these models often do not fully mimic human mutations or may show reduced sensitivity to *HNF1B* haploinsufficiency. Lokmane et al. (2010) reported that *HNF1B* plays a critical role during the early stages of urogenital development, as demonstrated in several mouse studies. To address these limitations, Niborski et al. (2021), generate a novel renal cysts and diabetes syndrome (RCAD) mouse model by introducing a human-specific hotspot mutation at the intron-2 splice donor site (<IVS2nt+1G>T) (Bingham et al., 2003; Harries et al., 2004). This model exhibited key features of *HNF1B* mutations in humans, including bilateral kidney cysts, tubular and glomerular abnormalities from embryonic day 15, and occasional genital/extrarenal defects. Unlike previous null allele mouse models where HNF1B protein levels were unchanged or increased (Kornfeld et al., 2013), protein levels in this cell model were reduced by 30-40%, leading to altered gene expression and disease phenotypes. These findings underscore the utility of mouse studies in investigating *HNF1B* function and associated diseases. The findings demonstrated that a reduced *Hnf1b* dosage in this mouse model led to differential expression of target genes, which in turn triggered the development of disease phenotypes (Niborski et al., 2021).

2.4 *HNF1B* GENE MUTATION LED TO CLINICAL DISEASE

Mutations in HNF1B can be familial or de novo, and results in a broad range of phenotypes. The cause of human renal tract malformations (RTMs) predominantly arises from heterozygous mutations in the *HNF1B* gene, as documented by Thomas et al. (2011) and Madariaga et al. (2018). *HNF1B* is crucial for visceral endoderm specification (Barbacci et al., 1999) and postmitotic reprogramming of many gene expressions linked to cystic kidney disease (Verdeguer et al., 2010). In humans, mutations in *HNF1B* cause cystic kidney diseases such as renal cysts and diabetes, multicystic dysplastic kidneys, glomerulocystic kidney disease, and autosomal dominant tubulointerstitial kidney disease (Shao et al., 2020). *HNF1A* and *HNF1B* DNA-binding domains are highly conserved, and the proteins recognise a similar DNA sequence (Shao et al., 2020). This study further highlighted that HNF1A expression is restricted to the renal proximal tubules, where it regulates genes associated with amino acid, phosphate, and organic acid transport. In contrast, HNF1B is expressed throughout all nephron segments. Heterozygous mutations in this gene produce a multisystem disorder that has been linked to RCAD, associated RTMs early onset of gout and hyperuricemia hypomagnesemia, and renal magnesium wasting (Adalat et al., 2009; Bingham et al., 2009).

Renal cysts were reported to be the main clinical aspect with *HNF1B* mutations and the linkage of renal cysts with diabetes steered to the description of renal cysts and diabetes syndrome (Chandra et al., 2021). Renal diseases are homogenous phenotype which is usually associated with the mutation in transcription factor. On the other hand, mutations in the *HNF1B* gene represent the most common monogenic causes of developmental kidney disorders. These mutations are associated with a broad spectrum of abnormalities, including congenital anomalies of the kidney and urinary tract (Nakayama et al., 2010) and autosomal dominant tubulointerstitial kidney diseases (Devuyst et al., 2019).

Previous studies also reported the microdeletions at chromosome 17q12 in patients, both with and without diabetes-associated renal disease. According to Mefford et al. (2007), the deletion of this specific chromosomal region typically leads to an early onset of multicystic dysplastic kidneys. According to Faguer et al. (2011), individuals with *HNF1B*-associated disease may experience a range of renal functions, from normal to end-stage renal disease (ESRD). A study conducted by Weber et al (2006) reported that out of 99 children with dysplastic or hypoplastic RTMs, eight were identified to carry *HNF1B* mutations. Furthermore, *HNF1B* mutations are most commonly observed in individuals with RTMs and the presence of cysts. According to Ulinski et al. (2006), there is a higher occurrence of *HNF1B* mutations in patients with hypoplastic or dysplastic kidneys, as well as those with cysts or bilateral illnesses.

A *PKHD1* autosomal recessive mutation causes polycystic kidneys, whereas a *HNF1B* mutation causes cysts in the glomeruli and renal tubules in transgenic mice (Hiesberger et al., 2004). This is due to the promoter region's evolutionary conserved *Pkhd1* binding location (Bingham and Hattersley, 2004). In addition, chronic kidney disease (CKD) typically progresses over time, resulting in a decline in glomerular filtration rate (GFR) and the presence of albuminuria representing key risk factors for the subsequent development of kidney failure (Fox et al., 2012). As mentioned above, HNF1B is required for UB branching and early nephrogenesis in mice. The presence of heterozygous mutations in the *HNF1B* gene has been acknowledged as a prevalent genetic cause underlying developmental kidney disease in human beings (Goea et al., 2022). Molecular genetic defects encompass a range of abnormalities, such as complete removal of a gene or mutations occurring within the gene itself. These mutations can take various forms, including missense mutations, frameshift mutations, nonsense mutations, and small insertions or deletions (Bellanné-Chantelot et al., 2005). Figure 2.5 presents the renal and extra-renal phenotypes commonly observed in patients with *HNF1B*-associated disease (Clissold et al., 2015).

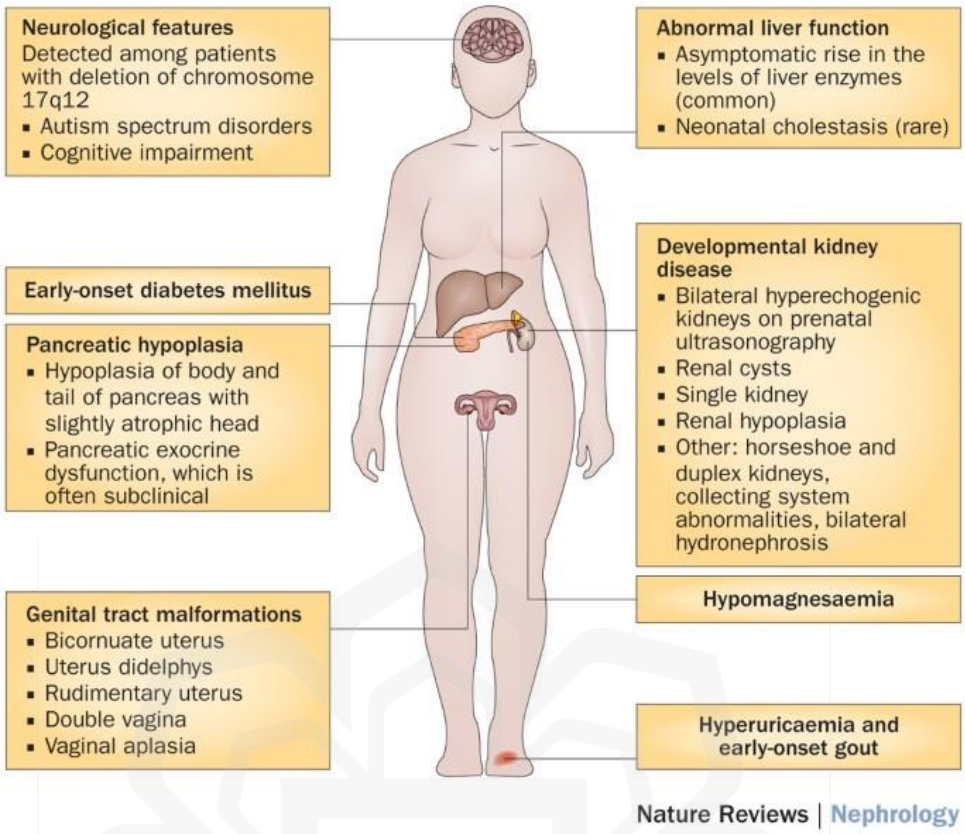

Figure 2.5 Renal and extra-renal phenotypes frequently observed among patients with *HNF1B* associated disease (Clissold et al., 2015)

2.5 GENE EDITING TECHNOLOGY

Genome editing is the modification of genomic DNA at a specific target site in a broad range of cell types and organisms, including insertion, deletion, and replacement of DNA, resulting in the inactivation of target genes, the acquisition of novel genetic traits, and the correction of pathogenic gene mutations (Xu & Li, 2020). Currently, there are four major approaches for genome editing, including meganucleases, transcription activator like effector nuclease (TALEN), zinc finger nuclease (ZFN) and clustered regularly interspaced short palindromic repeats (CRISPR) (Ben-David, 2013; Ramalingam et al., 2013; Kim, 2016). According to Gaj et al. (2013) & Gupta et al. (2019), the development of gene-editing technologies such as ZFN, TALEN and CRISPR, which belong to the third generation of gene-editing technology, has ushered in a new era of life science research. CRISPR/Cas-based techniques have revolutionized gene editing due to their simplicity, cost-effectiveness, and high efficiency.

The CRISPR-Cas9 system uses Cas9-sgRNA complexes to target specific genomic loci through the complementarity between the guide RNA and DNA sequences, achieving editing efficiencies exceeding 80% in some cases (Hwang et al., 2013, Kim et al., 2014, Higashijima et al., 2017). Unlike other systems, CRISPR/Cas9 requires only the modification of the guide RNA sequence to target different genomic sites, making it easier to design and construct (Charpentier & Doudna, 2014). Its efficiency in gene knockdown is unparalleled, with success rates surpassing 90% (Ni et al., 2014; Kalidasan & Theva Das, 2021). However, like other gene editing technologies, CRISPR/Cas9 carries a risk of off-target effects, highlighting the need for further refinement to improve specificity (Cruz & Freedman, 2018).

In comparison, ZFN were among the first genome-editing tools, but their design is challenging and costly due to the need to engineer specific DNA-binding module proteins for each target site (González et al., 2021). With editing success rates of approximately 24%, ZFN are less efficient and more labour-intensive than CRISPR/Cas9 (Kalidasan & Theva Das, 2021). However, TALEN offer better success rates, exceeding 99%, but also constrained from the same limitations as ZFN, including time-consuming and expensive construction processes (Gaj et al., 2013; Kalidasan & Theva Das, 2021). Despite their high efficiency, TALEN require significant protein engineering to recognize specific DNA sequences, making them less practical compared to CRISPR/Cas9 (Charpentier & Doudna, 2014). Overall, while ZFN and TALEN have contributed to advancements in gene editing, CRISPR/Cas9 stands out as the most efficient and accessible tool, transforming genetic research and therapeutic applications. Main gene editing approaches are further elaborated in Table 2.2 (Eid, A., & Mahfouz, M., 2016).

Table 2.2 Main gene editing approaches (Eid, A., & Mahfouz, M., 2016)

	Zinc-Finger Nuclease (ZFN)	**Transcription Activator Like Effcetor Nucleas (TALEN)**	**Clustered Regularly Interspaced Palindromic Repeats-CRISPR-Associated-9 (CRISPR-Cas9)**
Construction	Protein engineering for every single target	Protein engineering for every single target	20-Nucleotide sequence of single-guide RNA (sgRNA)
Target sequence recognition	Zinc fingers protein, protein-DNA interactions	Repeat variable diresidues (RVDs) repeats, protein-DNA interactions	sgRNA, RNA-DNA interactions
Endonuclease	FokI	FokI	Cas 9
Endonuclease construction	3-4 Zinc fingers domains	8-31 RVD repeats	sgRNA complementary to the target sequence with Cas9 protein
Delivery	Two ZFNs around the target sequence	Two TALENs around the target sequence are required	sgRNA complementary to the target sequence with Cas(protein
DNA sequence recognition size	(9 or 12bp) x 2	(8-31bp)x 2	17-20bp + NGG x 1
Targeting efficiency	Low	Moderate	High
Affordability	Resource intensive and time consuming	Affordable but time consuming	Highly affordable and rapid

2.6 ACTION AND REPAIR MECHANISM OF CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS (CRISPR)

According to Singh et al. (2022), the key two components of the CRISPR-Cas system are a synthetic single-stranded RNA, with approximately 105 bases of single-stranded nucleotide, known as guide RNA (gRNA) and the Cas9 protein derived from *Streptococcus pyogenes*. Makarova et al. (2011) reported that foreign DNA invades prokaryotes and is broken into short fragments by Cas proteins, which are then integrated into the CRISPR array as spacers. When the same invader returns, CRISPR RNA (crRNA) quickly recognizes and pairs with foreign DNA, guiding the Cas protein to cleave specific sequences of foreign DNA, protecting the host (Makarova et al., 2011).

Cas9 nuclease and sgRNA combine to generate a Cas9 ribonucleoprotein (RNP) capable of binding and cleaving a specific DNA target (Makarova et al, 2011). Cas9 is a programmable RNA-guided DNA endonuclease, and the Cas9-crRNA-tracrRNA complex cleaves double-stranded DNA targets according to the crRNA's 20-nucleotide guide sequence (Gasiunas et al., 2012; Jinek et al., 2012). Cas9 is made up of two lobes known as endonuclease domains, HNH and RuvC, which cleave complementary (target DNA strand) and non-complementary (non-target DNA strand) DNA strands to the crRNA guide, respectively (Chen et al, 2014). An early study showed that catalytically active dCas9 alone could effectively bind to target DNA and interfere with the binding activity of other proteins including RNA polymerase and transcription factors, thereby blocking transcription initiation and elongation in *E. coli* and mammalian cells (Qi et al., 2013).

There are three major delivery methods for CRISPR/Cas, including physical, viral, and non-viral approaches (Rani & Prabhu, 2022). Physical methods, including electroporation, microinjection, and mechanical cell deformation, are the simplest ways to deliver CRISPR-Cas components. They are simple and efficient, can increase gene expression, and are commonly used in *in vitro* investigations (Kotterman et al., 2015; Zu et al., 2019). Furthermore, due to their excellent delivery effectiveness, viral vectors such as adenovirus, adeno-associated virus (AAV), and lentivirus viral vectors are commonly employed for both *in vitro/ex vivo* and *in vivo* delivery. They are commonly utilised for gene delivery in gene therapy, and some have obtained clinical approval (Liu et al. 2017; Yin et al 2014). Yin et al (2016) reported that many studies have focused on non-viral vectors such as nanoparticles, liposomes and polymers (Tang et al., 2022) gaining attention as the key methods for the delivery of CRISPR/Cas components due to the advantages of safety, availability, and cost-effectiveness. In addition, the CRISPR/Cas system has various other applications, including multiplex KO, conditional allele generation (floxed), and reporter gene insertion (Higashijima et al., 2017). In addition, CRISPR/Cas based technology can distinguish a single nucleotide difference and thus, offers great specificity for detecting different genetic variations, even with a single nucleotide polymorphism (SNP) (Li et al., 2021).

As mentioned above, the Cas9 protein is directed to the specific recognition site upstream of the genome's protospacer-adjacent motif (PAM) by the gRNA. The endonuclease Cas9 creates specific double-strand breaks (DSBs) three nucleotides prior to the PAM site (Singh et al., 2022). The subsequent repair of chromosomal double-strand breaks (DSBs) by the cell can be divided into two categories, non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Ghexraoui et al., 2014). According to Ghezraoui et al. (2014), NHEJ-break ends can be ligated without a template, but HDR-breaks require a template for repair. NHEJ is an effective and highly active cellular repair mechanism. Due to nucleotide insertions and deletions (indels)., it is also susceptible to frequent mutation errors. This may result in frameshift mutations and, as a result, gene function loss (Wang et al., 2022).

HDR is the predominant mechanism for precise DSB repair and can be challenging because it requires greater sequence similarity between the damaged and intact donor DNA strands. HDR utilises DNA sequence homology to accurately repair DSB at the specific location within the genome (Xue et al., 2021) unlike NHEJ, which simply joins any two broken ends of DNA, the proteins involved in the HDR pathway specifically identify homologous sequences of DNA near the DSB. These homologous sequences are then utilised as a template for accurately repairing the damage (Rein et al., 2018). However, to ensure controlled modifications, HDR-edited DNA is preferable.

Repair mechanism via NHEJ implicates in random nucleotide insertions and deletions (indels) at the sites where the DNA strands have been cleaved and are being repaired (Graham et al., 2021). In addition, NHEJ proves to be advantageous in the creation of gene knockouts due to its tendency to induce frameshift mutations through random insertions and deletions (indels), as well as the occurrence of substantial undesired deletions and rearrangements (Kosicki et al., 2018). As a result, this DNA repair mechanism is most effective to knockout a gene of interest because it regularly generates tiny (1 to10 base pair) indels mistakes. According to the Song et al. (2017), the NHEJ pathway arises as the primary mechanism for DNA repair in the absence of a sister chromatid as a repair template. Therefore, Johnson (2022) reported that to generate these KO utilizing NHEJ, Cas9 nuclease (protein or plasmid delivery), single guide RNAs (sgRNA, complexed with Cas9 either in vitro or endogenously via plasmid), and two sets of PCR primers are required to validate the editing via sequencing. Figure 2.6 illustrates schematic representation of NHEJ and HDR repair mechanism.

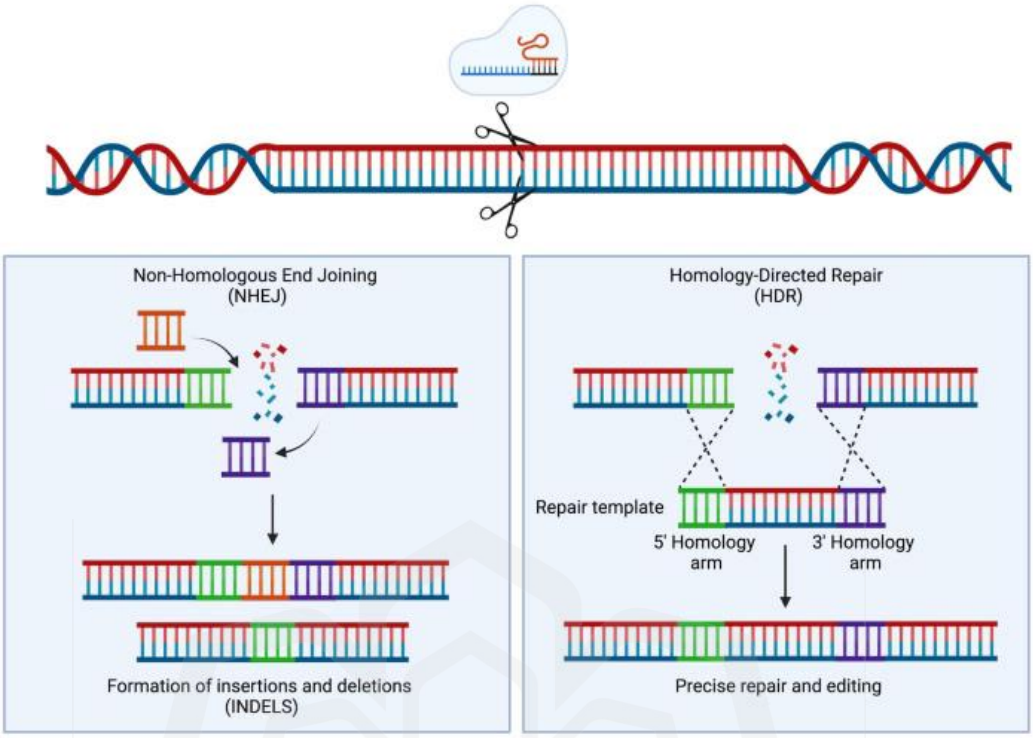

Figure 2.6 Schematic representation of NHEJ and HDR repair mechanism

2.7 CRISPR IN GENE THERAPY AND DISEASE MODELLING

The ZFN, TALEN, and CRISPR editing tools show great promise in the treatment of human diseases, especially genetic disorders that cannot be effectively treated using conventional methods. Several new technologies have been developed in recent decades to screen, detect, and diagnose various diseases, including cancer. Additionally, the applications of CRISPR/Cas9 technology have been identified in the fields of basic and clinical research, therapeutics, drug development, agriculture, and the environment (High & Roncarolo, 2019). CRISPR/Cas9 genome editing technology also has been utilised in the research and treatment of a variety of disorders, including monogenic diseases, central nervous system (CNS) diseases, and AIDS (Knott & Doudna, 2018). Numerous organisms, including mice, rats, monkeys, pigs, cows, rabbits, frogs, zebrafish, fruit flies, worms, yeast, and bacteria, have already been genetically modified (Gersbach, 2014). These species have contributed to the studies of genetics, genomics, gene function, and disease modelling.

CRISPR technology has been widely utilised in disease modelling, including cancer (Torres-Ruiz & Rodriguez-Perales, 2015) and neurological disorders (Heidenreich & Zhang, 2016), to better understand the molecular mechanisms driving disease progression. For instance, in cancer research, Maresch et al. (2016) demonstrated the use of CRISPR-Cas9 for the simultaneous modification of multiple genes involved in pancreatic cancer, employing a transfection-based multiplex delivery system directly targeting the pancreas. Apart from that, Sánchez-Rivera et al. (2014) applied CRISPR-Cas9 to inactivate tumour suppressor genes associated with loss-of-function mutations in human lung cancer and successfully inducing lung adenocarcinomas in adult mouse models. In neurological study, Tabebordbar et al. (2016) employed a CRISPR-based in vivo approach to edit the mouse genome. AAV-mediated delivery of Cas9 and sgRNAs targeting exon 23 of the mutated *Dmd* gene resulted in exon deletion, generating a truncated yet functional protein. This approach partially improved muscle function in Duchenne muscular dystrophy (DMD) models. Liu et al. (2016) utilised CRISPR-Cas9 genome engineering in an iPSC-based model to investigate epilepsy linked to *SCN1A* loss-of-function mutations. Their findings revealed that Nav1.1 protein was predominantly expressed in GABAergic neurons, with minimal expression in glutamatergic neurons within the differentiated neuronal system.

Gene therapy is the introduction of foreign genes into target cells to cure specific diseases caused by altered or defective genes (Xu & Li, 2020). The CRISPR technology has demonstrated its efficacy in addressing genetic mutations linked to multiple disorders, including thalassemia, cystic fibrosis, and Duchenne muscular dystrophy (Ginn et al., 2018). In addition, clinical research also has shown potential utilization for CRISPR in diseases such as sickle cell disease, cancer, AIDS, Huntington's disease and Duchenne muscular dystrophy (Khadempar et al., 2018). According to Khadempar et al. (2019), the CRISPR/Cas9 system functions as a bacterial defence mechanism against viral infections, where the Cas9 nuclease, guided by a single-stranded RNA, binds to specific complementary DNA sequences to induce targeted genetic modifications. In 2016, Chew et al. conducted a study demonstrating the production of Cas9-specific antibodies and T cell responses in mice. These responses were observed when Cas9 was delivered through intramuscular electroporation or adeno-associated virus (AAV) during experimental trials of CRISPR-based gene therapy.

A research conducted by Charlesworth et al. (2019), have collectively discovered that healthy individuals who donate blood possess antibodies and T cells that exhibit reactivity towards Cas9 in the human body. Clinical trials are ongoing to evaluate the effectiveness of CRISPR engineered cell therapies in treating various types of cancers and immunologic syndromes (Cruz & Freedman, 2018). This innovative approach shows potential for application in the treatment of inflammatory conditions, including lupus nephritis (Khadempar et al., 2018). CRISPR has been rapidly develop and adopted as a diagnostic tool for various human diseases due to its special characteristics and it has gotten a lot of attention from scientific communities and companies for treating human genetic diseases since it was regarded as a genome editing technology. According to Hille & Charpentier (2016), CRISPR-Cas recognizes and cleaves foreign DNA or RNA in a sequence-specific manner, and it has the potential to treat genetic disorders caused by single gene mutations, which is one of the most promising applications.

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Over the past decades, CRISPR/Cas genome editing technology has advanced significantly in both preclinical and clinical settings. These developments have enhanced disease screening and diagnosis, facilitated the treatment of genetic disorders, and contributed to fundamental biomedical research. (Li et al., 2021). Yin et al. (2017) conducted a recent study involving mice that were implanted with human immune cells and employed CRISPR technology in order to hinder the replication of the human immunodeficiency virus (HIV) by removing the integrated virus from the DNA. Notably, this approach proved effective even in mice that were currently experiencing acute infection. Additionally, CRISPR also has been employed in vivo to address various genetic disorders in mice and rats, including Duchenne muscular dystrophy (Bengtsson et al., 2017; Nelson et al., 2016; Tabebordbar et al., 2016) and Huntington disease (Yang et al., 2017). Schwank et al (2013) explored CRISPR/Cas9 as a potential treatment for cystic fibrosis (CF). The study successfully corrected a common CF-related mutation in intestinal organoids using adult intestinal stem cells from two CF patients. The CRISPR technology also been utilised in pluripotent stem cells or primary somatic stem cells to treat diseases such as thalassemia. Xie et al. (2014) demonstrated that the thalassemia-causing mutation could be repaired ex vivo in human induced pluripotent stem cells.

CRISPR/Cas also has advantages in identifying various pathogens, including the SARS-CoV-2 virus, because it has greater sensitivity and specificity compared to standard PCR and real-time quantitative reverse transcription-PCR (qRT-PCR) (Kumar et al., 2020). There have been various publications on SARS-CoV-2 diagnostics and detection using CRISPR/Cas-based therapeutic tools (Ali et al., 2020; Broughton et al., 2020; Chen et al., 2018; Ding et al., 2020; Islam and Iqbal, 2020; Javalkote et al., 2020; Wang M et al., 2020; Wang XJ et al., 2020).

In the field of cancer research, Huo et al. (2017) used CRISPR/Cas9 to successfully knock out the miRNA-21 (miR21) gene in ovarian cancer SKOV3 and OVCAR3 cell lines. The altered cell lines demonstrated that the miR21 knockout significantly inhibited cancer cell proliferation, migration, and invasion, revealing a primer target for cancer gene therapies (Huo et al., 2017). CRISPR/Cas systems also have been utilised in cancer treatment to repair oncogenic genome/epigenome alterations in tumour cells and animal models, resulting in suppression of tumour cell growth and promotion of cell death, consequently inhibiting tumour growth (Baylis et al., 2017; Wan et al., 2017; Yin et al., 2019). Therefore, the CRISPR-Cas9 system is set to be widely employed in human clinical and research applications in future (Higashijima et al., 2017). Figure 2.7 shows an overview of CRISPR technology applications in research.

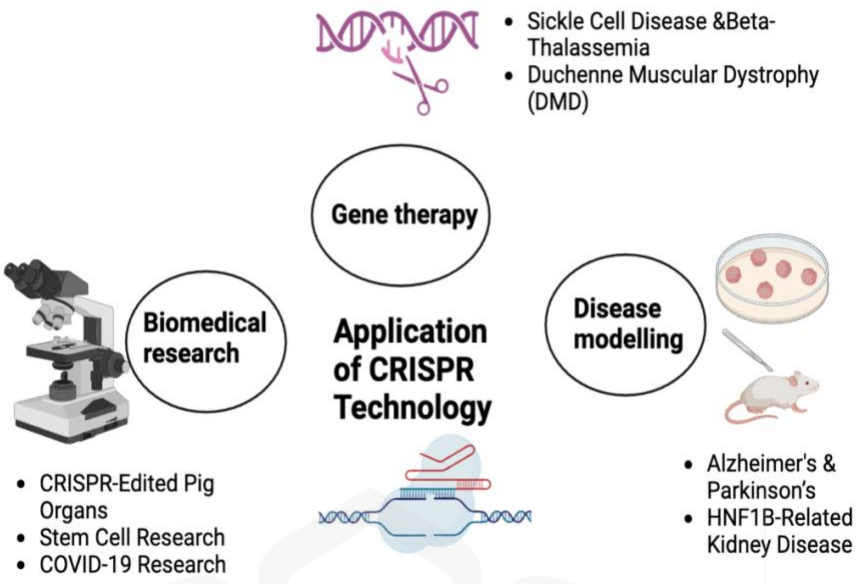


Figure 2.7 Overview of CRISPR technology applications in research

2.8 CONTRIBUTIONS OF GENETIC RESEARCH TO GLOBAL PLANETARY HEALTH AND SUSTAINABILITY

Planetary survival and sustainable wellbeing are closely connected to advancements in science and technology that aim to improve human health and quality of life. Addressing global health challenges through innovative research is essential to building a more resilient and sustainable future. Scientific studies that explore the genetic basis of diseases contribute to a deeper understanding of complex health conditions, enabling the development of more effective interventions (Vasileiou et al., 2024). Consequently, this contributes to the alleviation of long-term healthcare burdens and the enhancement of population health, both of which are critical elements in the pursuit of sustainable development.

In this context, research involving genome editing technologies such as CRISPR plays a significant role in driving progress toward sustainable wellbeing. By enhancing the understanding of biological processes and disease mechanisms, such studies contribute to medical innovation, inform public health strategies, and support the responsible use of scientific knowledge. The generation of genetic models to study human diseases not only advances biomedical research but also aligns with global goals of improving health outcomes while minimizing environmental and societal impacts (Lu et al., 2014). Ultimately, the integration of cutting-edge science into health research contributes meaningfully to both human wellbeing and the broader goals of planetary sustainability.

CHAPTER 3

METHODOLOGY

3.1 FLOW CHART OF THE STUDY

Research lab works was conducted at the UKM Medical Molecular Biology Institute (UMBI). This study began with the culture and maintenance of HEK 293T cells to obtain multiple cell passages. Subsequently, three sgRNAs targeting the *HNF1B* gene were designed using the CRISPick web tool. The sgRNAs were then cloned into the pKLV-U6gRNA(BbsI)-PGKhygro2ABFP vector, and successful insertion was confirmed through Sanger sequencing. However, only two sgRNAs were used in this experiment, as the cloning of the sgRNA 3 was unsuccessful. Following successful cloning of sgRNA 1 and sgRNA 2, lentiviral production was carried out separately for Cas9 and the sgRNAs. HEK 293T cells were transduced with Cas9 lentivirus and followed by blasticidin selection, as the Lenti-Cas9-Blast plasmid consist of blasticidin antibiotic resistance gene. The sgRNA 1 and sgRNA 2 lentivirus was then introduced into Cas9-expressing HEK 293T cells, followed by hygromycin selection as the plasmids for sgRNAs contained of hygromycin antibiotic resistance gene. Finally, western blot analysis was performed to validate the knockout at protein level. The flow chart of this study is shown in Figure 3.1. This study obtained approval from the Institutional Biosafety and Biosecurity Committee (IBBC). The form is attached in the appendix for reference (Appendix 10).

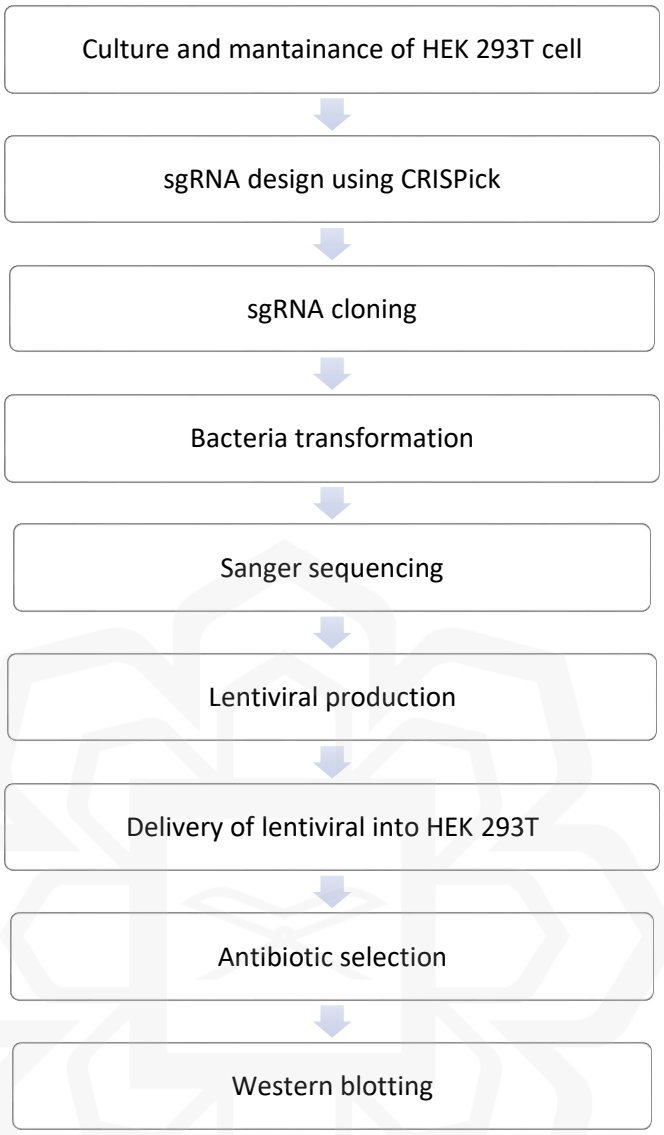


Figure 3.1 Flowchart of experimental design

3.2 MATERIALS

3.2.1 HEK 293T cell

The Human Embryonic Kidney cell (HEK 293T) is a cell line exhibiting epithelial morphology that was isolated from human embryo kidney tissue. This cell line was obtained from UMBI laboratory with unknown passage. Although the exact passage number was not documented, cell health was monitored through regular morphological observation under the microscope, consistent growth rate before proceeding with experiments.

3.2.2 Chemical reagents

In cell culture procedure, several essential chemicals utilised to ensure optimal growth and maintenance of HEK293T cells. The primary culture medium used was Dulbecco's Modified Eagle Medium, High Glucose (DMEM) #08458-16 (Nacalai Tesque, Japan) which provided the necessary nutrients for cell proliferation. For cell detachment and passaging, TrypLE Express #12604-013 (Gibco™, by Thermo Fisher Scientific, Massachusetts, USA), a gentle enzyme alternative to trypsin was employed. Phosphate-buffered saline (PBS) (Nacalai Tesque, Japan) was used for washing cells, while Dimethyl sulfoxide (DMSO) # D12345 (Molecular Probes™, United States) which is used in cryopreservation. Additionally, Fetal Bovine Serum (FBS) # SH30070.03 (HyClone™ by Cytiva, United States) was supplemented to support cell growth, and Penicillin-Streptomycin (10,000 U/mL) (Gibco™, by Thermo Fisher Scientific, Massachusetts, USA) was added to prevent bacterial contamination, ensuring a sterile and stable culture environment.

For sg*HNF1B* cloning, chemicals and reagents used including T4 Polynucleotide Kinase, Cat #M020 (New England Biolabs, Massachusetts, United States), along with T4 Ligase Buffer, Cat #B0202S (New England Biolabs, Massachusetts, United States) for ligation reactions. The top and bottom oligos (Apical Scientific, United States) were cloned into the pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector plasmid #50946 (Addgene, United States). Digestion of the plasmid was performed using the BbsI-HF restriction enzyme (New England Biolabs, Massachusetts, United States) in the presence of CutSmart Buffe, #B7204S (New England Biolabs, Massachusetts, United States). To prevent self-ligation of the digested plasmid, Antarctic Phosphatase Enzyme (New England Biolabs, Massachusetts, United States) and Antarctic Phosphatase Buffer (10X) (New England Biolabs, Massachusetts, United States) were used. DNA purification was carried out using the PCR Purification Kit (1st BASE, Malaysia) and the PrimeWay Gel Extraction/PCR Purification Kit #9050-50 (PrimeWay, China). Finally, the ligation step was completed using T4 Ligase, Cat #M0202 (New England Biolabs, Massachusetts, United States) with T4 Ligase Buffer (10X) (New England Biolabs, Massachusetts, United States) ensuring the successful assembly of the sg*HNF1B* construct.

In bacteria transformation, chemically competent *E. coli* (DH5α), Cat #C2897) (New England Biolabs, Massachusetts, United States) was employed to ensure efficient plasmid uptake. The transformed bacteria were cultured in LB broth, #L3522 (Sigma-Aldrich, United States) which provided essential nutrients for bacterial growth. To select for successfully transformed colonies, 100 mg/mL ampicillin #A5354 (Sigma-Aldrich, United States) incorporated into the culture medium, ensuring the growth of plasmid-containing bacteria only. Additionally, Nutrient Agar was used to prepare agar plates for bacterial culture, providing a solid medium for colony formation. This optimized transformation protocol facilitated high efficiency cloning and plasmid propagation.

For lentiviral production, three plasmids were used including PsPAX2 packaging plasmid #12260 (Addgene, United States), pMD2.G envelope plasmid (Addgene, United States) and lentiCas9-Blast plasmid #52962 (Addgene, United States). Transfection into HEK293T cells was facilitated using Attractene, Cat #301005 (Qiagen, Germany) to enhance plasmid delivery. The cells were maintained in high-glucose DMEM #08458-16 (Nacalai Tesque, Japan) media to provide optimal culture conditions. Following viral production, the lentiviral particles were used to transduce HEK293T cells, with blasticidin selection applied to establish successfully transduced cell populations for further analysis. For sgRNA viral particles, hygromycin (Nacalai Tesque, Japan) was used for antibiotic selection to ensure that the surviving cells contained plasmid.

The BCA assay was conducted using the Pierce™ BCA® Protein Assay Kit and (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's protocol to ensure accurate protein quantification before proceed with the western blot analysis. For Western blot analysis, the SDS-PAGE preparation involved the use of several chemicals, including MilliQ water, 30% acrylamide and bis-acrylamide solution #1610158 (Bio-Rad, California, USA), 1.5M Tris (pH 8.8) and 1.5M Tris (pH 6.8) (Bio-Rad, California, USA) 10% SDS solution #1610416 (Bio-Rad, California, USA), ammonium persulfate (APS) #1610700 (Bio-Rad, California, USA), and N,N,N',N'-tetramethylethylenediamine (TEMED) #33401-85 (Nacalai Tesque, Japan) along with deionized water (dH2O) and 10X SDS running buffer prepared by UMBI UKM.

Additionally, an enhanced 3-color high-range protein marker (9–245 kDa), PM2700 (ExcelBand™, SMOBIO Technology, Inc, Taiwan) was used. During the transfer protocol, 1× transfer buffer was used to facilitate protein transfer onto the membrane. In the post-transfer process, the membrane was stained with Ponceau S Staining Solution prepared by UMBI UKM to assess transfer efficiency, followed by blocking in 5% milk (2.5 g skimmed milk powder dissolved in 1× TBS-T) (unknown brand). The membrane was then incubated with primary and secondary antibodies, specifically Santa Cruz Biotechnology HNF-1b (94.8): sc-130407. (Santa Cruz Biotechnology, Dallas, Texas) and Rabbit Anti-Mouse IgG Antibody #AP160 (Sigma-Aldrich, United States), respectively. Finally, protein detection was performed using the Thermo Scientific™ Pierce™ ECL Plus Western Blotting Substrate #11527271 (Thermo Fisher Scientific, Massachusetts, USA).

3.2.3 Equipments

This study utilised all necessary equipment to culture HEK293T cells in ensuring proper maintenance and handling throughout the process. The equipment used included a serological pipette (Thermo Fisher Scientific, Massachusetts, USA) for accurate liquid transfer, along with a 6-well plate (Sigma-Aldrich, Merck, Darmstadt, Germany) and, both 25 cm^2 and 75 cm^2 flasks (Sigma-Aldrich Merck, Darmstadt, Germany). for cell cultivation. Additionally, 1000 µL micropipette and pipette tips (Eppendorf, Hamburg, Germany) was employed for precise volume measurements. A 100 mL beaker was used for solution preparation, while a centrifuge machine (Eppendorf, Hamburg, Germany) used to facilitated cell pelleting and media changes. Observations and imaging of the cultured cells were conducted using the Invitrogen EVOS XL Core inverted microscope (Thermo Fisher Scientific, Massachusetts, USA) to monitored cell growth and morphology.

For sg*HNF1B* cloning, various essential laboratory equipment were utilised for molecular cloning procedures such as 1.5 mL centrifuge tube (Eppendorf, Hamburg, Germany) for sample preparation and storage, while a micropipette facilitated liquid handling. DNA quantification was performed using the NanoDrop™ 2000/2000c Spectrophotometers (ND2000) (Thermo Fisher Scientific, Massachusetts, USA), ensuring optimal DNA concentration for downstream applications. PCR amplification was carried out using the T100 Thermal Cycler (Bio-Rad, California, USA), which provided temperature control for successful DNA amplification. Additionally, the MiniSpin® (Eppendorf, Hamburg, Germany) was employed for quick spin-down of reaction mixtures, and the Machine Vortex Mixer (Waltham, Massachusetts, USA) ensured thorough mixing of reagents for cloning workflow.

In bacterial transformation process, few laboratory equipment used to ensure successful plasmid uptake and bacterial culture. The centrifuge machine (Eppendorf, Hamburg, Germany) was used for pelleting and resuspending bacterial cells during the transformation process. The digital dry bath or block heaters (Thermo Scientific, Massachusetts, USA) provided temperature control, which was crucial for the heat-shock transformation method. Additionally, 1.5 mL centrifuge tubes (Eppendorf, Hamburg, Germany) were used to handle bacterial cultures and transformation reactions. After transformation, the bacteria were plated onto agar plates containing the Ampicilin, allowing only successfully transformed colonies to grow and for further analysis.

For lentiviral production, equipment used such as 1 mL syringe and 0.45 µm syringe filters (Sigma-Aldrich, Darmstadt, Germany) for sterilizing the viral supernatant. Additionally, 2 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) were used for sample collection and storage. HEK293T cells were cultured in 6-well plates (Thermo Scientific, Massachusetts, USA) to facilitate transfection and viral particle production under controlled conditions.

The BCA assay was conducted using Eppendorf micropipettes (0.1–2.5 µL, 10–100 µL, and 100–1000 µL) (Eppendorf, Hamburg, Germany), 1.5 mL microcentrifuge tubes (Eppendorf, Hamburg, Germany) for sample handling, micropipette tips (10 µL, 100 µL, and 1000 µL) (Eppendorf, Hamburg, Germany) for accurate liquid dispensing, a 96-well plate for assay setup and analysis, and a microplate Reader (BioTek Instruments, Vermont, United States) for absorbance measurements.

Western blot was performed using various equipment, including 0.75–1.5 mm glass plates (Bio-Rad, California, USA) a gel holder and clipper (Bio-Rad, California, USA), 1.0 mm gel combs (Bio-Rad, California, USA), an SDS-PAGE tank (Bio-Rad, California, USA), micropipettes and loading tips (Eppendorf, Hamburg, Germany), and a Vortex mixer (IKA, Staufen, Germany). For the transfer protocol, essential equipment included sponges, Whatman filter paper (Cytiva, Massachusetts, USA), a nitrocellulose membrane (Cytiva, Massachusetts, USA), a protein transfer roller, a gel holder cassette (Bio-Rad, California, USA), ice packs, and a magnetic stir bar and stirrer (Daihen Labtech Stirrer, Japan). During post-transfer steps, a plastic container, shaker, aluminium foil, 15 mL falcon tubes (Corning, New York, USA). The final incubation step involved viewing the membrane using the iBright Imager (Thermo Fisher Scientific, Massachusetts, USA) to detect protein bands.

3.3 METHODOLOGY

3.3.1 Culture of Human Embryonic Kidney Cell (HEK293T)

Cryovials with frozen cells of HEK 293T obtained from UKM Medical Molecular Biology Institute (UMBI) laboratory with an unidentified passage number were removed from liquid nitrogen storage and thawed in a water bath until only a small amount of ice remained in the vial. All cells from the cryovial were collected and put in a 15mL centrifuge tube, which was then centrifuged at 500 x g for 5 minutes to check the clarity of the supernatant and the visibility of the cell pellet. The cells were then placed in a 75cm^2 flask containing Dulbecco's Modified Eagle Medium (DMEM), which supplemented with 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 10% Fetal Bovine Serum (FBS). The flask was then incubated in a 5% CO_2 incubator at 37°C .

To maintain the viability of the cells, it was necessary to employ subculture techniques. The confluent HEK 293T cells underwent a triple wash using 3 mL of Phosphate Buffered Saline (PBS). Subsequently, the flask was filled with a precise volume of 1 mL of Gibco™ Trypsin-EDTA (0.5%), devoid of phenol red. The flask was incubated at room temperature for a duration of 5 minutes. Subsequently, it was imperative to conduct an examination of the cells under the inverted microscope to observe and assess the process of cell detachment. Three flasks with 7 mL of complete media were prepared, and a volume of 2 mL was transferred from the initial flask to each of the subsequent three flasks to maintain the growth process.

3.3.2 Maintenance of HEK 293T cell

The HEK 293T cells, was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin (10,000 IU). Confluent HEK 293T cells in a 75 cm^2 flask are washed three times with 1X PBS, and 1 mL of 0.5 mM EDTA was added. The flask is incubated at room temperature for 5 minutes to dislodge the cells. Then, 5 mL of complete media was added to the flask, and the mixture is centrifuged at 500 x g for 5 minutes. The supernatant was aspirated, the cell pellet was flicked, and 9 mL of freezing media containing 10% dimethyl sulfoxide (DMSO) and 90% Fetal Bovine Serum (FBS) was added. The cells were transferred to cryovials and stored at –80°C freezer, overnight before being transferred to liquid nitrogen tank.

3.3.3 SgRNA Design

The sgRNA was designed using the CRISPick Webtool, available at the Broad Institute's GPP Web Portal (https://portals.broadinstitute.org/gppx/crispick/public). Initially, the desired reference genome, such as Human GRCh38 (NCBI), was selected. The mechanism of CRISPR action, specifically CRISPR knockout (CRISPRko), was chosen to match the experimental needs. Next, SpCas9 (NGG) was selected from the CRISPR enzyme dropdown list. The gene of interest, HNF1B, was entered in the "Target(s)" section (Figure 3.2). Subsequently, the pick quota was set to display only the five best sgRNAs, while the option to report unpicked sequences was left unchecked to avoid unnecessary data. Validation was performed by clicking the “Validate” button, followed by submission. Once the sgRNA picking process was completed, the results were downloaded, and the file was unzipped (Figure 3.3). The extracted content was then opened in Excel for further analysis.

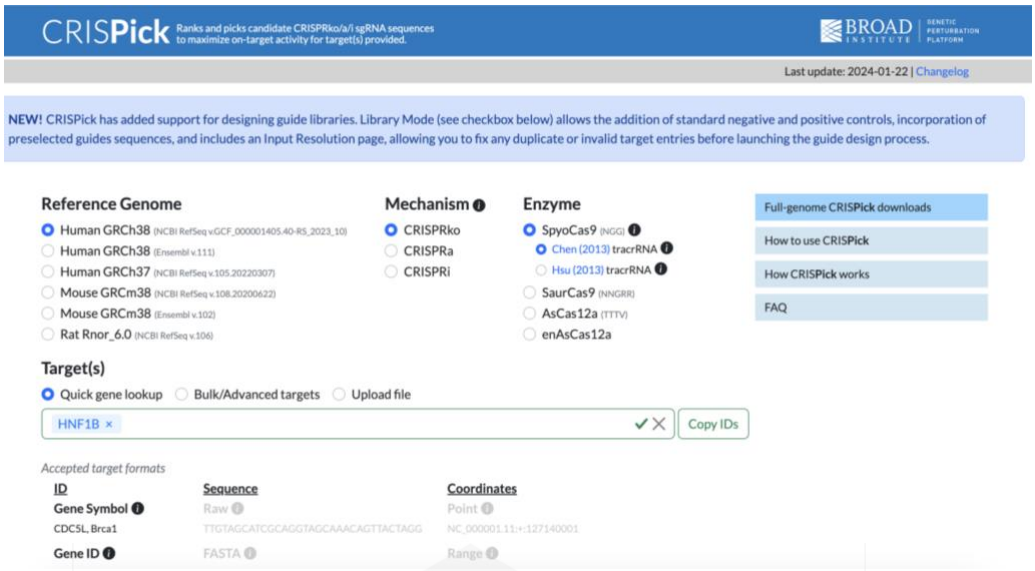


Figure 3.2 CRISPick web tool select *HNF1B* as the target gene for CRISPR-based genome editing

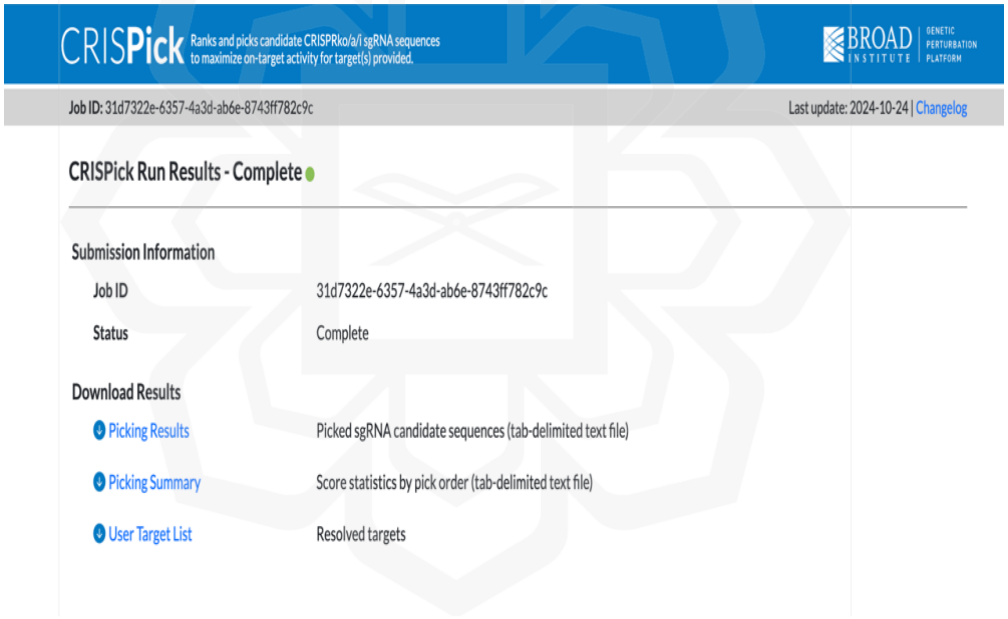


Figure 3.3 Final output for candidate sequences

To extract information regarding the genomic region targeted by the sgRNA, the Ensembl database (www.ensembl.org or https://asia.ensembl.org/index.html) was accessed. The species was specified as human, and the gene symbol, such as *HNF1B,* was entered in the search bar. The target gene was selected from the search results, and the transcript table was displayed by clicking the respective option. The transcript with a verified sequence (e.g., CCDS) was chosen, and for *HNF1B*, the specific transcript ID ENST00000617811.5 was selected. Data export was carried out using the Export Data feature on the left panel. Configuration settings included ensuring the correct transcript ID and selecting the FASTA sequence output. Additional options included specifying the strand as "Feature strand" and enabling cDNA, coding sequences (CDS), exons, and introns (if required for the guide RNA design). The output was saved as a text file and opened in a word processing document for further review.

Alternatively, the genomic data could be downloaded in a compressed text format (gz). The file was unzipped and accessed using word processing software or specialized DNA viewing programs such as SnapGene Viewer. SnapGene Viewer version 8.0.2 was employed to map and visualize the sgRNA target sites within the gene of interest. Features such as exons, introns, and start and stop codons were annotated as needed. This software can be downloaded for free from the SnapGene website (https://www.snapgene.com/snapgene-viewer).

3.3.3.1 In-vitro Oligos Annealing and Phosphorylation for sgRNA Cloning

The preparation of sgRNA strands began by resuspending the lyophilized individual top and bottom sgRNA strands in molecular-grade water to obtain a 100 µM stock solution. A 10 µL reaction was subsequently prepared, containing 1 µL of 100 µM top sgRNA oligos, 1 µL of 100 µM bottom sgRNA oligos, 1 µL of T4 Polynucleotide Kinase, 1 µL of T4 Ligase buffer, and 1 µL of water, bringing the total reaction volume to 6 µL. The reaction mixture were performed using thermocycler with prior incubation at 37°C for 30 minutes to allow enzymatic activity, followed by heat inactivation at 95°C for 5 minutes. Afterward, the temperature was gradually reduced from 25°C to 5°C at a rate of 1°C per minute to ensure proper annealing of the top and bottom sgRNA oligos. To finalize the preparation, Antarctic phosphatase treatment was performed on the digested plasmid using thermocycler. Alternatively, the digested plasmid was stored at -20°C for future use, ensuring its stability until required for downstream applications.

3.3.3.2 sgRNA expression plasmid digestion (pKLV-U6gRNA (BbsI)-PGKhygro2ABFP) with Bbs1 restriction enzyme

For the sgRNA expression plasmid digestion with Bbs1 restriction enzyme, the concentration and purity of the plasmid were first determined using a NanoDrop spectrophotometer to ensure the sample's suitability for enzymatic reactions. A digestion reaction was then prepared in a total volume of 50 µL, consisting of 4.2 µL of sgRNA expression plasmid (2 µg), 1 µL of BbsI-HF (20 U/µL), 5 µL of CutSmart Buffer (10X), and 39.8 µL of molecular-grade water. The reaction mixture was incubated at 37°C for 2 hours to achieved complete digestion by the restriction enzyme. To inactivate the enzyme, the reaction was then heated to 65°C for 20 minutes. Following the digestion, the plasmid was either treated with Antarctic phosphatase for further processing or stored at -20°C for later use.

3.3.3.3 Antarctic Phosphate (ANP) treatment

For the Antarctic phosphatase (AnP) treatment, a reaction mixture with a total volume of 60 µL was prepared, consisting of 50 µL of digested plasmid, 2 µL of Antarctic phosphatase (5 units/µL), 6 µL of Antarctic phosphatase buffer (10X), and 2 µL of water. The reaction was incubated in thermocycle at 37°C for 1 hour to allow dephosphorylation, followed by incubation at 70°C for 5 minutes to inactivate the enzyme. The treated plasmid was subsequently purified using a PCR purification kit to ensure removal of residual reagents. For purification, up to 100 µL of the reaction product was transferred into a 1.5 mL microcentrifuge tube. A PrimeWay Gel/PCR Column was placed into a collection tube, and the sample was loaded onto the column.

The sample was centrifuged at 11,000 × g for 30 seconds, and the flow-through was discarded. The column was then returned to the collection tube. A washing step was performed by adding 750 µL of Wash Buffer (pre-mixed with ethanol) to the column, followed by centrifugation at 11,000 × g for 30 seconds. The flow-through was discarded, and the column was centrifuged again at maximum speed (~18,000 × g) for 3 minutes to remove any residual wash buffer. The column was subsequently transferred to a new 1.5 mL microcentrifuge tube, and 40 µL of Elution Buffer was added to the centre of the column. The sample was allowed to stand for 1 minute before being centrifuged at maximum speed of ~18,000 × g for 1 minute. The purified plasmid was either used immediately for the ligation step or stored at -20°C for future applications. This procedure ensures the plasmid is prepared with high purity and ready for subsequent analysis.

3.3.3.4 Ligation of the annealed sqRNA into Bbsl digested-sgRNA expression plasmid

To perform the ligation of annealed sgRNA into the BbsI-digested sgRNA expression plasmid, a reaction mixture was prepared, containing 2.25 µL of digested plasmid (50–100 ng), 10 µL of annealed sgRNA diluted at a 1:200 ratio, 1 µL of T4 ligase, 2 µL of T4 ligase buffer (10X), and 4.75 µL of water, making up a total volume of 20 µL. The ligation reaction was incubated in thermocycler at 25°C for at least 1 hour to allow efficient ligation of the annealed sgRNA into the plasmid. For enhanced ligation efficiency, the incubation time at 25°C could be extended for several hours, or the reaction could be carried out overnight at 16°C. Following the ligation step, the reaction mixture was inactivated by heating at 65°C for 10 minutes. This prepared ligation mixture was then ready for subsequent transformation into competent cells or could be stored at -20°C until further processing. This step ensures successful incorporation of the sgRNA sequence into the plasmid backbone.

3.3.3.5 Bacteria Transformation

Bacteria transformation was conducted using DH5α competent *E. coli* to introduce the ligated plasmid. The competent cells were thawed on ice prior to the procedure. Subsequently, 20 µL of the ligation reaction mixture was gently added to the tube containing the thawed bacteria. The solution was mixed gently by flicking the tube 4–5 times without vortexing, ensuring minimal disruption to the competent cells. The mixture was incubated on ice for 30 minutes to facilitate the interaction between the plasmid DNA and the bacterial cells. Following this incubation, a heat shock was performed at 42°C for 30 seconds to promote the uptake of the plasmid DNA. The bacterial cells were immediately returned to ice for an additional 5 minutes to stabilize the transformed cells. Afterward, 300 µL of LB broth was added to the mixture, and the bacteria were incubated at 37°C with shaking at 250 rpm for 1 hour to allow recovery and expression of antibiotic resistance genes.

A negative control was also transformed following the same procedure to validate the efficiency of the transformation process. The transformed cells were plated onto LB agar plates containing 100 µg/mL of ampicillin. The plates were incubated overnight at 37°C in a bacterial incubator to facilitate colony formation. The following day, individual colonies were picked and cultured in LB broth containing 100 µg/mL of ampicillin. These cultures were grown overnight to amplify the transformed plasmids, which were subsequently extracted using a plasmid extraction kit. Finally, the extracted plasmids were sent for Sanger sequencing to verify the presence and correct insertion of the sgRNA sequence within the plasmid backbone. This step was to ensure the successful construction of the sgRNA expression plasmid for downstream applications.

3.3.4 Cells delivery and antibiotic selection

3.3.4.1 Cas 9 Lentiviral production

Cas9 lentiviral production was initiated by plating HEK293T cells in a six-well plate and incubating them overnight to achieve 70 to 80% confluency the following day. A transfection mix was prepared in a 1.5 mL Eppendorf tube by combining the required plasmids and serum-free media. Specifically, 10.8 µL of psPAX plasmid (1.3 µg), 0.5 µL of pMD2.G plasmid (0.5 µg), and 7.45 µL of lenti Cas9-Blast plasmid (1.5 µg) were added to 181.25 µL serum-free media to achieve a total reaction volume of 200 µL. To facilitate transfection, 10 µL of Attractene Trawas added dropwise into the prepared mixture. The solution was then incubated at room temperature for 30 minutes to allow the formation of transfection complexes. During this incubation period, the media on the HEK 293T cells was replaced with 1.8 mL of fresh DMEM media to ensure optimal conditions for transfection. The prepared transfection mixture was added dropwise onto the HEK 293T cells, resulting in a final media volume of 2 mL, including the transfection mix. The cells were incubated in CO_2 incubator at 37°C for 48 to 72 hours to allow lentiviral particle production. After the incubation period, the lentiviral particles were collected for downstream applications.

3.3.4.2 Transduction of HEK 293T cell with the Cas9 lentiviral particles

To transduce mammalian cell lines with Cas9 lentiviral particles, HEK293T cells were first plated in a six-well plate and incubated overnight to achieve 70 to 80% confluency. The following day, the media was replenished, and 1.6 µL of polybrene transfection reagent was added to each well, achieving a final concentration of 8 µg/mL. The plate was swirled gently to ensure thorough mixing. Next, 500 µL of harvested lentiviral particles was added dropwise onto the plated cells, followed by gentle swirling to distribute the virus evenly. The cells were then incubated overnight CO_2 incubator at 37°C to allow for successful transduction. On the following day, the media was removed, and the cells were washed once with 1× PBS before adding fresh media into the wells. The cells were incubated for an additional 24 hours or until they were about reach 70% confluency in a well. For selection, 30 µg/mL of Blasticidin was used. Specifically, 6 µL of Blasticidin was added to the plated cells to eliminate untransduced cells. The antibiotic treatment was maintained until all untransfected cells died, which typically occurred within 3 to 5 days. Once the transduced cells reached confluency, cryopreservation was conducted and stored at –80°C freezer for future use, such as sgRNA transduction.

3.3.4.3 sgHNF1B lentiviral production

For the production of sgRNA lentiviral particles, HEK 293T cells were plated in six-well plates and incubated overnight to achieve a confluency of 70 to 80% by the following day. On the day of transfection, a transfection mixture was prepared in a 1.5 mL Eppendorf tube by combining 1.3 µg of psPAX plasmid, 0.5 µg of pMD2.G plasmid, 1.5 µg of sgRNA 1 plasmid, and 1.5 µg of sgRNA 2 plasmid with 185 µL of serum-free media, making a total volume of 200 µL. Around 10 µL of Attractene was added dropwise to the mixture, and the solution was incubated at room temperature for 30 minutes to allow for complex formation. Following this, the media of the HEK 293T cells was replenished with 1.8 mL fresh DMEM. The transfection mixture was then added dropwise onto the cells, ensuring thorough mixing. The total volume of media, including the transfection mix, was adjusted to 2 mL per well. The cells were then incubated at 37°C for 48 to 72 hours to facilitate the production of lentiviral particles. The following day, lentiviral particles were harvested by passing the conditioned media through a 1 mL or 10 mL syringe fitted with a 0.45 µm syringe filter. The filtered media containing the lentiviral particles was collected into a 15 mL falcon tube. To facilitate long-term storage and ease of use, the filtered lentiviral media was aliquoted into multiple cryovial tubes. The aliquots were either stored at –80°C for future use or used directly for transduction of mammalian cells.

3.3.4.4 Transduction of CRISPR-targeted cell with the sgHNF1B lentiviral particles

To transduce CRISPR-targeted cells with sg*HNF1B* lentiviral particles, the targeted cells were plated in a 6 cm plate and incubated overnight to achieve 70 to 80% confluency by the following day. On the day of transduction, the media was replenished, and 3.2 µL of Polybrene transfection reagent was added to the cells to achieve a final concentration of 8 µg/mL. The mixture was gently swirled to ensure even distribution. Subsequently, 250 µL of the harvested lentiviral particles was added dropwise onto the plated cells and mixed by swirling the plate. The cells were then incubated overnight at 37°C in CO_2 incubator. The following day, the media was removed, and the cells were washed once with 1X PBS to eliminate any residual viral particles. Fresh media was then added to the cells, which were further incubated for 24 hours or until it reached about 50% on confluency for antibiotic selection. For selection, 100 µL of hygromycin a final concentration of 1000 µg/mL was added to each plated cell culture. The antibiotic selection process was continued until all untransfected cells died, by observing whether the cell are floating and unattached which typically taking around 5 to 7 days.

3.3.5 Validation of CRISPR Edited Cell

3.3.5.1 Western Blot

a) Cell Harvest

The Western blotting procedure involved several steps, including cell harvesting, lysis, gel electrophoresis, protein transfer, and antibody detection. The cell culture dishes were placed on ice to prevent proteolysis. About 2.36 X 10^6 of cells were used for this procedure. The culture medium was discarded, and the cells were gently washed twice with 2× cold PBS. For a 6-well plate, 2 mL of PBS was used. During the final wash, cells were scraped into 200 µL per well of fresh PBS and transferred to a microcentrifuge tube. The cell suspension was centrifuged at 500 x g for 5 minutes at 4 °C. After centrifugation, the supernatant was discarded, and the cell pellet was either snap-frozen in liquid nitrogen for storage at -80 °C or lysed immediately using the appropriate buffer.

b) Cellular Lysis

Cell pellets were placed on ice, or if frozen, thawed in icy water. Denaturing (urea) lysis buffer was used, with the buffer volume approximately four times the pellet size (40 to 50 µL of buffer for the cell pellet). The cell pellet was resuspended by pipetting up and down, and the mixture was incubated on ice for 30 minutes. Subsequently, the lysate was centrifuged at maximum speed of 1800 x g for 15 minutes at 4°C. The supernatant, containing the protein was transferred to a fresh tube on ice, ensuring the pellet, if any, was undisturbed. The lysate could then be snap-frozen in liquid nitrogen for storage at -80°C if required. Protein concentration was determined using Bicinchoninic (BCA) assay (Shen, 2023). Based on the concentration, proteins were diluted to the desired amount and brought to a uniform volume with distilled water. The sample was mixed 1:1 with 2X sample buffer, as such 10 µL sample and 10 µL buffer. The proteins were denatured by heating the mixture at 95 °C for 5 minutes if β-mercaptoethanol was included or at 85 °C if dithiothreitol (DTT) was used.

c) SDS-PAGE

The resolving and stacking gels were prepared as in Table 3.1.

Table 3.1 The ingredients for resolving and stacking gel

12% Resolving gel	Volume
MilliQ Water	4.9 mL
30% acrylamide mix	6.0 mL
1.5M Tris (PH 8.8)	3.8 mL
10% SDS	150 µL
10% ammonium persulfate	150 µL
TEMED	6 µL

5% stacking gel	Volume
MilliQ Water	3.4 mL
30% acrylamide mix	830 µL
1.5M Tris (PH 6.8)	630 µL

10% SDS	50 µL
10% ammonium persulfate	50 µL
TEMED	5 µL

TEMED was added lastly as it initiates polymerization. The gel solution was poured between glass plates, and distilled water (dH_2O) was layered on top of the resolving gel to ensure even polymerization. After solidification, the water was removed, and the stacking gel was poured. A comb was inserted to create wells for loading samples. After the gel solidified, it was set in a gel electrophoresis apparatus, and 1× SDS running buffer was added. Around of 60 µL (50µg) of samples and a 5 µL protein marker were loaded into the wells, and electrophoresis was run at 140 to 180 V for approximately 1 hour.

d) Protein Transfer

The gel was carefully removed from the plates, and 3 cm from the top and bottom of the gel were trimmed to remove the wells. A transfer sandwich was assembled using wetted sponges, filter paper, the nitrocellulose membrane, and the gel. The membrane was placed closest to the positive plate, while the gel was closest to the negative plate. The sandwich was carefully assembled in the transfer cassette, ensuring no air bubbles were present. Ice packs were added around the tank to maintain low temperatures, and the transfer was performed at 300 mA for 2 hours. After post-transfer, the membrane was stained with Ponceau S to confirm successful protein transfer and destained with MilliQ water.

e) Blocking and Imaging

The membrane was blocked with 5% milk in 1× TBS-T for 1 hour at room temperature or overnight at 4 °C. Primary antibody diluted 1:2000 in 5% milk was added to the membrane, ensuring the target protein areas were covered. The membrane was incubated overnight at 4 °C in cold room. It was then washed three times with 1× TBS-T, each wash lasting 5 minutes. A secondary antibody diluted 1:2000 in 5% milk was applied, and the membrane was incubated for 1 hour in room temperature. After three additional washes with 1× TBS-T, an enhanced chemiluminescence (ECL) mixture with 1:1 ratio of ECL I and ECL II was applied to the membrane for 1 minute. The membrane was then imaged using the Amersham Imager 600 to detect the presence of protein bands. For loading control, B-actin detection was performed using the same protocol.

3.3.6 Quantification of Western Blot band using iBright Analysis Software

Protein quantification from western blot images was performed using the iBright Analysis Software (Thermo Fisher Scientific, Massachusetts, USA), which is available in both desktop and cloud-based versions. The software was downloaded from the Thermo Fisher Scientific website (www.thermofisher.com). This software provides a comprehensive platform for analysing protein gels and western blots band. The process began by importing the blot image into the software, followed by adjustments such as cropping, rotating, and contrast enhancement to optimize visualisation. Automatic lane and band detection features were utilised, with manual corrections applied where necessary. Densitometry analysis was then conducted to quantify the intensity of protein bands, and background subtraction tools were employed to minimize non-specific signals. For accurate interpretation, signal intensities were normalized against a loading control, such as B-actin. Finally, the quantified data were exported in formats such as CSV or Excel for further analysis and documentation.

CHAPTER 4

RESULT

4.1 SGRNA DESIGN USING CRISPICK WEBTOOL

Table 4.1 displayed five best selection for sense and antisense oligonucleotides of *HNF1B*-gRNA by utilizing CRISPick Webtool. However, only the top three oligos were selected for cloning in this study. Even though single guide is commonly sufficient but testing multiple sgRNAs increases the chance of a successful knockout. The oligos were modified by adding CACCG to the 5' end of the sequence and CAAAAT to the 5' end of the complementary sequence. Out of the three oligos chosen only two were further selected, sgRNA 1 and sgRNA 2, as the sgRNA 3 cloning was unsuccessful despite multiple attempts. Figure 4.1 shows sg*HNF1B* 1 targeting exon 3 of *HNF1B* gene and figure 4.2 shows sg*HNF1B* 2 targeting exon 4 of *HNF1B* gene. The red square are the *HNF1B*-gRNA oligos selected for knockout experiment.

Table 4.1 oligos for *HNF1B*-gRNA

sgRNA label	Sense oligonucleotides	Antisense oligonucleotides
sgRNA 1	CACCGAGAGAGAGAGGCCTTAGTGGGT	TAAAACCCACTAAGGCCTCTCTCTCTC
sgRNA 2	CACCG GTTGGAGCTATAGGCGTCCAGT	TAAAACTGGACGCCTATAGCTCCAACC
sgRNA 3	CACCGGTCTAGTGAGGACCCTTGGAGT	TAAAACTCCAAGGGTCCTCACTAGACC
sgRNA 4	CACCGGATGAAAACACTTACGTCGGGT	TAAAAACCCGACGTAAGTGTTTTCATC
sgRNA 5	CACCGGGTGCCCTTGTTGAGATGCTGT	TAAAAACAGCATCTCAACAAGGGCAC

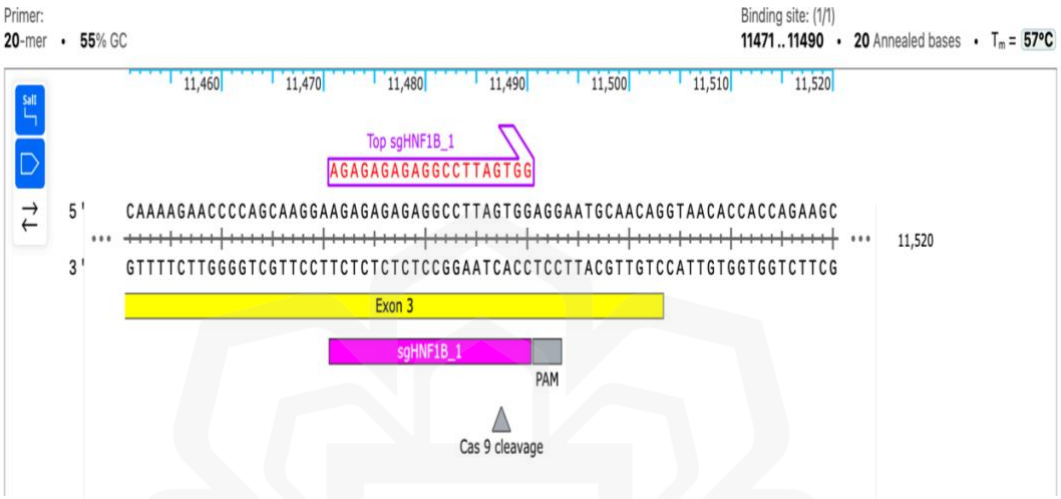


Figure 4.1 sg*HNF1B* 1 targeting exon 3 of *HNF1B* gene

Figure 4.2 sg*HNF1B* 2 targeting exon 4 of *HNF1B* gene

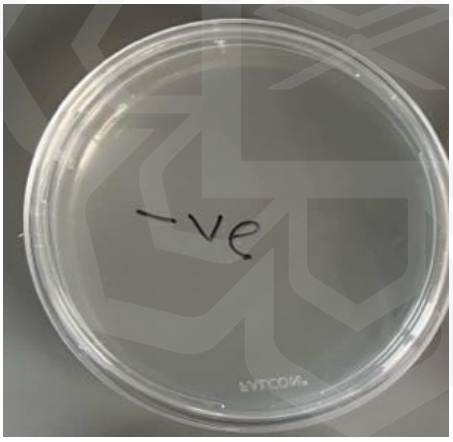
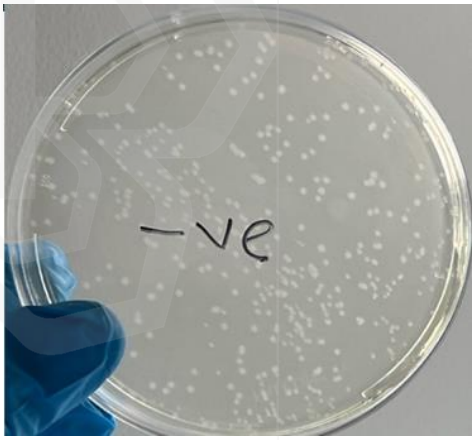
4.2 CLONING OF SG*HNF1B* INTO PKLV-U6GRNA (BBS1)-PGK HYGRO2BFP VECTOR

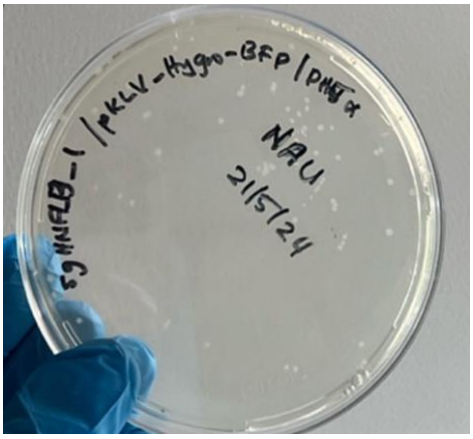
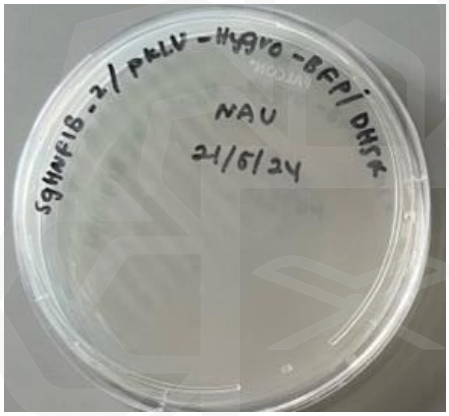
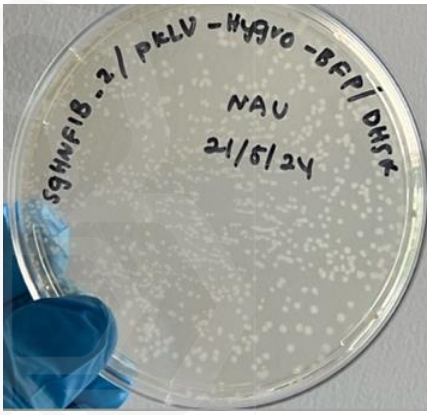
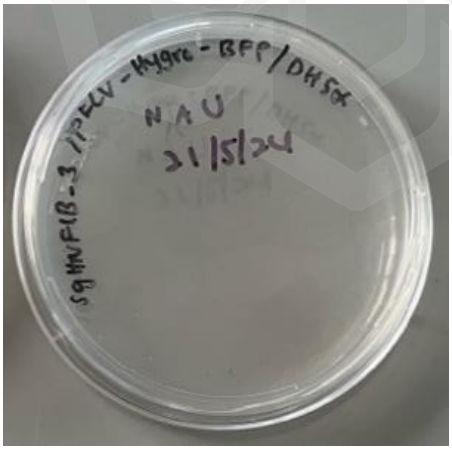
The pKLV-U6gRNA(BbsI)-PGKhygro2ABFP plasmid was utilised in this research as a vector to clone the sgHNF1B, serving as a hygromycin resistance cassette.

4.2.1 Incubation and bacterial growth on agar plate

Following the procedure, bacterial colonies were observed on all agar plates (Table 4.2). sgRNA 3 plates had the highest number of colonies, followed by sgRNA 2 and sgRNA 1. However, colonies also presented on the negative control plate.

Table 4.2 The condition of the agar plate observed both before incubation, prior to colony growth, and after incubation, showing colony growth

	Pre-incubation	Post-incubation
Negative control		

sgRNA 1		
sgRNA 2		
sgRNA 3		

Following the observation on the growth of colonies, individual colonies were carefully selected from each plate and cultured in LB medium supplemented with ampicillin. Overnight incubation resulted in cloudy and turbid cultures, indicating successful bacterial growth (Figure 4.3). Plasmid extraction was performed and quantified for sequencing analysis (Table 4.3).

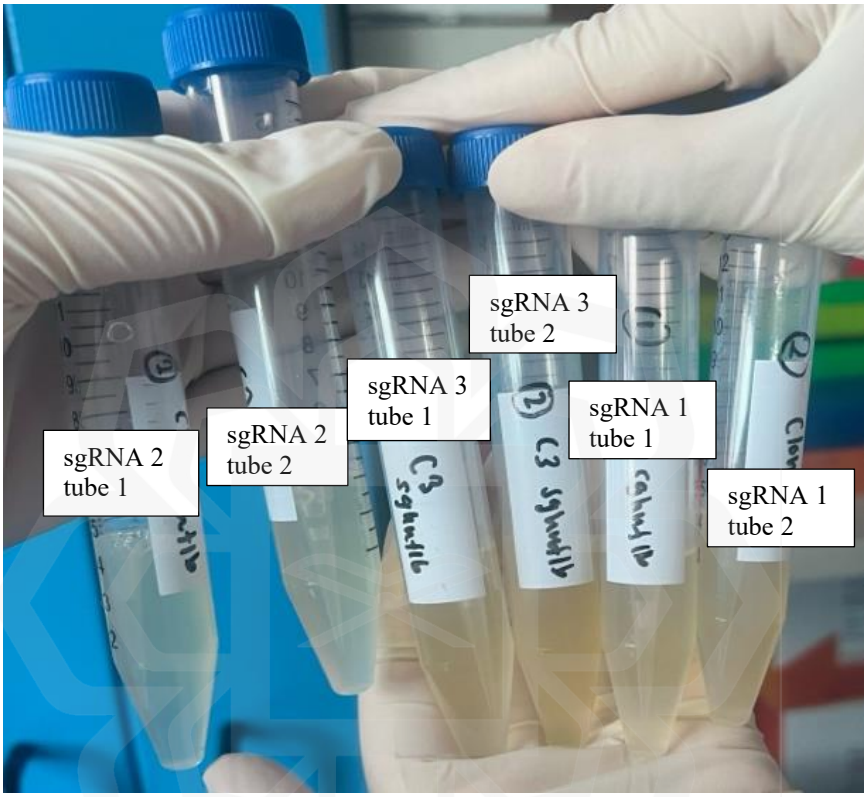


Figure 4.3 Condition of the LB broth observed on the following day after incubation, illustrating the growth of colonies

Table 4.3 Plasmid concentration for all sgRNA tubes

Tube sgRNA	Concentration (ng/ µL)
Tube A sgRNA 1	554.2
Tube B sgRNA 1	469.6
Tube A sgRNA 2	517.3
Tube B sgRNA 2	440.4

Tube A sgRNA 3	522.2
Tube B sgRNA 3	439.9

4.2.2 Gel electrophoresis

To purify the digested plasmid, two methods are commonly recommended: PCR purification and gel extraction kits. Initially, this study employed the PCR purification kit for plasmid purification. However, gel extraction was later performed as an alternative approach, as the PCR purification method resulted in colonies in the negative control and unsuccessful cloning of sgRNA 3. Two types of plasmids were utilised, pKLV-U6gRNA (BbsI)-PGKhygro2ABFP (plasmid-Hygro) and pKLV-U6gRNA (BbsI)-PGKPuro2ABFP (plasmid-Puro). However, this study proceed with pKLV-U6gRNA (BbsI)-PGKhygro2ABFP (plasmid-Hygro). This procedure was to ensure efficient recovery of the DNA fragments and the purified plasmids was measured (Table 4.4). Gel electrophoresis showing bands for both plasmid is demonstrated in Figure 4.6.

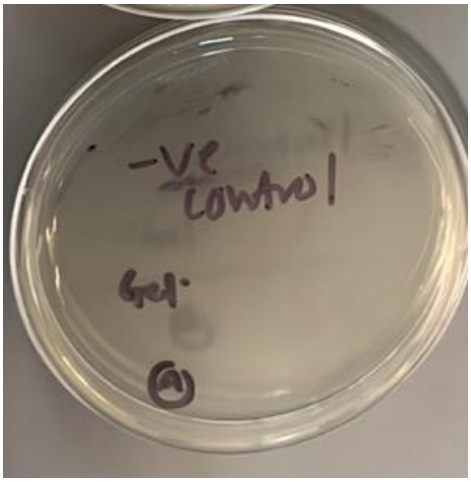
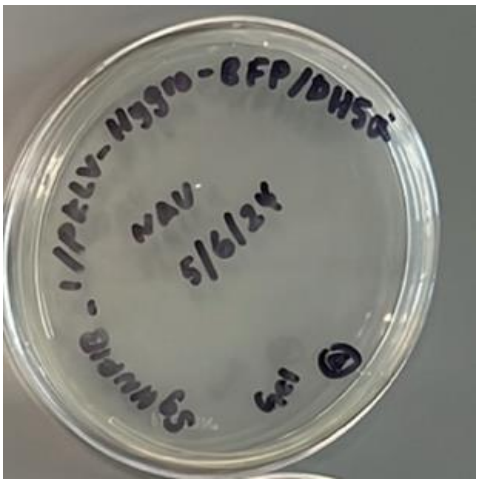
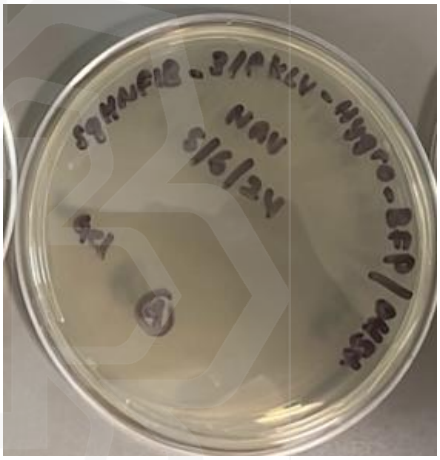
Table 4.4 Purified plasmid concentration

Plasmid	plasmid-Hygro	plasmid-Puro
Concentrations of plasmid (ng/μL)	52.1	39.2

Figure 4.4 Gel electrophoresis showing bands for plasmid-Hygro and plasmid-Puro

In this experiment, only the plasmid-Hygro was utilised for agar plating and cloning due to the higher concentration compared to plasmid-Puro. The digested plasmid obtained from gel extraction was used for ligation process. However, no bacterial colonies were observed on the agar plates, indicating an unsuccessful transformation (Table 4.5). This step has been repeated two times but still no result of colony form. The images of agar plate condition can be refer in Appendix for reference (Appendix 3).

Table 4.5 Condition of the agar plate following gel electrophoresis showing no colonies form

	
Negative control	sgRNA 1
	
sgRNA 2	sgRNA 3

4.3 SANGER SEQUENCING RESULT FOR THE GENOMIC DNA TO CONFIRM THE MUTATION OF THE HNF1B GENE

As seen in Figure 4.5 and Figure 4.7, analysis of the plasmid using SnapGene confirmed the successful cloning of the sgRNA 1 sequence (*CACCGAGAGAGAGAGGCCTTAGTGGGT*) and sgRNA 2 sequence (*CACCGGTTGGAGCTATAGGCGTCCAGT*) into the pKLV-U6gRNA(BbsI)-PGKhygro2ABFP plasmid. These findings validate the successful incorporation of both guide RNA sequences into the plasmid of interest, ensuring its suitability for CRISPR-mediated gene editing applications. Figure 4.6 and 4.8 shows the presence of sgRNA 1 and sgRNA 2 sequence, respectively in pKLV-U6gRNA (BbsI)-PGKhygro2ABFP plasmid.

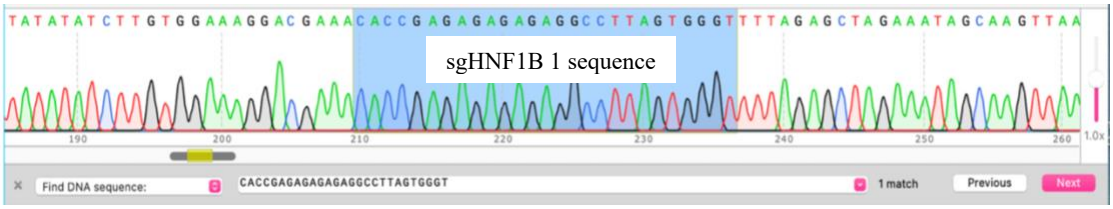


Figure 4.5 Successful cloning of sg*HNF1B* 1 into lenti pKLV-U6gRNA (BbsI)-PGKhygro2ABFP backbone

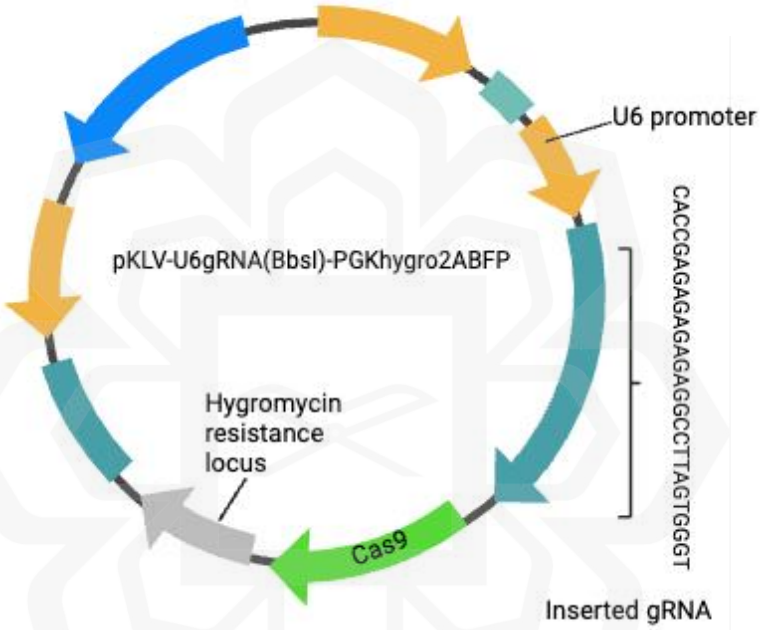


Figure 4.6 The inserted sgRNA 1 into expression plasmid

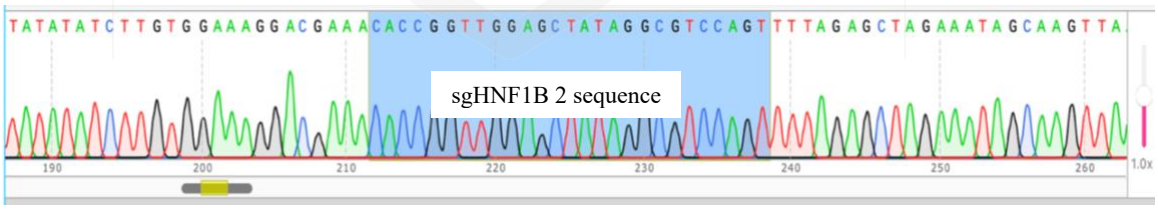


Figure 4.7 Successful cloning of sg*HNF1B* 2 into lenti pKLV-U6gRNA (BbsI)-PGKhygro2ABFP backbone

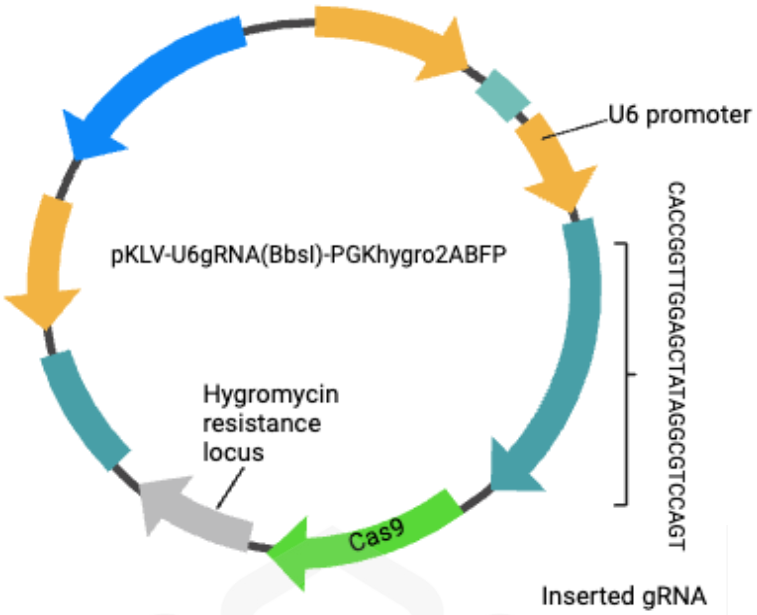


Figure 4.8 The inserted sgRNA 2 into expression plasmid

4.4 TRANSDUCTION OF LENTIVIRUS INTO HEK 293T CELL

Lenti-Cas9-Blast, psPAX2, and pMD2.G plasmids are co-transfected into HEK 293T cells to generate lentiviral particles that contain the Cas9 gene along with a Blasticidin resistance marker. psPAX2 serves as a packaging plasmid that encodes essential viral proteins required for lentiviral assembly. pMD2.G serves as the envelope plasmid, supplying the envelope glycoprotein that enables the virus to infect a cell. For producing cell containing Cas9-blast, HEK 293T cell were plated in 6 cm plate and transduced with Lenti-Cas9-Blast, psPAX2, and pMD2.G plasmids. Figure 4.9 shows the structural map of the lenti-Cas9-Blast vector employed in the CRISPR/Cas9 experiment. Figure 4.10 display Wild-type HEK 293T cells with 70% confluency used for Cas9 lentivirus production using 4X magnification.

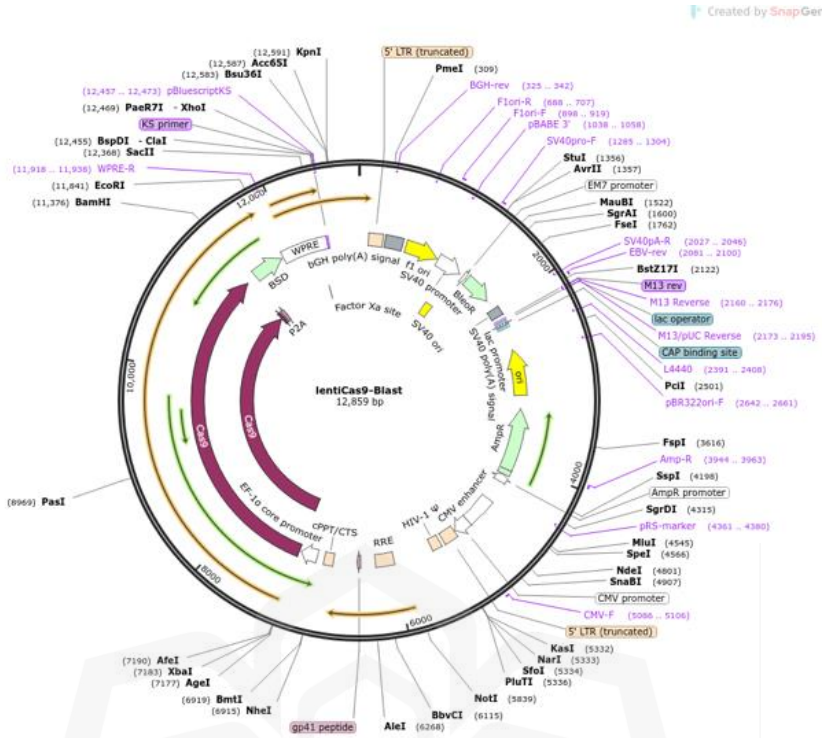


Figure 4.9 Map of the lenti-Cas9-Blast vector utilised in the CRISPR experiment (source: Addgene plasmid # 52962)

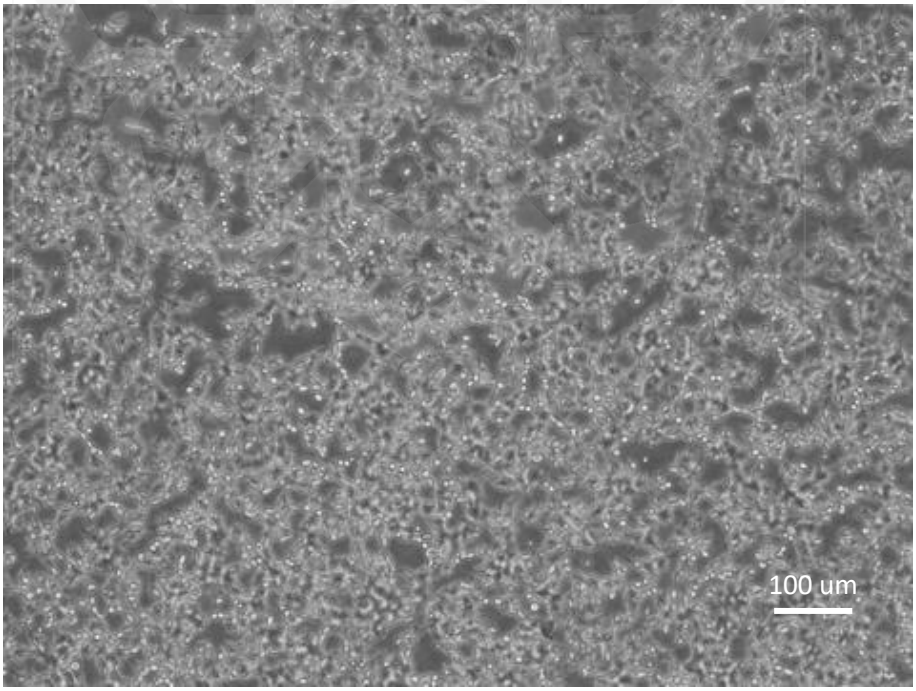
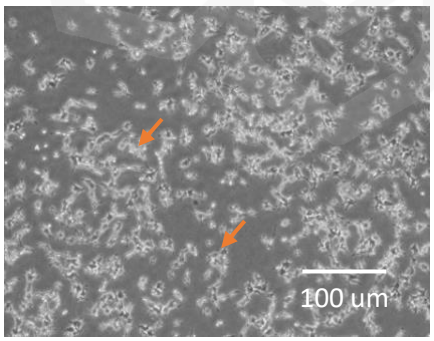
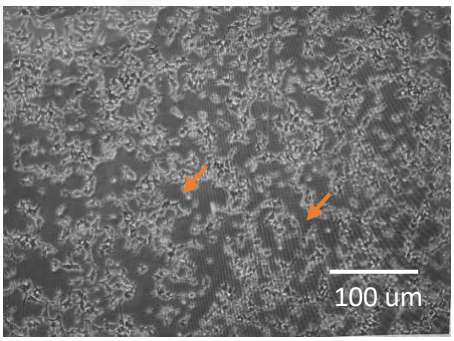
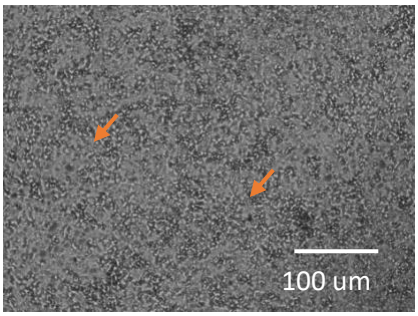
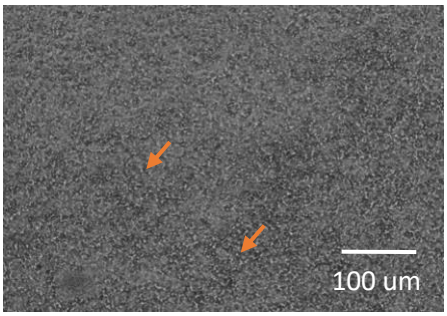
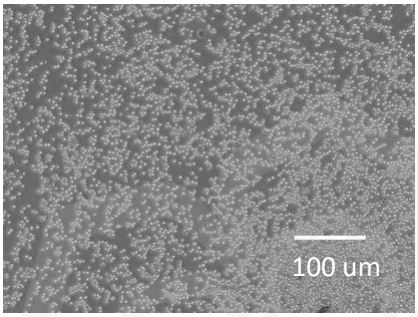
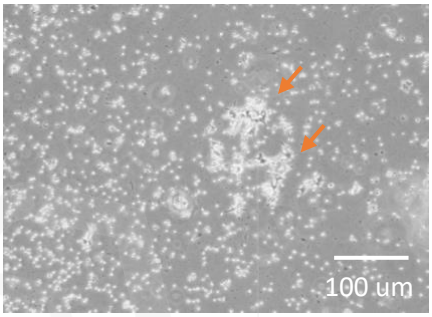
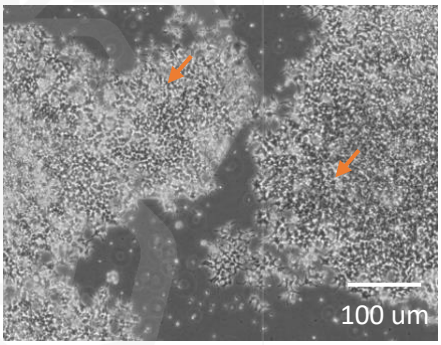
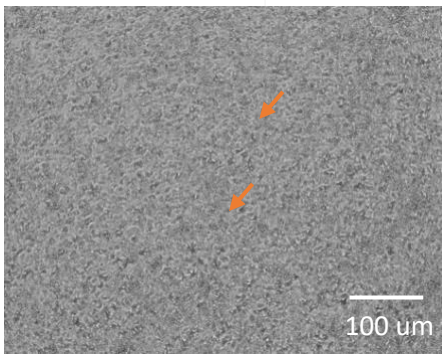


Figure 4.10 Wild-type HEK 293T cells with 70% confluency used for Cas9 lentivirus production using 4X magnification

This HEK 293T is cultured in DMEM with 10% of FBS and 1% PenStrep. Smaller area make it easier to cell attached to each other and grow. The transduction of Cas9 lentivirus into HEK293T take one day to be done. The next day, few cells seems to floating but many cell survived. The cell continue to grow until ready for antibiotic selection using Blasticidin antibiotic. Table 4.6 shows condition of untransduced HEK 293T cells and HEK 293T cell transduced with Cas9-Blast lentivirus prior and post antibiotic selection.

Table 4.6 Morphology changes of untransduced HEk293T cell and HEK 293T cell transduced with Cas9-Blast lentivirus on day 1, day 6, day 9 and day 11 using 4X magnification. Orange arrows indicate the viable cells that survived blasticidin selection.

Day	Untransduced HEK 293T cells	HEK 293T cell transduced with Cas9-Blast lentivirus
Prior to anti-biotic selection		

Day 1 anti-biotic selection		
Day 6 anti-biotic selection		
Day 9 anti-biotic selection	Cell death	
Day 11 anti-biotic selection	Cell death	

Based on this transduction, it was observed that confluency of untransfected cells declined commencing day 1 after antibiotic treatment and reached 0% by day 6 (Table 4.6). This indicates a complete cell death and an inability to proliferate under antibiotic conditions. In comparison, the lenti-Cas9 transduced cells exhibited a similar declining trend at the start of antibiotic treatment but gradually recovered, reaching approximately 90% confluency by day 11. This observation suggests that the transduced cells were able to adapt prolonged treatment and capable to express antibiotic resistance gene in the construct. The survived cells were cultured to confluency, collected and seeded into larger culture vessels to allow cell expansion. Cells were passaged at least three times to allow stable culture and as preparation (cell stock) before second transduction (sgRNA lentivirus delivery).

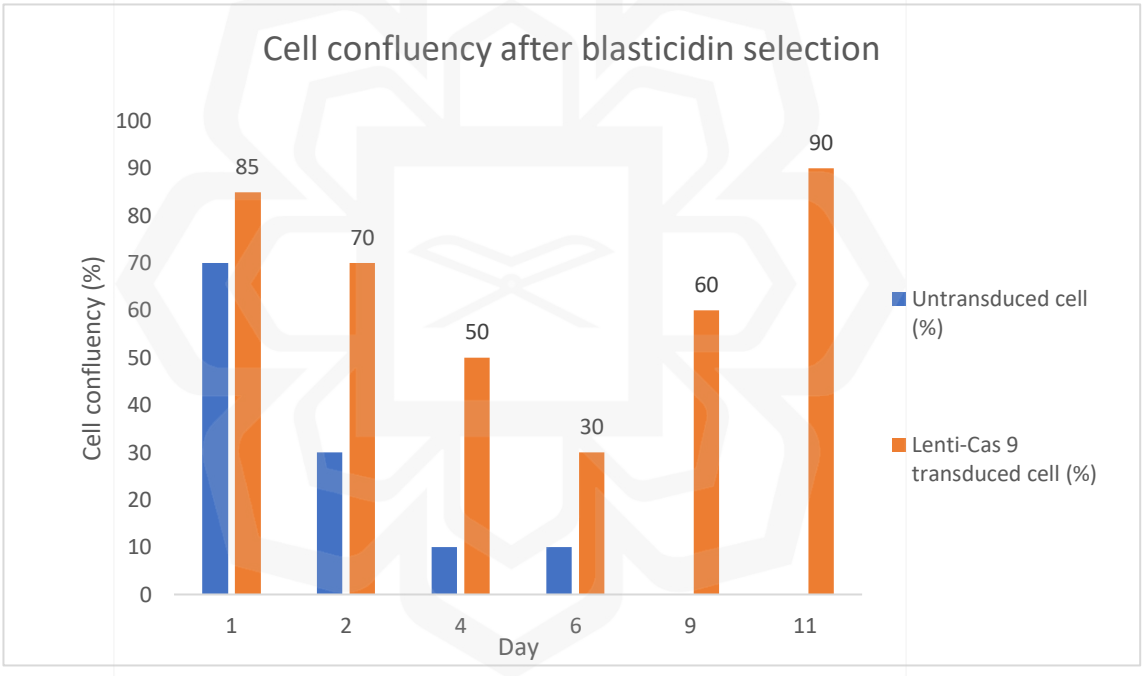


Figure 4.11 Percentage of confluency (%) during blasticidin selection

While maintaining the lenti-Cas9 transduced cells, lentivirus production was initiated for sgRNA 1 and sgRNA 2. Lentiviral particles carrying sgRNA 1 and sgRNA 2 were harvested and transduced into the lenti-Cas9 transduced cells at approximately 70% confluency. Antibiotic selection was performed using hygromycin after post-transduction, with expression plasmid contained a hygromycin resistance gene. As illustrated in Figure 4.12, untransduced cells exhibited a steady decline in confluency from Day 1, ultimately detaching and floating by Day 6, indicating complete cell death. However, the sgRNA 1 and sgRNA 2-transduced cells also showed a reduction in confluency but remained viable up to day 6, suggesting successful integration with expression plasmid. The morphology of cells in each condition are included in the appendix for reference (Appendix 4 and 5). Table 4.7 shows images cell condition of untransduced cell (control) and HEK 293T transduced with sgRNA 1 and sgRNA 2 on day 1 and day 6 at 4X magnification.

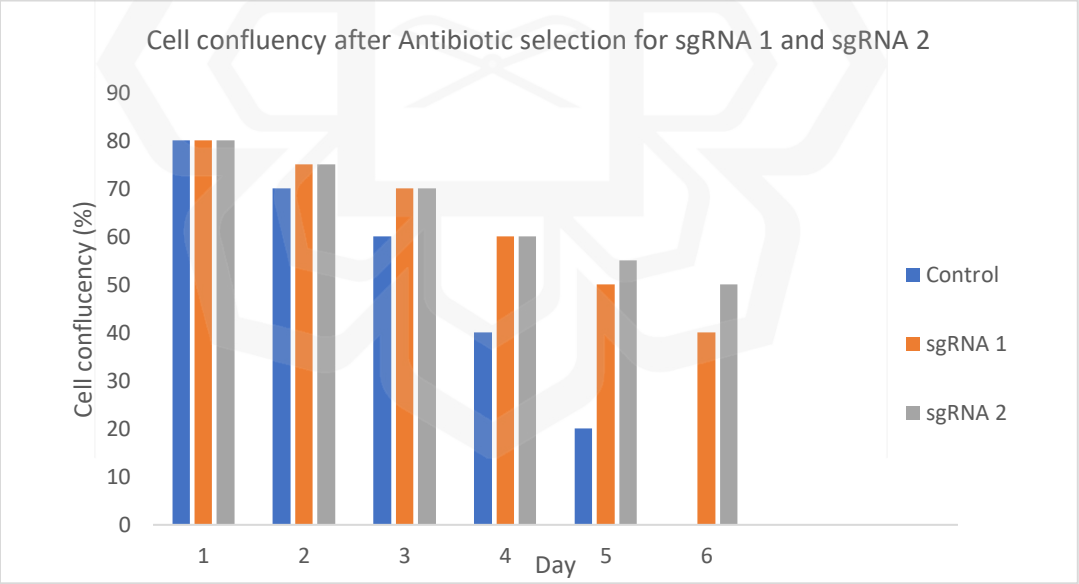
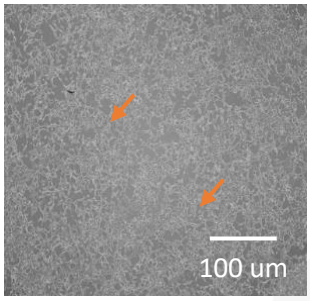
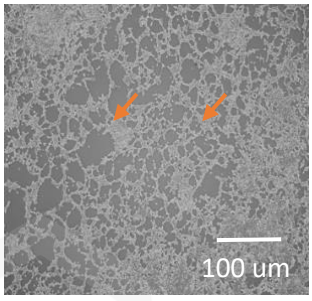
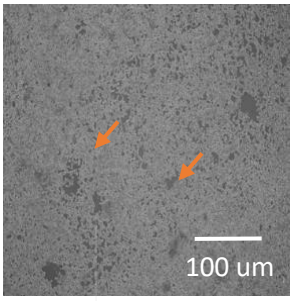
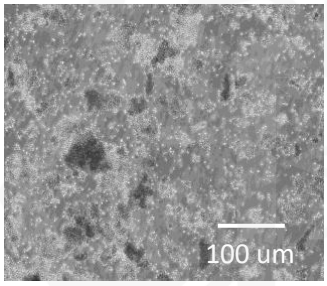
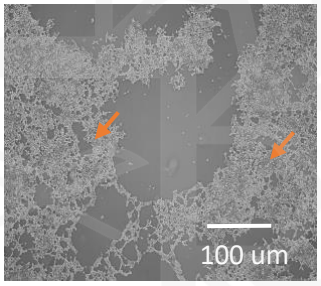
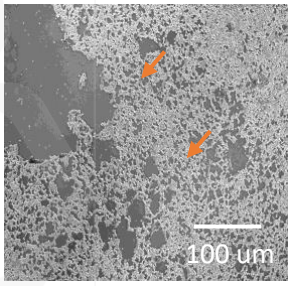


Figure 4.12 Percentage of confluency (%) during antibiotic selection using Hygromycin for sgRNA 1, sgRNA 2 and control cells

Table 4. 7 Images cell condition of untransduced cell (control) and HEK 293T transduced with sgRNA 1 and sgRNA 2 on day 1 and day 6 using 4X magnification. Orange arrows indicate the viable cells that survived hygomycin selection.

Day	Untransduced (WT)	HEK 293T transduced with sgRNA 1	HEK 293T transduced with sgRNA 2
Day 1 anti-biotic selec-tion			
Day 6 anti-biotic selec-tion			

4.5 WESTERN BLOT ANALYSIS FOR PROTEIN EXPRESSION VALIDATION

To assess the efficiency of protein transfer, Ponceau S staining was performed (Figure 4.13).

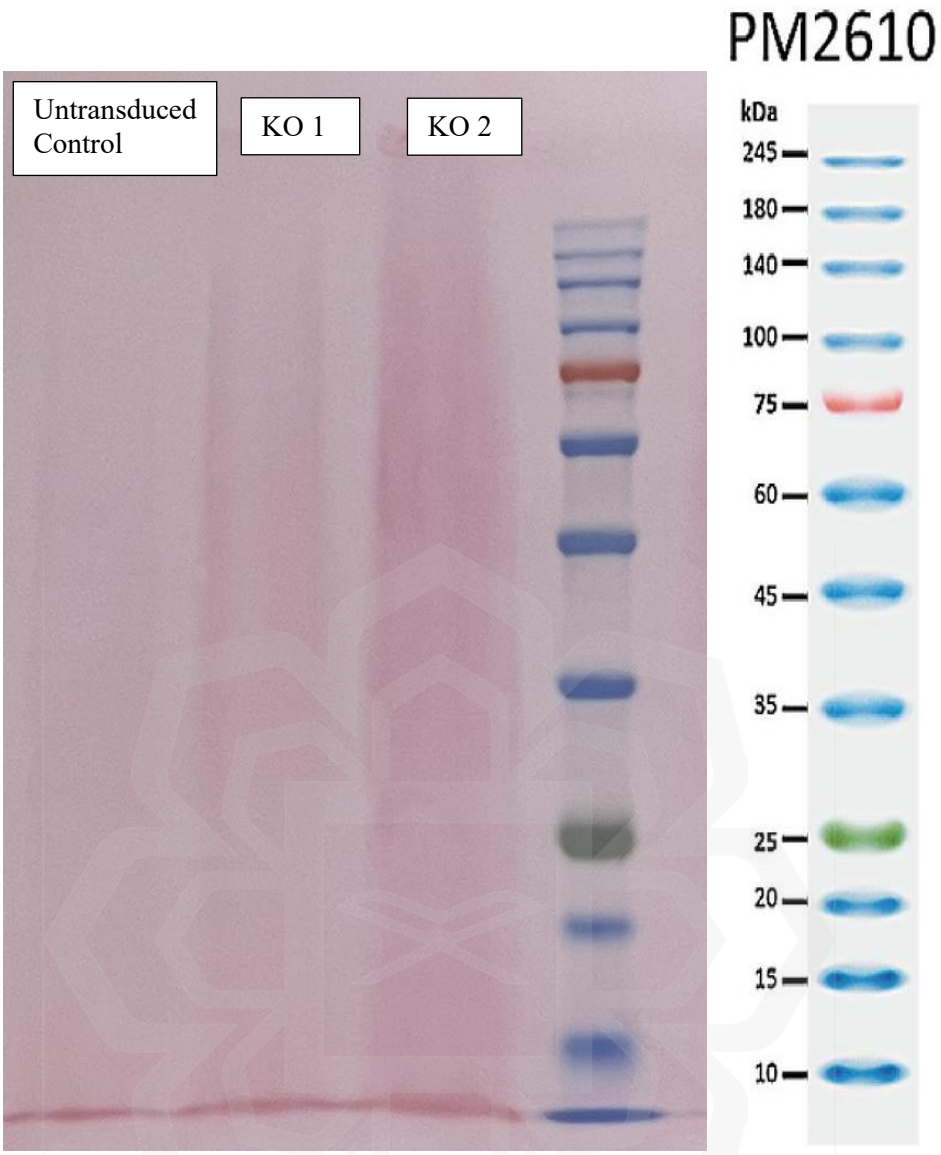


Figure 4.13 Ponceau S result

In this experiment, western blot assays were performed three times due to some technical issues, and the third blot produced the clearest results with better band visibility. The images for western blots 1 and 2 are included in the appendix for reference (Appendix 1 and Appendix 2). To confirm that protein loading was consistent across all samples, the blot was incubated with B-actin as a loading control. The expected protein sizes for Cas9, HNF1B, and B-actin are 160 kDa, 56 kDa, and 42 kDa, respectively.

The results in Figure 4.14 showed a visible band for HNF1B protein in the untransduced Control sample though slightly unclear, while no bands were observed in the KO 1 and KO 2 samples. 50 µg protein were loaded for each samples. In addition, bands for Cas9 and B-actin were observed, confirming successful delivery of the CRISPR/Cas9 system even the intensity of bands is inconsistent. The results showed that the unstransduced control sample had a stronger B-actin signal compared to KO 1 and KO 2, indicating that unequal protein loading may have contributed to the observed differences. The higher intensity in the wild-type sample suggests that more total protein was loaded into that lane compared to the KO samples. Figure 4.15 illustrated Protein expression analysis (intensity, %) of Cas9 and HNF1B for unstransduced control, KO1 and KO 2 cells.

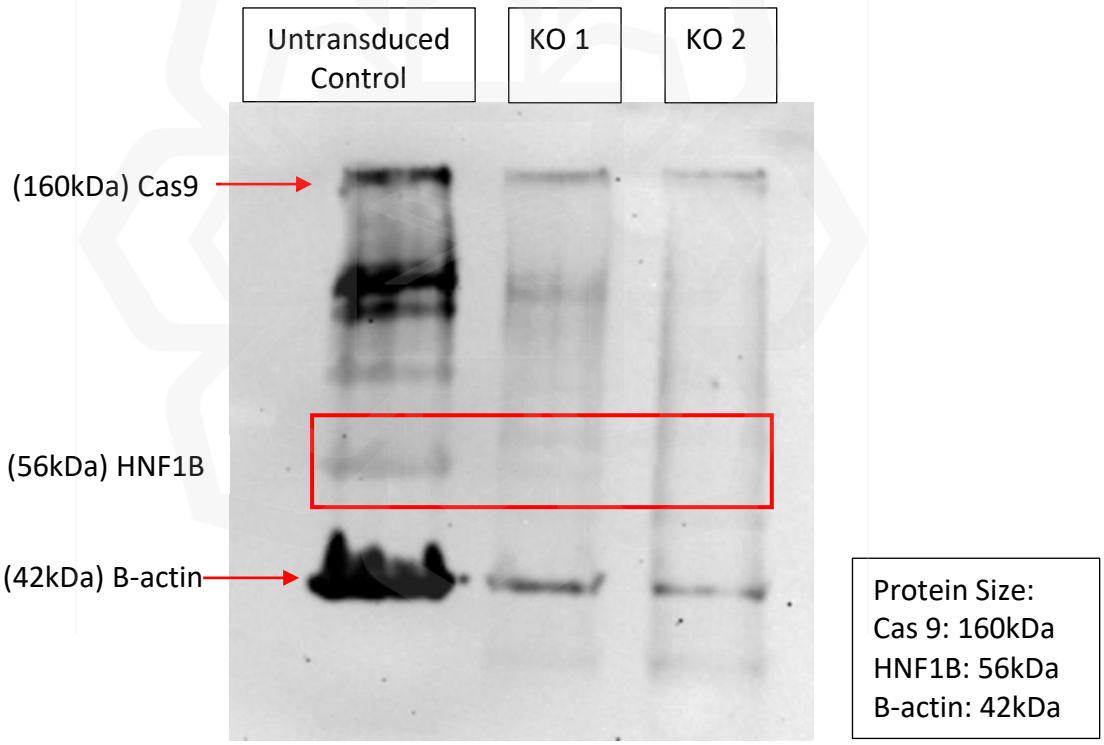


Figure 4.14 Protein expression of Cas9 and HNF1B in untransduced Control and HNF1B+/- in HEK 293T using sgRNA 1 (KO 1) and sgRNA 2 (KO 2)

Figure 4.15 and figure 4.16 represent protein expression analysis (intensity, %) of Cas9 and HNF1B for untransduced control, KO 1 and KO 2 cells. The graph represents HNF1B and Cas9 protein intensities normalized to B-actin using ImageJ analysis. For Cas9, the untransduced control cells shows the highest signal intensity (100%), whereas KO 1 and KO 2 show markedly reduced intensities of 26% and 13%, respectively. A similar pattern shown for HNF1B. Untransduced control cells recorded highest signal (100%), followed by KO 1 (60%) and KO 2 (34%). These findings indicate that both knockout lines display considerably lower protein expression levels of Cas9 and HNF1B compared to the untransduced control.

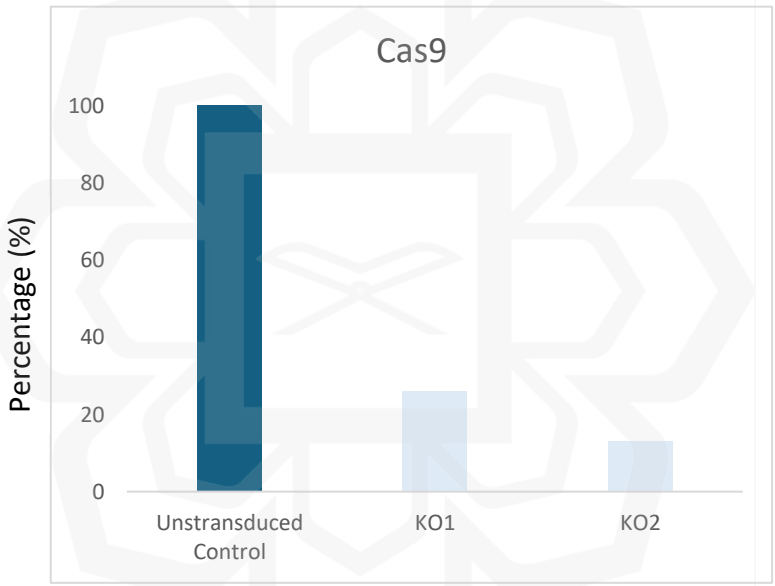


Figure 4.15 Protein expression analysis (intensity, %) of Cas9 for unstransduced control, KO 1 and KO 2 cells

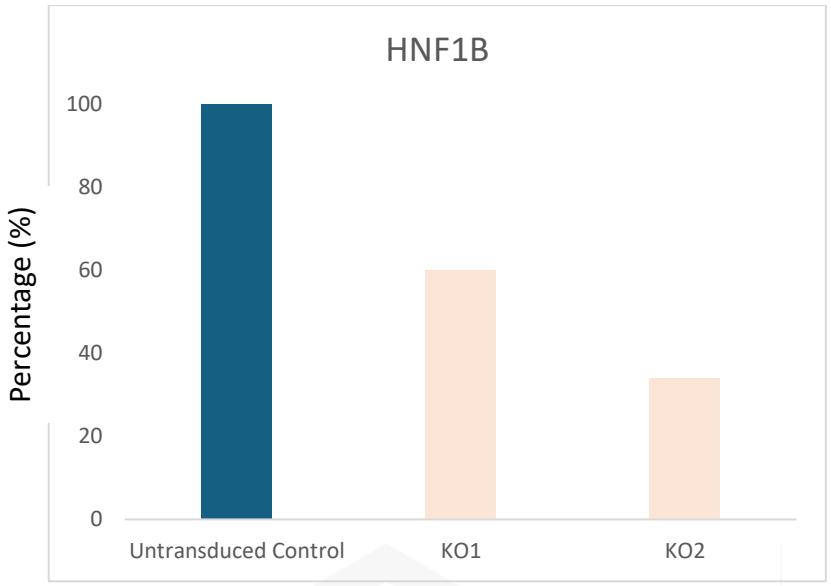


Figure 4.16 Protein expression analysis (intensity, %) of HNF1B for unstransduced control, KO 1 and KO 2 cells

CHAPTER 5

DISCUSSION

5.1 SGRNA DESIGN USING CRISPICK WEBTOOL

In this study, the CRISPICK web tool was utilised to design single guide RNAs (sgRNAs) for the CRISPR/Cas9 knockout experiment. As reported in the results, only sgRNA 1 and sgRNA 2 were successfully cloned into the target plasmid and subsequently selected for further experiments. Despite multiple attempts, the cloning of sgRNA 3 was unsuccessful using both PCR purification and gel electrophoresis approaches. While PCR purification resulted in colony formation, subsequent Sanger sequencing revealed that the sgRNA 3 sequence was absent from the expression plasmid, indicating unsuccessful cloning. This confirmed the inability to integrate sgRNA 3 into the target plasmid. Consequently, no further DNA extraction or sequencing could be performed for sgRNA 3. Therefore, the first two sgRNAs are sufficient to proceed with the knockout experiment, and no additional attempts to clone sgRNA 3 were made.

Before ordering the oligonucleotides for all sgRNAs, specific overhang sequences were incorporated to facilitate efficient and accurate cloning. The CACCG sequence was added to the 5′ end of the top strand, while CAAAAT was added to the 3′ end of the bottom strand. The inclusion of the CACCG overhang ensures that, upon digestion, the oligonucleotides generate compatible sticky ends that allow for directional ligation into the vector (Nageshwaran et al., 2018). Directional cloning is crucial to ensure the correct orientation of the sgRNA insert, which is essential for its proper expression and functional activity in the knockout experiment (Nageshwaran et al., 2018). This strategic design and cloning approach aimed to maximize the success of sgRNA integration while ensuring the constructs were correctly oriented for optimal performance in downstream CRISPR/Cas9-mediated gene editing.

As demonstrated in the results section (Figure 4.1 and Figure 4.2), sgRNA 1 and sgRNA 2 were designed to target exon 3 and exon 4, respectively. Targeting early exons is a common strategy in gene knockout experiments, as these regions often encode critical functional domains of the protein. Disrupting an early exon is more likely to prevent the synthesis of a functional protein by causing a frameshift mutation or introducing premature stop codons, ultimately leading to nonsense-mediated mRNA decay or the production of a truncated, non-functional protein (Hsu et al., 2014).

In the case of HNF1B, the DNA-binding domain is a crucial functional region composed of two key segments, one of which is the POU-homeodomain (POU_H). This domain is encoded by sequences located within exons 3 and exon 4 and plays an essential role in enabling HNF1B to bind to DNA and regulate the transcription of its target genes (Clissold et al., 2015). Disruption of these exons through CRISPR/Cas9-mediated gene editing is expected to impair the integrity of the POU_H domain, effectively abolishing the DNA-binding capacity of HNF1B.

The deletion of exon 3 and exon 4 is expected to result in the functional inactivation of the HNF1B protein (Bellanné-Chantelot et al., 2004). Consequently, this loss is expected to reduce HNF1B protein expression and compromise its regulatory role in cellular processes. This targeted strategy aligns with the goal of achieving an effective and complete knockout of *HNF1B*, maximizing the chances of disrupting its biological function.

5.2 CLONING STRATEGY OF SGHNF1B INTO PKLV-U6GRNA (BBS1)-PGK HYGRO2BFP VECTOR

In this study, PKLV-U6gRNA (Bbs1)-PGK hygro2BFP plasmid was used for cloning of sgHNF1B. A key feature of the PKLV-U6gRNA (Bbs1)-PGK hygro2BFP vector is the U6 promoter, which drives the transcription of guide RNAs (gRNAs) (Koike-Yusa et al., 2014). This plasmid contains the U6 promoter, a robust RNA polymerase III promoter that drives efficient guide RNA expression, along with a BbsI restriction site to facilitate the cloning of custom gRNAs into the plasmid (Koike-Yusa et al., 2014). PKLV-U6gRNA (Bbs1)-PGK hygro2BFP plasmid contains 8103 base pairs (Koike-Yusa et al., 2014). The PKLV plasmid serves as the backbone for stable integration into the genome during lentiviral transduction, to ensure the long-term expression of the desired genetic elements (Metzakopian et al., 2017). Additionally, the PGK promoter is ensuring the expression of downstream genes, such as the hygromycin resistance gene meaning that cells that contain this plasmid will survive and remain viable after hygromycin selection. (Koike-Yusa et al., 2014).

The plasmid also incorporates the 2BFP marker, which produces a blue fluorescent protein, providing a visual indicator of successful plasmid integration (Koike-Yusa et al., 2014). Its efficiency in facilitating targeted gene modifications underscores its utility in both basic and translational biomedical research. Furthermore, its effective use in genome-wide CRISPR screening underscores its significance in enhancing the comprehension of gene functions and facilitating targeted genetic modifications. Figure 5.1 illustrates vector map of pKLV-U6gRNA (BbsI)-PGKhygro2ABFP vector.

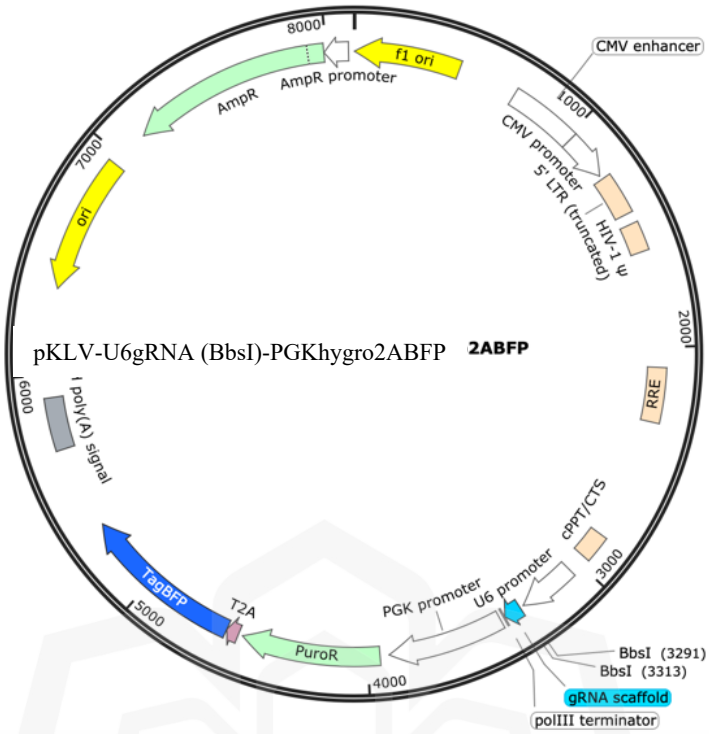


Figure 5.1 Vector map of pKLV-U6gRNA (BbsI)-PGKhygro2ABFP vector (Addgene Plasmid #50946) (Koike-Yusa et al., 2014)

This study utilised DH5a competent *E.coli* in to clone the sgHNF1B into the expression plasmid. Competent cells are bacterial cells with the ability to efficiently uptake foreign DNA. *Escherichia coli* (*E. coli)* is commonly used for this purpose due to its capacity to facilitate DNA amplification and cloning during bacterial transformation (Fakruddin et al., 2012). During the bacteria transformation, the mixture were incubated for 30 minutes on ice followed by heat shock at 42°C for 30 second, after the addition of ligated plasmid into the bacteria. This process helps to increase the efficiency of bacterial transformation by temporarily increasing the permeability of the cell membrane, allowing plasmid DNA to enter (Froger and Hall, 2007). The cold incubation helps maintain the cells in a competent state, while the heat shock briefly disrupts the membrane structure, making it easier for the DNA to pass through (Panja et al., 2006). Turbidity in a broth culture serves as an indirect indicator of microbial growth, indicating the successful proliferation of microorganisms in the culture medium (Bonnet et al., 2019). Such turbidity serves as a visual confirmation of active bacterial replication and the presence of a substantial number of cells within the broth (Bonnet et al., 2019; Hossain, 2024). Before sending plasmids for sequencing, it is essential to perform plasmid purification to ensure high-quality DNA. Plasmid concentration is a critical parameter, as sufficient DNA yield and purity are required for successful sequencing.

5.2.1 Incubation and bacterial growth on agar plate

In a negative control experiment involving digested plasmid and *E. coli DH5a* (without an sgRNA insert), colonies are expected not to form. However, the presence of colonies can be attributed to two main factors. First, self-ligation of the vector may occur. After digestion, the plasmid backbone might re-circularize without incorporating an insert (Sorida et al., 2023). This happens when the ends of the digested plasmid are compatible and ligation proceeds either due to the presence of ligase or because the ends are sufficiently close in compatibility (Lessard, 2013; Sorida et al., 2023). Such self-ligated plasmids retain their ability to transform the bacteria, resulting in colonies. Secondly, incomplete digestion of the plasmid vector can leave some circular, undigested plasmids intact (Kostylev, et al 2015). These undigested plasmids remain functional and can transform competent *E. coli DH5a* cells, leading to the growth of colonies. (Figure 4.4.). Despite the potential impact on study reliability, the colony was sent for Sanger sequencing to confirm whether the intended sequence was successfully inserted. This step helps to validate the cloning process and clarify whether the observed colony was due to contamination, incomplete digestion, or other factors, hence maintaining transparency and reliability in data interpretation.

5.2.2 Gel electrophoresis

Transformations revealed discrepancies between gel electrophoresis and PCR analyses. It was apparent that there were no colonies are visible on agar plate after gel electrophoresis and gel extraction (Table 4.5) indicating a potential failure in ligation or annealing (Sorida & Bonasio, 2023). However, colonies were observed in agar plates that using PCR purification kit (Figure 4.4), suggesting that ligation may have been partially successful since colonies were amplified. This inconsistency points toward a possible issue during the annealing phase of the experiment, where the sg*HNF1B* fragments may not have properly ligated with the plasmid vector. To address this annealing challenge, the annealed sample of sgRNA 1, sgRNA 2 and sgRNA 3 were subjected to a troubleshooting procedure involving a ramp-down heating method. The samples was heated to 95°C for 10 minutes in a microwave, ensuring complete denaturation of DNA strands. This step was followed by a gradual cooling process to room temperature, allowing the DNA strands to anneal properly (Figure 5.2) .

This method aims to enhance the annealing efficiency by facilitating proper alignment and hybridization of complementary DNA sequences, potentially increasing the success rate of the ligation process (Evans, 2009). Despite the annealing adjustments, colonies observed when using PCR purification kit method. Two colonies from each plate were picked followed by plasmid DNA extraction. All three sgRNA (sgRNA 1, sgRNA 2, and sgRNA 3) are 20 nucleotides in length, ensuring precise binding to the target DNA while minimizing non-specific annealing (Hsu et al., 2013). According to Ran et al., (2013), Cas9 can be easily retargeted to new DNA sequences by simply purchasing a pair of oligos encoding the 20 nucleotide guide sequences.

In addition, SpCas9 typically cleave the DNA about 3 bp 5' of the PAM, which corresponds to the junction between the 17th and 18th nucleotides of the target sequence when using a 20 nucleotide guide. Hence, designing an sgRNA with 20 nucleotides ensures that the Cas9 cleavage site falls within the region recognized by the sgRNA–Cas9 complex (Ran et al., 2013). This provides sufficient length for specific binding. If the guide was shorter or longer, it could reduce targeting specificity (Ran et al., 2013).

The sgRNA 1 and sgRNA 2 melting temperatures (Tm) are 57 °C and 58°C respectively. The Tm between 50°C to 60°C is suitable for efficient annealing during the reaction. Melting temperature (Tm) is affected by GC content and nucleotide composition, both of which play a critical role in the stability of sgRNA binding (Doench et al., 2016; Xu et al., 2015). A high GC content, which increases Tm, can lead to excessively strong binding, potentially disrupting the formation of the ribonucleoprotein (RNP) complex. On the other hand, a low GC content may result in weak sgRNA binding (Xu et al., 2015). Thus, in this study, sgRNA 1 and sgRNA 2 were both designed to have a GC content of 55%, which is within the optimal range for stable binding. This ensure an efficient Cas9 binding and precise genome editing. Figure 5.2 display annealing process achieved through natural cooling method instead of thermocycler machine.

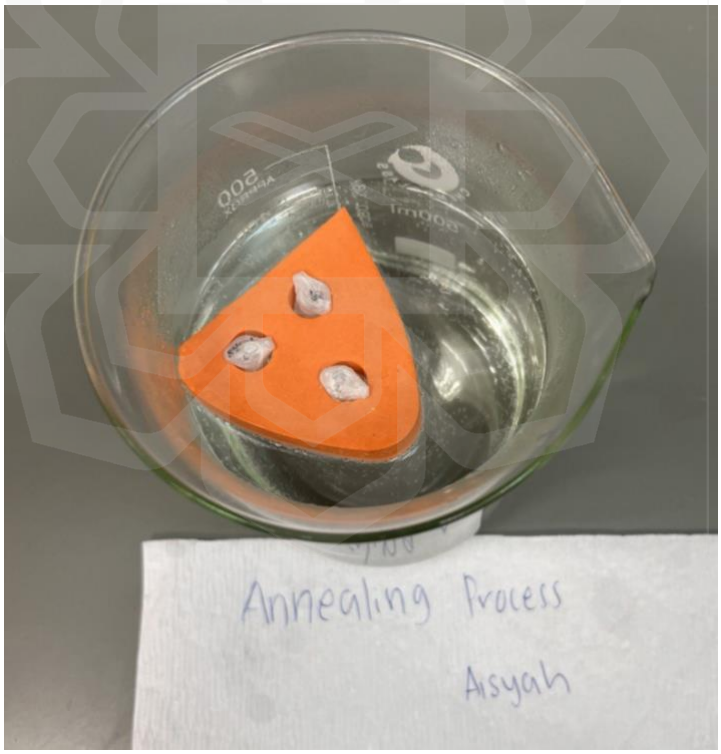


Figure 5.2 Annealing process done through natural cooling instead of using a PCR thermocycler

5.3 TRANSDUCTION OF LENTIVIRUS INTO HEK 293T CELL

5.3.1 Optimization of Lentiviral Transduction in HEK293T Cells

To deliver CRISPR components into cells, lentiviral vectors have emerged as an efficient and versatile tool. In this study, lentiviral produced from lentiCas9-Blast (Plasmid #52962) was utlised. Lentiviruses are engineered to carry the CRISPR-Cas9 system, along with selectable markers or fluorescent proteins, enabling stable integration into the host genome sustain (Hsu et al., 2014). This process, known as transduction, ensures long-term expression of the introduced genetic material, making lentiviral delivery particularly advantageous for experiments requiring sustained gene editing in hard-to-transfect cells or in vivo applications (Jankowsky & Harris, 2017). Lentiviral transduction was selected instead of transient transfection to establish stable Cas9 expression, facilitating consistent and long-term gene editing. In contrast to transient methods that limit Cas9 expression to a few days, the use of lentiviral integration facilitates long-term expression (Hsu et al., 2014). This method enhances editing efficiency and ensures consistent expression throughout the cell population, reducing variability and achieving a complete gene knockout (Sanjana et al., 2014; Shalem et al., 2014).) Lentivirus effectively transduces both dividing and non-dividing cells, making it appropriate for a wider range of cell types, including those that prove difficult to transfect.

Transfection methods for delivering CRISPR/Cas9 components can be categorized into non-viral and viral systems. Non-viral methods, such as electroporation and liposome-mediated delivery, are relatively safe and straightforward but often lack efficiency, particularly for *in vivo* applications (Ramamoorth & Narvekar, 2015). In contrast, viral delivery methods, including adenoviral, adeno-associated viral (AAV), and lentiviral vectors, leverage the natural ability of viruses to efficiently deliver genetic material into host cells, making them a preferred choice for complex or *in vivo* experiments. This research utilised lentivirus as transduction method in HEK293T cells.

While lentiviral vectors are highly effective for CRISPR/Cas9 delivery, their use comes with specific safety concerns. One major issue is the potential risk of insertional mutagenesis, where the integration of the viral genome into the host DNA could disrupt critical genes or regulatory regions, leading to unintended consequences (Cornu et al., 2017; Milone & O'Doherty, 2018). Moreover, since lentiviruses originate from retroviruses, there remains a minimal yet potential risk of reverting to a replication-competent state, which may lead to unintended infections (Uddin et al., 2020). To mitigate these risks, lentiviral production and handling must be performed by certified individual in a controlled laboratory environment. This includes adherence to strict biosafety protocols, such as working in biosafety level 2 (BSL2), using proper personal protective equipment (PPE), and following decontamination procedures to ensure safe handling and disposal of viral materials.

The current study employed a dual-vector system for CRISPR/Cas9 gene editing where HEK 293T cells were utilised to produce lentivirus for sgRNA. This is due to availability of SV40 large T antigen in the genome, which enables them to produce recombinant proteins within plasmid vectors containing the SV40 promoter (Dong & Kantor, 2021). In this case, the Cas9 protein and sgRNA were delivered separately into HEK 293T cells via lentiviral transduction. This approach allows for greater flexibility in controlling the expression of Cas9 and sgRNA, while minimizing potential off-target effects. Initially, the Cas9 lentivirus was transduced into HEK 293T cells, followed by antibiotic selection using blasticidin, as the Cas9 construct included a blasticidin-resistance gene.

The antibiotic selection process was carried out over a six-day period, or until all untransduced control cells were completely eliminated. This duration was chosen based on standard protocols for blasticidin selection, ensuring sufficient time for the antibiotic to effectively eliminate non-resistant cells while allowing successfully transduced cells to survive and proliferate. Throughout the selection phase, HEK293T cell cultures were closely monitored for changes in confluency, which served as an indicator of cell viability and growth. A gradual decline in confluency was observed in the untransduced control cells, ultimately leading to their complete detachment from the culture surface and accumulation as floating cells in the medium. The presence of these floating cells was indicative of cell death due to either unsuccessful transduction or the absence of the blasticidin resistance gene.

In contrast, a small population of adherent cells remained viable and continued to proliferate. These surviving cells were presumed to be those that had been successfully transduced with the Cas9-Blast construct, conferring resistance to blasticidin. The presence of this resistant population confirmed the functional expression of the blasticidin resistance gene, which is co-expressed alongside the Cas9 protein in the lentiviral construct. This selection step was crucial for enriching the cell population with successfully transduced cells, ensuring a purified population for downstream analyses and functional experiments. To promote the growth and attachment of these surviving cells, they were cultured in a 6 well plate, enabling rapid expansion and confluence. Once stable growth was established, the cells were further expanded to larger culture plates (10 cm plate).

5.3.2 Handling and Culturing of Surviving Cells After sgRNA Lentivirus Transduction

Despite following the selection of CRISPR protocols, the experiment encountered a critical limitation, as no cells remained viable following the dual antibiotic selection process. During the hygromycin selection process, both edited cell lines failed to survive, while the untransduced control cells, as expected, did not survive either. This outcome compromised the recovery of successfully edited cell. Specifically, all plates, including those containing the control cells, sgRNA1 transduced cells, and sgRNA2 transduced cells, shows floating cells with no detectable adherence or viability. To further validate this observation, the cells were collected via centrifugation at 500 x g resuspended, and re-plated in 6 well plates. However, after a two day incubation period, no adherent cells were observed and all cells remained non-viable, floating in the culture medium. A potential issue was identified is the concentration of hygromycin used. The hygromycin stock solution was prepared at 50,000 µg/mL, and 100 µL was added to a 5 mL volume of DMEM, resulting in a final concentration of 1,000 µg/mL. This concentration may have been excessively high for HEK 293T cells, leading to cytotoxic effects, even in cells that were potentially transduced with the sgRNA construct (Taha et al., 2022). However, even after reducing the concentration of antibiotics to 500 µg/mL for selection, both the edited and control cells could not survive.

Hence, to optimize the selection process, the CRISPR sgRNA transduced cells were not immediately exposed to antibiotic treatment post-transduction. Instead, the transduced cells were maintained in culture for three weeks, during which they underwent several passages to ensure stable expression of cell. Following this maintenance period, the cells were seeded onto 6 well plate alongside non-edited control cells. Antibiotic selection was performed using 700 µg/mL of the antibiotic, and the selection process was stopped once all control cells were completely eliminated. The culture medium was replenished every two to three days, depending on the observed colour change, which indicated changes in nutrient availability or pH. The surviving cells after the antibiotic selection were subsequently subjected to validation via western blot to confirm editing efficiency.

5.4 WESTERN BLOT ANALYSIS FOR PROTEIN EXPRESSION VALIDATION

Ponceau S was performed prior to gel electrophoresis to ensure the presence of protein and uniform loading of samples across lanes. Normalization of western blot data is an essential step to minimize inaccuracies that arise from uneven sample loading across gel lanes, inconsistencies in sample preparation, and experimental variability. Previous study by Sander et al., (2009) have proposed that the total protein normalization may offer a more reliable approach compared to the use of housekeeping proteins for data normalization in western blotting. Ponceau S is the most commonly used stains for normalizing western blot data. Additionally, errors in western blotting can originate from various sources, including inconsistencies during sample preparation, unequal loading of sample onto the gel or inconsistency protein transfer to the membrane. The efficiency of protein transfer from the gel to the membrane can be compromised by factors such as the presence of air bubbles during the transfer process(Ghosh et al., 2014; Mishra et al., 2017). These errors, which have the potential to result in inaccurate data interpretation, can be mitigated by implementing a normalization control.

The blot was probed for three proteins, Cas9 (~160 kDa) (Thermo Fisher Scientific, Massachusetts, USA), HNF1B (~56 kDa) (Santa Cruz Biotechnology, Dallas, Texas), and B-actin (~42 kDa) (Thermo Fisher Scientific, Massachusetts, USA), which serves as the loading control. A molecular weight marker is included for size reference in figure 4.13, with highlighted bands corresponding to the expected sizes of the probed proteins. The untransduced Control sample shows slightly clear bands of Cas9 protein, indicating its expression. However, these bands are slightly detectable in the HNF1B KO1 and HNF1B KO2 lanes, suggesting minimal Cas9 expression in the knockout samples. Interestingly, a faint band is visible at ~56 kDa in the untransduced control sample, corresponding to HNF1B protein expression. This band is nearly absence in the HNF1B KO1 and KO2 lanes expected lower protein expression. However, B-actin bands at ~42 kDa are not comparable between untransduced control and KO samples, suggesting diminished B-actin function in KO. This could happen due to unequal loading of protein samples or incomplete transfer during blotting. Further optimization should include normalization of protein concentrations, increasing transfer time and voltage settings (Liu et al., 2023). Moreover, HNF1B functions as a transcription factor that regulates various genes, and its mutation may impact B-actin expression. For example, it could influence the ACTB gene, which encodes B-actin, leading to reduce the protein levels. Additionally, since this study involves a heterozygous knockout, the partial loss of HNF1B may alter the expression of housekeeping genes, resulting in decreased B-actin levels in certain samples.

The HNF1B protein band in the untransduced control cells appeared unclear and smeared, potentially due to variations in protein transfer efficiency from the gel to the membrane. This inconsistency may have also impacted the B-actin signal, leading to variability in normalization (Pillai-Kastoori et l., 2020). Uneven transfer efficiency can lead to variations in band intensity, emphasizing the need to optimize transfer time and voltage. Additionally, overexposure of Enhanced Chemiluminescence (ECL) reagent can affect the band signals, making it difficult to interpret results accurately. In this experiment, ECL exposure time was set to one minute before visualization. Therefore, optimization should include varying ECL exposure time and reagent distribution.

The Cas9 antibody was included to confirm the expression of the Cas9 protein, a crucial component of the CRISPR/Cas9 system responsible for introducing targeted double-strand breaks in the DNA. Detection of the Cas9 protein serves as evidence that the LentiCas9-plasmid was successfully delivered into the cell ensuring that the knockout process was conducted correctly (Santoz et al., 2016). Additionally, the B-actin antibody was used as a loading control. B-actin is an expressed housekeeping protein, providing a baseline for assessing equal protein loading across all lanes of the gel. Gilda et al. (2015) and Moritz (2017) described the use of housekeeping proteins, such as B-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for normalization in Western blot analysis. These proteins are constitutively expressed at relatively high levels and are typically probed alongside the target protein using separate antibodies (Gilda et al., 2015; Moritz, 2017). Alternatively, housekeeping proteins can be detected after the blot is stripped following initial probing for the target protein. Its consistent signal ensures that any variations observed in protein expression, such as the target protein (HNF1B), are not due to differences in sample loading or transfer efficiency. Together, the use of these antibodies strengthens the efficacy of the experiment by confirming both the efficiency of the CRISPR system and the integration of the protein samples analysed.

The unclear and smeared band for HNF1B could be attributed to several factors. First, it is possible that partial degradation of the HNF1B protein occurred during sample preparation, leading to fragmented protein products that appear as a smear rather than a distinct band (Mahmood & Yang, 2012). Additionally, the HNF1B antibody optimization may not have reached optimal levels such as suboptimal antibody concentration or insufficient specificity which may have resulted in weak or non-specific binding, contributing to the smearing effect (Pillai-Kastoori et al., 2020).

HNF1B protein may undergoes several post-translational modifications (PTMs), including phosphorylation, acetylation, and ubiquitination, which are essential for regulating its function (Clissold et al., 2015). However, these modifications can alter the epitope recognized by specific antibodies, potentially hindering protein detection. To improve the clarity of the HNF1B protein band and obtain more conclusive evidence regarding the knockout's success, additional troubleshooting steps should be considered. These include using a fresh protease inhibitor during lysis, optimizing antibody concentrations, or selecting an alternative HNF1B antibody that recognizes the modified or unmodified form of the protein better (Igarashi et al., 2005). Another potential explanation for the variation in HNF1B protein detection is the presence of PTMs in wild-type cells, such as phosphorylation or glycosylation, which can lead to molecular weight heterogeneity and smeared appearance on the western blot (Zhong et al., 2023).

Additionally, based on information from datasheet of this antibody, sc-130407 is raised against an epitope within amino acids 1-282 of human HNF1B, meaning it targets the N-terminal region of the protein. HNF1B protein contains distinct functional domains, including the DNA-binding domain (DBD) at the N-terminus and a transactivation domain at the C-terminus (Igarashi et al., 2005). If an antibody is designed to target the N-terminal region, structural changes or proteolytic cleavage of the protein could prevent detection. Conversely, antibodies recognizing the C-terminal region might still detect the protein if the N-terminal region is altered (Igarashi et al., 2005). Other possible reason is proteins are denatured in western blot analysis, meaning they unfold into a linear structure (Ferrè & Igarashi, 2018). Some antibodies are only capable of recognizing the native (folded) conformation of HNF1B protein, making them more suitable for applications such as immunofluorescence (IF) or immunoprecipitation (IP) rather than western blot (Ferrè & Igarashi, 2018). While some commercial antibodies are specifically designed to detect both denatured and native forms of the protein, not all antibodies share this capability.

Therefore, selecting the appropriate antibody is essential for obtaining reliable results. In this case, the HNF1B Antibody (94.8) (sc-130407) from Santa Cruz Biotechnology is a mouse monoclonal antibody designed to detect human HNF1B protein. According to the manufacturer's datasheet, it is validated for Western blot where proteins are typically denatured. This suggests the antibody primarily recognizes linear epitopes in the denatured form. However, there is no explicit information from the datasheet on whether it can detect the native (folded) form of HNF1B protein. Therefore, it may be necessary to test multiple HNF1B antibodies, as some manufacturers design antibodies that can recognize both linear and folded forms of the protein for western blot applications.

CHAPTER 6

CONCLUSION

6.1 CONCLUSION

This study aim to generate *HNF1B* gene knockout cell in HEK293T via CRISPR/Cas9 and validate the successful knockout of the *HNF1B* gene via CRISPR experiment using western blot analysis. The *HNF1B* gene encodes a transcription factor essential for embryonic development, particularly in the formation and function of the kidneys, pancreas, liver, and genitourinary system. Genetic mutations in *HNF1B* have been linked to multispectrum disorders, highlighting its crucial regulatory role across multiple organ systems. Cystic kidney disease is among the most common renal symptoms linked to *HNF1B* mutations. The *HNF1B* gene is also an essential regulator of renal development, affecting nephron production and tubular differentiation. Mutations in this gene impair normal renal development while increasing susceptibility to diverse cystic kidney abnormalities. Early diagnosis and complete treatment are crucial for enhancing patient outcomes and preventing disease progression. CRISPR/Cas9 gene editing technology serves as an invaluable tool for the study of gene function and the elucidation of the molecular mechanisms that underlie diseases. The CRISPR/Cas9 system allows precise alterations to the genome through the introduction of targeted mutations, including knockouts, to assess the functional role of *HNF1B*.

This study successfully designed three single-guide RNAs (sgRNAs) targeting the *HNF1B* gene using the CRISPick web tool, ensuring compatibility for cloning into the pKLV-U6gRNA(BbsI)-PGKhygro2ABFP plasmid. The insertion of *HNF1B*-specific gRNA sequences into the plasmid was confirmed through Sanger sequencing. In this study, dual system approach was employed to generate edited cell lines. Hence, this study successfully generated HEK 293T/Cas9 expression cell line and *HNF1B* KO cell line. Western blot analysis confirmed the successful knockout of *HNF1B*, as evidenced by the reduced and nearly absence of the HNF1B band in knockout cells, whereas wild-type controls displayed a detectable band. Furthermore, the lower intensity of the HNF1B protein band in both KO 1 and KO 2 cells indicates a lower protein expression level compared to untransduced cells.

6.2 LIMITATIONS

Despite these achievements, several limitations were identified in this study. First, the cloning process could have been improved by incorporating gel extraction to purify DNA fragments, potentially enhancing cloning efficiency and sgRNA validation. The absence of this step may have introduced impurities, affecting downstream applications. Secondly, it was apparent that the CRISPR-edited cells suffered from low survival rates following knockout experiment. This outcome underscores the critical role of *HNF1B* in cellular functions, particularly in kidney-related mechanism, as the gene is essential for maintaining cellular homeostasis. The loss of HNF1B protein in HEK 293T cells likely disrupted key pathways, resulting in reduced viability. This suggests that *HNF1B* is an essential gene that may disrupt cell development and survival. A study conducted by Niborski et al. (2021) demonstrated that reduced HNF1B levels lead to developmental disease phenotypes through the deregulation of a subset of HNF1B target genes. This finding supports the notion that *HNF1B* functions as a critical regulatory factor, and its loss may result in significant functional and physiological disruptions, either at the cellular level or within broader disease contexts.

Other limitations includes unavailability of HEK 293T cells with a confirmed *HNF1B* knockout as a positive control. This hindered the ability to conclusively validate the knockout's efficiency, as observed results could not be fully distinguished from potential technical inconsistencies or off-target effects. Future studies should address this by generating or sourcing well-characterized *HNF1B*-knockout cell lines to enhance experimental accuracy. Lastly, non-specific antibody reactivity may cause a significant limitation, particularly in validation phase of this study. Such reactivity can result in non-specific binding on western blots, potentially producing bands that may be misinterpreted as HNF1B protein, even in cases where the gene has been successfully knocked out. This can affect the accuracy of the validation process and lead to erroneous conclusions regarding the knockout efficiency. External limitation includes lack of infrastructure which contributed to the delay of the downstream workplan, experimental repeats and analysis.

6.3 RECOMMENDATIONS

Future research should involve utilising this plasmid in mature human-derived kidney organoids and investigate its phenotypes. This can give a better understanding on how *HNF1B* defect may disrupt nephrogenesis and nephron functions. Ideally, heterozygous *HNF1B* mutation can be repaired using CRISPR, which could correct the mutation and serve as a reliable tool for treating *HNF1B*-derived pathologies as a first step towards personalised medicine. This study lays the groundwork for exploring the role of the *HNF1B* gene as a proof-of-concept of potential technology in treating genetic kidney diseases. By addressing the above limitations, future research can build on these proof-of-concept to advance the understanding of kidney development, pathophysiology and at the same time improve the precision and reliability of gene-editing experiments.

REFERENCES

Abaandou, L., Quan, D., & Shiloach, J. (2021). Affecting HEK293 Cell Growth and Production Performance by Modifying the Expression of Specific Genes. Cells, 10(7), 1667. https://doi.org/10.3390/cells10071667

Adalat, S., Woolf, A. S., Johnstone, K. A., Wirsing, A., Harries, L. W., Long, D. A., Hennekam, R. C., Ledermann, S. E., Rees, L., van't Hoff, W., Marks, S. D., Trompeter, R. S., Tullus, K., Winyard, P. J., Cansick, J., Mushtaq, I., Dhillon, H. K., Bingham, C., Edghill, E. L., Shroff, R., … Bockenhauer, D. (2009). HNF1B mutations associate with hypomagnesemia and renal magnesium wasting. Journal of the American Society of Nephrology : JASN, 20(5), 1123–1131. https://doi.org/10.1681/ASN.2008060633

Barbacci, E., Reber, M., Ott, M. O., Breillat, C., Huetz, F., & Cereghini, S. (1999). Variant a hepatocyte nuclear factor 1 is required for visceral endoderm specification. Development (Cambridge, England), 126(21), 4795–4805. https://doi.org/10.1242/dev.126.21.4795.

Bártů, M., Hojný, J., Hájková, N., Michálková, R., Krkavcová, E., Hadravský, L., Kleissnerová, L., Bui, Q. H., Stružinská, I., Němejcová, K., Čapoun, O., Šlemendová, M., & Dundr, P. (2020). Analysis of expression, epigenetic, and genetic changes of HNF1B in 130 kidney tumors. Scientific Reports, 10(1). https://doi.org/10.1038/s41598-020-74059-z

Baylis, F., & McLeod, M. (2017). First-in-human Phase 1 CRISPR Gene Editing Cancer Trials: Are We Ready?. Current gene therapy, 17(4), 309–319. https://doi.org/10.2174/1566523217666171121165935

Bellanné-Chantelot, C., Chauveau, D., Gautier, J. F., Dubois-Laforgue, D., Clauin, S., Beaufils, S., Wilhelm, J. M., Boitard, C., Noël, L. H., Velho, G., & Timsit, J. (2004). Clinical spectrum associated with hepatocyte nuclear factor-1beta mutations. Annals of internal medicine, 140(7), 510–517. https://doi.org/10.7326/0003-4819-140-7-200404060-00009

Bellanné-Chantelot, C., Clauin, S., Chauveau, D., Collin, P., Daumont, M., Douillard, C., Dubois-Laforgue, D., Dusselier, L., Gautier, J. F., Jadoul, M., Laloi-Michelin, M., Jacquesson, L., Larger, E., Louis, J., Nicolino, M., Subra, J. F., Wilhem, J. M., Young, J., Velho, G., & Timsit, J. (2005). Large genomic rearrangements in the hepatocyte nuclear factor-1beta (TCF2) gene are the most frequent cause of maturity-onset diabetes of the young type 5. Diabetes, 54(11), 3126–3132. https://doi.org/10.2337/diabetes.54.11.3126

Ben-David U. (2013). Flowing through the CRISPR-CAScade: Will genome editing boost cell therapies?. Molecular and cellular therapies, 1, 3. https://doi.org/10.1186/2052-8426-1-3

Bengtsson, N. E., Hall, J. K., Odom, G. L., Phelps, M. P., Andrus, C. R., Hawkins, R. D., Hauschka, S. D., Chamberlain, J. R., & Chamberlain, J. S. (2017). Corrigendum: Musclespecific CRISPR/Cas9 dystrophin gene editing ameliorates pathophysiology in a mouse model for Duchenne muscular dystrophy. Nature communications, 8, 16007. https://doi.org/10.1038/ncomms16007

Bingham, C., & Hattersley, A. T. (2004). Renal cysts and diabetes syndrome resulting from mutations in hepatocyte nuclear factor-1beta. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association, 19(11), 2703–2708. https://doi.org/10.1093/ndt/gfh348

Bingham, C., Bulman, M. P., Ellard, S., Allen, L. I., Lipkin, G. W., Hoff, W. G., Woolf, A. S., Rizzoni, G., Novelli, G., Nicholls, A. J., & Hattersley, A. T. (2001). Mutations in the hepatocyte nuclear factor-1beta gene are associated with familial hypoplastic glomerulocystic kidney disease. American journal of human genetics, 68(1), 219–224. https://doi.org/10.1086/316945

Bockenhauer, D., & Jaureguiberry, G. (2016). HNF1B-associated clinical phenotypes: the kidney and beyond. Pediatric nephrology (Berlin, Germany), 31(5), 707–714. https://doi.org/10.1007/s00467-015-3142-2

Bonnet, M., Lagier, J. C., Raoult, D., & Khelaifia, S. (2019). Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical

microbiology. New microbes and new infections, 34, 100622. https://doi.org/10.1016/j.nmni.2019.100622

Breshears, M. A., & Confer, A. W. (2017). The Urinary System. Pathologic Basis of Veterinary Disease, 617–681.e1. https://doi.org/10.1016/B978-0-323-35775-3.00011-4

Brown, D. L., & Mattix, M. E. (2016). Kidney development. In Atlas of Histology of the Juvenile Rat. Elsevier. (Original work published 2014 in Cancer Genomics).

Burnstock, G., & Loesch, A. (2017). Sympathetic innervation of the kidney in health and disease: Emphasis on the role of purinergic cotransmission. Autonomic neuroscience: basic & clinical, 204, 4–16. https://doi.org/10.1016/j.autneu.2016.05.007

Capone, V. P., Morello, W., Taroni, F., & Montini, G. (2017). Genetics of Congenital Anomalies of the Kidney and Urinary Tract: The Current State of Play. International journal of molecular sciences, 18(4), 796. https://doi.org/10.3390/ijms18040796

Cereghini S. (1996). Liver-enriched transcription factors and hepatocyte differentiation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 10(2), 267–282.

Chandra, S., Srinivasan, S., & Batra, J. (2021). Hepatocyte nuclear factor 1 beta: A perspective in cancer. Cancer medicine, 10(5), 1791–1804. https://doi.org/10.1002/cam4.3676

Charlesworth, C. T., Deshpande, P. S., Dever, D. P., Camarena, J., Lemgart, V. T., Cromer, M. K., Vakulskas, C. A., Collingwood, M. A., Zhang, L., Bode, N. M., Behlke, M. A., Dejene, B., Cieniewicz, B., Romano, R., Lesch, B. J., Gomez-Ospina, N., Mantri, S., Pavel-Dinu, M., Weinberg, K. I., & Porteus, M. H. (2019). Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nature Medicine, 25(2), 249–254. https://doi.org/10.1038/s41591-018-0326-x

Chen, H., Choi, J., & Bailey, S. (2014). Cut site selection by the two nuclease domains of the Cas9 RNA-guided endonuclease. The Journal of biological chemistry, 289(19), 13284–13294. https://doi.org/10.1074/jbc.M113.539726

Chew, W. L., Tabebordbar, M., Cheng, J. K., Mali, P., Wu, E. Y., Ng, A. H., Zhu, K., Wagers, A. J., & Church, G. M. (2016). A multifunctional AAV-CRISPR-Cas9 and its host response. Nature methods, 13(10), 868–874. https://doi.org/10.1038/nmeth.3993

Clissold, R. L., Hamilton, A. J., Hattersley, A. T., Ellard, S., & Bingham, C. (2015). HNF1B-associated renal and extra-renal disease-an expanding clinical spectrum. Nature reviews. Nephrology, 11(2), 102–112. https://doi.org/10.1038/nrneph.2014.232

Cockwell, P., & Fisher, L. A. (2020). The global burden of chronic kidney disease. Lancet (London, England), 395(10225), 662–664. https://doi.org/10.1016/S0140-6736(19)32977-0

Coffinier, C., Barra, J., Babinet, C., & Yaniv, M. (1999). Expression of the vHNF1/HNF1beta homeoprotein gene during mouse organogenesis. Mechanisms of development, 89(1-2), 211– 213. https://doi.org/10.1016/s0925-4773(99)00221-x.

Coralie Bingham, Andrew T. Hattersley, Renal cysts and diabetes syndrome resulting from mutations in hepatocyte nuclear factor-1β, Nephrology Dialysis Transplantation, Volume 19, Issue 11, November 2004, Pages 2703–2708, https://doi.org/10.1093/ndt/gfh348

Cornu, T. I., Mussolino, C., & Cathomen, T. (2017). Refining strategies to translate genome editing to the clinic. Nature Medicine, 23(4), 415–423. https://doi.org/10.1038/nm.4313

Cruz, N. M., & Freedman, B. S. (2018). CRISPR Gene Editing in the Kidney. American journal of kidney diseases : the official journal of the National Kidney Foundation, 71(6), 874–883. https://doi.org/10.1053/j.ajkd.2018.02.347

Cyranoski D. (2016). CRISPR gene-editing tested in a person for the first time. Nature, 539(7630), 479. https://doi.org/10.1038/nature.2016.20988

Daim, N. (2022, March 12). Alarming rise in kidney problems in Malaysia. New Straits Times. Retrieved from https://www.nst.com.my/news/nation/2022/03/779327/alarming-rise-kidney-problems-malaysia

Davidson, A. J., Lewis, P., Przepiorski, A., & Sander, V. (2019). Turning mesoderm into kidney. Seminars in cell & developmental biology, 91, 86–93. https://doi.org/10.1016/j.semcdb.2018.08.016

De Stefani, D., Patron, M., & Rizzuto, R. (2015). Structure and function of the mitochondrial calcium uniporter complex. Biochimica et biophysica acta, 1853(9), 2006–2011. https://doi.org/10.1016/j.bbamcr.2015.04.008

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K., Chao, Y., Pirzada, Z. A., Eckert, M. R., Vogel, J., & Charpentier, E. (2011). CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature, 471(7340), 602–607. https://doi.org/10.1038/nature09886

Desgrange, A., Heliot, C., Skovorodkin, I., Akram, S. U., Heikkilä, J., Ronkainen, V. P., Miinalainen, I., Vainio, S. J., & Cereghini, S. (2017). HNF1B controls epithelial organization and cell polarity during ureteric bud branching and collecting duct morphogenesis. Development (Cambridge, England), 144(24), 4704–4719. https://doi.org/10.1242/dev.154336

Devuyst, O., Olinger, E., Weber, S., Eckardt, K. U., Kmoch, S., Rampoldi, L., & Bleyer, A. J. (2019). Autosomal dominant tubulointerstitial kidney disease. Nature reviews. Disease primers, 5(1), 60. https://doi.org/10.1038/s41572-019-0109-9

Doench, J. G., Hartenian, E., Graham, D. B., Tothova, Z., Hegde, M., Smith, I., Sullender, M., Ebert, B. L., Xavier, R. J., & Root, D. E. (2014). Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. Nature biotechnology, 32(12), 1262–1267. https://doi.org/10.1038/nbt.3026

Dong, W., & Kantor, B. (2021). Lentiviral Vectors for Delivery of Gene-Editing Systems Based on CRISPR/Cas: Current State and Perspectives. Viruses, 13(7), 1288. https://doi.org/10.3390/v13071288

Doudna, J. A., & Charpentier, E. (2014). Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science (New York, N.Y.), 346(6213), 1258096. https://doi.org/10.1126/science.1258096

Dressler G. R. (2011). Patterning and early cell lineage decisions in the developing kidney: the role of Pax genes. Pediatric nephrology (Berlin, Germany), 26(9), 1387–1394. https://doi.org/10.1007/s00467-010-1749-x

DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., & Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Molecular and cellular biology, 7(1), 379–387. https://doi.org/10.1128/mcb.7.1.379-387.1987

Eid, A., & Mahfouz, M. (2016). Genome editing: The road of CRISPR/Cas9 from bench to clinic. Experimental & Molecular Medicine, 48(1), e265. https://doi.org/10.1038/emm.2016.111

Elmore, S. A., Kavari, S. L., Hoenerhoff, M. J., Mahler, B., Scott, B. E., Yabe, K., & Seely, J. C. (2019). Histology Atlas of the Developing Mouse Urinary System With Emphasis on Prenatal Days E10.5-E18.5. Toxicologic pathology, 47(7), 865–886. https://doi.org/10.1177/0192623319873871

Evans M. F. (2009). The polymerase chain reaction and pathology practice. Diagnostic histopathology (Oxford, England), 15(7), 344–356. https://doi.org/10.1016/j.mpdhp.2009.04.001

Faa, G., Gerosa, C., Fanni, D., Monga, G., Zaffanello, M., Van Eyken, P., & Fanos, V. (2012). Morphogenesis and molecular mechanisms involved in human kidney development. Journal of cellular physiology, 227(3), 1257–1268. https://doi.org/10.1002/jcp.22985

Fakruddin, M., Mohammad Mazumdar, R., Bin Mannan, K. S., Chowdhury, A., & Hossain, M. N. (2012). Critical Factors Affecting the Success of Cloning,

Expression, and Mass Production of Enzymes by Recombinant E. coli. *ISRN biotechnology*, *2013*, 590587. https://doi.org/10.5402/2013/590587

Ferrè, S., & Igarashi, P. (2018). New insights into the role of HNF-1β in kidney (patho)physiology. Pediatric Nephrology. https://doi.org/10.1007/s00467-018-3990-7

Fox, C. S., Matsushita, K., Woodward, M., Bilo, H. J., Chalmers, J., Heerspink, H. J., Lee, B. J., Perkins, R. M., Rossing, P., Sairenchi, T., Tonelli, M., Vassalotti, J. A., Yamagishi, K., Coresh, J., de Jong, P. E., Wen, C. P., Nelson, R. G., & Chronic Kidney Disease Prognosis Consortium (2012). Associations of kidney disease measures with mortality and end-stage renal disease in individuals with and without diabetes: a meta-analysis. Lancet (London, England), 380(9854), 1662–1673. https://doi.org/10.1016/S0140-6736(12)61350-6

Froger, A., & Hall, J. E. (2007). Transformation of plasmid DNA into E. coli using the heat shock method. Journal of visualized experiments : JoVE, (6), 253. https://doi.org/10.3791/253

Gaj, T., Gersbach, C. A., & Barbas, C. F., 3rd (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends in biotechnology, 31(7), 397–405. https://doi.org/10.1016/j.tibtech.2013.04.004

Gantsova, E., Serova, O., Vishnyakova, P., Deyev, I., Elchaninov, A., & Fatkhudinov, T. (2024). Mechanisms and physiological relevance of acid-base exchange in functional units of the kidney. PeerJ, 12, e17316. https://doi.org/10.7717/peerj.17316

Gasiunas, G., Barrangou, R., Horvath, P., & Siksnys, V. (2012). Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proceedings of the National Academy of Sciences of the United States of America, 109(39), E2579–E2586. https://doi.org/10.1073/pnas.1208507109

Gersbach, C. A., Gaj, T., & Barbas, C. F. III. (2014). The emerging therapeutic potential of gene editing. Accounts of Chemical Research, 47(8), 2309–2318. https://doi.org/10.1021/ar500039w

Ghezraoui, H., Piganeau, M., Renouf, B., Renaud, J. B., Sallmyr, A., Ruis, B., Oh, S., Tomkinson, A. E., Hendrickson, E. A., Giovannangeli, C., Jasin, M., & Brunet, E. (2014). Chromosomal translocations in human cells are generated by canonical nonhomologous endjoining. Molecular cell, 55(6), 829–842. https://doi.org/10.1016/j.molcel.2014.08.002

Ghosh, R., Gilda, J. E., & Gomes, A. V. (2014). The necessity of and strategies for improving confidence in the accuracy of western blots. Expert review of proteomics, 11(5), 549–560. https://doi.org/10.1586/14789450.2014.939635

Gilda, J. E., Ghosh, R., Cheah, J. X., West, T. M., Bodine, S. C., & Gomes, A. V. (2015). Western Blotting Inaccuracies with Unverified Antibodies: Need for a Western Blotting Minimal Reporting Standard (WBMRS). PloS one, 10(8), e0135392. https://doi.org/10.1371/journal.pone.0135392

Gimpel, C., Avni, F. E., Bergmann, C., Cetiner, M., Habbig, S., Haffner, D., König, J., Konrad, M., Liebau, M. C., Pape, L., Rellensmann, G., Titieni, A., von Kaisenberg, C., Weber, S., Winyard, P. J. D., & Schaefer, F. (2018). Perinatal Diagnosis, Management, and Follow-up of Cystic Renal Diseases: A Clinical Practice Recommendation With Systematic Literature Reviews. JAMA pediatrics, 172(1), 74–86. https://doi.org/10.1001/jamapediatrics.2017.3938

Ginn, S. L., Amaya, A. K., Alexander, I. E., Edelstein, M., & Abedi, M. R. (2018). Gene therapy clinical trials worldwide to 2017: An update. The journal of gene medicine, 20(5), e3015. https://doi.org/10.1002/jgm.3015

Goea, L., Buisson, I., Bello, V., Eschstruth, A., Paces-Fessy, M., Le Bouffant, R., Chesneau, A., Cereghini, S., Riou, J. F., & Umbhauer, M. (2022). Hnf1b renal expression directed by a distal enhancer responsive to Pax8. Scientific Reports, 12(1). https://doi.org/10.1038/s41598https://doi.org/10.1038/s41598-022-21171-x022-21171-x

Gómez-García, F., Martínez-Pulleiro, R., Carrera, N., Allegue, C., & Garcia-Gonzalez, M. A. (2022). Genetic Kidney Diseases (GKDs) Modeling Using Genome Editing Technologies. Cells, 11(9), 1571. https://doi.org/10.3390/cells11091571

González Castro, N., Bjelic, J., Malhotra, G., Huang, C., & Alsaffar, S. H. (2021). Comparison of the Feasibility, Efficiency, and Safety of Genome Editing Technologies. International journal of molecular sciences, 22(19), 10355. https://doi.org/10.3390/ijms221910355

Graham, C., & Hart, S. (2021). CRISPR/Cas9 gene editing therapies for cystic fibrosis. In Expert Opinion on Biological Therapy (Vol. 21, Issue 6, pp. 767–780). Taylor and Francis Ltd. https://doi.org/10.1080/14712598.2021.1869208

Granatiero, V., De Stefani, D., & Rizzuto, R. (2017). Mitochondrial Calcium Handling in Physiology and Disease. Advances in experimental medicine and biology, 982, 25–47. https://doi.org/10.1007/978-3-319-55330-6_2

Gupta, D., Bhattacharjee, O., Mandal, D., Sen, M. K., Dey, D., Dasgupta, A., Kazi, T. A., Gupta, R., Sinharoy, S., Acharya, K., Chattopadhyay, D., Ravichandiran, V., Roy, S., & Ghosh, D. (2019). CRISPR-Cas9 system: A new-fangled dawn in gene editing. Life sciences, 232, 116636. https://doi.org/10.1016/j.lfs.2019.116636

Haldorsen, I. S., Vesterhus, M., Raeder, H., Jensen, D. K., Søvik, O., Molven, A., & Njølstad, P. R. (2008). Lack of pancreatic body and tail in HNF1B mutation carriers. Diabetic medicine : a journal of the British Diabetic Association, 25(7), 782–787. https://doi.org/10.1111/j.1464-5491.2008.02460.x

Harries, L. W., Bingham, C., Bellanne-Chantelot, C., Hattersley, A. T., & Ellard, S. (2005). The position of premature termination codons in the hepatocyte nuclear factor -1 beta gene determines susceptibility to nonsense-mediated decay. Human genetics, 118(2), 214–224. https://doi.org/10.1007/s00439-005-0023-y

Heidenreich, M., & Zhang, F. (2016). Applications of CRISPR-Cas systems in neuroscience. Nature reviews. Neuroscience, 17(1), 36–44. https://doi.org/10.1038/nrn.2015.2

Higashijima, Y., Hirano, S., Nangaku, M., & Nureki, O. (2017). Applications of the CRISPRCas9 system in kidney research. In Kidney International (Vol. 92, Issue 2). https://doi.org/10.1016/j.kint.2017.01.037

High, K. A., & Roncarolo, M. G. (2019). Gene Therapy. The New England Journal of Medicine, 381(5), 455–464. https://doi.org/10.1056/NEJMra1706910

Hille, F., & Charpentier, E. (2016). CRISPR-cas: Biology, mechanisms and relevance. Philosophical Transactions of the Royal Society B: Biological Sciences, 371(1707). https://doi.org/10.1098/RSTB.2015.0496

Hossain T. J. (2024). Methods for screening and evaluation of antimicrobial activity: A review of protocols, advantages, and limitations. European Journal of Microbiology & Immunology, 14(2), 97–115. https://doi.org/10.1556/1886.2024.00035

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. Cell, 157(6), 1262–1278. https://doi.org/10.1016/j.cell.2014.05.010

Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nature Biotechnology, 31(9), 827–832. https://doi.org/10.1038/nbt.2647

Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., Peterson, R. T., Yeh, J. R., & Joung, J. K. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. Nature biotechnology, 31(3), 227–229. https://doi.org/10.1038/nbt.2501

Igarashi, P., & Somlo, S. (2007). Polycystic kidney disease. Journal of the American Society of Nephrology : JASN, 18(5), 1371–1373. https://doi.org/10.1681/ASN.2007030299

Igarashi, P., Shao, X., McNally, B. T., & Hiesberger, T. (2005). Roles of HNF-1beta in kidney development and congenital cystic diseases. Kidney International, 68(5), 1944–1947. https://doi.org/10.1111/j.1523-1755.2005.00625.x

Iliuta, I. A., Kalatharan, V., Wang, K., Cornec-Le Gall, E., Conklin, J., Pourafkari, M., Ting, R., Chen, C., Borgo, A. C., He, N., Song, X., Heyer, C. M., Senum, S. R., Hwang, Y. H., Paterson, A. D., Harris, P. C., Khalili, K., & Pei, Y. (2017). Polycystic Kidney Disease without an Apparent Family History. Journal of the

American Society of Nephrology : JASN, 28(9), 2768–2776. https://doi.org/10.1681/ASN.2016090938

Ishibashi, A., Saga, K., Hisatomi, Y. et al. A simple method using CRISPR-Cas9 to knock-out genes in murine cancerous cell lines. Sci Rep 10, 22345 (2020). https://doi.org/10.1038/s41598-020-79303-0

Israni, A. K., Zaun, D. A., Gauntt, K., Schaffhausen, C. R., McKinney, W. T., Miller, J. M., & Snyder, J. J. (2023). OPTN/SRTR 2021 Annual Data Report: Deceased Organ Donation. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 23(2 Suppl 1), S443–S474. https://doi.org/10.1016/j.ajt.2023.02.010

Jacob, M., Yusuf, F., & Jrgen, H. (2012). Development, Differentiation and Derivatives of the Wolffian and Müllerian Ducts. InTech. doi: 10.5772/34351

Jankowsky, E., & Harris, M. E. (2017). Mapping specificity landscapes of RNA-protein interactions by high throughput sequencing. Methods (San Diego, Calif.), 118-119, 111–118. https://doi.org/10.1016/j.ymeth.2017.03.002

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.), 337(6096), 816–821. https://doi.org/10.1126/science.1225829

Johnson, C. (2022, October 28). Differences between HDR vs Nhej. InVivo Biosystems. Retrieved February 6, 2023, from https://invivobiosystems.com/news-announcements/differences-between-hdr-vs-nhej/

Kalidasan, V., & Theva Das, K. (2021). Is Malaysia Ready for Human Gene Editing: A Regulatory, Biosafety and Biosecurity Perspective. In Frontiers in Bioengineering and Biotechnology (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.649203

Kassab, G.H., Perez-Rossello, J.M., Servaes, S., & Darge, K. (2021). Urinary Tract. In H.J. Paltiel & E.Y. Lee (Eds.), Pediatric Ultrasound (pp. 369-392). Springer, Cham. https://doi.org/10.1007/978-3-030-56802-3_17

Kavsan, V. M., Iershov, A. V., & Balynska, O. V. (2011). Immortalized cells and one oncogene in malignant transformation: old insights on new explanation. BMC Cell Biology, 12, 23. https://doi.org/10.1186/1471-2121-12-23

Kettunen, J. L. T., et al. (2017). Biliary anomalies in patients with HNF1B diabetes. Journal of Clinical Endocrinology and Metabolism, 102, 2075–2082. https://doi.org/10.1210/jc.2017https://doi.org/10.1210/jc.2017-0006100061

Khadempar, S., Familghadakchi, S., Motlagh, R. A., Farahani, N., Dashtiahangar, M., Rezaei, H., & Gheibi Hayat, S. M. (2019). CRISPR-Cas9 in genome editing: Its function and medical applications. Journal of cellular physiology, 234(5), 5751–5761. https://doi.org/10.1002/jcp.27476

Kim J. S. (2016). Genome editing comes of age. Nature protocols, 11(9), 1573–1578. https://doi.org/10.1038/nprot.2016.104

Kim, S., Kim, D., Cho, S. W., Kim, J., & Kim, J. S. (2014). Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome research, 24(6), 1012–1019. https://doi.org/10.1101/gr.171322.113

Knott, G. J., & Doudna, J. A. (2018). CRISPR-Cas guides the future of genetic engineering. Science (New York, N.Y.), 361(6405), 866–869. https://doi.org/10.1126/science.aat5011

Koike-Yusa, H., Li, Y., Tan, E. P., Velasco-Herrera, M. del C., & Yusa, K. (2014). Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nature Biotechnology, 32(3), 267–273. https://doi.org/10.1038/nbt.2800

Kornfeld, J. W., Baitzel, C., Könner, A. C., Nicholls, H. T., Vogt, M. C., Herrmanns, K., Scheja, L., Haumaitre, C., Wolf, A. M., Knippschild, U., Seibler, J., Cereghini, S., Heeren, J., Stoffel, M., & Brüning, J. C. (2013). Obesity-induced

overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b. Nature, 494(7435), 111–115. https://doi.org/10.1038/nature11793

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. Nature biotechnology, 36(8), 765–771. https://doi.org/10.1038/nbt.4192

Kostylev, M., Otwell, A. E., Richardson, R. E., & Suzuki, Y. (2015). Cloning Should Be Simple: Escherichia coli DH5α-Mediated Assembly of Multiple DNA Fragments with Short End Homologies. PloS one, 10(9), e0137466. https://doi.org/10.1371/journal.pone.0137466

Kotterman, M. A., Chalberg, T. W., & Schaffer, D. V. (2015). Viral Vectors for Gene Therapy: Translational and Clinical Outlook. Annual review of biomedical engineering, 17, 63–89. https://doi.org/10.1146/annurev-bioeng-071813-104938

Krause, M., Rak-Raszewska, A., Pietilä, I., Quaggin, S. E., & Vainio, S. (2015). Signaling during Kidney Development. Cells, 4(2), 112–132. https://doi.org/10.3390/cells4020112https://doi.org/10.3390/cells4020112

Kumar P, Malik YS, Ganesh B, et al., 2020. CRISPR-Cas system: an approach with potentials for COVID-19 diagnosis and therapeutics. Frontiers in Cellular and Infection Microbiology https://doi.org/10.3389/fcimb.2020.576875

Lanini, I., Samoni, S., Husain-Syed, F., Fabbri, S., Canzani, F., Messeri, A., Mediati, R. D., Ricci, Z., Romagnoli, S., & Villa, G. (2022). Palliative Care for Patients with Kidney Disease. Journal of clinical medicine, 11(13), 3923. https://doi.org/10.3390/jcm11133923

Lanktree, M. B., Haghighi, A., Guiard, E., Iliuta, I. A., Song, X., Harris, P. C., Paterson, A. D., & Pei, Y. (2018). Prevalence Estimates of Polycystic Kidney and Liver Disease by Population Sequencing. Journal of the American Society of Nephrology : JASN, 29(10), 2593–2600. https://doi.org/10.1681/ASN.2018050493

Lessard, J. C. (2013). Molecular cloning. In Methods in Enzymology (Vol. 529, pp. 85–98). Elsevier. https://doi.org/10.1016/B978-0-12-418687-3.00007-0

Li, C., Brant, E., Budak, H., & Zhang, B. (2021). CRISPR/Cas: a Nobel Prize award-winning precise genome editing technology for gene therapy and crop improvement. Journal of Zhejiang University: Science B, 22(4), 253–284. https://doi.org/10.1631/jzus.B2100009

Li, H., Hohenstein, P., & Kuure, S. (2021). Embryonic kidney development, stem cells and the origin of wilms tumor. In Genes (Vol. 12, Issue 2, pp. 1–21). MDPI AG. https://doi.org/10.3390/genes12020318

Lipsitch, M., Tchetgen Tchetgen, E., & Cohen, T. (2010). Negative controls: a tool for detecting confounding and bias in observational studies. Epidemiology (Cambridge, Mass.), 21(3), 383–388. https://doi.org/10.1097/EDE.0b013e3181d61eeb

Liu, C., Zhang, L., Liu, H., & Cheng, K. (2017). Delivery strategies of the CRISPR-Cas9 geneediting system for therapeutic applications. Journal of controlled release : official journal of the Controlled Release Society, 266, 17–26. https://doi.org/10.1016/j.jconrel.2017.09.012

Liu, J., Gao, C., Chen, W., Ma, W., Li, X., Shi, Y., Zhang, H., Zhang, L., Long, Y., Xu, H., Guo, X., Deng, S., Yan, X., Yu, D., Pan, G., Chen, Y., Lai, L., Liao, W., & Li, Z. (2016). CRISPR/Cas9 facilitates investigation of neural circuit disease using human iPSCs: Mechanism of epilepsy caused by an *SCN1A* loss-of-function mutation. Translational Psychiatry, 6(1), e703. https://doi.org/10.1038/tp.2015.203

Lokmane, L., Heliot, C., Garcia-Villalba, P., Fabre, M., & Cereghini, S. (2010). vHNF1 functions in distinct regulatory circuits to control ureteric bud branching and early nephrogenesis. Development (Cambridge, England), 137(2), 347–357. https://doi.org/10.1242/dev.042226

Lu, Y. F., Goldstein, D. B., Angrist, M., & Cavalleri, G. (2014). Personalized medicine and human genetic diversity. Cold Spring Harbor perspectives in medicine, 4(9), a008581. https://doi.org/10.1101/cshperspect.a008581

Ludwig, K. S., & Landmann, L. (2005). Early development of the human mesonephros. Anatomy and embryology, 209(6), 439–447. https://doi.org/10.1007/s00429-005-0460-3

Madariaga, L., García-Castaño, A., Ariceta, G., Martínez-Salazar, R., Aguayo, A., Castaño, L., & Spanish group for the study of HNF1B mutations (2018). Variable phenotype in HNF1B mutations: extrarenal manifestations distinguish affected individuals from the population with congenital anomalies of the kidney and urinary tract. Clinical kidney journal, 12(3), 373–379. https://doi.org/10.1093/ckj/sfy102

Mahadevan, V. (2019). Anatomy of the kidney and ureter. Surgery (Oxford), 37(7), 359–364. https://doi.org/10.1016/j.mpsur.2019.04.005

Mahmood, T., & Yang, P. C. (2012). Western blot: technique, theory, and trouble shooting. North American journal of medical sciences, 4(9), 429–434. https://doi.org/10.4103/1947-2714.100998

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F. J., Wolf, Y. I., Yakunin, A. F., van der Oost, J., & Koonin, E. V. (2011). Evolution and classification of the CRISPR-Cas systems. Nature reviews. Microbiology, 9(6), 467–477. https://doi.org/10.1038/nrmicro2577

Mallamaci, F., & Tripepi, G. (2024). Risk Factors of Chronic Kidney Disease Progression: Between Old and New Concepts. Journal of clinical medicine, 13(3), 678. https://doi.org/10.3390/jcm13030678 Mout, R., Ray, M., Lee, Y. W., Scaletti, F., & Rotello, V. M. (2017). In Vivo Delivery of CRISPR/Cas9 for Therapeutic Gene Editing: Progress and Challenges. Bioconjugate chemistry, 28(4), 880–884. https://doi.org/10.1021/acs.bioconjchem.7b00057

Mandarim-de-Lacerda, C. A., Aguila, M. B., & Younes-Ibrahim, M. (2014). The number of nephrons in the kidney: A relevant question implicated with arterial hypertension. International Journal of Medical and Surgical Sciences, 1(1), 5–11.

Maresch, R., Mueller, S., Veltkamp, C., Öllinger, R., Friedrich, M., Heid, I., Steiger, K., Weber, J., Engleitner, T., Barenboim, M., Klein, S., Louzada, S., Banerjee,

R., Strong, A., Stauber, T., Gross, N., Geumann, U., Lange, S., Ringelhan, M., Varela, I., … Rad, R. (2016). Multiplexed pancreatic genome engineering and cancer induction by transfection-based CRISPR/Cas9 delivery in mice. *Nature communications*, *7*, 10770. https://doi.org/10.1038/ncomms10770

Massa, F., Garbay, S., Bouvier, R., Sugitani, Y., Noda, T., Gubler, M. C., Heidet, L., Pontoglio, M., & Fischer, E. (2013). Hepatocyte nuclear factor 1β controls nephron tubular development. Development (Cambridge, England), 140(4), 886–896. https://doi.org/10.1242/dev.086546

McKnight, A. J., Currie, D., & Maxwell, A. P. (2010). Unravelling the genetic basis of renal diseases; from single gene to multifactorial disorders. The Journal of Pathology, 220(2), 198– 216. https://doi.org/10.1002/path.2639

McMahon A. P. (2016). Development of the Mammalian Kidney. Current topics in developmental biology, 117, 31–64. https://doi.org/10.1016/bs.ctdb.2015.10.010

Mefford, H. C., Clauin, S., Sharp, A. J., Moller, R. S., Ullmann, R., Kapur, R., Pinkel, D., Cooper, G. M., Ventura, M., Ropers, H. H., Tommerup, N., Eichler, E. E., & Bellanne-Chantelot, C. (2007). Recurrent reciprocal genomic rearrangements of 17q12 are associated with renal disease, diabetes, and epilepsy. American journal of human genetics, 81(5), 1057–1069. https://doi.org/10.1086/522591

Metzakopian, E., Strong, A., Iyer, V. et al. Enhancing the genome editing toolbox: genome wide CRISPR arrayed libraries. Sci Rep 7, 2244 (2017). https://doi.org/10.1038/s41598-017-01766-5

Milone, M. C., & O'Doherty, U. (2018). Clinical use of lentiviral vectors. Leukemia, 32(7), 1529–1541. https://doi.org/10.1038/s41375-018-0106-0

Mishra, M., Tiwari, S., & Gomes, A. V. (2017). Protein purification and analysis: next generation Western blotting techniques. Expert review of proteomics, 14(11), 1037–1053. https://doi.org/10.1080/14789450.2017.1388167

Montoli, A., Colussi, G., Massa, O., Caccia, R., Rizzoni, G., Civati, G., & Barbetti, F. (2002). Renal cysts and diabetes syndrome linked to mutations of the hepatocyte

nuclear factor-1 beta gene: description of a new family with associated liver involvement. American journal of kidney diseases : the official journal of the National Kidney Foundation, 40(2), 397–402. https://doi.org/10.1053/ajkd.2002.34538

Moritz C. P. (2017). Tubulin or Not Tubulin: Heading Toward Total Protein Staining as Loading Control in Western Blots. Proteomics, 17(20), 10.1002/pmic.201600189. https://doi.org/10.1002/pmic.201600189

Mruk, D. D., & Cheng, C. Y. (2011). Enhanced chemiluminescence (ECL) for routine immunoblotting: An inexpensive alternative to commercially available kits. *Spermatogenesis*, *1*(2), 121–122. https://doi.org/10.4161/spmg.1.2.16606

Nakayama, M., Nozu, K., Goto, Y., Kamei, K., Ito, S., Sato, H., Emi, M., Nakanishi, K., Tsuchiya, S., & Iijima, K. (2010). HNF1B alterations associated with congenital anomalies of the kidney and urinary tract. Pediatric nephrology (Berlin, Germany), 25(6), 1073–1079. https://doi.org/10.1007/s00467-010-1454-9

Nelson, C. E., Hakim, C. H., Ousterout, D. G., Thakore, P. I., Moreb, E. A., Castellanos Rivera, R. M., Madhavan, S., Pan, X., Ran, F. A., Yan, W. X., Asokan, A., Zhang, F., Duan, D., & Gersbach, C. A. (2016). In vivo genome editing improves muscle function in a mouse model of Duchenne muscular dystrophy. Science (New York, N.Y.), 351(6271), 403–407. https://doi.org/10.1126/science.aad5143

Ni, W., Qiao, J., Hu, S., Zhao, X., Regouski, M., Yang, M., Polejaeva, I. A., & Chen, C. (2014). Efficient gene knockout in goats using CRISPR/Cas9 system. PloS one, 9(9), e106718. https://doi.org/10.1371/journal.pone.0106718

Nie, C., Wang, B., Wang, B., Lv, N., & Zhang, E. (2020). Integrative Analysis of HNF1B mRNA in Human Cancers Based on Data Mining. International journal of medical sciences, 17(18), 2895–2904. https://doi.org/10.7150/ijms.51213

Nishigori, H., Yamada, S., Kohama, T., Tomura, H., Sho, K., Horikawa, Y., Bell, G. I., Takeuchi, T., & Takeda, J. (1998). Frameshift mutation, A263fsinsGG, in the hepatocyte nuclear factor-1β gene associated with diabetes and renal dysfunction. Diabetes, 47(8), 1354–1355. https://doi.org/10.2337/diab.47.8.1354

Obrycki, Ł., Sarnecki, J., Lichosik, M., Sopińska, M., Placzyńska, M., Stańczyk, M., Mirecka, J., Wasilewska, A., Michalski, M., Lewandowska, W., Dereziński, T., Pac, M., Szwarc, N., Annusewicz, K., Rekuta, V., Ažukaitis, K., Čekuolis, A., Wierzbicka-Rucińska, A., Jankauskiene, A., Kalicki, B., … Litwin, M. (2022). Kidney length normative values in children aged 0-19 years - a multicenter study. Pediatric nephrology (Berlin, Germany), 37(5), 1075–1085. https://doi.org/10.1007/s00467-021-05303-5

Panja, S., Saha, S., Jana, B., & Basu, T. (2006). Role of membrane potential on artificial transformation of E. coli with plasmid DNA. Journal of biotechnology, 127(1), 14–20. https://doi.org/10.1016/j.jbiotec.2006.06.008

Przepiorski, A., Crunk, A. E., Espiritu, E. B., Hukriede, N. A., & Davidson, A. J. (2020). The Utility of Human Kidney Organoids in Modeling Kidney Disease. Seminars in Nephrology, 40(2), 188–198. https://doi.org/10.1016/J.SEMNEPHROL.2020.01.009

Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., & Lim, W. A. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell, 152(5), 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022

Ramalingam, S., Annaluru, N., & Chandrasegaran, S. (2013). A CRISPR way to engineer the human genome. Genome biology, 14(2), 107. https://doi.org/10.1186/gb-2013-14-2-107

Ramamoorth, M., & Narvekar, A. (2015). Non viral vectors in gene therapy- an overview. Journal of clinical and diagnostic research : JCDR, 9(1), GE01–GE6. https://doi.org/10.7860/JCDR/2015/10443.5394

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nature Protocols, 8(11), 2281–2308. https://doi.org/10.1038/nprot.2013.143

Rani, V., & Prabhu, A. (2022). CRISPR-Cas9 based non-viral approaches in nanoparticle-elicited therapeutic delivery. Journal of Drug Delivery Science and Technology, 76, 103737. https://doi.org/10.1016/j.jddst.2022.103737

Redman, M., King, A., Watson, C., & King, D. (2016). What is CRISPR/Cas9?. Archives of disease in childhood. Education and practice edition, 101(4), 213–215. https://doi.org/10.1136/archdischild-2016-310459

Rein, L. A. M., Yang, H., & Chao, N. J. (2018). Applications of gene editing technologies to cellular therapies. *Biology of Blood and Marrow Transplantation, 24*(6), 1050–1058. https://doi.org/10.1016/j.bbmt.2018.03.021

Röck, R., Rizzo, L., & Lienkamp, S. S. (2022). Kidney development: Recent insights from technological advances. Physiology, 37(4), 207–215. American Physiological Society. https://doi.org/10.1152/physiol.00041.2021

Sampogna, R. V., Schneider, L., & Al-Awqati, Q. (2015). Developmental Programming of Branching Morphogenesis in the Kidney. Journal of the American Society of Nephrology : JASN, 26(10), 2414–2422. https://doi.org/10.1681/ASN.2014090886

Sánchez-Rivera, F. J., Papagiannakopoulos, T., Romero, R., Tammela, T., Bauer, M. R., Bhutkar, A., Joshi, N. S., Subbaraj, L., Bronson, R. T., Xue, W., & Jacks, T. (2014). Rapid modelling of cooperating genetic events in cancer through somatic genome editing. *Nature*, *516*(7531), 428–431. https://doi.org/10.1038/nature13906

Sander, H., Wallace, S., Plouse, R., Tiwari, S., & Gomes, A. V. (2019). Ponceau S waste: Ponceau S staining for total protein normalization. Analytical biochemistry, 575, 44–53. https://doi.org/10.1016/j.ab.2019.03.010

Sanjana, N. E., Shalem, O., & Zhang, F. (2014). Improved vectors and genome-wide libraries for CRISPR screening. Nature Methods, 11(8), 783–784. https://doi.org/10.1038/nmeth.3047

Sanna-Cherchi, S., Westland, R., Ghiggeri, G. M., & Gharavi, A. G. (2018). Genetic basis of human congenital anomalies of the kidney and urinary tract. The Journal of clinical investigation, 128(1), 4–15. https://doi.org/10.1172/JCI95300

Saxén, L., & Sariola, H. (1987). Early organogenesis of the kidney. Pediatric nephrology (Berlin, Germany), 1(3), 385–392. https://doi.org/10.1007/BF00849241

Schnaper H. W. (2014). Remnant nephron physiology and the progression of chronic kidney disease. Pediatric nephrology (Berlin, Germany), 29(2), 193–202. https://doi.org/10.1007/s00467-013-2494-8

Schwank, G., Koo, B. K., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., Sasaki, N., Boymans, S., Cuppen, E., van der Ent, C. K., Nieuwenhuis, E. E., Beekman, J. M., & Clevers, H. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell stem cell, 13(6), 653–658. https://doi.org/10.1016/j.stem.2013.11.002

Sconocchia, T., Foßelteder, J., Köhnke, T., Majeti, R., & Reinisch, A. (2023). Engineering oncogenic heterozygous gain-of-function mutations in human hematopoietic stem and progenitor cells. Journal of Visualized Experiments, (64558). https://doi.org/10.3791/64558

Seely J. C. (2017). A brief review of kidney development, maturation, developmental abnormalities, and drug toxicity: juvenile animal relevancy. Journal of toxicologic pathology, 30(2), 125–133. https://doi.org/10.1293/tox.2017-0006

Shalem, O., Sanjana, N. E., Hartenian, E., Shi, X., Scott, D. A., Mikkelson, T., Heckl, D., Ebert, B. L., Root, D. E., Doench, J. G., & Zhang, F. (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science, 343(6166), 84–87. https://doi.org/10.1126/science.1247005

Shao, A., Chan, S. C., & Igarashi, P. (2020). Role of transcription factor hepatocyte nuclear factor-1β in polycystic kidney disease. Cellular signalling, 71, 109568. https://doi.org/10.1016/j.cellsig.2020.109568

Short, K. M., Combes, A. N., Lefevre, J., Ju, A. L., Georgas, K. M., Lamberton, T., Cairncross, O., Rumballe, B. A., McMahon, A. P., Hamilton, N. A., Smyth, I. M., & Little, M. H. (2014). Global quantification of tissue dynamics in the developing mouse kidney. Developmental cell, 29(2), 188–202. https://doi.org/10.1016/j.devcel.2014.02.017

Singh, R., Chandel, S., Ghosh, A., Gautam, A., Huson, D. H., Ravichandiran, V., & Ghosh, D. (2022). Easy efficient HDR-based targeted knock-in in Saccharomyces cerevisiae genome using CRISPR-Cas9 system. Bioengineered, 13(6), 14857–14871. https://doi.org/10.1080/21655979.2022.2162667

Smith, J., Petrovic, P., Rose, M., De Souz, C., Muller, L., Nowak, B., & Martinez, J. (2021). Placeholder Text: A Study. The Journal of Citation Styles, 3. https://doi.org/10.10/X

Song M. (2017). The CRISPR/Cas9 system: Their delivery, in vivo and ex vivo applications and clinical development by startups. Biotechnology progress, 33(4), 1035–1045. https://doi.org/10.1002/btpr.2484

Sorida, M., & Bonasio, R. (2023). An efficient cloning method to expand vector and restriction site compatibility of Golden Gate Assembly. Cell reports methods, 3(8), 100564. https://doi.org/10.1016/j.crmeth.2023.100564

Tabebordbar, M., Zhu, K., Cheng, J. K. W., Chew, W. L., Widrick, J. J., Yan, W. X., Maesner, C., Wu, E. Y., Xiao, R., Ran, F. A., Cong, L., Zhang, F., Vandenberghe, L. H., Church, G. M., & Wagers, A. J. (2016). In vivo gene editing in dystrophic mouse muscle and muscle stem cells. Science (New York, N.Y.), 351(6271), 407–411. https://doi.org/10.1126/science.aad5177

Taha, E. A., Lee, J., & Hotta, A. (2022). Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges. Journal of Controlled Release, 343, 345-361. https://doi.org/10.1016/j.jconrel.2022.01.013

Tang, X., Wang, Z., Zhang, Y., Mu, W., & Han, X. (2022). Non-viral nanocarriers for CRISPR-Cas9 gene editing system delivery. Chemical Engineering Journal, 435(Part 2), 135116. https://doi.org/10.1016/j.cej.2022.135116

Tebas, P., Stein, D., Tang, W. W., Frank, I., Wang, S. Q., Lee, G., Spratt, S. K., Surosky, R. T., Giedlin, M. A., Nichol, G., Holmes, M. C., Gregory, P. D., Ando, D. G., Kalos, M., Collman, R. G., Binder-Scholl, G., Plesa, G., Hwang, W. T., Levine, B. L., & June, C. H. (2014). Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. The New England journal of medicine, 370(10), 901–910. https://doi.org/10.1056/NEJMoa1300662

Thomas, P., & Smart, T. G. (2005). HEK293 cell line: a vehicle for the expression of recombinant proteins. Journal of pharmacological and toxicological methods, 51(3), 187–200. https://doi.org/10.1016/j.vascn.2004.08.014

Torres-Ruiz, R., & Rodriguez-Perales, S. (2015). CRISPR-Cas9: A Revolutionary Tool for Cancer Modelling. *International journal of molecular sciences*, *16*(9), 22151–22168. https://doi.org/10.3390/ijms160922151

Tuttle, A. R., Trahan, N. D., & Son, M. S. (2021). Growth and Maintenance of Escherichia coli Laboratory Strains. Current protocols, 1(1), e20. https://doi.org/10.1002/cpz1.20

U.S. National Library of Medicine. (n.d.). HNF1B gene: Medlineplus genetics. MedlinePlus HNF1B gene. Retrieved January 29, 2023, from https://medlineplus.gov/genetics/gene/hnf1b/#conditions

Uddin, F., Rudin, C. M., & Sen, T. (2020). CRISPR Gene Therapy: Applications, Limitations, and Implications for the Future. Frontiers in oncology, 10, 1387. https://doi.org/10.3389/fonc.2020.01387

Ulinski, T., Lescure, S., Beaufils, S., Guigonis, V., Decramer, S., Morin, D., Clauin, S., Deschênes, G., Bouissou, F., Bensman, A., & Bellanné-Chantelot, C. (2006). Renal phenotypes related to hepatocyte nuclear factor-1beta (TCF2) mutations in a pediatric cohort. Journal of the American Society of Nephrology: JASN, 17(2), 497–503. https://doi.org/10.1681/ASN.2005101040

Uy, N., & Reidy, K. (2016). Developmental Genetics and Congenital Anomalies of the Kidney and Urinary Tract. Journal of pediatric genetics, 5(1), 51–60. https://doi.org/10.1055/s-0035https://doi.org/10.1055/s-0035-15584231558423

Vasileiou, A., Sfakianaki, E., & Tsekouropoulos, G. (2024). Exploring Sustainability and Efficiency Improvements in Healthcare: A Qualitative Study. Sustainability, 16(19), 8306. https://doi.org/10.3390/su16198306

Veikkolainen, V. (2014). Regulation of cell growth, death, and polarization by ERBB4 [Scientific figure]. ResearchGate.

https://www.researchgate.net/figure/Development-of-the-metanephros-A-B-Metanephric-mesenchyme-induces-ureteric-bud-to_fig5_265076735

Verdeguer, F., Le Corre, S., Fischer, E., Callens, C., Garbay, S., Doyen, A., Igarashi, P., Terzi, F., & Pontoglio, M. (2010). A mitotic transcriptional switch in polycystic kidney disease. Nature medicine, 16(1), 106–110. https://doi.org/10.1038/nm.2068

Verhave, J. C., Bech, A. P., Wetzels, J. F., & Nijenhuis, T. (2016). Hepatocyte Nuclear Factor 1β-Associated Kidney Disease: More than Renal Cysts and Diabetes. Journal of the American Society of Nephrology : JASN, 27(2), 345–353. https://doi.org/10.1681/ASN.2015050544

Vivante, A., & Hildebrandt, F. (2016). Exploring the genetic basis of early-onset chronic kidney disease. Nature reviews. Nephrology, 12(3), 133–146. https://doi.org/10.1038/nrneph.2015.205

Wagner, D. L., Amini, L., Wendering, D. J., Burkhardt, L. M., Akyüz, L., Reinke, P., Volk, H. D., & Schmueck-Henneresse, M. (2019). High prevalence of Streptococcus pyogenes Cas9reactive T cells within the adult human population. Nature medicine, 25(2), 242–248. https://doi.org/10.1038/s41591-018-0204-6

Wan, L., Wen, H., Li, Y., Lyu, J., Xi, Y., Hoshii, T., Joseph, J. K., Wang, X., Loh, Y. E., Erb, M. A., Souza, A. L., Bradner, J. E., Shen, L., Li, W., Li, H., Allis, C. D., Armstrong, S. A., & Shi, X. (2017). ENL links histone acetylation to oncogenic gene expression in acute myeloid leukaemia. Nature, 543(7644), 265–269. https://doi.org/10.1038/nature21687

Wang, S. W., Gao, C., Zheng, Y. M., Yi, L., Lu, J. C., Huang, X. Y., Cai, J. Bin, Zhang, P. F., Cui, Y. H., & Ke, A. W. (2022). Current applications and future perspective of CRISPR/Cas9 gene editing in cancer. In Molecular Cancer (Vol. 21, Issue 1). https://doi.org/10.1186/s12943https://doi.org/10.1186/s12943-022-01518-8022-01518-8

Weber, S., Moriniere, V., Knüppel, T., Charbit, M., Dusek, J., Ghiggeri, G. M., Jankauskiené, A., Mir, S., Montini, G., Peco-Antic, A., Wühl, E., Zurowska, A. M., Mehls, O., Antignac, C., Schaefer, F., & Salomon, R. (2006). Prevalence of

mutations in renal developmental genes in children with renal hypodysplasia: results of the ESCAPE study. Journal of the American Society of Nephrology : JASN, 17(10), 2864–2870. https://doi.org/10.1681/ASN.2006030277

Webster, A. C., Nagler, E. V., Morton, R. L., & Masson, P. (2017). Chronic Kidney Disease. Lancet (London, England), 389(10075), 1238–1252. https://doi.org/10.1016/S0140-6736(16)32064-56736(16)32064-5

World Health Organization. Mortality and global health estimates: Causes of death; Projections for 2015–2030; Projection of death rates. http://apps.who.int/gho/data/node.main. PROJRATEWORLD?lang=en (accessed 20 August, 2023).

Xie, F., Ye, L., Chang, J. C., Beyer, A. I., Wang, J., Muench, M. O., & Kan, Y. W. (2014). Seamless gene correction of β-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. Genome research, 24(9), 1526–1533. https://doi.org/10.1101/gr.173427.114.

Xu, H., Xiao, T., Chen, C. H., Li, W., Meyer, C. A., Wu, Q., Wu, D., Cong, L., Zhang, F., Liu, J. S., Brown, M., & Liu, X. S. (2015). Sequence determinants of improved CRISPR sgRNA design. Genome research, 25(8), 1147–1157. https://doi.org/10.1101/gr.191452.115

Xu, Y., & Li, Z. (2020). CRISPR-Cas systems : Overview , innovations and applications in human disease research and gene therapy. Computational and Structural Biotechnology Journal, 18, 2401–2415. https://doi.org/10.1016/j.csbj.2020.08.031

Yang, H., Sieben, C. J., Schauer, R. S., & Harris, P. C. (2023). Genetic Spectrum of Polycystic Kidney and Liver Diseases and the Resulting Phenotypes. Advances in kidney disease and health, 30(5), 397–406. https://doi.org/10.1053/j.akdh.2023.04.004

Yang, S., Chang, R., Yang, H., Zhao, T., Hong, Y., Kong, H. E., Sun, X., Qin, Z., Jin, P., Li, S., & Li, X. J. (2017). CRISPR/Cas9-mediated gene editing ameliorates neurotoxicity in mouse model of Huntington's disease. The Journal of clinical investigation, 127(7), 2719–2724. https://doi.org/10.1172/JCI92087

Yin, C., Zhang, T., Qu, X., Zhang, Y., Putatunda, R., Xiao, X., Li, F., Xiao, W., Zhao, H., Dai, S., Qin, X., Mo, X., Young, W. B., Khalili, K., & Hu, W. (2017). In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. Molecular therapy : the journal of the American Society of Gene Therapy, 25(5), 1168–1186. https://doi.org/10.1016/j.ymthe.2017.03.012

Yin, H., Kanasty, R. L., Eltoukhy, A. A., Vegas, A. J., Dorkin, J. R., & Anderson, D. G. (2014). Non-viral vectors for gene-based therapy. Nature reviews. Genetics, 15(8), 541–555. https://doi.org/10.1038/nrg3763

Yin, H., Song, C. Q., Dorkin, J. R., Zhu, L. J., Li, Y., Wu, Q., Park, A., Yang, J., Suresh, S., Bizhanova, A., Gupta, A., Bolukbasi, M. F., Walsh, S., Bogorad, R. L., Gao, G., Weng, Z., Dong, Y., Koteliansky, V., Wolfe, S. A., Langer, R., … Anderson, D. G. (2016). Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nature biotechnology, 34(3), 328–333. https://doi.org/10.1038/nbt.3471

Yin, H., Xue, W., & Anderson, D. G. (2019). CRISPR-Cas: a tool for cancer research and therapeutics. Nature reviews. Clinical oncology, 16(5), 281–295. https://doi.org/10.1038/s41571-019-0166-8

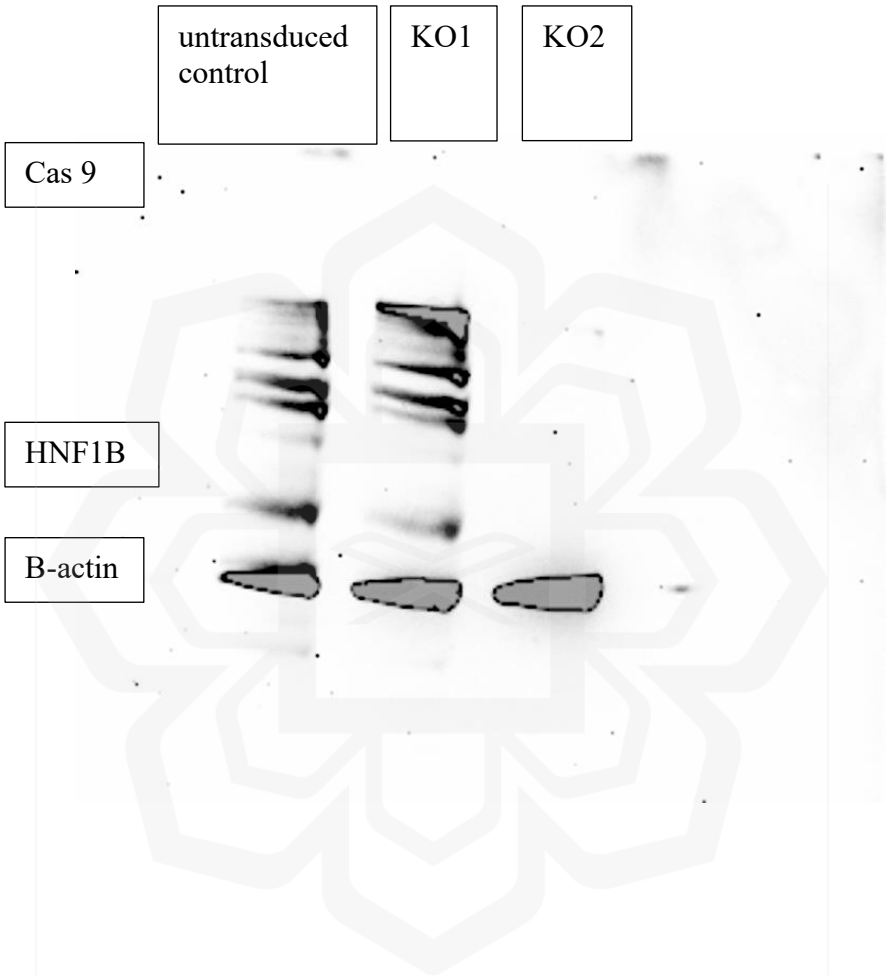
Yoshimura, Y., Muto, Y., Ledru, N., Wu, H., Omachi, K., Miner, J. H., & Humphreys, B. D. (2023). A single-cell multiomic analysis of kidney organoid differentiation. Proceedings of the National Academy of Sciences of the United States of America, 120(20), e2219699120. https://doi.org/10.1073/pnas.2219699120

Zhang, W., Xiao, D., Shan, L., Zhao, J., Mao, Q., & Xia, H. (2017). Generation of apoptosisresistant HEK293 cells with CRISPR/Cas mediated quadruple gene knockout for improved protein and virus production. Biotechnology and bioengineering, 114(11), 2539–2549. https://doi.org/10.1002/bit.26382

Zhong, Q., Xiao, X., Qiu, Y., Xu, Z., Chen, C., Chong, B., Zhao, X., Hai, S., Li, S., An, Z., & Dai, L. (2023). Protein posttranslational modifications in health and diseases: Functions, regulatory mechanisms, and therapeutic implications. MedComm, 4(3), e261. https://doi.org/10.1002/mco2.261

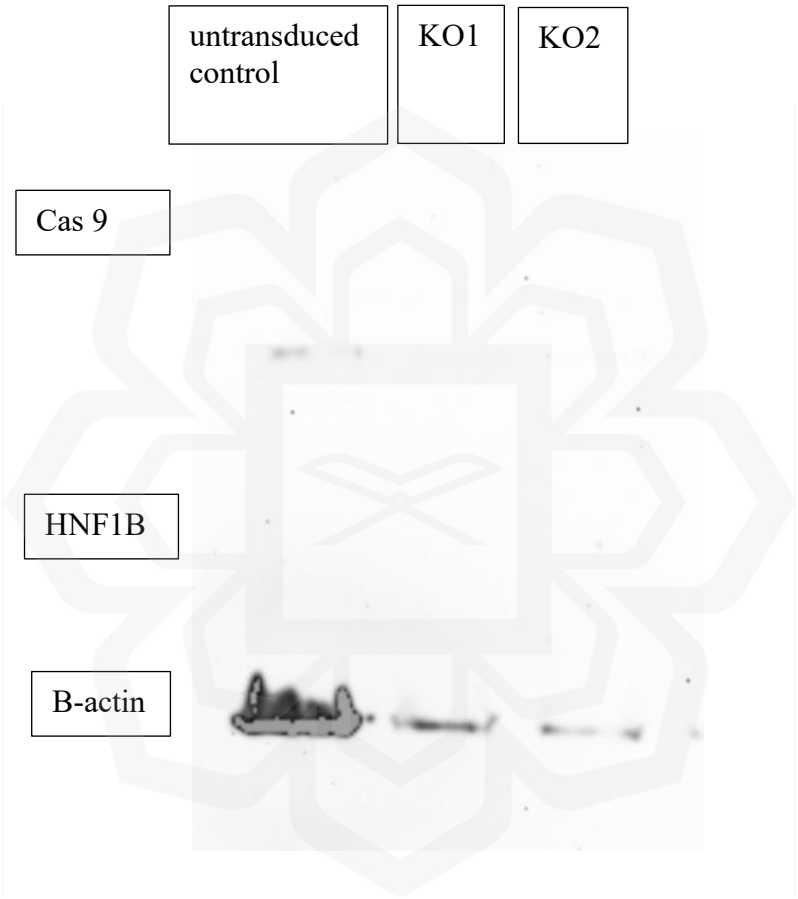
APPENDIX 1

Chemiblot image showing only B-actin band visible. No HNF1B protein band detected



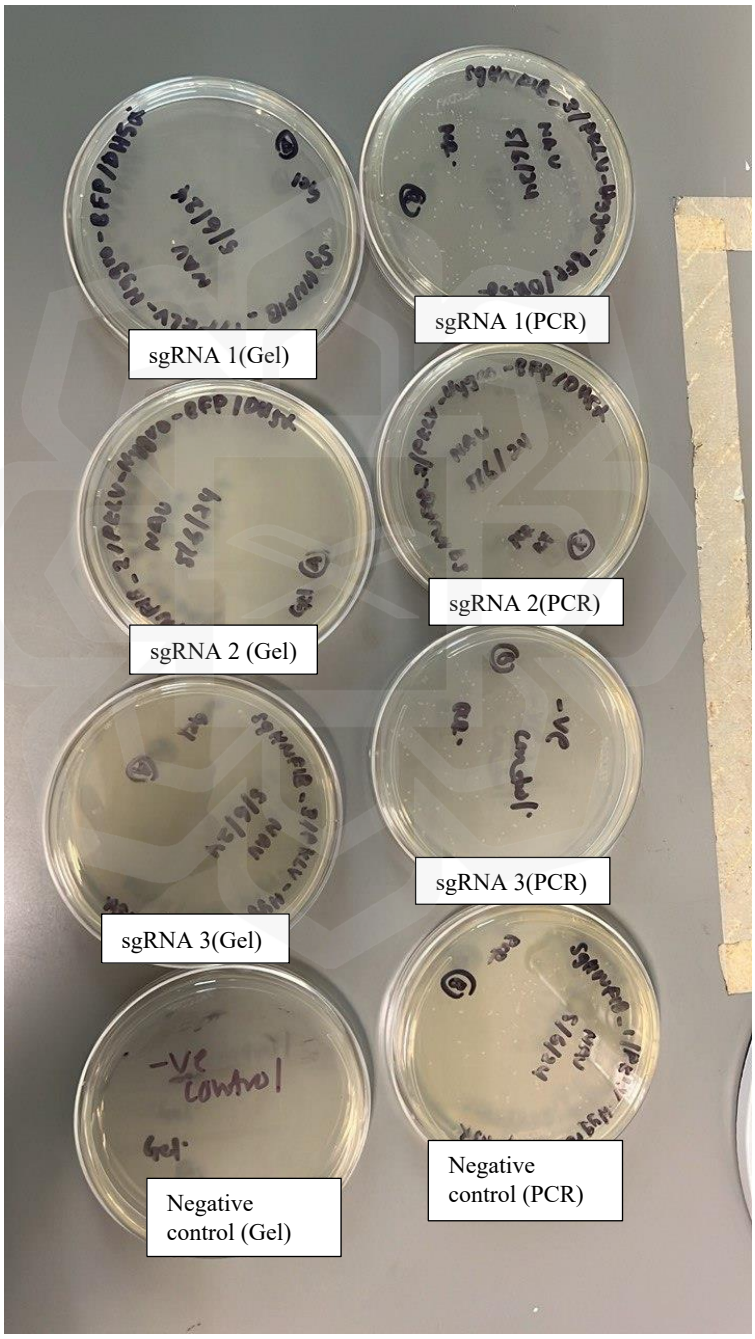
APPENDIX 2

Chemiblot image showing only B-actin band visible but not clear. Not even single band of HNF1B detected



APPENDIX 3

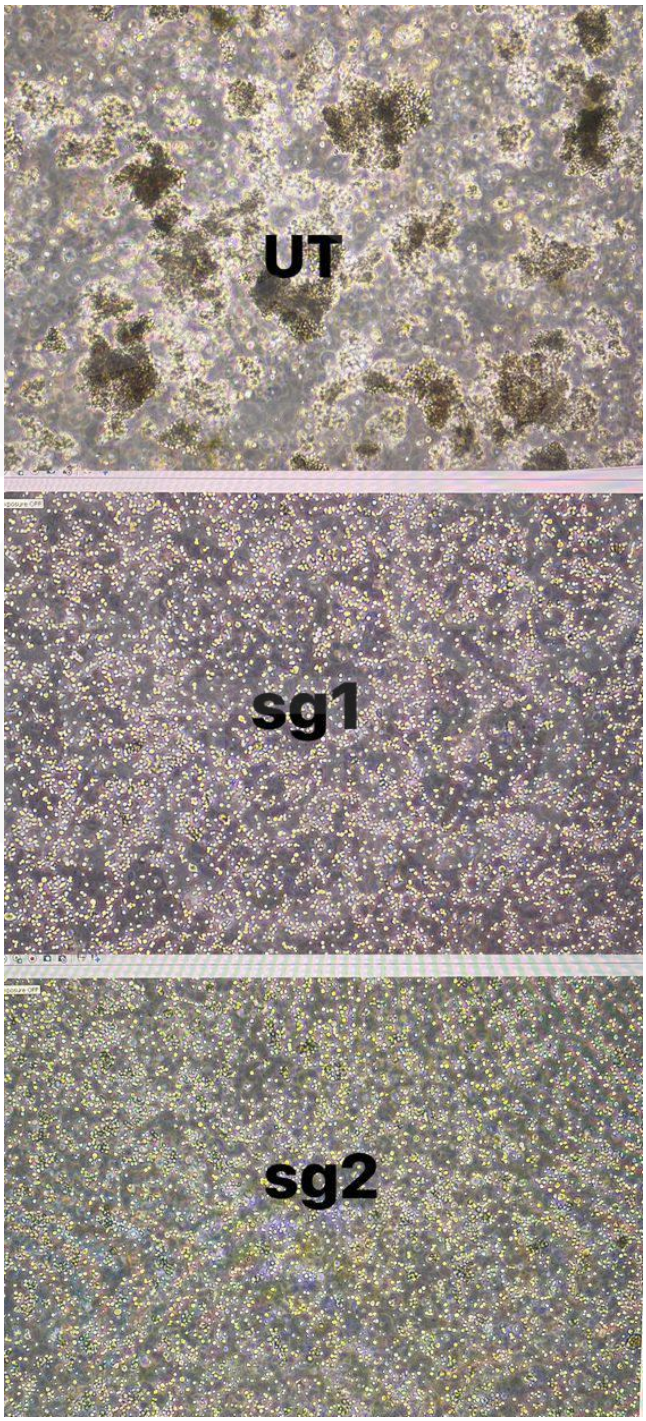
MOLECULAR CLONING OF HNF1B GENE



PCR purification method confirms the presence of colonies, despite no visible colonies on the agar plate while using gel electrophoresis (for gel extraction method)

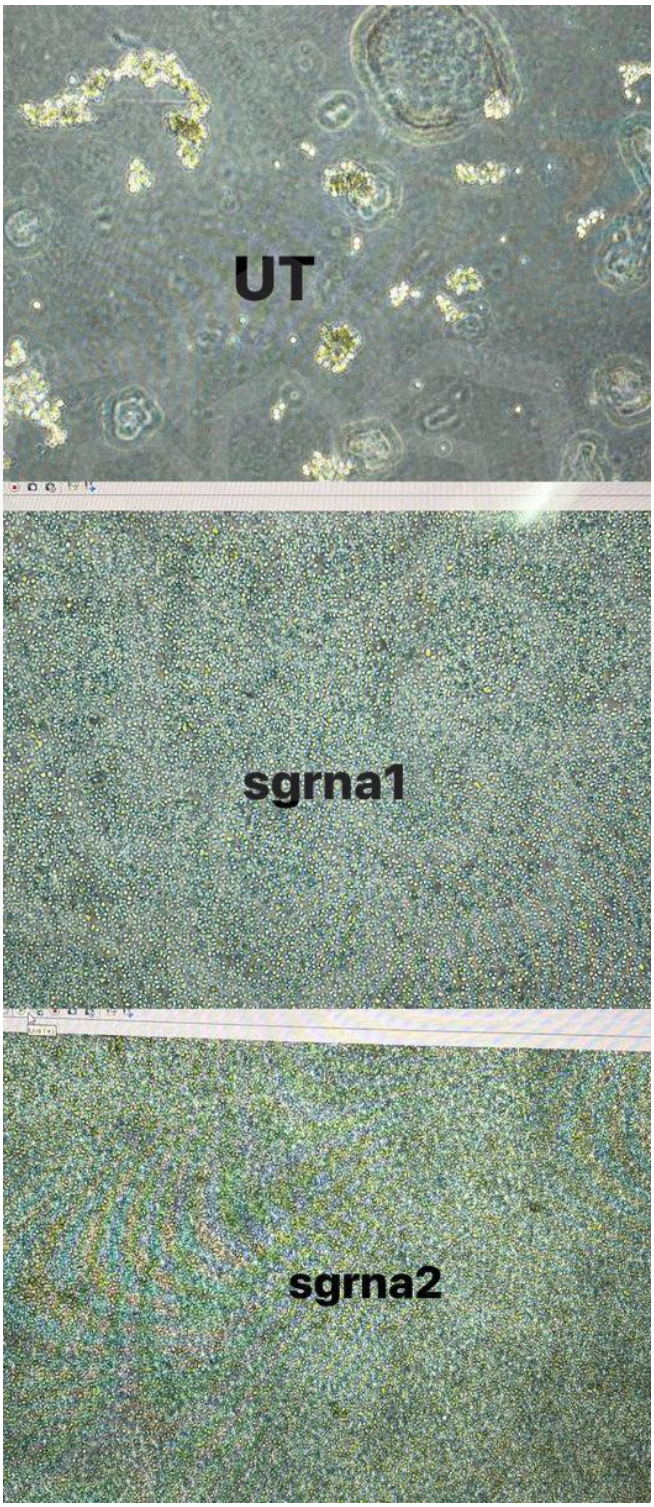
APPENDIX 4

Antibiotic selection for sgrna 1 and sgrna 2. The condition of untransduced control ,sgRNA1 and sgRNA 2 after day 7 antibiotic selection



APPENDIX 5

The condition of Untransduced cell (untransduced control), sgRNA1 and sgRNA 2 after day 6 antibiotic selection



APPENDIX 6

Abstract for oral presentation for IIUM PG colloquium 2024 at Kulliyyah of Nursing, IIUM Kuantan on September 23rd, 2024

GENERATING HETEROZYGOUS HNF1B CRISPR-KNOCKOUT LINES TO UNDERSTAND KIDNEY PATHOPHYSIOLOGY

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ABSTRACT

The Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated system (CRISPR/Cas9) is a widely used gene-editing tool to study gene function. In this study, we employed the CRISPR/Cas9 technology to target the hepatocyte nuclear factor 1β (HNF1B) gene that encodes human transcription factor HNF1 which regulates formation of healthy kidney. Heterozygous mutations in this gene was known as the monogenic cause of developmental kidney disease. To achieve this, we cultured HEK293T (human embryonic kidney) cell line under normal condition and introduced the knockout using two guide RNAs (gRNAs) via non-homologous end-joining (NHEJ) repair mechanism. In this study, we utilized the CRISPick web tool to design sgRNAs targeting the HNF1B gene, incorporating BbsI overhangs into the sequences before oligos preparation. The sgHNF1B was cloned into the pKLV-U6gRNA(BbsI)-PGKpuro2ABFP vector, transformed into E. coli and successful cloning were validated by Sanger sequencing. For a stable, long-term expression of sgRNA and Cas9, lentiviral particles were produced (dual vector system) and transduced into HEK293T cells cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin (10,000 IU). Post-transduction of Cas9 lentiviral construct, cells were selected with 30 mg/mL Blasticidin, and the modified cells were isolated. Subsequently, these cells (containing Cas9) were transduced with sgHNF1B and further selected using Hygromycin. Cells that survive indicates an efficient sgHNF1B integration and Cas9-mediated gene editing. In conclusion, our study provides the proof-of- concept that demonstrates utility of genome editing in understanding the role of a gene and essentially modelling human pathophysiology.

Keywords:
CRISPR/Cas9, HEK293T, HNF1B, sgRNA cloning, kidney, lentivirus

Acknowledgement:
Ministry of Higher Education (MOHE Grants)-FRGS21-207-0816, Institute of Planetary Survival for Sustainable Well-being (PLANETIIUM), International Islamic University (IIUM), UKM Medical Molecular Biology Institute (UMBI)


LEADING THE WAY
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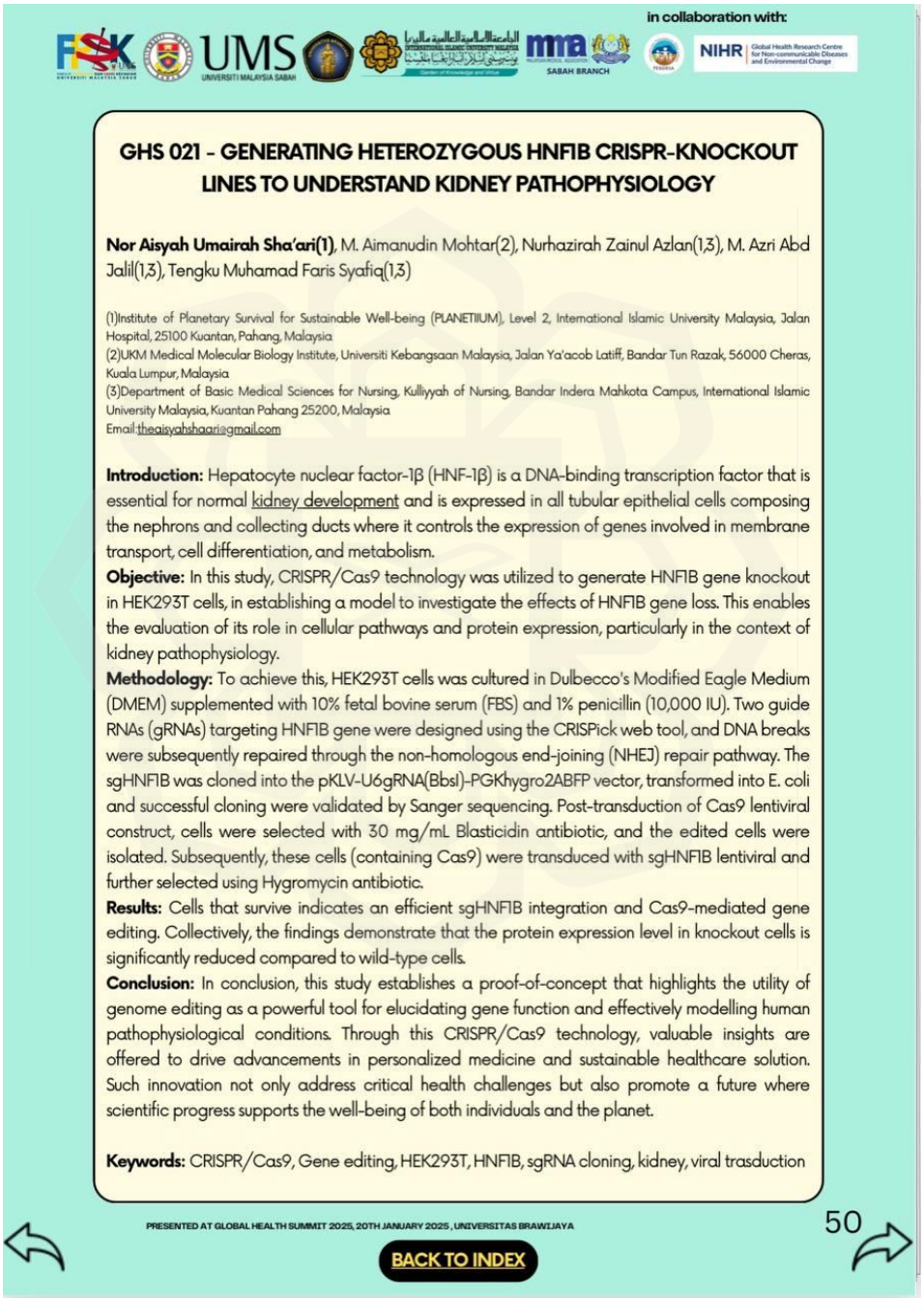
for his/her participation as an Oral Presenter at the
IIUM Postgraduate Colloquium 2024
held on
23 September 2024 at Kulliyyah of Nursing
International Islamic University Malaysia
PROF. DR. SHAHRUL NA'IM SIDEK
Dean
Centre for Postgraduate Studies
IIUM

APPENDIX 7

Abstract for oral presentation for Global Health Summit 2025 at Universitas Brawijaya, Indonesia on January 20th, 2025.



in collaboration with:

GHS 021 - GENERATING HETEROZYGOUS HNF1B CRISPR-KNOCKOUT LINES TO UNDERSTAND KIDNEY PATHOPHYSIOLOGY

Nor Aisyah Umairah Sha'ari(1), M. Aimanudin Mohtar(2), Nurhazirah Zainul Azlan(1,3), M. Azri Abd Jalil(1,3), Tengku Muhamad Faris Syafiq(1,3)

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(3)Department of Basic Medical Sciences for Nursing, Kulliyyah of Nursing, Bandar Indera Mahkota Campus, International Islamic University Malaysia, Kuantan Pahang 25200, Malaysia
Email:theaisyahshaari@gmail.com

Introduction: Hepatocyte nuclear factor-1β (HNF-1β) is a DNA-binding transcription factor that is essential for normal kidney development and is expressed in all tubular epithelial cells composing the nephrons and collecting ducts where it controls the expression of genes involved in membrane transport, cell differentiation, and metabolism.
Objective: In this study, CRISPR/Cas9 technology was utilized to generate HNF1B gene knockout in HEK293T cells, in establishing a model to investigate the effects of HNF1B gene loss. This enables the evaluation of its role in cellular pathways and protein expression, particularly in the context of kidney pathophysiology.
Methodology: To achieve this, HEK293T cells was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (10,000 IU). Two guide RNAs (gRNAs) targeting HNF1B gene were designed using the CRISPick web tool, and DNA breaks were subsequently repaired through the non-homologous end-joining (NHEJ) repair pathway. The sgHNF1B was cloned into the pKLV-U6gRNA(BbsI)-PGKhygro2ABFP vector, transformed into E. coli and successful cloning were validated by Sanger sequencing. Post-transduction of Cas9 lentiviral construct, cells were selected with 30 mg/mL Blasticidin antibiotic, and the edited cells were isolated. Subsequently, these cells (containing Cas9) were transduced with sgHNF1B lentiviral and further selected using Hygromycin antibiotic.
Results: Cells that survive indicates an efficient sgHNF1B integration and Cas9-mediated gene editing. Collectively, the findings demonstrate that the protein expression level in knockout cells is significantly reduced compared to wild-type cells.
Conclusion: In conclusion, this study establishes a proof-of-concept that highlights the utility of genome editing as a powerful tool for elucidating gene function and effectively modelling human pathophysiological conditions. Through this CRISPR/Cas9 technology, valuable insights are offered to drive advancements in personalized medicine and sustainable healthcare solution. Such innovation not only address critical health challenges but also promote a future where scientific progress supports the well-being of both individuals and the planet.

Keywords: CRISPR/Cas9, Gene editing, HEK293T, HNF1B, sgRNA cloning, kidney, viral trasduction

PRESENTED AT GLOBAL HEALTH SUMMIT 2025, 20TH JANUARY 2025 , UNIVERSITAS BRAWIJAYA

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BACK TO INDEX


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CERTIFICATE
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This certificate is proudly presented to
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In recognition of your exceptional contributions and dedication in
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held in
Universitas Brawijaya, Indonesia
on January 20th, 2025
PROF. DATUK DR. KASIM HJ MANSOR
VICE-CHANCELLOR,
UNIVERSITI MALAYSIA SABAH
PROF. WIDODO, S.Si., M.Si., Ph.D.Med.Sc.
RECTOR,
UNIVERSITAS BRAWIJAYA
PROF. EMERITUS DATUK DR. OSMAN BAKAR
RECTOR,
INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

APPENDIX 8

Certificate for participation in Basic To Real Time PCR Workshop 2023 In Conjugation with the 15th Malaysia International Genetics Congress (MiGC15) at Faculty of Medicine and Health Science, University Putra Malaysia.

APPENDIX 9

Certificate for participation in Essential Molecular Biology Techniques Webinar Series 2023. Organized by School of Dental Sciences, Health Campus, Universiti Sains Malaysia.

APPENDIX 10

Approval form from Instituitonal Biosafety and Biosecurity Committee (IBBC) to conduct this research. Approval date on 18/1/2023.

IIUM | IBBC | PRELIM
Preliminary Assessment Form

For IBBC use only
Registration No.: IBBC.Prelim
Rev. no.: Rev2
Effective Date: 22/02/2022

INSTITUTIONAL BIOSAFETY & BIOSECURITY COMMITTEE (IBBC)

Instruction:

Preliminary assessment form is used to identify new proposal(s) or activity involving the use of infectious and potentially infectious agents/materials, biological toxins, living modified organism/genetically modified organism (LMO/GMO) and other biological materials. Submission is to be made by email to: ibc@iium.edu.my

SECTION A: PRINCIPAL INVESTIGATOR'S (PI's) INFORMATION	
Name: Dr Tengku Muhamad Faris Syafiq bin Tengku Zakaria	
Faculty/Center/Institute: Kulliyyah of Nursing	
Postal Address: Department of Basic Medical Sciences for Nursing, Kulliyyah of Nursing, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang.	
Office Phone No: 09-570 7335	**Mobile Phone No:** 012-720 1752
Staff No: 9893	**Email:** fariss@iium.edu.my
Project Title: Modelling genetic kidney malformations using CRISPR/Cas9-edited 3D organoids derived from pluripotent stem cells to understand pathomechanism of human kidney disorders	

SECTION B : PROJECT INFORMATION

1. Purpose: [/] Research [ ] Teaching [ ] Clinical Trial [ ] Service [ ] Diagnostic

Others (Please Specify):

2. Project Status [ ] New Project [ ] On-Going [/] Funded

If funded, please provide grant no:

3. Brief summary of the project (including objectives and expected outcome):

Some human renal tract malformations (RTMs) are caused by mutations of genes that are active during formation of the metanephric kidney, so these are direct outcomes of perturbed kidney differentiation. Indeed, classic dissections by Edith Potter, 60 years ago, showed that human dysplastic kidney cysts are dilated segments of poorly branched collecting ducts derived from the ureteric bud (UB). How these genes drive specific stages of human kidney development is unclear. Moreover, the precise biological aberrations in human RTMs associated with defined mutations are unknown. Recently, several new human pluripotent stem cell (hPSCs) protocols have been developed to generate human cells of the major kidney lineages, generating nephrons and collecting tubules. Hepatocyte nuclear factor 1-beta (HNF1B), a homeodomain-containing superfamily of transcription factor, is known to regulate embryonic kidney development. Mutation in HNF1B gene leads to 'renal cysts and diabetes syndrome' (RCAD) generating poorly branched collecting ducts terminating in cysts, and lack of functional nephrons. In this project, we aim to employ stem cell biology and the cutting-edge gene editing CRISPR/Cas9 tools to understand pathomechanism of RTMs. The wild-type hPSCs and CRISPR-edited lines that harbour HNF1B mutation will be differentiated to 3D organoids in parallel and phenotypically compared to study roles of HNF1B in kidney development. Formation of 3D disease model of RTMs in vitro are expected to be used in testing novel therapies that ameliorate aberrant cellular functions. We predict that the project will recapitulate morphological aspect of kidney with a full complement of cell lineages and components displaying key physiological functions, such as glomerular filtration. Essentially, in the future, application of CRISPR/Cas9 to correct HNF1B mutation will facilitate understanding of molecular and cellular pathobiology with a view to impede or prevent kidney disease progression.

4. Classification and name of biological agent/material to be used in the study:

i.	Infectious or potentially infectious agent/material	✓
ii.	Biological toxin	
iii.	LMO/GMO/CRISPR/Synthetic DNA (Proceed to part 5)	✓
iv.	Others	

5. Description of the LMO(s)

No.	Common and scientific name of donor organism	Common and scientific name of parent/ recipient organism	Vector(s) or method of genetic modification	Identity and function of gene(s) of donor organism responsible for the modified trait	Target organism(s) of the LMO	Target tissues for genetic modification
1.	Sendai Virus	Murine respirovirus/ murine parainfluenza virus type 1 or hemagglutinating virus of Japan (HVJ)	CytoTune™-iPS 2.0 Sendai system	polycistronic Klf4–Oct3/4–Sox2, cMyc, and Klf4.	Mammalian cells	Human induced pluripotent stem cells
2.	CRISPR	Bacteria (Streptococcus pyogenes)	Neon electroporation system	Cas9 nuclease	HEK293T and iPSCs	Kidney organoids

6. Risk group of agent/material or toxin (refer to Classification of Microorganisms into Risk Group at http://iium.edu.my/media/48111/IIUM%20Biosafety%20Guideline.pdf)	7. Biosafety level where the work will be performed:
☑ 1 ☐ 2 ☐ 3 ☐ 4 ☐ Unknown	☐ 1 ☑ 2 ☐ 3 ☐ 4

I hereby declare that all information provided in this application is accurate to the best of my knowledge.

Assoc. Prof. Dr Tengku Faris
Department of Basic Medical Sciences
Kulliyyah of Nursing
International Islamic University Malaysia

Signature and stamp of PI **Date 16/11/2022**

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Decision by IBBC

☐ Notification Form (Form E) submission is required

☑ Exemption from Notification Form (Form E) submission

Assoc. Prof. Dr. Raha Ahmad Raus
Chairperson
IIUM Biosafety & Biosecurity Committee
International Islamic University Malaysia

Signature and stamp of IBBC Chairman **Date 18/1/2023**