Modulation of DNA repair pathways after CRISPR/Cas9 mediated Double Stranded Break in Porcine Cells

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Abstract (Academic)

The CRISPR/Cas9 system has become the predominant tool for genome editing. Targeted modifications can be introduced while repairing double strand breaks (DSBs), induced by the CRISPR/Cas9 system. The DSB is repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), and the repair is commonly processed through NHEJ because it is the dominant repair pathway in most cell types. The goal of this study is to modulate DNA repair system of somatic cells to increase the frequency of homology-directed repair (HDR) through HR by chemical treatment and the frequency of NHEJ by serum starvation. CRISPR/Cas9 systems targeting *RAG2* gene and donor DNA to replace endogenous *RAG2* were transfected into porcine fetal fibroblast (PFF) cells and the cells were treated with various chemicals that were known to inhibit NHEJ or stimulate HR. Among the chemical treated groups, cells treated with thymidine showed an average of 5.85-fold increase in HDR compared to the control group; the difference ranged from 1.37 to 9.59. There was no positive effect on the frequency of HDR after treating transfected cells with other chemicals. Placing PFFs under low amount of serum (serum deprivation) could enrich the cells in G0/G1 phase, but there was little difference in the frequency of NHEJ. Our results indicate that modulating DNA repair pathways during CRISPR/Cas9-mediated gene targeting could change the outcome of the targeted events.

Abstract (public)

The CRISPR/Cas9 system is the newest generation of genetic engineering tool for genome editing. Genetic modifications can be introduced while repairing double strand breaks (DSBs) on DNA, induced by the CRISPR/Cas9 system. The DSB is repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), and the repair is commonly processed through NHEJ because it is the dominant repair pathway in most cell types. The goal of this study is to modulate DNA repair system of somatic cells to increase the frequency of homology-directed repair (HDR) through HR by chemical treatment and the frequency of NHEJ by serum starvation. Among the chemical treated groups, cells treated with thymidine showed an average of 5.85-fold increase in HDR compared to the control group; the difference ranged from 1.37 to 9.59. There was no positive effect on the frequency of HDR after treating transfected cells with other chemicals. Placing pig fibroblast under low amount of nutrient could enrich the cells in G0/G1 phase of cell cycle, but there was little difference in the frequency of NHEJ. Our results indicate that modulating DNA repair pathways during CRISPR/Cas9-mediated gene targeting could change the outcome of the targeted events.

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Abstract(public)

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List of abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein-9 nuclease
CMAH	Cytidine mophosphate-N-acetylneuraminic acid hydroxylase-like protein encoding gene
CFTR	Cystic fibrosis transmembrane conductance regulator
DNA	Deoxyribonucleic acid
B	Double stranded break
ES	Embryonic stem
ESC	Embryonic stem cell
FACS	Fluorescence-activated cell sorting
GE	Genetically engineered
GGTA1	Glycoprotein galactosyltransferase alpha 1, 3
GTKO	Glycoprotein galactosyltransferase alpha 1, 3 Knock Out
G0	Gap 0
G1	Gap 1
G2	Gap 2
HAR	Hyper acute rejection
HR	Homologous recombination
HDR	Homology directed repair

IL2RG	Interleukin 2 Receptor Subunit Gamma
KO	Knock out
NHEJ	Non homologous end joining
NT	Nuclear transfer
PAM	Protospacer adjacent motif
PCR	Polymerase chain reaction
PFF	Porcine fetal fibroblast
PPAR-γ	proliferator-activated receptor gamma
RAG2	Recombination activating gene 2
RNA	Ribonucleic acid
S	Synthesis
SCID	Severe combined immunodeficiency
SCNT	Somatic cell nuclear transfer
TALEN	Transcription activator-like effector nuclease
TALE	Transcription activator-like effector
ZFN	Zinc finger nuclease
ZFP	Zinc finger protein

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Chapter 1: Literature Review

Pigs as a research model

The pig has been an attractive model for a wide range of scientific and medical research due to its similarities in anatomical and physiological characteristics shared with humans (VodiČKa et al., 2005). Pigs are also known to require minimal environmental control for housing, and effective management tools are available amongst livestock animals (Rehbinder et al., 1998). Moreover, pigs are highly reproductive, displaying early sexual maturity within 5-8 months, a short gestational period (114 days), and an all-season breeder (Wolf et al., 2000).

The most significant use of pigs in current scientific research is for biomedical research and xenotransplantation. The mouse has been the leading model for biomedicine because of the ability to alter its genome (Thomas and Capecchi, 1987), but the ability to modify the genome in pigs, i.e. production of genetically engineered (GE) pigs, has expanded the use of pigs in various research fields (Dai et al., 2002; Lai et al., 2002). Broad application of GE pig models is used in immunology and disease studies with using severe immuno-deficient pigs known as SCID pigs. SCID pigs are being well used for developing new therapies for human diseases and regenerative medicines (Fan and Lai, 2013; Prather et al., 2013; Suzuki et al., 2016). Another popular application of GE pig models is in xenotransplantation studies. Xenotransplantation refers to the interspecies transplantation of living cells, organs, or tissues (Prather et al., 2013). It is one of the most commonly proposed alternatives to allograft, same-species transplant organs, to resolve the current donor organ shortage issues (UNOS, http://www.unos.org). The concept of xenotransplantation was

discovered centuries ago, however, the procedure is technically challenging that advancement of xenotransplantation study was difficult. Nonetheless, it was tried in several occasions but failed due to strong immune responses. Suppression of this severe immune responses were required for xenotransplantation study to advance further. To resolve this issue, researchers developed a way to suppress the immune responses caused by xenotransplantation by inactivating the genes which are suspected to cause the severe rejection and immune responses (Whyte and Prather, 2011). However, its progress has been slow, until recently when gene editing with meganucleases was discovered. This technology will be explained later in this literature review.

1. Conventional approach to generate GE pigs

The first generation of transgenic pigs was generated for agricultural purposes more than three decades ago. Direct introduction of genetic materials by pronuclear injection was used to produce the animals (Hammer et al., 1985). However, the efficiency of this approach was poor (1-10%), and many founder animals were required to establish a line of transgenic pigs carrying intended phenotype. Another disadvantage of this approach was that it could only introduce exogenous expression of foreign genes. Therefore, targeted modifications (i.e. knockout models) were not yet possible. The biggest limitation for GE pig production has been the lack of authentic embryonic stem (ES) cells, which is the key for generating knockout mice. Combination of ES cells and homologous recombination was critical in establishing a system for the knockout mice (Thomas and Capecchi, 1987). There has been extensive effort to establish ES cells in pigs, but until now there is no sign of true ES cells in the pigs. Porcine induced pluripotent stem (iPS) cell was suggested to be an alternative to ES cells (Esteban et al., 2009). However, production of chimeric offspring by introduction of

cells into host blastocysts has shown its limitations again (West et al., 2010). There was no germ-line contribution from the iPS cells which then it was suggested that the iPS cells should be a better donor for SCNT because they would require less reprogramming to reach full pluripotency state. However, current publications do not support this notion (Fan et al., 2013; Yuan et al., 2014; Lei et al., 2016).

Study for transgenic pigs became more efficient as utilization of SCNT allowed the first generation of mammal using adult cell. Birth of the first cloned mammal, Dolly, was an eye-opener for many researchers and became the pioneer in cloning technologies. Researchers found SCNT as an opportunity to introduce site-specific modifications in livestock. By combining SCNT and homologous recombination in somatic cells, the first GE pigs carrying site-specific modifications were reported (Lai et al., 2002). The approach revolutionized the use of pigs as an animal model in research and clinical studies. Although direct microinjection of engineered nuclease could generate GE pigs carrying targeted (Hai et al., 2014; Whitworth et al., 2014), it is not commonly applied.

1.1. *Somatic Cell Nuclear Transfer (SCNT)*

Somatic cell nuclear transfer (SCNT) is a technology applied in cloning, stem cell research, and regenerative medicine, by donating nuclei from a somatic cell to an enucleated egg. Use of SCNT in generating genetically engineered pigs was first successfully reported by two groups in 2002 (Dai et al., 2002; Lai et al., 2002).

The base technique of SCNT was first successfully attempted with *Xenopus laevi* in 1958 (Gurdon et al., 1958) by transferring a extracted nuclei of adult frog cell to an enucleated egg. This was an extension of work which transplanted nuclei from

embryonic blastula cells into an enucleated frog eggs in 1952 (Briggs and King, 1952). Successful attempt to use SCNT by Gurdon et al. (1958), demonstrated the possibilities of nuclear transfer and eventually lead to the development of nuclear transfer and cloning technologies used today.

Even with the success of nuclear transfers, scientists still struggled to perform nuclear transfer in mammals. Several attempts were given to nuclear transfer in higher mammal species. However, successful nuclear transfer reports were only made when early embryonic cells were used as the donor (Willadsen, 1986; Prather et al., 1987; Prather et al., 1989); it seemed impossible to clone a mammal from differentiated somatic cells. The birth of the first mammal cloned from laboratory cultured cells was achieved in 1996, followed by the successful birth of the first cloned sheep, Dolly (Campbell et al., 1996; Wilmut et al., 1997). This was the first report demonstrating that DNA of embryonic cell can be 'reprogrammed' and develop into a full term animal. This report encouraged other laboratories to utilize the SCNT technology, however, the efficiency of SCNT was extremely low and the process was labor intensive. The efficiency of cloning procedure is studied to be around 1% and efficiency of modified cell line generation is around 1 out of million.

The first piglets born from SCNT were reported in 2000, which used a slightly different technique from the sheep cloning used to generate Dolly (Betthauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000). When donor cell is transferred enucleated oocyte, it requires a signal to start its growth and division. However, due to the artificial insertion of genetic material, oocyte lacks its signal. Therefore, artificial activation also known as electrofusion, was require to stimulate oocyte with a shock and signals it to start its division. However, the artificial activation signal from the original method was sought to be insufficient to stimulate full term development of the porcine embryos. Polejaeva et al.

(2000) applied double NT strategy, where they have used pronuclei from NT fused oocyte to transfer to another enucleated oocyte. By doing this, they were able to achieve sufficient activation stimulus create a reconstructed zygote to develop full term. The report suggested that pig cloning might be more difficult than other species. However, within the same year, another report on the birth of cloned pigs by directly injecting fetal fibroblast nuclei into an enucleated porcine oocyte proved that the double NT strategy was unnecessary (Onishi et al., 2000).

1.2 GE pigs generated through conventional approaches

One of the most important accomplishments in GE pig study was the successful production of GE pigs carrying heterozygous mutation on the α1,3-galactosyltransferase gene (Dai et al., 2002; Lai et al., 2002). Glycoprotein α-galactosyltransferase 1 (GGTA1) or α1,3-galactosyltransferase gene is an enzyme responsible for the synthesis of α1,3 galactose epitopes. The importance of GGTA1 gene in study comes from where it is lost during evolution in humans and primates. With primates and human not having the GGTA1 epitope, they have developed antibodies which would react to show a strong immunological response to the tissues or cells transferred from animals with functional GGTA1 gene. Therefore, if tissues or organs from different species such as pigs are transplanted, pre-formed anti-gal antibodies will immediately recognize the α1,3 galactose epitopes, resulting in hyper acute rejection (HAR) of the transplanted tissues or organs. By disrupting GGTA1 gene, it was predicted to reduce or eliminate the production of α1,3 gal epitopes, leading pig organs or tissues to avoid HAR. As expected, organs from homozygous GGTA1 knock-out pigs, produced based on the heterozygous knock-out lines (Phelps et al., 2003a; Kolber-Simonds et al., 2004), demonstrated that the disruption of GGTA1 could extend the life of kidney and

heart without the HAR when transplanted into non-human primates (Chen et al., 2005; Kuwaki et al., 2005; Tseng et al., 2005; Yamada et al., 2005).

Another significant GE pig model is cystic fibrosis transmembrane conductance regulator (CFTR) KO pigs. Cystic fibrosis is one of the most common genetic diseases in North America. Patients with mutated CFTR have altered movement of chloride ions across the membrane, leading to trouble with mucous secretion in many of their organs. Accumulation of mucus could frequently lead to infections and inflammations in organs, such as the lungs and pancreas. Even though they were previously introduced in mice models, they did not exhibit all of the expected phenotypes (Grubb and Gabriel, 1997). Thus, a new model to study cystic fibrosis was required. Heterozygote animals with KO has been created in 2008 (Rogers et al., 2008; Welsh et al., 2009) and both CFTR KO pig and F508 deletion pig successfully expressed expected phenotypes of cystic fibrosis in humans, providing a physically relevant model of cystic fibrosis for humans (Stoltz et al., 2010). This study demonstrated that previously unusable animal models could now be used along with deletion of the 508th amino acid, pheynylalanine, which is the most common mutation resulted in CF in human.

2. Application of meganucleases in GE pig production

2.1. Meganucleases (engineered endonucleases)

Meganucleases are endodeoxyribonucleases that recognize longer recognition sites compared to conventional restriction endonucleases. Typical recognition lengths of meganucleases are about 12 to 40 base pairs versus 4 to 8 bases for conventional endonucleases. Meganuclease consist of two main function, recognition and cleavage. The recognition sites of

meganucleases can be engineered to multi-base pair sequences on the genome. Incorporation of cleavage domain (FokI or Cas9) could cleave the target sequence. The engineered meganucleases can be used as a molecular DNA scissor to cleave a specific region of the genome and induce a double stranded break (DSB) at the location. Because DSB is harmful to cell's survivability, formation of DSB will trigger the DNA repair pathway, which could then introduce targeted modifications. During the DSB repair process, the presence of template DNA can induce site-specific recombination. Without the available template DNA, the DSB is repaired by non-homologous end joining (NHEJ), which often introduces short DNA insertions or deletions, called indels. The indels could induce targeted gene knockouts by frameshift. For instance, a functional protein is formed with group of amino acids, which is formed with nucleotide triplet codons. Due to this characteristic, frameshift in nucleotides could change the order of nucleotides coding amino acids to make proteins. This will eventually lead to form a premature stop codon thus inactivate the function of protein (Christian, 2010).

The first generation of meganucleases, zinc finger nucleases (ZFN), combines the non-specific cleavage domain of FokI endonuclease with zinc finger proteins (ZFPs) that can recognize specific sequences on the genome. The ZFNs can deliver a site-specific DSB to the genome (Urnov, 2005). Each zinc-finger is designed to recognize a three base pair. By connecting multiple zinc-fingers, zinc-finer molecules will recognize longer base pair spans. These finger-like modules formed with multiple zinc-fingers also contains the non-specific cleavage domain FokI. When zinc-fingers recognize and bind to a specific target sequence, FokI domain's function is to cleave the recognized sequence (Miller et al., 2005). However, FokI is only functional when they form dimers. In order for FokI domain to dimerize, two individual ZFNs would have to be introduced to the cell at the same time, each binding to opposite strands of the DNA to form FokI dimers to cleave the region. This requirement of

dimerization of FokI domain is also known to increase the specificity of gene targeting (Doyon et al., 2011).

Another novel DNA binding domain was described in a family of proteins known as transcription activator-like effectors (TALEs) (Boch et al., 2009). TALEs are proteins that are secreted from Xanthomonas bacteria which cause disease on many types of plants. Xanthomonas bacteria's type III secretion system secretes TALE to infect plants (Boch and Bonas, 2010). TALE consist of 30-35 amino acid sequences which can be fused with the same cleave domain FokI to form TALE nuclease (TALEN). TALEN can act as an engineered endonuclease to recognize specific sequences on the genome and induce DSB using the same mechanism of FokI domain from ZFN to cleave the specific site

Both ZFNs and TALENs offer a similar systematic approach to engineer DNA; however, both systems are not extremely specific and often lead to off-target cleavages. This off-target cleavage may lead to the production of enough double-strand breaks to overwhelm the repair machinery and consequently yield chromosomal rearrangements and/or cell death (Claudio Mussolino, 2011). Also, these two generations of meganucleases are costly and time-consuming to engineer.

More recently, a new tool based on a bacterial CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR associated protein 9 (Cas 9) from Streptococcus pyogene was developed. CRISPR/Cas9 genome editing tool derived from a type II CRISPR system, which is an essential adaptive immunity mechanism to eliminate invading genetic materials in select bacteria and archaea (Garneau et al., 2010).

There are two distinct and required components to this system, guideRNA (gRNA) and Cas 9 endonucelase (Ran et al., 2013b). Invading DNA from viruses or plasmids, will be cut into small pieces and memorized in the CRISPR locus as a series of short repeats. These loci

can be processed to generate small RNAs known as protospacers, which can memorize and recognize invading DNA in the future encounters (Jinek et al., 2012; M et al., 2012; Srivastava et al., 2012). When genomes are excised as protospacers, locations of excision are not randomly selected. Instead, they were found adjacent to a short DNA sequence, which is now known as protospacer adjacent motif (PAM) sequence (Bolotin et al., 2005; Deveau et al., 2008). Thus, PAM sequence which is known to be in order of 5'-NGG in type II CRISPR system, is required for precise sequence excision. This mechanism is known to be developed to maintain the regular spacer size in CRISPR array (Erdmann and Garrett, 2012; Diez-Villasenor et al., 2013).

gRNA consist two important RNA pieces called CRISPR targeting RNA(crRNA) and trans-activating crRNA(tracrRNA) (Ran et al., 2013b). crRNA is the targeting sequence transcribed from the protospacers. Transcribed array of crRNA is processed to each individual recognition sequence between the repeats. Processing of crRNA sequence is dependent on tracrRNA which has sequence complementary to the palindromic repeats. When gRNA is bound to the recognition site, tracrRNA hybridize with palindromic repeats to recruit and trigger the binding of Cas 9 endonuclease to form gRNA/Cas9 complex (Jinek et al., 2012; M et al., 2012; Srivastava et al., 2012). Formation of gRNA/Cas9 complex will cleave the targeted region of the genome, inducing DSB.

By developing and engineering this system, now we are able to choose and design a gRNA to be recognized as a foreign genetic material. The gRNA sequence of choice must be a sequence immediately followed by PAM sequence to deliver the excision when Cas 9 endonuclease is recruited. When Cas 9 endonuclease induce DSB in target region, the location can be used for genetic mutation or modification.

2.2 GE pigs generated utilizing meganucleases

One of the most important models for GE pig study, GGTA1 KO pig, have developed to become a better model as GGTA1/CMAH KO pig, GTKO/hCRP pigs, and GTKO+additional gene KO, after multiple gene manipulation became possible after utilization of meganucleases (McGregor et al., 2012; Kwon et al., 2013; Lutz et al., 2013; Azimzadeh et al., 2015). These models have proven its effectiveness and efficiency of meganucleases and its multigene manipulation capabilities. Meganucleases' benefits integrated in many future studies and became the most important focus of the GE pig production (McGregor et al., 2012; Azimzadeh et al., 2015).

Severe combined immunodeficiency (SCID) pig models are also a good model that is actively being studied. One of the most studied target gene is recombination activating gene 2(RAG2), which is associated with V(D)J recombination initiation during B and T cell development and have also used in our study. *RAG2* KO pigs are known to be immunodeficient, which could be used as a novel model for biomedical research (Huang et al., 2014; Suzuki et al., 2016). Even further, *RAG1/RAG2* KO (Huang et al., 2014) and *RAG2/IL2RG* (Lei et al., 2016; Suzuki et al., 2016) which has delivered more severe immunological defects than RAG2 KO models have developed after multi-gene manipulation became possible with engineered meganucleases.

Only a few additional models had been reported since the first report of GE pigs carrying site-specific modification using only SCNT (Dai et al., 2002; Lai et al., 2002). However, since the first report of GE pigs using meganucleases, more than ten different models have been reported. Therefore, the development of meganucleases is revolutionizing the way GE pigs are produced and used in research fields.

3. Genetic engineering of somatic cells by transfection

There are two main categories of methods to introduce exogenous genetic materials into eukaryotic, transduction and transfection. Transduction is a methods mediate delivery of the gene by utilizing the ability of a virus to inject its DNA to a host cell (Cepko and Pear, 2001). However, there are some drawbacks of using viruses, being labor intensiveness, risks of newly formed virus and mutagenesis, and some risk of random insertion. Therefore, transfection is more commonly used. Transfection is a process of introducing exogenous genetic material, such as DNA by a non-viral method into eukaryotic cells (Medicine, 2011). There are various methods of transfection available. Two main categories of these methods can be divided in to chemical based and non-chemical based methods (Kamimura et al., 2011).

3.1 Chemical-based transfection

Chemical based transections are based on types of chemicals to deliver genetic materials into cells. Chemicals include cyclodextrin (Menuel et al., 2008), polymers (Fischer et al., 2002), or a nanoparticle known as liposome (Whitt et al., 2001). All chemical based transfection behaves in similar ways to activate or modify cell-membranes to uptake its particles with genetic materials such as DNA or RNA, so the cell can integrate newly delivered genetic materials.

One of the most often used and efficient methods in chemical based transfection is lipofection or liposome transfection. It is a technique used to insert genetic materials into a nanoparticle called liposome, which behaves as a spherical vehicle that can merge into the cell membrane and deliver functional DNA into the cell, since both liposome and the cell membrane are formed by a phospholipid bi-layer (Felgner et al., 1987).

3.2 Non-chemical methods

Compared to chemical methods that are available, non-chemical methods mostly rely on physical treatment including cell squeezing (Sharei et al., 2013), microinjection (Ittner and Gotz, 2007), electroporation (Neumann et al., 1982).

One of the most popular methods for non-chemical methods being used recently is electroporation. Electroporation (gene electro-transfer) will transiently increase the cell membrane permeability by exposing the cell population to single or multiple short pulses of an intense electric field. Delivery of an electric field is done by electroporation, which enables adjustment of an electric field, that are suitable for certain type of cells (Neumann et al., 1982).

4. DNA Repair pathway and cell cycle association

4.1 DNA repair pathway

Double stranded breaks on DNA are the most critical damage to the cell that could easily induce genomic mutations, instability, and apoptosis, eventually leading to cell death (Rich et al., 2000). The DSBs can be generated by DNA damaging agents and conditions, endogenous and environmental stresses, and as recombination intermediates. With such critical consequences of DSBs, repair system of DNA DSBs has evolved and triggered to increase cell's survivability. There are two major repair pathways to resolve such damage, NHEJ or HR (Helleday et al., 2007).

NHEJ modifies the broken DNA ends and re-ligate each end together without requiring a template or sister chromatids (Moore and Haber, 1996). However, NHEJ often generate insertions or deletions during re-ligation (Heidenreich et al., 2003). On the other hand, HR uses

an undamaged DNA template as a homology to direct DNA repair. Present sister chromatid or given template DNA will be used as a homologous pair to repair the break, leading to reconstruct the original sequence back (Thompson and Schild, 2001). With characteristic of using homology, HR is known to be error-free and starts to be employed in the late S to G2 phase (Takata et al., 1998).

These two general DNA repair pathways are carried out by multiple step reactions, requiring different core components. NHEJ in eukaryotes carries its unique family of components to be recruited to become functional. Eukaryotic Ku, consist of DNA end-binding protein Ku70 and Ku80, which will interact and bind with free ends of DNA breaks (Smith and Jackson, 1999). Binding of Ku proteins will lead to the recruitment of the catalytic subunit DNA-PKcs, which is essential for efficient progression of NHEJ reaction, to form a complex (Kurimasa et al., 1999). The complex of Ku and DNA-PKcs will bring two broken ends closely and join them. This joining will interact with a catalytic subunit DNA ligase IV its cofactor XRCC4 to form a complex. This DNA ligase IV-XRCC4 complex will interact with XRCC4-like factor (XLF), to form tighter complex and stabilize (Bryans et al., 1999; Yano et al., 2008). XLF is reported   to promote re-adenylation of DNA ligase IV, which allows it to catalyze the next ligation step (Riballo et al., 2009).

Homologous recombination also has its own family of components to accurately repair a double strand break. Core components of HR widely vary among different types of organisms and cell types, although they all share the same basic steps and similar functions.

When DSB occurs in eukaryotes, a complex combined of MRE11/RAD50/NBS1 (MRN) binds to DNA on either side of the break. Binding of MRN will recruit Sae2 protein where two protein trim out the 5'ends of either side and provides resection of a DSB, 3′ single-stranded DNA (ssDNA) overhangs (Mimitou and Symington, 2009). When 3' ssDNA overhangs are created,

RAD52 will recognize and bind to the end of those overhangs to promote assembly of Rad51. In this reaction, Rad51 protein will start searching for a homologous template DNA and form joint molecules between the overhangs (Bhattacharyya et al., 2000). Proteins known as Rad54, Rad55, Rad57, BRCA1, and BRCA2 are required for promotion in formation of sub-nuclear Rad51 complexes. Collectively, these proteins contribute to the damage repair by homologous recombination (Sung, 1997; Bhattacharyya et al., 2000; Yang et al., 2005). When a homologous template is available, Rad51 proteins will assemble with ssDNA to form the helical nucleoprotein filament, which promotes DNA strand invasion (Ogawa et al., 1993; Sung and Robberson, 1995). DNA strand invasion will occur between the 3' ssDNA overhang and the homologous template available, which will create a displacement loop (D-loop). After this invasion, a DNA polymerase starts to synthesize new DNA, extending the end of 3' ssDNA overhang, which will lead to create a cross-shaped structure called a Holliday junction (Schwacha and Kleckner, 1995). After the formation of the Holliday junction, DNA polymerase will continuously synthesize DNA, effectively restoring the homologous template strand that was displaced during strand invasion. After both strands are stable, a nicking endonuclease cuts one strand of DNA, resolute to two separate repaired DNA strands.

4.2 Cell cycle

Cell cycle is an essential mechanism for cell survivability and sustainability, by regulating the timing and frequency of cell division and DNA replication (Reece and Campbell, 2011). The mechanism ensures that cells are accurately replicated and control its quality. It is controlled by a complex network of signaling pathways and proteins which are responsible to intra and extracellular signals about cell size and developmental program.

Although the cell cycle is complex at the molecular level, it can be categorized into two stages, interphase and mitosis. Interphase refers to the resting stage between cell divisions and prepares the cell to be accurately divided during the mitosis period. Mitosis is stage of cell cycle where division of nucleus occurs. During mitosis, duplicated chromosomes from interphase will condense and attach to spindle fibers that pulls one copy of each chromosomes to the opposite side of cells. These two identical daughter nuclei will then go through cytokinesis, where cell divides and produce two daughter cells (Reece and Campbell, 2011). Mitosis lasts for a comparatively short period of time, taking approximately 5% of the cell cycle, where the remaining time is spent in interphase.

Interphase prepares cells for cell division. There are four phases of interphase: gap0 (G0), gap1 (G1), DNA synthesis (S) and gap2 (G2) (Reece and Campbell, 2011). In G1, the cells are metabolically active to grow and synthesize RNA and proteins required for various cell functions and for progressing through the next phases. During the G1 phase, it has an important check-point mechanism also known as G1/S check-point, which ensure cells are ready to pass on to the S phase. This mechanism recognizes that if cells are not necessary to be duplicated, it can lead to delay G1 or enter G0 phase, also known as resting stage. In G0 phase, cells machinery dismantles and remains until there is reason for them to divide. If this check-point confirms that DNA replication is successful and cell division is necessary, then it will let cells move on to the S phase. Then during the S phase, DNA replication occurs. In this process, enzyme helicase will unbind the available DNA double helix for DNA polymerase to bind to each strand. DNA polymerase will then recruit free-floating nucleotides and bind to the separated strands, which creates two identical DNA. After accurate DNA replication, cells enter the G2 phase. G2 phase is known as a stage where cell will rapidly grow and stabilize to readies themselves for the next mitosis. G2 also carries its own check-point mechanism known as

G2/M check-point, which examines the genotoxic stress such as oxidation, UV radiation stress, DNA intercalating agents, etc. If the G2/M check-point recognize the cells are healthy and if DNA replication from S phase has occurred accurately. Finally, during the M phase, cell division occurs and divides them into two similar daughter cells. M phase also carries its own check point to ensure that all components are ready for its division.

4.3 Relation between the cell cycle and double strand break

DNA repair pathway is known to have good association with cell cycle although relative contribution on cell cycle phases are not fully understood. There are important characteristics that is thought to distinguish the frequency of NHEJ and HR throughout the cell cycle. NHEJ is thought to be the predominant pathway in all phases of the cell cycle, while frequency of HR is at the peak at the late-S and G2 phases (Rothkamm et al., 2003).

NHEJ does not require additional materials to repair the template, rather detect its damage and repair itself (Panier and Boulton, 2014). Therefore, DNA is predominantly chosen out of two throughout the cell cycle mainly due to lack of availability of chromatin homology. This characteristic makes NHEJ more prone to be utilized for repairing most of DNA impairment. As cells process through, HR becomes active in S and G2 phase, where sister chromatids and DNA templates are more easily found due to replication. However, as cells progress into G2 and M phase, condensation of chromosomes is increased, which makes it difficult to search for chromatin homology, where NHEJ becomes predominant again. If cell cycle is synchronized to G0/G1 phase, cells will dominantly take NHEJ as a repair pathway.

Homologous template would be predominantly present during and after S phase since it is where DNA replication begins and a template DNA is present(Mali et al., 2013b; Wang et

al., 2013; Yang et al., 2013). By synchronizing the cell cycle to S phase, there will be a higher chance for the genome to use HR as their repair pathway, which could lead to specific gene modification and error-free knock out.

4.4 Effect of cell cycle on SCNT

The relation between cell cycle and the outcome of SCNT became significant when Campbell et al. and Wilmut et al. successfully used the cell cycle synchronization approach to clone a mammal (Campbell et al., 1996; Wilmut et al., 1997). Their approach involved the use of serum deprivation to force cells to exit their cell division phase and synchronize themselves at the G0 stage. Their hypothesis was that these quiescent G0 stage somatic cells would be favorable to SCNT by providing a greater opportunity to reprogram DNA, given their altered chromatin state and reduced transcriptional profile, which could improve the chance of maintaining normal ploidy and prevent DNA damage (Campbell et al., 1996; Wilmut et al., 1997).

5. Manipulating cell cycle and DNA repair pathway

5.1 Chemical inhibition

The Efficiency of Cas9 mediated modification through NHEJ in murine genome from embryo is reported to vary between 20% -60% (Wang et al., 2013; Yang et al., 2013). Inheritance of HDR is comparatively lower at an efficiency of 0.5-20% (Wang et al., 2013; Yang et al., 2013). This could be a big difficulty for some applications where precise genome editing is required instead of random mutation occurrence.

Even though HR mainly occurs in G2 and S phases (Wang et al., 2013; Yang et al., 2013), occurrence is concurrent with NHEJ, that its occurrence is increased in NHEJ-deficient cells (Pierce et al., 2001). Manipulating this characteristic, few recent studies have reported that inhibition of NHEJ would improve the frequency of HDR in Cas 9 mediated mutations, using chemical based inhibition (Chu et al., 2015; Maruyama et al., 2015).

There are different chemicals that are known to inhibit or stimulate the important components of each phase of the cell cycle, which would allow synchronization to take effect in different phases. If cell cycle synchronization become easier, it could be used to manipulate the occurrence of either NHEJ or HR after a DSB. Ability to do so, it could be possible to choose desired repair system's occurrence frequency. Therefore, if we are able to manipulate and choose to increase or decrease NHEJ and HDR concurrence rate, application of genetic modification using meganucleases would likely to be more productive.

SCR7 is a known ligase IV inhibitor, which joins DSB during the NHEJ of repair pathway (Srivastava et al., 2012; Chu et al., 2015; Maruyama et al., 2015; Song et al., 2016). Similarly, Wortmannin is a known as PIKK inhibitor, which regulates DNA damage responses during NHEJ (Zhu and Gooderham, 2006). NU7026 is a known DNA-PKcs inhibitor, which is a catalytic subunit, which binds to the Ku protein to direct it to DNA ends to trigger its kinase activity to successfully perform NHEJ (Willmore et al., 2004; Zhu and Gooderham, 2006). These three chemicals above can be used to inhibit critical components of NHEJ pathway to reduce the chance of NHEJ to be a predominant pathway. By suppressing the DNA to take NHEJ as their repair pathway, the HR frequency could possibly increase.

Oppositely, there are some chemicals that can stimulate the HR key components' activities or synchronize cells at the S phase, when HR frequency is at the peak. Vanillin is a dietary antimutagen known to reduce random mutagenesis by enhancing recombination repair

(Lirdprapamongkol et al., 2005; Ho et al., 2009). Chemical thymidine is a well-known chemical that is used to arrest the cell cycle at S phase, which could possibly induce HR as pathway, as HR occurs only in G2 and S phase of cell cycle (Bootsma et al., 1964; Thomas and Lingwood, 1975; Griffin, 1976; Harper, 2005). RS-1 is a known RAD51 stimulator, which carries a central role in HR by catalyzing strand transfer from a damaged sequence and a undamaged sequence to allow resynthesize the DSB region (Jayathilaka et al., 2008; Song et al., 2016). These three chemicals above can be used to stimulate HR frequency, predominantly by stimulating critical HR components and environment.

5.2 Serum deprivation

Serum deprivation is done by eliminating serum in cell medium, which provides nutritional environment for cell cultures to grow. Severe deprivation (>1%) of nutrient, however, would rather provide insufficient environment for cells' survival, leading to cell death. With appropriate amount of deprivation (1%-2%), we could synchronize cells in G0/G1 phase of cell cycle without losing their proliferation capacity (Kues et al., 2000). This study suggests that a short period of serum deprivation terminates the mitotic activities after 48 hours, which significantly increased population of cells at G0/G1. Growth factor called mitogens are required in mammalian fibroblasts to progress through the G1 phase of cell cycle. However, when mitotic signals and activities are absent, cells could exit from the cell cycle into a quiescence state, which is known as G0 phase (Holley and Kiernan, 1968; Iyer et al., 1999).

The information is used in our study to design strategies to manipulate the activity of each DNA repair pathway (NHEJ or HR). First experiment is to increase the frequency of HDR, thus providing precise error-free gene editing, by introducing chemicals that can inhibit

NHEJ or increase the activity of HR. Second experiment utilizes cell cycle synchronization approach to maximize the activity of NHEJ because the activity of NHEJ is high when cells are in G0/G1 stage.

Chapter 2: Use of chemicals to increase the frequency of homology-directed repair after CRISPR/Cas9-mediated Double Strand Break

Abstract

CRISPR/Cas9 system has become the major tool for genome editing. Targeted modifications can be introduced while repairing double strand breaks (DSBs), induced by the CRISPR/Cas9 system. The DSB is repaired by either non-homologous end joining (NHEJ) or homologous recombination (HR), and the repair is commonly through NHEJ because it is the dominant repair pathway in most cells. However, targeted mutations introduced by NHEJ do not always silence target genes. The HR pathway, on the other hand, can introduce error-free mutations at the nucleotide level. The goal of this study is to increase the frequency of homology-directed repair (HDR) through HR by introducing chemicals that can either inhibit NHEJ or stimulate HR. A total of six chemicals were selected for this purpose; SCR7, RS-1, wortmannin, thymidine, vanillin, and NU7026. CRISPR/Cas9 systems targeting *RAG2* and donor DNA to replace endogenous *RAG2* through HDR were transfected into porcine fetal fibroblast (PFF) cells and the cells were divided into seven groups (six chemical groups and control) to examine the effect of the chemicals. When PCR was used to measure the frequency of HDR, there was an average of 5.85-fold increase compared to the control; the difference ranged from 1.37 to 9.59. Thymidine is known to interrupt the deoxy-nucleotide metabolism pathway, thereby halting DNA replication and arresting cell cycle at the S phase. There was no positive effect on the frequency of HDR after treating transfected cells with the other chemicals. Further studies will focus on how the thymidine treatment increased the efficiency of HDR.

Introduction

Use of a CRISPR/Cas9 system can dramatically increase the efficiency of gene targeting in cells (Cong et al., 2013; Wang et al., 2013). The CRISPR/Cas9 system utilizes the endogenous DNA repair system to introduce targeted genetic modifications. Specifically, target-specific double stand breaks (DSBs), generated by the CRISPR/Cas9 system, trigger the endogenous DNA repair mechanism to protect integrity of the genome (Figure 2-1(a)).

When DNA is damaged in the cell, DNA is repaired by two major pathways, homologous recombination (HR) or non-homologous end joining (NHEJ). DNA is predominantly repaired by NHEJ, since NHEJ does not require additional materials to repair the template. Due to this characteristic, NHEJ sometimes create insertion or deletion of nucleotides in sequence by mistake. Although this makes NHEJ more prone to DNA impairment, NHEJ can be easily be applied for causing genetic mutation and knock out (Panier and Boulton, 2014).

However, in S phase of the cell cycle, where replication occurs, the activity of HR is at the highest because it utilizes a repair template or sister template to repair its damage (Mali et al., 2013b; Wang et al., 2013; Yang et al., 2013). This remains to be true in repairing DSBs created by the CRISPR/Cas9 system. Even though only a small percentage of HR occurs in the natural environment, demand for homology-directed repair (HDR) is higher during CRISPR/Cas9-mediated gene targeting because it is required for specific gene modification and error-free gene-editing.

To stimulate the frequency of HDR during CRISPR/Cas9–mediated gene targeting, some studies introduced certain chemicals (Chu et al., 2015; Maruyama et al., 2015). These chemical treatment approaches either inhibit the crucial components of NHEJ so the prevalence of NHEJ could be lowered, or stimulate the crucial components of HR so there is a higher

chance of HDR to occur (Figure 2-2).

Other approaches such as cell cycle synchronization can affect the ratio between NHEJ and HDR because G0/G1 cells will preferably use NHEJ and S phase cells will have higher activity of HR. Six different chemicals (SCR7, Thymidine, Vanillin, RS-1, NU7026, wortmannin), known to inhibit NHEJ, stimulate HR, or synchronize cells at S phase (Willmore et al., 2004; Zhu and Gooderham, 2006), are used in this study to stimulate HDR after CRISPR/Cas9-mediate DSB.

Materials and methods

Construction of CRISPR/Cas9 Vector targeting *RAG2*

PX330 vector was obtained from Addgene (https://www.addgene.org/). The vector was digested with restriction enzyme BbsI (NEB) to linearize at 37°C water bath for 30 minutes. Digested linear PX330 vector was loaded on 0.8% agarose gel and electrophoresed at 100V for 40 minutes. After confirming the size of DNA, the linear PX330 was recovered through gel extraction using Thermo Scientific gel extraction kit. The extracted linear PX330 was used to ligate specific CRISPR sequences targeting porcine *RAG2*. Primers used in this study are listed on Table 2-1.

Ligation of *RAG2* CRISPR sequence into the PX330

Two CRISPR sequences, 20-bp sequence upstream of PAM sequence (5'-NGG) in *RAG2* exon 2, were selected for this experiment (Figure 2-3(a)). Forward and reverse oligo-primers corresponding to the identified guide sequences were placed into the linearized PX330

vector following a previous publication (Wang et al., 2013). These oligo-primers are designed to create overhangs by annealing. One µL of forward oligo-primer and one µL of reverse oligo-primer were diluted with 8 µL of annealing buffer, making 10 µL of reaction to be annealed using a PCR Thermal Cycler (Veriti). Product from this reaction was ligated into the linear vector using T4 ligase. The ligation mixture (1 backbone: 3 insert ratio) was incubated with T4 ligase (Thermo Scientific) solution over overnight at 17ºC.

Transformation (overnight)

Competent cells were mixed with 1 µL of the ligated product. First, the mixed solutions were put in ice for 30 minutes, then heat-shocked at 42°C for 30 seconds. After the heat shock, the mixture was placed back on ice for 2 minutes. Then 300 – 400 µL of LB medium was added. The mixture was placed at 37°C for 1 hour at 170rpm in a shaker. After an hour, 100uL to 250 µL of the transformant were plated on a pre-warmed LB agar plate with ampicillin; the volume depended on the growth and concentration of transformant in the media. After transformants were plated, the plates were incubated in an incubator at 37°C overnight. Bacterial colonies from the plates were inoculated with a toothpick, then transferred into 3 mL-5 mL LB solution for growth at 37ºC overnight.

Identification of PX330-RAG2 plasmids

From the previous liquid culture, DNA was isolated through miniprep using the Thermo Scientific mini prep kit. Potential PX330-RAG2 DNA from the miniprep were digested with EcoRI and BbsI. BbsI restriction enzyme sites were where they were digest to ligate annealed oligonucleotides to introduce gRNA sequence to PX330. EcoRI restriction enzyme

site exists within the site of BbsI restriction enzyme site. This characteristic can be used to analyze the successful ligation of annealed oligos. The digestion was done in water bath at 37°C for an hour, then the DNA was loaded on 0.8% gel for analysis. A single band indicated successful ligation of the annealed oligos, whereas two bands meant unsuccessful ligation. To confirm, the plasmid DNA was also sequenced to verify the *RAG2* CRISPR sequence in the vector. Using LB media from the confirmed final product, midiprep was done with Thermo Scientific midiprep kit, to gather a sufficient amount of DNA for transfection.

Porcine fetal fibroblast culture

Porcine fetal fibroblast (PFF) cells, established from a day 40 fetus, were used for the following experiments. Frozen cell stock vial from lab's previous experiments were used which was stored at passage number ranging from 1 to 2. Passage numbers of the cells used in this study was between 4 to 7. This range of passage numbers were set to minimize the difference in cellular age and growth capability. A frozen vial of the cells was quickly taken to a 37 ºC water bath for thawing. The cells were re-suspended and were split into 3 100 mm dishes with pipette, then 10-12 ml of cell culture medium were added in each dish for culture. The dishes were then incubated in CO_2 incubator until cells reached 70% confluency. Cell culture medium for these experiments was made by adding 15% of FBS and 1000x gentamycin into the base medium DMEM. The cell culture media was sterilely filtered through Bottletop Filters, 0.22μm PES, to remove any possible contaminants or substances.

Passaging the PFF

The PFF cells were grown to 70% confluency to obtain consistency in growth and

physiological characters. For a fresh stock from a frozen vial to reach 70% confluence, it generally took 4-5 days. When the cells reached 70% confluence, the cell cultures were passaged.

First, cell culture medium was removed, then the culture was washed with DPBS solution. DPBS wash is essential to remove protease inhibitors from FBS. 5mL of 0.25% Trypsin (Sigma Aldrich) were diluted with 5mL of DPBS to obtain a working concentration. The trypsin was then applied to the wash cell culture dish to detach cells from the culture dish. The digestion lasted for 30 seconds at maximum to reduce potential toxicity to the cells. Trypsin treated cells were then incubated for 3-5 minutes at 37 ºC to be disassociated from the culture dish and each other. Completely detached cells were washed with 10 mL of DPBS, then pipetted to 10mL flask for centrifugation. The cells were centrifuged at 1900rpm for 5 minutes. Then supernatants were removed carefully to obtain cell pellets. The washing step was repeated 2 times to remove any harmful or toxic substances. In the remaining cell pellets, 1 mL of culture medium was added to distribute passage cells to new cell culture dishes or flasks. Then 10 mL of 15% FBS cell culture medium were added in each dish. All passaged cell culture dishes were incubated at 37 ºC under 5% CO_2.

Measuring potential toxicity from chemical treatments

To observe viability and toxicity from chemicals used in this study, PFF cells were exposed to the chemicals. The cells were cultured in a 100mm dish at the concentration of $2x10^5$ then the culture was split into a 6 well dish. Each well was treated with different chemicals at given working concentration from Table 2-2. Observations were done at 48 hours and after another 48 hours for recovery. Images were captured for comparison.

Optimization of transfection system

Neon transfection system was used for transfection procedure. Generally recommended settings and optimization were available at Thermo Fischer Scientific website (https://www.thermofisher.com/); however, specific settings for porcine embryonic fibroblast were not available. To obtain the highest transfection efficiency, optimization of voltage, wave length, and number of pulses were conducted. GFP vector was used to optimize the setting. Settings for common types of cells were chosen from the available list and adjusted to 3 different optimum settings; 1400V/30ms/1pulse, 1600V/30ms/1pulse, and 1650V/30ms/1pulse (voltage/duration/number of pulses). These 3 settings were used to transfect GFP vectors, then the efficiency of transfection was observed by the expression of GFP under a fluorescence microscope. Five mg of GFP vector per $1x10^6$ cells were used in this transfection. The observations were done at 24hrs, 48hrs, and 72hrs, and images were captured to compare its efficiency. The most efficient setting was used in the further transfection procedures.

PX330-RAG2 Transfection

On day 0, cell cultures were inspected to have 70%-90% confluency and no signs of contaminants. First, cell culture medium was removed from the culture plate, then the cells were washed with DPBS without calcium and magnesium. The cells were trypsinized as previously described, then 10mL of DPBS solution was added to each cell plate and tapped gently to dislodge the cells from plate. When cells were dislodged, the cells were transferred into an empty 15 mL conical tube. The cells were centrifuged at 2000rpm for 5 minutes to obtain a cell pellet and the supernatant solution was discarded. After re-suspending cell pellets with 1 mL of DPBS, the concentration of the cells was measured using a cell counter (Bio-Rad) and diluted to obtain the concentration of $1x10^6$ per transfection. After the measurement, the

cell pellet was washed once more with DPBS.

PX330-RAG2 vector 1 and 2, and RAG2 HR vector (1500 bp) were used for the transfection. Five mg of each vector per 1.0×1^6 cells were used. Cell pellets prepared were re-suspended with 100ul of resuspension solution from Neon transfection kit, per 1×10^6 of samples. Then, transfer total volume of re-suspended solutions was mixed with RAG2 vectors.

Below are the settings on the Neon

Pulse Voltage	Pulse Width	Pulse Number	Cell density	Tip Type
1600V	30ms	1	1×10^6 cells/0.1mL	100uL

The cells were then transfected with the Neon following the manufacturer's instructions.

Chemical treatment

The transfected cells were carefully transferred to new 6 well dishes; equal number of cells were plated into each well. Three mL of cell culture medium with 15% FBS were added to each well for cells to enrich and recover. Then, after 4 hours, the cells were exposed to the chemical treatment; the concentration of chemicals was adjusted to the reported studies of each. (Table 2-2). One control group was set to have no chemical treatment. The cells were incubated with the presence of the chemicals for 2 days (Figure 2-4). Then the chemicals were removed from the culture and incubated for 3 days to recover. After the recovery, genomic DNA from each group was extracted using a genomic mini isolation kit (Invitrogen). The extracted DNAs from each sample were used to measure the frequency of HDR.

Homologous Recombination Analysis

The frequency of HDR on *RAG2* locus was measured by amplifying homology junction using PCR. HR donor primers were designed to flank each end of the homology arms to amplify the homology junction (Figure 2-3(b)). An equal amount (30ng) of DNA from each sample was used for the PCR reaction to compare the difference in HDR frequency among different groups. The amount of DNA was measured using a spectrophotometer. An endogenous gene *GGTA1* was used as a control to standardize any potential biased amplification from each genomic DNA sample. This process can also confirm the quality of DNA to show uniformity in result and the concentration measured with spectrophotometer was correct. PCR was carried out in the following condition; initial denaturation 95 ºC: 2minutes, 34 cycles of '95 ºC: 30 seconds 55 ºC: 30 seconds, 72 ºC: 30 seconds, and final extension at 72 ºC for 5 minutes. The products were analyzed on 2% gel electrophoresis. Intensity of each amplicon was calculated using Image J (NIH). The intensity of PCR products, indicating the frequency of HDR on *RAG2* locus, was divided by the intensity of *GGTA1* PCR products.

Statistical analysis

Differences in the frequency of targeting events were determined after analysis of variance (ANOVA) by using the PROC GLM procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were analyzed using the Tukey's test. All data are presented as mean±S.E.M.; significance was determined at the $P < 0.05$ level.

Results

PX330-RAG2 vector construction

Ligated PX330-RAG2 was double digested with BbsI and EcoRI. A single band shows successful ligation of the annealed oligos (lane 3 and 5 in figure 2-5(a). The double band shows original vectors with unsuccessful ligation (lane 1,2,4, and 6 in figure 2-5(a). Sequencing results of those ligated are in figure 2-5(b), where the sequence indicated by the blue box is the CRISPR sequence inserted into the linearized PX330.

Chemical viability and toxicity.

Treating the PFF cells with the chemicals for 48 hours limited their cell growth compared to the control group. However, when the chemicals were removed from the culture, all groups were able to recover at least enough to extract DNA for analysis. Some groups recovered slower compared to other treatments (Figure 2-6). The results suggest that the toxicity from the chemicals exist, but does not kill the entire population.

GFP Vector analysis – optimization of transfection

Efficiency of transfection was examined following three different settings (1400V/30ms/1pulse, 1600V/30ms/1pulse, 1650V/30ms/1pulse) on the Neon system as shown in Figure 2-7. The 1400V/30ms/1pulse setting gave significantly lower level of transfection efficiency than the other two. To optimize further, 2 additional trials of 1500V/30ms/1pulse, 1600V/30ms/1pulse, 1650V/30ms/1pulse, and 1700V/30ms/1pulse were done. 1500V/30ms/1pulse also showed similar result as 1400V/30ms/1pulse. However, as previously observed, 1600V/30ms/1pulse and 1650V/30ms/1pulse shown to be the most effective setting.

The 1700V/30ms/1pulse setting on the other hand, was too harsh that only minimal number of successful transfection was detected. During the transfection procedure, visible electric spark was observed in the higher voltage setting, such as 1650V/30ms/1pulse, 1700V/30ms/1pulse, which resulted in complete cell death. Based on the optimization, 1600V/30ms/1pulse were selected for transfection experiments to calculate the frequency of HDR during *RAG2* targeting.

HDR analysis using PCR

Mutations introduced by PX330-RAG2 and frequency of HDR was measured by PCR amplification followed by Sanger's sequence (Figure 2-8(c)). When PCR analysis was used to measure the efficiency HDR after 6 chemical treatments, gel electrophoresis analysis showed varying intensity for each chemical. However, an increase in the frequency of HDR was only detected from thymidine treated group compared to the control (Figure 2-8(a) and 2-8(b)). The difference was more noticeable when the intensity of PCR products was measured and graphed (Figure 2-9). The thymidine treatment increased the frequency of HDR by 5.85 fold on average. The other five chemicals (SCR7, Wortmannin, Vanillin, RS-1, NU-7026) had no change to the level of HDR in CRISPR/Cas9-mediated targeting.   However, there was no statistical difference among all group (P>0.05).

Discussion

The published knock-out efficiency using the CRISPR/Cas9 system ranges from 10% to 100% in vitro and 32.1 to 83.3% in vivo (Yang et al., 2014). However, the frequency of HDR is known to be much lower than the frequency of NHEJ. For instance, HR to NHEJ ratio

calculated by the number of random integrations over specific knock-in events is reported to be below 10% in the rabbit and other species (Wang et al., 2013; Zhu et al., 2014; Song et al., 2016). This limits broader application of CRISPR/Cas9 system because disease and research models often require a specific gene modification to be introduced, rather than random base-pair changes through NHEJ.

In this study, the goal was to develop an approach to increase the frequency of HDR during CRISPR/Cas9-mediated targeting by treating PFF cells with chemicals that are known to either inhibit NHEJ or stimulate HR. However, the majority of the chemicals that we used in this study were not as effective as we had predicted. RS-1, for example, did not significantly improve the frequency of HDR in porcine PEF cells although the RS-1 was reported to stimulate RAD51 protein, a key player in the HR complex (Jayathilaka et al., 2008). RS-1 was also reported recently to increase both TALEN- and CRISPR/Cas9-mediated knock-in efficiencies in the rabbit; approximately 2 to 4 fold increase in HDR efficiency was reported in two genes examined (Song et al., 2016). However, it was reported in 2005 that the overall rate of HR events was significantly reduced at least by 10 fold, when RAD51 was overexpressed at high level in Saccharomyces cerevisiae (Paffett et al., 2005). And the differences could be that Song et al. (2016) used rabbit embryos whereas this study was conducted using somatic cells which may have different DNA repair machinery active in the system. Their study also applied 2 different concentrations of RS-1, 7.5 uM and 15uM respectively, but only 7.5 uM treatment was effective whereas 15 uM had shown cytotoxicity and no significant improvement in knock-in ratio, implicating that the effect of RS-1 is species-specific and concentration dependent.

NHEJ inhibitor SCR7, also had no significant effects on improving the overall HDR rate in PEF cells. This ineffectiveness also differs from several recent reports, where SCR7 treatment on mouse embryos and mammalian cells has increased the HDR frequency by as

high as 10 folds (Chu et al., 2015; Maruyama et al., 2015; Singh et al., 2015). Our study also used the same concentration of SCR7 as in these studies, however, similar to RS-1, the effect of SCR7 also seemed to be specie-specific and concentration depended. Another reprot from Chu et al. (2015) showed that coexpression of adenovirus 4 E1B55K and E4orf6 were required along with the SCR7 treatment to improve the efficiency of HDR, which was not conducted in our experiment. In addition, a study reporting the effect of use of SCR7 in porcine somatic cell to increase the HDR efficiency recently was published. However, this study co-applied transient incubation of cells at low temperature also known as 'transient cold shock' to see some improvement (Park et al., 2016). This method has previously reported to increase the efficiency of gene targeting in both ZFN and TALEN (Doyon et al., 2010; Miller et al., 2011). Therefore, the effect of SCR7 of its own could be difficult to validate.

Among the 6 chemicals that were used in this study, NU7026 (Nutley et al., 2005; Urushihara et al., 2012; Bee et al., 2013), wortmannin (Delacôte et al., 2002), vanillin (Durant and Karran, 2003), are known to be DNA-PK inhibitors. The three chemicals act a similar way by inhibiting DNA-PKs family, which is a crucial component of NHEJ pathway. Although NU7026 is reported to effectively inhibit the NHEJ pathway (Veuger et al., 2003; Nutley et al., 2005; Urushihara et al., 2012; Bee et al., 2013), increase in HDR rate by NU7026 treatment has not been published. It has also shown in a previous study that overall DNA repair rate through both NHEJ and HR has significantly decreased by the NHEJ inhibition (Urushihara et al., 2012). We could speculate that NHEJ inhibition led to more apoptotic cells, rather than to take another repair pathway, HR. This is consistent with cytotoxicity we observed from the chemical treatment.

Thymidine treatment, on the other hand, showed to significantly improve the frequency of HDR in our study. Even though numerical difference in analysis was detected,

there were no significant difference in statistical analysis ($p>0.05$). We believe this is due to the heterogeneous variation and human error, by having 1 out of 4 replicates having minimal difference, and 1 out of 4 replicates having no difference among all groups of chemicals. Chemical thymidine is known to help synchronize cells at the S phase. Piedrahita et al (2004), used thymidine to enhance gene targeting efficiency in primary fetal fibroblast cell in cows, which has decreased the random integration by 54-fold and increased targeting efficiency by 6-fold (Mir and Piedrahita, 2004). Their results were consistent with earlier observations that thymidine treatment synchronized cells at the S-phase and enhanced the frequency of HDR (Lundin et al., 2002). Our result, consistent with the previous reports, showed that thymidine treatment could significantly increase the HDR frequency by average of 5.85 fold. Unfortunately, the difference was not statistically different ($p>0.05$). We suspect that the high heterogeneous variance on the thymidine group result in no statistical difference. Additional replication could provide a statistically significant difference. It is interesting that cell cycle synchronization approach led to the highest increase in HDR frequency. It would be that the chemical treatment was harsh enough to induce apoptosis in the cells whereas the cell cycle synchronization by thymidine is less toxic to the cells.

Overall, our findings have important implications for gene targeting in porcine fibroblast cells because it shows a potential to increase the frequency of HDR during CRISPR/Cas9-mediate gene targeting. This approach could have a broader application in

gene targeting in somatic cells, which can be used to generate genetically engineered animal models.

Table 2-1. List of primers

Primers	Sequence
RAG2 Oligo primer F	5'-ACACCGTATAGTCGAGGGAAAAGTAG-3'
RAG2 Oligo primer R	5'-AAAACTACTTTTCCCTCGACTATACG-3'
Genomic RAG2 F	5'-AAGGATTCCTGCTACCTTCCTCCT-3'
Genomic RAG2 R	5'-AGATAGCCCATCTTGAAGTTCTGG-3'
Genomic GGTA1 F	5'-GATAATAGCATATTGTCTCCTCTAGAAATCCCAGAGG-3'
Genomic GGTA1 R	5'-GAGTGATGTTTAGAACCTGAGTGGGTGTTC-3'
RAG2 HR Primer R	5'-CGTCTCAGACTCATCTTCCTCATCATCTT-3'
RAG2 HR Primer MidF	5'-GTTCCTTAAATAGATGACCCGGGCTAGCTG-3'

Table 2-2. List of chemicals, functions, solubility and final working concentration of each.

Chemicals	Functions	Solubility	Working Concentration
SCR7	NHEJ inhibitor(a Ligase IV inhibitor)	DMSO	50uM
NU7026	DNA-PKCs inhibitor	DMSO(soluble 3mg/mL at 60C)	10uM
Wortmannin	PIKK(DNA-PK family) inhibitor	DMSO	10uM
Vanillin	Reduces mutagenesis by enhancing recombinational repair.	Ethanol	0.5mM
Thymidine	Arrest of cell cycle at S phase	H2O	2mM
RS-1	RAD51 Stimulator	DMSO	10uM

(a)
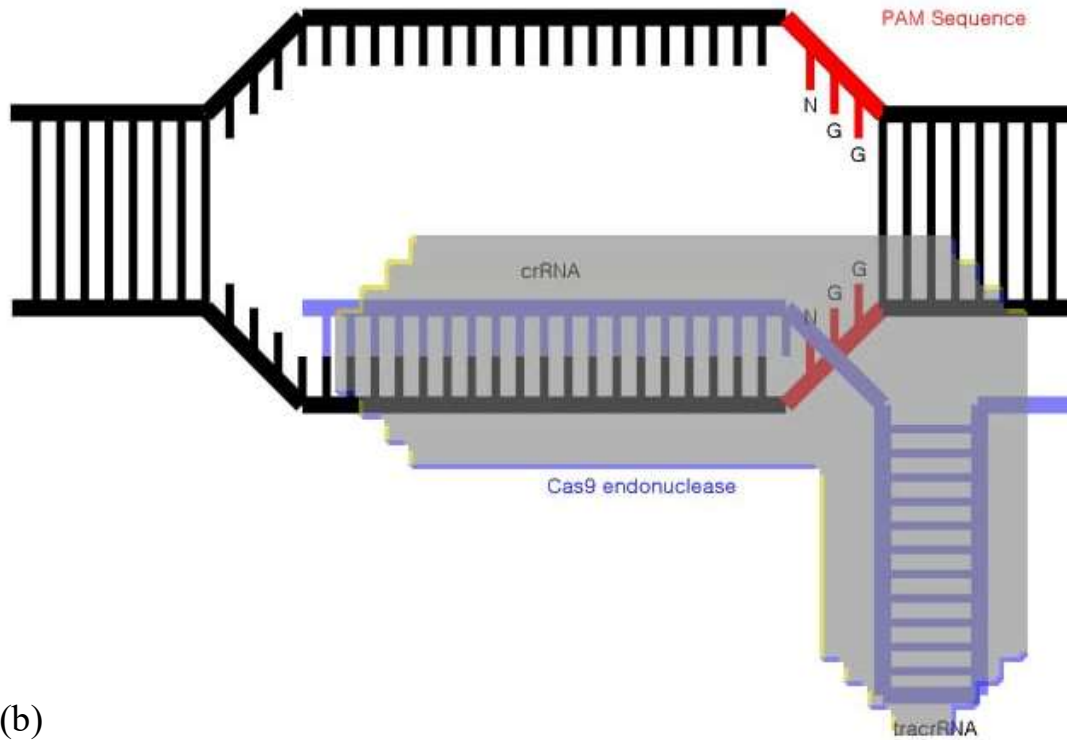
(b)
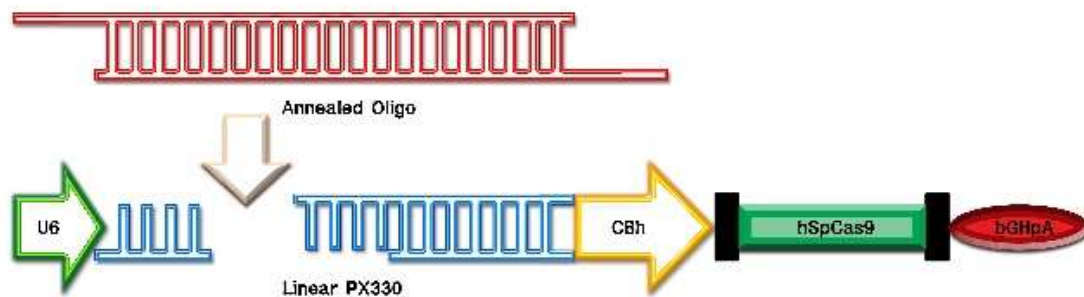
Figure 2-1. Construction of PX330-RAG2 vector to disrupt porcine RAG2 in PFF cells: (a)CRISPR/Cas9 Genome Editing: Cas9 endonuclease (gray) is recruited by a guide RNA (blue) which is composed of crRNA and tracrRNA. Target recognition is facilitated by protospacer-adjacent motif (PAM) sequence (red), where Cas9 mediates a double stranded break(DSB) -3bp upstream of the PAM.(b)Px330: Commercial form of CRISPR/Cas9 vector (PX330), which can be digested to a linear form and ligate with annealed oligo containing targeting sequence. Oligos representing porcine *RAG2* sequences were inserted into the vector to generate PX330-RAG2 vectors.
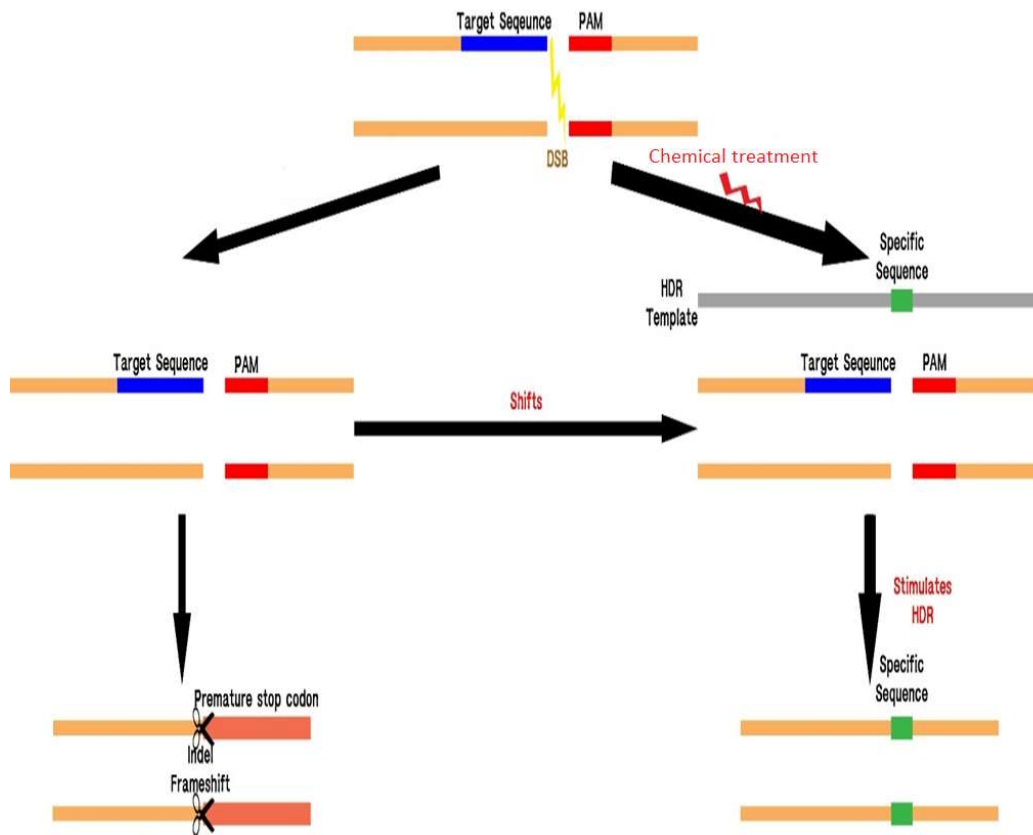
Figure 2-2. Schematic diagram of chemical treatment to either suppress NHEJ or stimulate HR: Chemical treatment will increase the chance of HDR occurrence during CRISPR/Cas9-mediated gene targeting, eventually leading to higher frequency of DNA repair through HR.
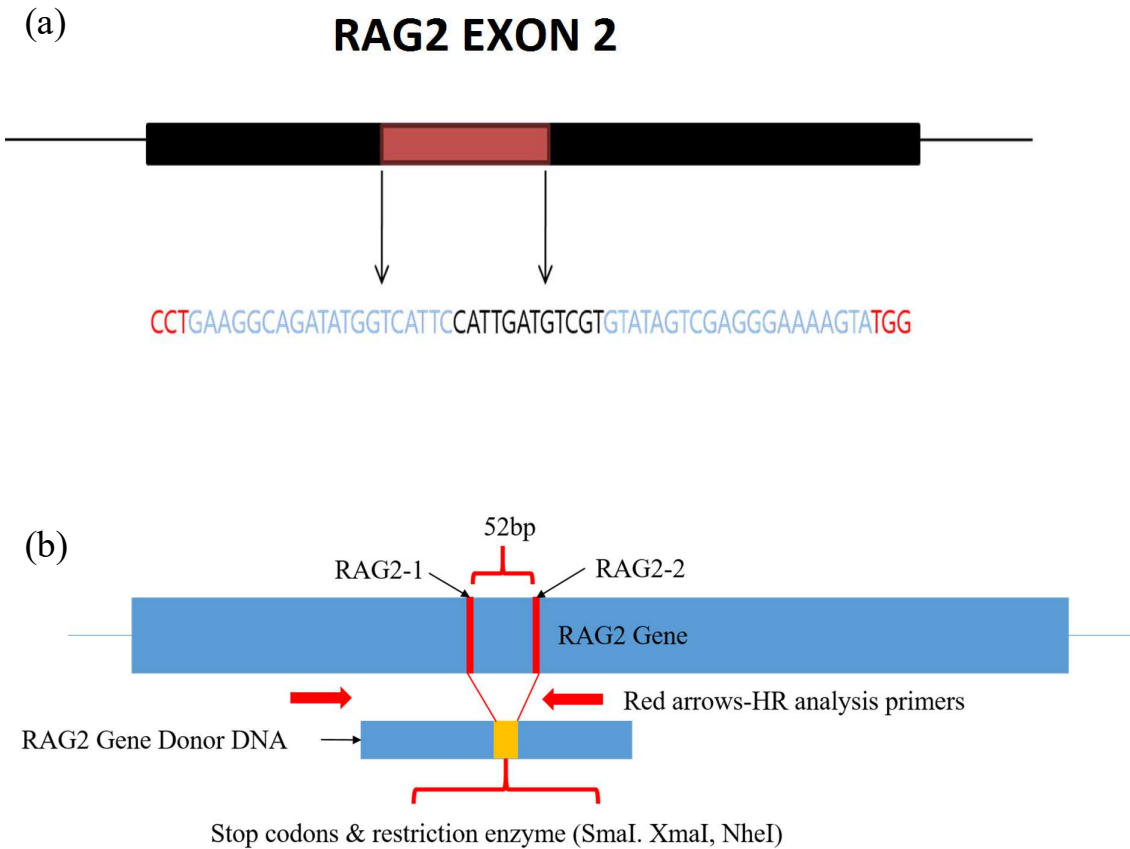
(a) **RAG2 EXON 2**

CCTGAAGGCAGATATGGTCATTCCATTGATGTCGTGTATAGTCGAGGGAAAAGTATGG

(b)

52bp

RAG2-1 RAG2-2

RAG2 Gene

Red arrows-HR analysis primers

RAG2 Gene Donor DNA →

Stop codons & restriction enzyme (SmaI. XmaI, NheI)

Figure 2-3. Strategy to disrupt porcine *RAG2*: (a) Designed CRISPR sequences on the *RAG2* exon 2. Blue sequences are two target sites (sgRNA), and red sequences are PAM (Protospacer adjacent motif) sites. (b) two RAG2 targeting vectors are designed to increase efficiency of targeting and induce intended deletion; two CRISPR sequences are 52 bp apart. RAG2 donor DNA will contain stop codons and restriction enzymes that will lead to complete knock out of the gene. Red arrows indicate the location of primers used to amplify a fragment of RAG2 gene flanking CRISPR/Cas9 induced DSB sites for genotyping.
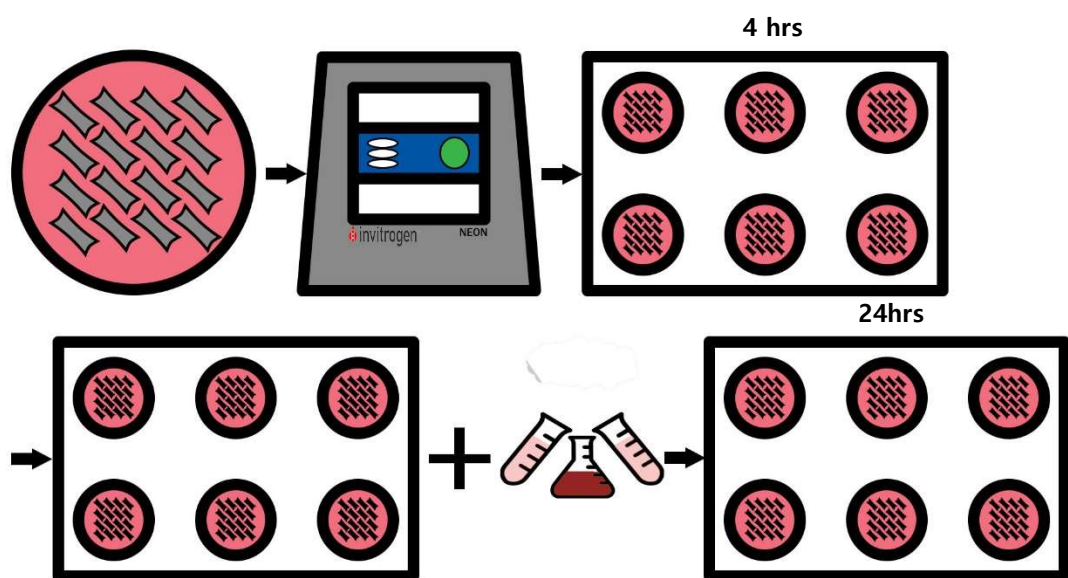
Figure 2-4. Experimental design to increase the frequency of HDR using chemical treatment: PFF cells were cultured and transfected using the Neon system. The transfected cells were split into a 6 well dish and each dish were chemically treated with chemicals listed in table 2.
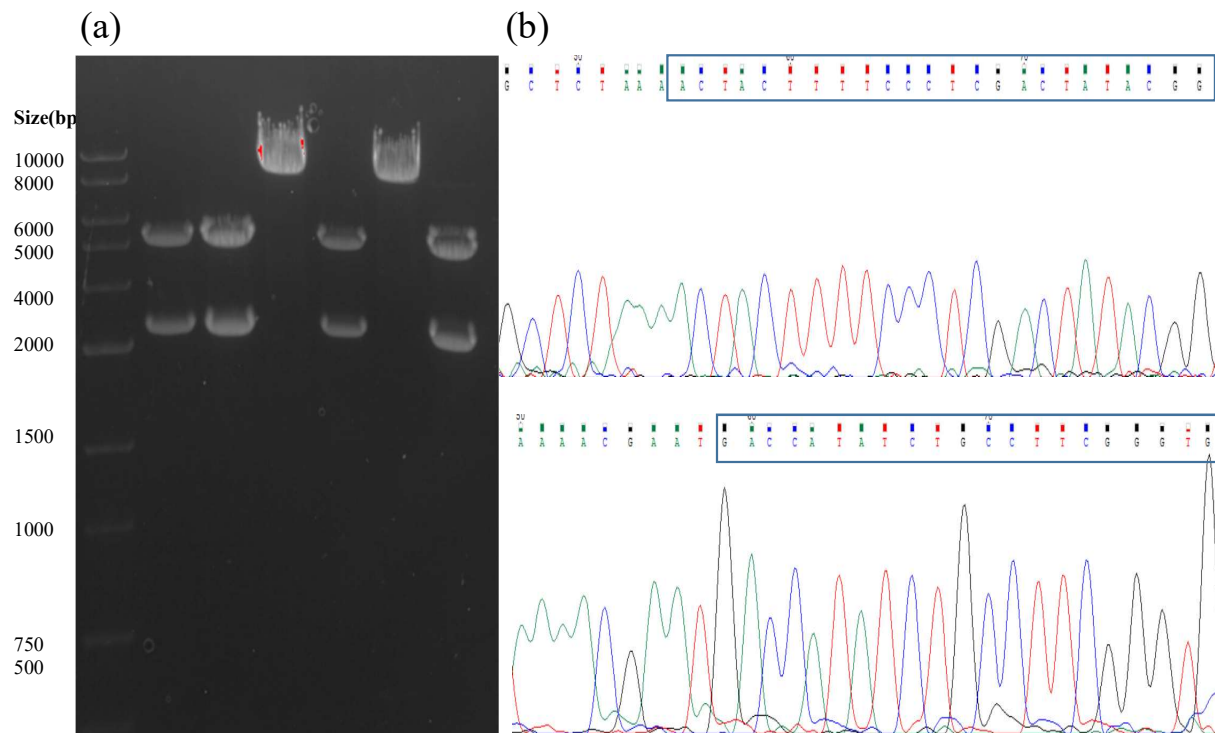
Figure 2-5. Analysis of PX330-RAG2 vector: (a)Gel analysis: Isolated DNA from each bacterial colony are double-digested with BbsI and EcoRI. Single band indicates successful ligation of the annealed oligo into the linearized PX330 (Lane 3 and 5), whereas double band shows original vector only with unsuccessful ligation (Lane 1,2,4, and 6). (b)Genomic RAG2-1 and 2-2 vector: Highlighted regions are the ligated RAG2 targeting CRISPR sequence.
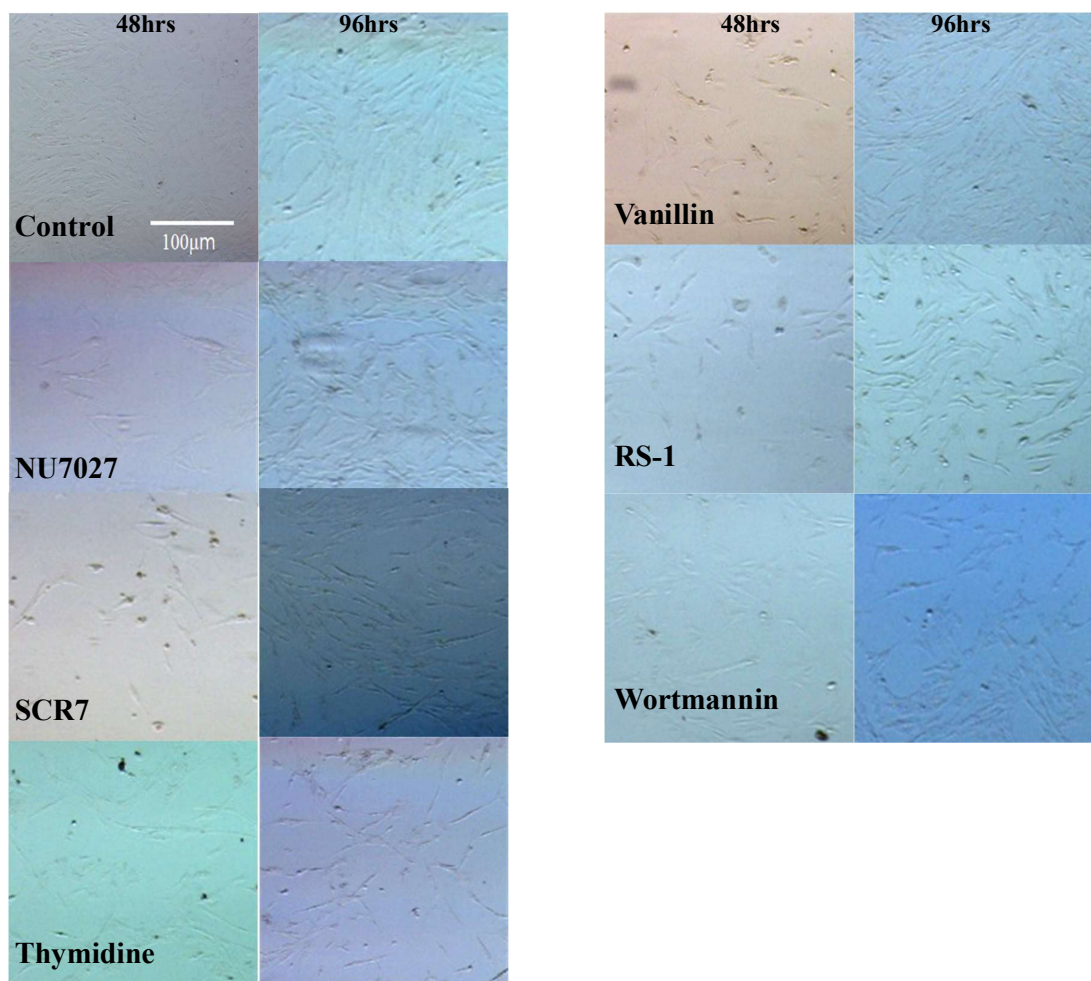
Figure 2-6. Toxicity of the chemical treatment: Toxicity of the introduced chemicals was tested. The PFF cells did not grow as well as the control group under the presence of the chemicals. Microscopic image was captured at 48hrs and 96hrs after introducing the chemicals.
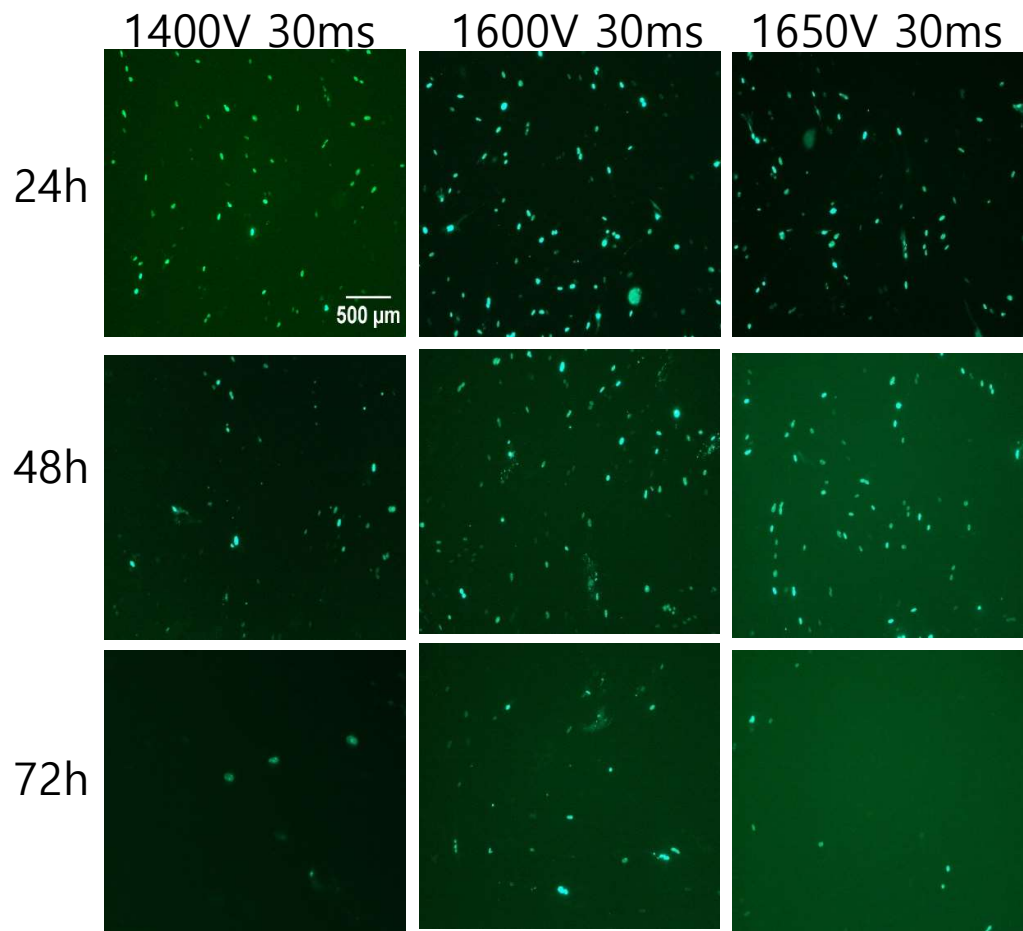
Figure 2-7. GFP transfection to optimize the transfection system: Transfection using GFP vectors were done to optimize Neon transfection system settings. Three different settings (1400v/30ms/1pulse, 1600v/30ms/1pulse, 1650v/30ms/1pulse) were used and the effects were examined. Pictures were taken in 24h interval to see the efficacy of each condition.
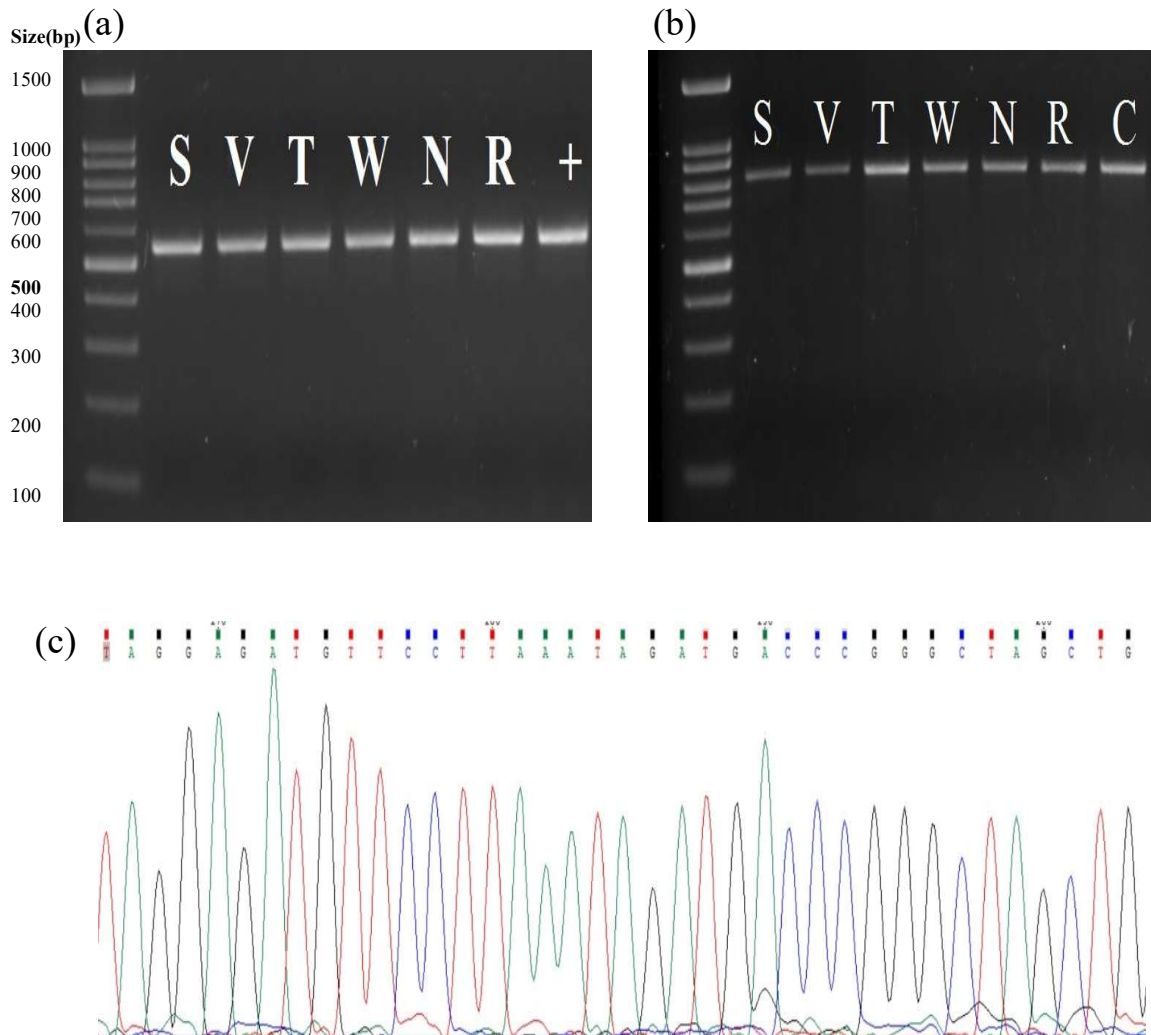
Figure 2-8. PCR amplifications to measure the efficiency of HDR after the chemical treatments: S (SCR7), V (Vanillin), T (Thymidine), W (Wortmannin), N (NU7026), R(RS-1), C (Control). (a)Agarose gel electrophoresis analysis of PCR amplicons with GGTA1 primers. (b) Agarose gel electrophoresis PCR with RAG2 HR primers. Density of the bands shows the relative frequency of HR. (c)Chromatogram of genotyping result indicating successful HDR event.
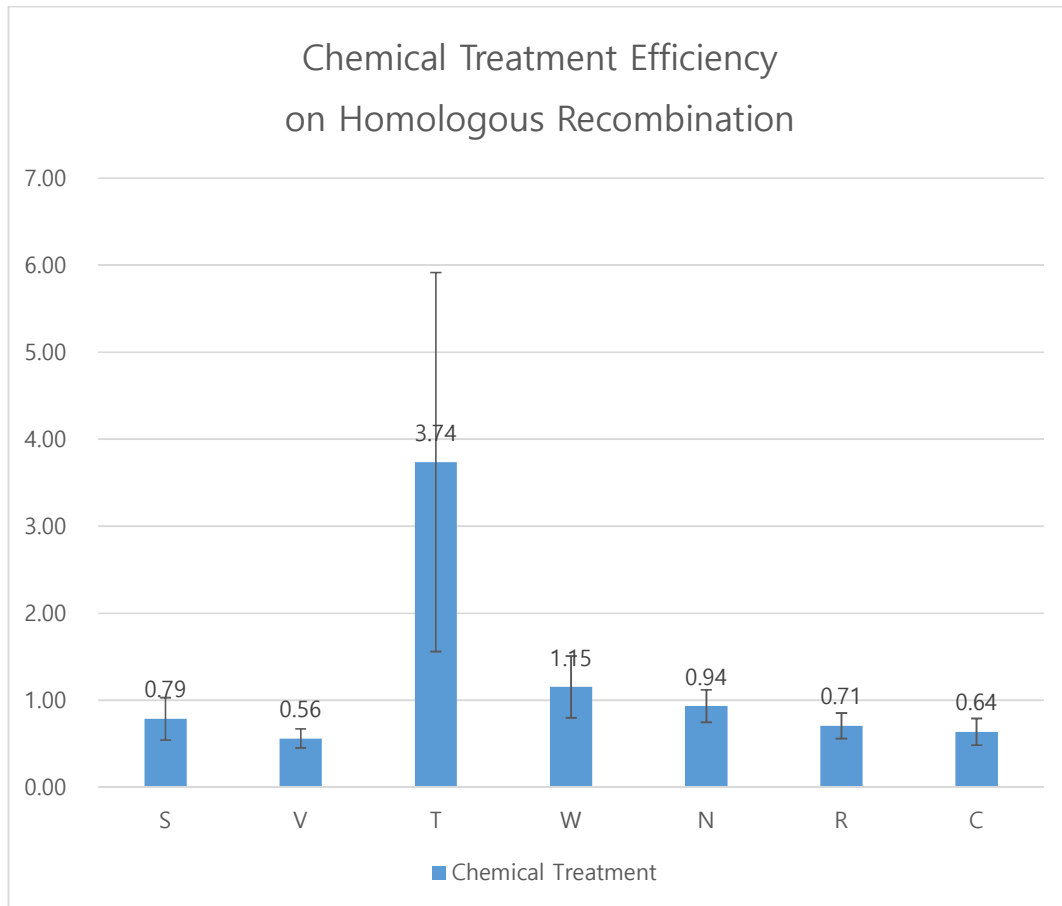
Figure 2-9. Chemical Treatment Efficiency on Homologous Recombination: All groups were measured to compare chemical treatment efficiency on homologous recombination. Thymidine (T) shows 5.85-fold higher efficiency than the control group although the difference is not statistically significant. Other groups, showed minimal numerical difference. There was no significant statistical difference between the groups (p>0.05).

Chapter 3: Effect of serum deprivation on the DNA repair pathway after CRISPR/Cas9-mediated Double Stranded Break

Abstract

DNA damage is known to be predominantly repaired by NHEJ pathway throughout the cell cycle, and its activity increases when the cells progresses from G1 to G2/M. In contrast, HR is nearly absent in the G1 phase, most active in the S phase, and the activity declines in G2/M. Although high efficiency of gene targeting in somatic cells is achievable with the use of CRISPR/Cas9 system, the efficiency is not high enough that the selection process can be ignored. Therefore, it is important to develop a strategy to increase the overall mutation efficiency which could easily be applied in everyday practical lab settings. Serum deprivation has been used to synchronize cells in G0/G1 phase without losing their proliferation capacity. In this study, we attempted to increase the efficiency of NHEJ and its successful mutation rate of the CRISPR/Cas9 system by synchronizing cells at G0/G1 stage with the serum deprivation strategy to the cell culture medium. However, the serum deprivation treatment did not have any influence on the efficiency of NHEJ. Interestingly, efficiency of HDR seemed to decrease after the serum starvation treatment although no statistical difference was found. The results indicate that cell cycle synchronization through serum starvation do not have any significant effect on the frequency of NHEJ or HDR during CRISPR/Cas9-mediated gene targeting.

Introduction

CRISPR/Cas9-mediated gene targeting can introduce targeted mutations at higher efficiency than previous versions of engineered meganucelases (Cong et al., 2013; Wang et al., 2013). The system relies on an endogenous DNA repair mechanism, specifically through either non-homologous end-joining (NHEJ) or homologous recombination (HR). DNA breaks are predominantly repaired by NHEJ because the NHEJ pathway does not require additional materials or donor DNA to repair the damage (Panier and Boulton, 2014). For that reason, NHEJ is a faster and more efficient DSB repair pathway than HR (Mao et al., 2008). If the purpose is to inactivate a target gene, it is easier and more efficient to achieve the mutation by NHEJ because mutations through NHEJ can often generate a premature stop codon. In fact, most of CRISPR/Cas9-mediated gene targeting reports utilize NHEJ. Although CRISPR/Cas9 can effectively induce targeted modification, published success rates vary due to a given gRNA targeting sequence could have additional sites throughout the genome by containing partial homology. Therefore this requires additional strategies to increase its specificity and avoid off-targeting activities (Mali et al., 2013a; Ran et al., 2013a; Fu et al., 2014). Therefore, it is also important to develop a strategy to increase the activity of NHEJ.

The NHEJ pathway is the major DNA repair system throughout the whole cell cycle, regardless of its phase. In the S phase, however, HR becomes more frequent with the presence of template DNA for replication (Mali et al., 2013b; Wang et al., 2013; Yang et al., 2013). These findings suggest that synchronizing cells out of S phase and toward G0/G1 phase can force the cells to predominantly rely on NHEJ pathway to repair their genome.

Serum deprivation has been used for cell synchronization for decades. With appropriate amounts of serum (1%-2%) in the media, synchronization of cells in G0/G1 phase is successful without losing their proliferation capacity (Kues et al., 2000). It has also been

suggested that a short period of serum deprivation significantly increased the population of cells at G0/G1, following with termination of mitotic activities after 48h, which will lead cells to exit from the cell cycle into a non-division state, which is known as G0 phase (Holley and Kiernan, 1968; Iyer et al., 1999).

Based on the reports, we hypothesized that an increase in the NHEJ activity can be achieved by synchronizing cells at G0/G1 phase using serum deprivation approach (Figure 3-1). Frequency of HDR and NHEJ was measured following the serum deprivation treatments.

Materials and methods

Porcine fetal fibroblast (PFF) culture

The culture of PFF cells was conducted following the same protocols described in the chapter 2.

Cell culture medium

Cell culture media used for these experiments were prepared separately by adding different percentages of FBS (15%, 10%, and 2%) and 1000x gentamycin into the base medium (DMEM). Cell culture media were then sterilely filtered through bottle-top Filters, 0.22 um PES, to remove any possible contaminants or substances. Treating PFF cells with 2% FBS should synchronize the cells at G0/G1 phase.

Cell passage

PFF cells were grown to 70% confluence to obtain consistency in growth and physiological characters. When 70% confluence was reached, which typically took 4-5 days from a fresh stock of seed cells, the cell cultures were passaged. First, cell culture medium was removed, then washed with DPBS solution. The DPBS wash is essential as cell culture medium contains fetal bovine serum (FBS), which has protease inhibitors. After the wash, 5 mL of 0.125% Trypsin was added into the culture for digestion. The Trypsin digestion was done for 30 seconds at maximum to minimize any potential the toxicity to the cells. Trypsinized cells were then washed with 10 mL of DPBS, then pipetted to 10mL flask for centrifugation. Cells were centrifuged at 1900rpm for 5 minutes to obtain a pellet. Supernatants were removed carefully and obtained remaining cell pellets. The washing process was done two times to ensure cells are free of any harmful or toxic substances. One milliliter of culture medium was added to the cell pellet to distribute passage cells to new cell culture dishes or flasks. Then 10 mL of 15% FBS cell culture medium were added in each dish. All passaged cell culture dishes were incubated in 37 ºC with 5% CO_2 incubator.

Cell Transfection using PX330-RAG2 and RAG2 donor DNA

An identical approach was used to deliver PX330-RAG2 constructs and RAG2 donor DNA into PFF cells by using the Neon system. Detailed procedures are described in the chapter 2.

Serum starvation treatment

The transfected cells were carefully transferred to new 6 well dishes with the samples

separated in three different wells. Each well equally received 3 ml of culture media containing 15% FBS, 10% FBS, and 2% FBS. The amount of FBS in normal cell culture medium is 15%, thus the 15% FBS group served as a positive control. After 48 hours, media in the all groups were replaced with 15% FBS for recovery up to 5 days. After the cell population recovers, Genomic DNA mini kit (Invitrogen) was used to extract DNA from the groups. Extracted DNA from each sample were used to measure the frequency of HDR and NHEJ using PCR and T7E1 (NEB), respectively (Figure 3-2).

Cell cycle detection using FACS

To measure the distribution of cell cycles after the serum treatments, FACS (fluorescent-activated cell sorting) analysis was used. To perform the FACS, cell DNA was stained. To perform the FACS analysis on the cells treated with different concentration of FBS for 48hrs, we harvested the cells after 48hrs of incubation by trypsinization following the same procedures as described in the *cell passage*. All the harvested cells were washed with 2 mL of PBS two times. Then the washed cells were centrifuged at 300 x g for 5 minutes, and then fixed with 1mL of 70% EtOH. The cell pellets were well-suspended for complete fixation and to minimize clumping. The fixation was done for at least 30 minutes in 4°C before they were used for staining and FACS analysis. The fixed cells were washed with PBS to remove any trace of EtOH. The cells were then resuspended in 200 μl PI/Triton X-100 staining solution; the staining solution contained 0.1% Triton X-100, 0.2 mg/ml RNAse A, and 20 μg/ml Propidium Iodide (PI, diluted from 1mg/ml solution from Sigma Aldrich) in PBS. The cells were incubated in the solution in room temperature for 30 minutes in the dark. Then the tubes were transferred on ice and used for FACS analysis or stored at 4°C. Three biological replicates were used to obtain consistent effect of the serum treatment on the cell cycle.

Detection of NHEJ events using T7E1 analysis

First, extracted DNA from serum starvation populations were measured by Sanger's sequence to confirm transfection and NHEJ.

Then, as shown in the previous publications (Auer et al., 2014; Vouillot et al., 2015), T7E1 endonuclease analysis can be used to detect the frequency of overall NHEJ. Since T7E1 endonuclease cleaves hetero-duplex double strand DNA, digesting reannealed PCR products obtained from using primers flanking CRISPR/Cas9 target region on *RAG2* can represent frequency of NEHJ in a population. First, various amount of PCR product (200 ng, 500 ng, 1 mg, and 2 mg) from a *RAG2* heterozygous pig (Lei et al., 2016) were used to determine sensitivity of the assay. Digested product following instructions from the manufacturer was analyzed on gel electrophoresis on 1.5% agarose gel. Although the manufacturer's protocol suggested that 200 ng of reannealed PCR product was sufficient for the analysis, the preliminary analysis indicated that higher amount of DNA was needed. Based on the results, 1mg of reannealed PCR products were used in this experiment.

DNAs isolated from the transfected cells treated with different concentration of FBS were used as a template. The PCR products were amplified by RAG2 R1 primer (20 pg/ul) and RAG2 F1 primer (20 pg/ul). The expected size of amplified region was around 450bp. After the PCR, products were purified using PCR purification kit (Thermo Scientific GeneJet PCR Purification kit). Concentration of the isolated DNAs was measured using a photo-spectrometer, where 1mg of DNA was used for T7E1 digestion. A reaction mixture, containing 2 μL NEB 2 buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @ 25°C), 1 mg of purified DNA, and total volume of 19 μL with adding H_2O, were prepared for the T7E1 analysis. The reaction mixtures were then placed through denature/annealing process by using PCR

cycle design. This process was required to detect mismatch-sensitive nuclease assay using T7E1; the DNA denature and reannealing process generated a mixture of homoduplex and heteroduplex, where heteroduplex would be detected and cleaved by T7E1 endonuclease. DNA was denatured at 95°C for 2 minutes then reannealed as the temperature was continuously decreased by -2°C per second to 85°C, -0.1°C per second to 25°C, 16°C infinitely. These reaction mixtures were stored in 4°C for 1~2 days for further analysis. Reannealed products were then mixed with 1 µL of T7 endonuclease I, making 20 µL of final product. The final product was placed in a 37°C water bath for 15 minutes for complete digestion. Then the products were visualized on a 1.5% agarose gel through electrophoresis. The DNA sequences of PCR primers are shown in Table 2-1.

Homology-directed repair (HDR) Analysis

The frequency of HDR was detected by PCR by amplifying a junction between the HR donor vector and the HR donor primer. HR donor primers used in this analysis were designed to flank each end of the HR region, amplifying only HDR events (Figure 2-3(b)). DNAs extracted from the transfected cells were used as a template for the PCR. An equal amount (30 ng) of DNA was used in each PCR reaction, to compare the difference in the frequency of HDR after the serum treatment. To further control any potential differences in the quality of the extracted DNAs, amplification of an endogenous gene, *GGTA1*, was also conducted; the endogenous *GGTA1* would be amplified regardless of the HDR frequency in the *RAG2* locus, thus was used as an internal control. The PCR conditions for this analysis was in following condition; [GGTA1: initial denaturation 95 ºC:2minutes, 34 cycles of '95 ºC:30 seconds 55 ºC:30 seconds, 72 ºC:30 seconds' and final extension at 72 ºC for 5 minutes] [RAG2: initial denaturation 95 ºC:2minutes, 34 cycles of '95 ºC:30 seconds 55 ºC:60 seconds, 72 ºC:30

seconds' and final extension at 72 ºC for 5 minutes]. The products were analyzed on 2% gel electrophoresis and the intensity of the PCR products were measured by the Image J software. The frequency of HDR was calculated by getting a ratio between PCR products between *GGTA1* (internal control) and *RAG2* (HDR frequency).

Statistical analysis

Differences in the frequency of targeting events were determined after analysis of variance (ANOVA) by using the PROC GLM procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were analyzed using the Tukey's test. All data are presented as mean±S.E.M.; significance was determined at the *P* < 0.05 level.

Result

Cell cycle analysis using FACS system

The distribution of cells cycles in the cells treated with 15% or 10% serum were similar. Most of cell populations were in G0/G1 phase, being 81.98% and 81.35% respectively (Figure 3-3(a) and (b)). Higher distribution of G2 phase was observed in 15% serum samples than the other two groups, having 9.79% of cell population at G2. Comparatively, 10% serum samples had less G2 at 7.60%, but higher population in S phase at 10.26% versus 7.90% compared to the 15% group. Interestingly, cells that received 2% serum presented higher cell population arrested in G0/G1 phase at 87.96%. Cells also showed lower population of cells in S phase and G2 phase, at 6.39% and 4.04% respectively, compared to the other treatments. There was a

group of dead cells detected in all populations; however, the difference of the dead cells was trivial among the groups (Figure 3-3(b)).

Activity of NHEJ after the serum starvation treatment

Genomic DNAs from gene targeted PFF cell lines were used to amplify a fragment of *RAG2* gene flanking the CRISPR/Cas9 target locations. Sanger sequencing results of the PCR products showed unstable population of nucleotides in and around the CRISPR/Cas9 target sequence, confirming the effectiveness of transfection and mutations generated through NHEJ (Figure 3-4). The intensity of cleaved PCR products after T7E1 digestion was different among the groups (15%, 10%, and 2%). DNA from the 10% group showed the most digested population, whereas 2% group showed the least. (Figure 3-5(a)). Differences in numerical values of the digested amount of PCR products were 0.31 versus 0.27 versus 0.26 (Figure 35(d)), but there was no statistical difference in the frequency of NHEJ among all groups (P>0.05).

Activity of HDR after the serum starvation treatment

Serum starvation treatment had a similar effect on the frequency of HDR as the NHEJ analysis. The highest frequency of HDR was detected from the 10% group, and the least level of HDR was observed from the 2% treatment (Figure 3-5(b), 3-5(c)). There were differences in numerical values of the HDR frequency in each group (15%:.56 v 10%:.58 v 2%:0.38) (Figure 3-5(e)). However, there was no statistical difference among all group (P>0.05).

Discussion

In this study, we employed serum starvation to synchronize the cell cycle at G0/G1 to alter the activity of NHEJ and HR. The purpose was to increase the activity of NHEJ, thus increase the overall efficacy of CRISPR/Cas9 system on introducing targeted mutations. Cell cycle synchronization through serum starvation is a simple approach and practical approach if effective.

However, results from this study were unexpected. The overall activity of both NHEJ and HR was decreased after serum starvation with 2% FBS in cell culture medium. The FACS analysis showed that the cell cycle arrest using the cell deprivation was successful. However, majority of the cells (>80%) in all groups were already in G0/G1 stage, as detected by the FACS analysis, indicating that there was little room to further synchronize the cells in G0/G1 stage using the serum deprivation approach.

Similarly, it is likely that lower population of cells at S and G2 phase after the serum deprivation decreased the frequency of HDR during the CRISPR/Cas9-mediated gene targeting compared to the 15% and 10% groups. The overall decrease in both NHEJ and HDR rate after treating PFF cells with 2% FBS indicates that the severe cell cycle arrest could lead to poor overall mutation efficiency, possibly due to the arrest in cell cycle progression machinery. Although cell cycle synchronization using the serum starvation approach was not successful, another approach to arrest cell cycle at G0/G1 phase more effectively might increase the efficiency of NHEJ. Mao et al, reported that cells grown to confluence, had significant cell cycle arrest at G1 phase by 97%, 1% in s phase and 2% in G2/M phase respectively (Mao et al., 2008). In respect to this study, we should be able to employ cell growth confluence instead of serum starvation, to achieve a more severe cell cycle arrest at G1 phase to see its relation to mutation efficiency in future studies.

It is also interesting to see the corresponding increase of HR rate in 10% FBS group, where our FACS analysis has shown the most population in S phase. In respect to the result in chapter 2, where cell cycle synchronization at S phase was achieved by thymidine, we can see the similar result in rate of HR, that rate of HR and cell cycle synchrony might have a more valuable relation. This result also indicate that cells should be in replicating state to induce HDR.

Our results suggest that cell cycle synchronization using the serum starvation approach may not be an ideal approach to increase the efficacy of CRISPR/Cas9-mediated gene targeting approach. However, our findings suggest that cell cycle is closely related to the outcome of gene targeting using CRISPR/Cas9 system. Future studies should focus on more effective way of altering cell cycle in PFF cells to effectively utilize either NHEJ or HDR pathways during CRISPR/Cas9-mediated gene targeting.
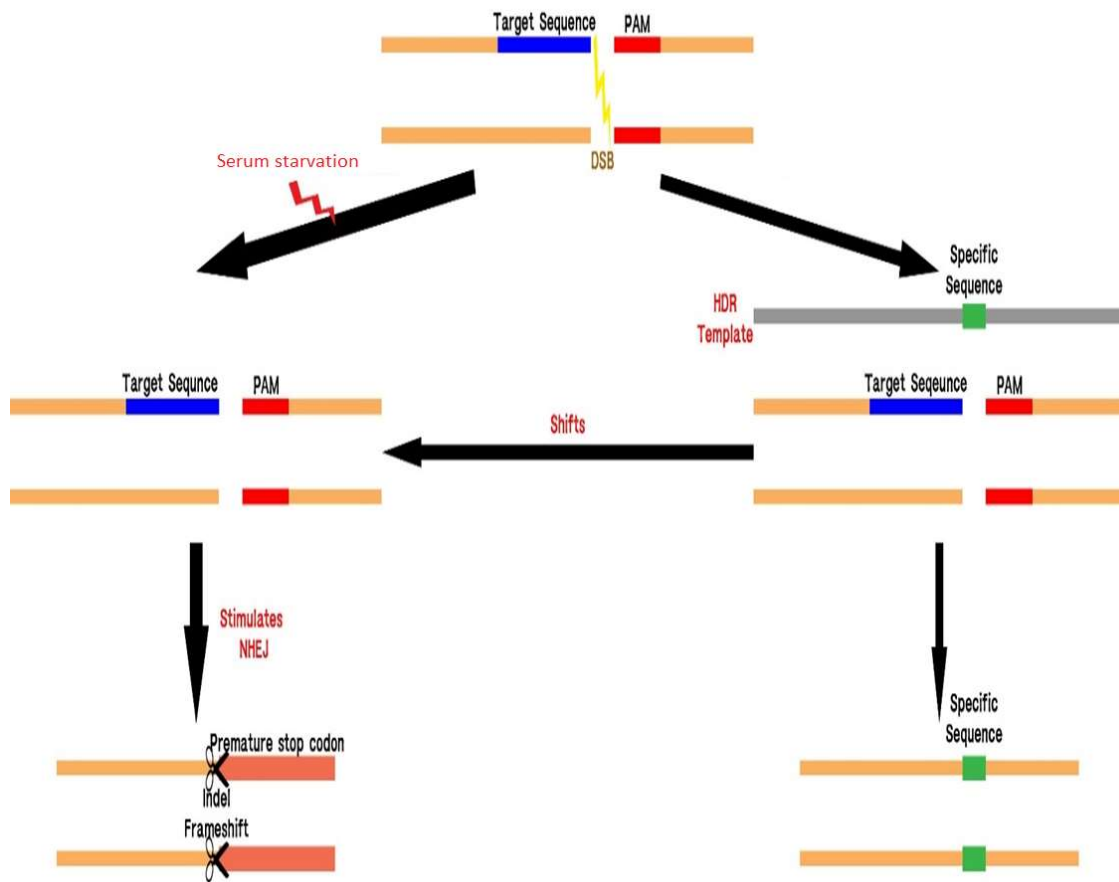
Figure 3-1. Schematic diagram of serum starvation to increase the NHEJ activity: Serum starvation will synchronize the cells at G0/G1, which could increase the frequency of NHEJ by halting chance for cells at S phase to take HDR as their repair pathway.
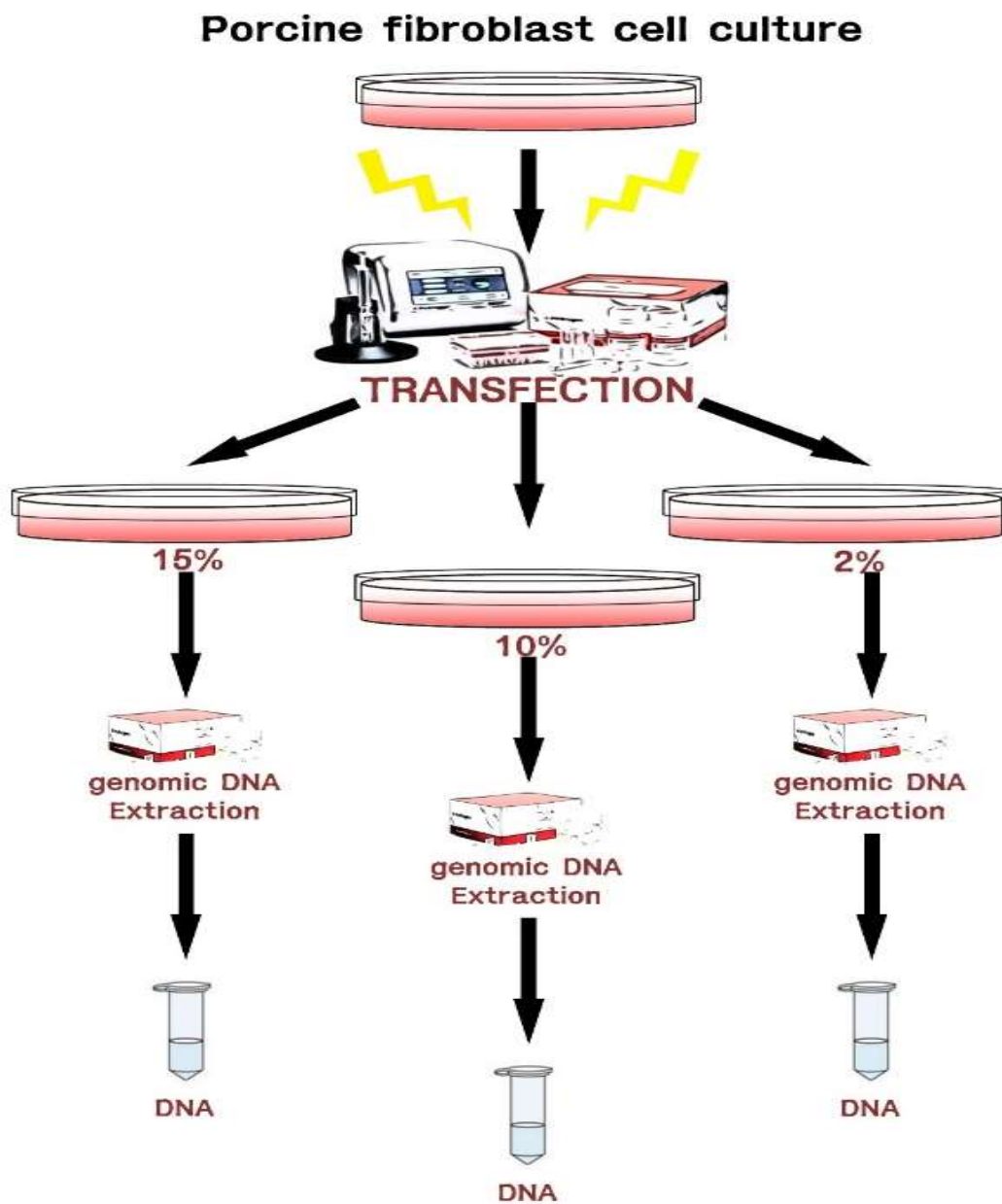
Figure 3-2. Schematic diagram of serum starvation approach used in this study: PFF cells are transfected using as optimized setting described in material & methods at 1 pulse of 1600voltage wave in 30ms width. The transfected PEF cells are split in to 3 dishes, containing FBS concentration of 15%,10%, and 2%.
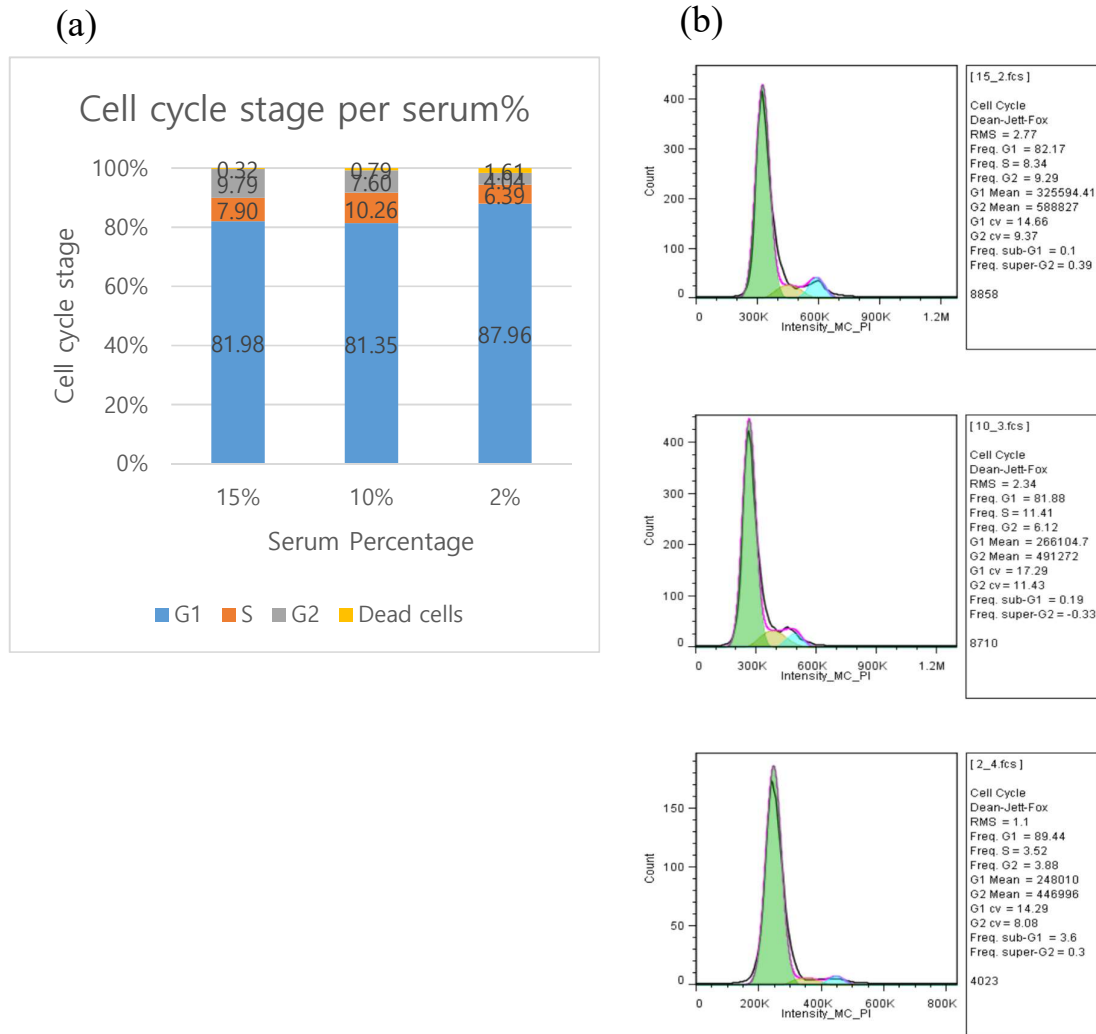
Figure 3-3. Effect of serum deprivation on cell cycle distribution: (a) FACS analysis to identify cell cycle distribution of PFF cells grown in cell culture medium with different serum concentration (15%,10%,2%) are stained and analyzed through FACS. (b) Cell cycle stage per serum%: there are less cell populations in G2 stage as serum concentration decrease in cell culture medium, which shows that the serum deprivation is successfully.
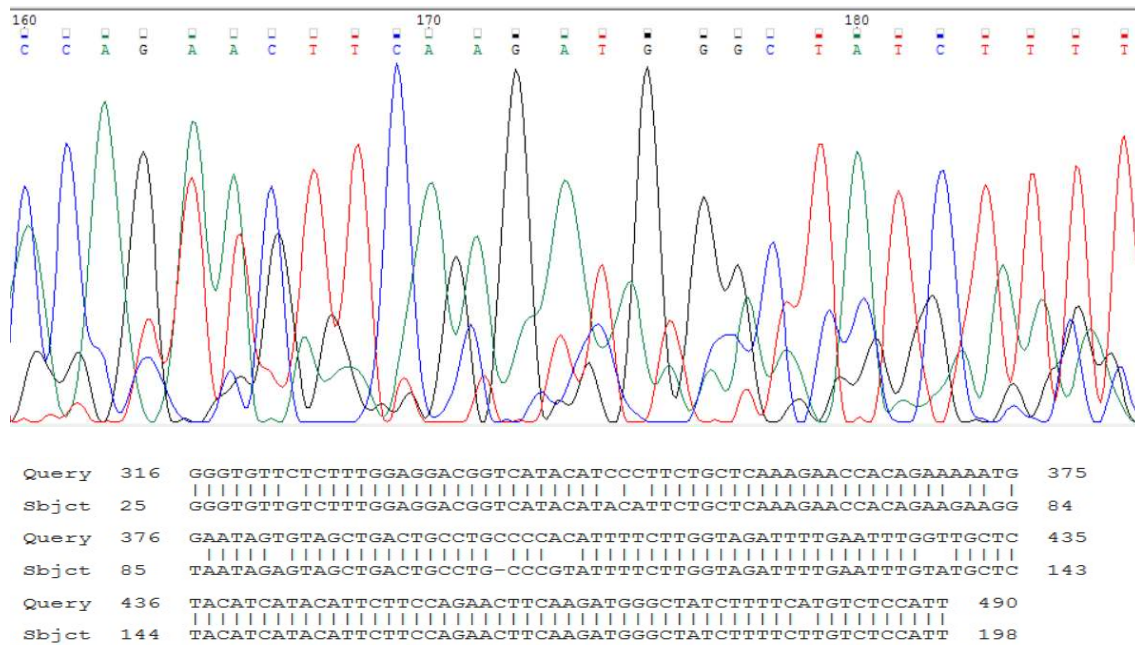
```
Query  316  GGGTGTTCTCTTTGGAGGACGGTCATACATCCCTTCTGCTCAAAGAACCACAGAAAAATG  375
            ||||||| |||||||||||||||||||||| | |||||||||||||||||||||| || |
Sbjct  25   GGGTGTTGTCTTTGGAGGACGGTCATACATACATTCTGCTCAAAGAACCACAGAAGAAGG  84

Query  376  GAATAGTGTAGCTGACTGCCTGCCCCACATTTTCTTGGTAGATTTTGAATTTGGTTGCTC  435
            ||||||  |||||||||||||||| |||     |||||||||||||||||||||  |||||
Sbjct  85   TAATAGAGTAGCTGACTGCCTG-CCCGTATTTTCTTGGTAGATTTTGAATTTGTATGCTC  143

Query  436  TACATCATACATTCTTCCAGAACTTCAAGATGGGCTATCTTTTCATGTCTCCATT   490
            |||||||||||||||||||||||||||||||||||||||||||| |||||||||||
Sbjct  144  TACATCATACATTCTTCCAGAACTTCAAGATGGGCTATCTTTTCTTGTCTCCATT   198
```

Figure 3-4. Chromatography of DNA sequencing results showing mutations induced by NHEJ pathway. Chromatography zoomed into the RAG2 exon 2. It shows there are nucleotides of heterogeneous mutated population detected along with original sequence, showing NHEJ has successfully led mutation.
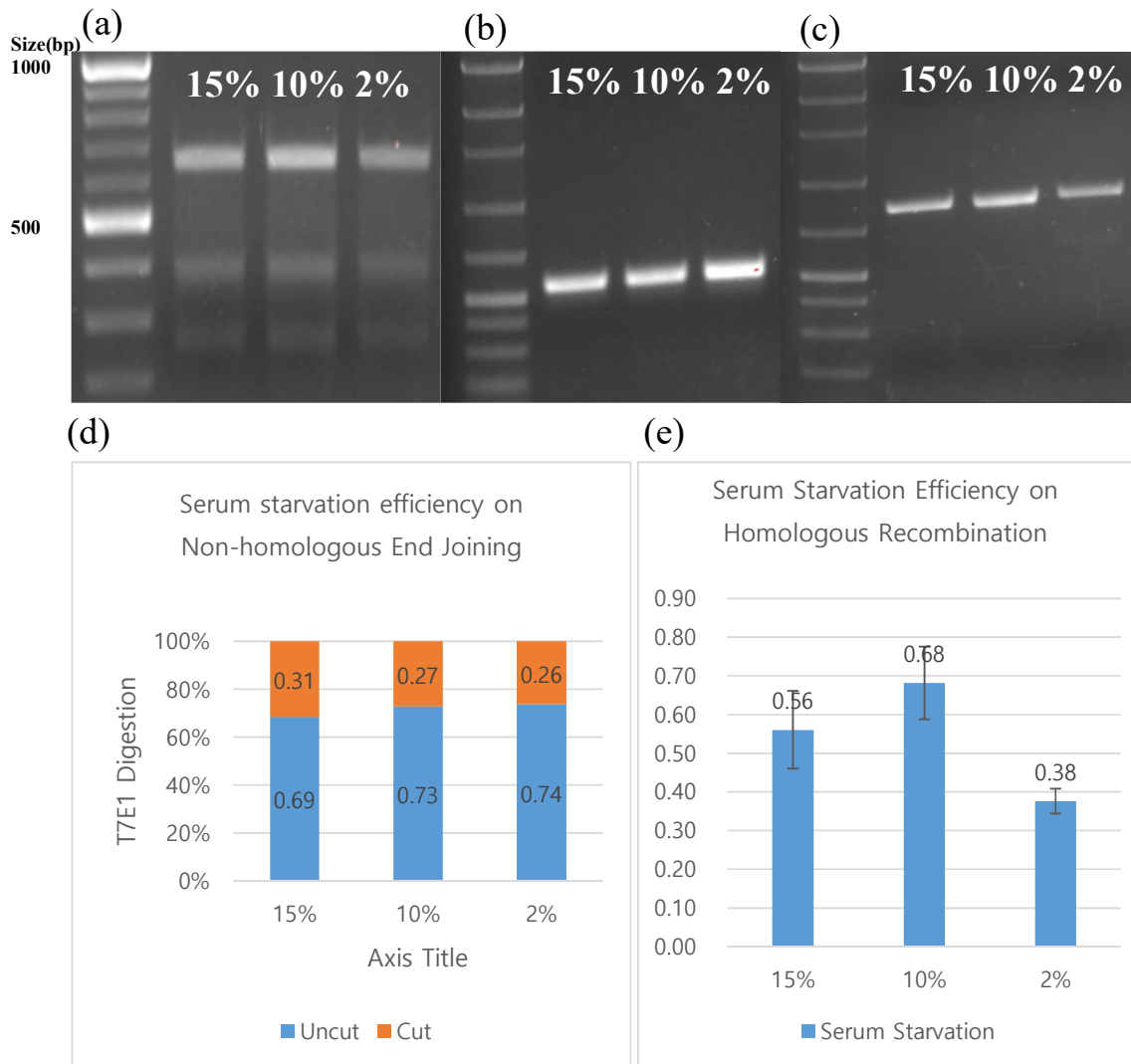
60

Figure 3-5. Analysis of the frequency of NHEJ and HDR after CRISPR/Cas9-mediated gene targeting: (a) Agarose gel electrophoresis analysis for T7E1 analysis for nonhomologous-end joining (NHEJ) detection. (b) Agarose gel electrophoresis analysis for PCR with GGTA1 primers for positive control. (c) Agarose gel electrophoresis analysis for PCR with RAG2 HR primers to homologous recombination (HR) detection. (d) Effect of serum starvation on the efficiency of NHEJ, calculated based on (a). (e) Effect of serum starvation on the efficiency of HDR, calculated based on (b) and (c). There was no significant statistical difference between the groups (p>0.05). (page 73)

Chapter 4. Future prospective of gene-editing technology in pigs

Our findings from this study indicate that modulation of the DNA repair system is not as easy as it logically seems. The results were not as significant as what we had expected nor previous literatures have demonstrated; in both chemical and non-chemical approaches, modification from the previous studies were required to successfully achieve higher efficiency of NHEJ or HDR.

Our attempt to increase the frequency of HDR by incubating PFF cells with chemicals was not consistent with previous studies (Song et al., 2016), although concentration of chemicals used in our study was identical as the previous reports. It could be that previous studies used various types of cells that are different than PFF cells, which might have also led to have less effect than the previous studies and require additional optimization. The frequency of HDR was elevated at an average of 5.85-fold compared to the control groups in cells that were treated with thymidine. Therefore, modulating the cell cycle at S phase would have been the effective approach to increase the frequency of HDR in PEF cells and maybe other chemical treatments may have cause some unknown toxicity. Further studies should be focused on the optimization of thymidine usage in PEF cells and how the thymidine treatment increased the efficiency of HDR. Applying this approach to different types of cells would be an interesting experiment to conduct.

Our serum deprivation to increase the efficiency of NHEJ was not as effective as we initially hypothesized. Our FACS result showed that although serum deprivation did affect their cell cycle distribution, however, not all cells were in G0/G1 stage; cell populations in G0/G1 phase were <80%. Further studies should focus on applying more severe form of cell cycle arrest to keep the cells within G0/G1 phase. One previous study have shown that contact

inhibition method shows more severe cell cycle arrest at G1 phase (Mao et al., 2008), that it could be a better approach than serum starvation as a non-chemical based method. Even further, study employing more severe form of approach to increase the frequency of NHEJ, other types of common non-chemical methods to arrest cell cycles or some chemical based on cell cycle arrest at G0/G1 is recommended.

Our results imply that cell cycle synchronization can be an effective approach to modulate the DNA repair pathway after CRISPR/Cas9 induced DSB. Although serum starvation was also an approach to induce cell cycle arrest at G0, more precise and efficient cell cycle arrest approach is required to expect to see better result. Chemical based cell cycle arrest approaches seem to be more effective, however, further studies are required to adjust the concentration of chemicals used for available cell type used and cell growth environment to see the optimum result. Interestingly, thymidine treatment, known to synchronize cells at S phase, was the most effective one among different treatment, further emphasizing differential presence of machineries related to either NHEJ or HR depending on cells cycle.

Compared to the first report of GE pigs (Hammer et al., 1985), efficiency of generating GE pigs has been dramatically increased since the development of meganucleases such as CRISPR/Cas9 system. The system is effective enough to introduce site specific mutations during porcine embryogenesis (Wang et al., 2013; Yang et al., 2013; Hai et al., 2014). However, utilization of the system is often hindered due to inefficiency in introducing mutations through HDR. Although this study could not find an optimal approach to utilize HDR, future studies should identify a better approach to induce HDR. Effective use of HDR should increase the use of GE pigs for biomedicine and agriculture.

Bibliography

Auer, T. O., K. Duroure, J.-P. Concordet, and F. Del Bene. 2014. CRISPR/Cas9-mediated conversion of eGFP- into Gal4-transgenic lines in zebrafish. Nat. Protocols 9: 2823-2840.

Azimzadeh, A. M. et al. 2015. Early graft failure of GalTKO pig organs in baboons is reduced by expression of a human complement pathway-regulatory protein. Xenotransplantation 22: 310-316.

Bee, L., S. Fabris, R. Cherubini, M. Mognato, and L. Celotti. 2013. The efficiency of homologous recombination and non-homologous end joining systems in repairing double-strand breaks during cell cycle progression. PloS one 8: e69061.

Betthauser, J. et al. 2000. Production of cloned pigs from in vitro systems. Nature biotechnology 18: 1055-1059.

Bhattacharyya, A., U. S. Ear, B. H. Koller, R. R. Weichselbaum, and D. K. Bishop. 2000. The breast cancer susceptibility gene BRCA1 is required for subnuclear assembly of Rad51 and survival following treatment with the DNA cross-linking agent cisplatin. The Journal of biological chemistry 275: 23899-23903.

Boch, J., and U. Bonas. 2010. Xanthomonas AvrBs3 family-type III effectors: discovery and function. Annual review of phytopathology 48: 419-436.

Boch, J. et al. 2009. Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors. Science (New York, N.Y.) 326: 1509-1512.

Bolotin, A., B. Quinquis, A. Sorokin, and S. D. Ehrlich. 2005. Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology (Reading, England) 151: 2551-2561.

Bootsma, D., L. Budke, and O. Vos. 1964. STUDIES ON SYNCHRONOUS DIVISION OF TISSUE CULTURE CELLS INITIATED BY EXCESS THYMIDINE. Experimental cell research 33: 301-309.

Briggs, R., and T. J. King. 1952. Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. Proceedings of the National Academy of Sciences of the United States of America 38: 455-463.

Bryans, M., M. C. Valenzano, and T. D. Stamato. 1999. Absence of DNA ligase IV protein in XR-1 cells: evidence for stabilization by XRCC4. Mutation research 433: 53-58.

Campbell, K. H. S., J. McWhir, W. A. Ritchie, and I. Wilmut. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380: 64-66.

Cepko, C., and W. Pear. 2001. Overview of the retrovirus transduction system. Current protocols in molecular biology Chapter 9: Unit9.9.

Christian, M. 2010. Targeting DNA Double-Strand Breaks with TAL Effector Nucleases

Genetics Society of America.

Chu, V. T. et al. 2015. Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. Nat Biotech 33: 543-548.

Claudio Mussolino, R. M., Fabienne Lütge, Nadine Dannemann, Thomas Lahaye and Toni Cathomen. 2011. A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity.

Cong, L. et al. 2013. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science (New York, N.Y.) 339: 819-823.

Dai, Y. et al. 2002. Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nature biotechnology 20: 251-255.

Delacôte, F., M. Han, T. D. Stamato, M. Jasin, and B. S. Lopez. 2002. An xrcc4 defect or

Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. Nucleic Acids Research 30: 3454-3463.

Deveau, H. et al. 2008. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. Journal of bacteriology 190: 1390-1400.

Diez-Villasenor, C., N. M. Guzman, C. Almendros, J. Garcia-Martinez, and F. J. Mojica. 2013. CRISPR-spacer integration reporter plasmids reveal distinct genuine acquisition specificities among CRISPR-Cas I-E variants of Escherichia coli. RNA biology 10: 792-802.

Doyon, Y. et al. 2010. Transient cold shock enhances zinc-finger nuclease-mediated gene disruption. Nature methods 7: 459-460.

Doyon, Y. et al. 2011. Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nature methods 8: 74-79.

Durant, S., and P. Karran. 2003. Vanillins--a novel family of DNA-PK inhibitors. Nucleic Acids Res 31: 5501-5512.

Erdmann, S., and R. A. Garrett. 2012. Selective and hyperactive uptake of foreign DNA by adaptive immune systems of an archaeon via two distinct mechanisms. Molecular microbiology 85: 1044-1056.

Esteban, M. A. et al. 2009. Generation of induced pluripotent stem cell lines from Tibetan miniature pig. The Journal of biological chemistry 284: 17634-17640.

Fan, N. et al. 2013. Piglets cloned from induced pluripotent stem cells. Cell Res 23: 162-166.

Fan, N., and L. Lai. 2013. Genetically Modified Pig Models for Human Diseases. Journal of Genetics and Genomics 40: 67-73.

Felgner, P. L. et al. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences of the United States of America 84: 7413-7417.

Fischer, D. et al. 2002. Copolymers of Ethylene Imine and N-(2-Hydroxyethyl)-ethylene Imine as Tools To Study Effects of Polymer Structure on Physicochemical and Biological Properties of DNA Complexes. Bioconjugate chemistry 13: 1124-1133.

Fu, Y., J. D. Sander, D. Reyon, V. M. Cascio, and J. K. Joung. 2014. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature biotechnology 32: 279-284.

Garneau, J. E. et al. 2010. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 468: 67-71.

Griffin, M. J. 1976. Synchronization of some human cell strains by serum and calcium starvation. In vitro 12: 393-398.

Grubb, B. R., and S. E. Gabriel. 1997. Intestinal physiology and pathology in gene-targeted mouse models of cystic fibrosis. The American journal of physiology 273: G258-266.

Gurdon, J. B., T. R. Elsdale, and M. Fischberg. 1958. Sexually Mature Individuals of Xenopus laevis from the Transplantation of Single Somatic Nuclei. Nature 182: 64-65.

Hai, T., F. Teng, R. Guo, W. Li, and Q. Zhou. 2014. One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res 24: 372-375.

Hammer, R. E. et al. 1985. Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315: 680-683.

Harper, J. V. 2005. Synchronization of cell populations in G1/S and G2/M phases of the cell cycle. Methods in molecular biology (Clifton, N.J.) 296: 157-166.

Heidenreich, E., R. Novotny, B. Kneidinger, V. Holzmann, and U. Wintersberger. 2003. Non-homologous end joining as an important mutagenic process in cell cycle-arrested cells. The EMBO journal 22: 2274-2283.

Helleday, T., J. Lo, D. C. van Gent, and B. P. Engelward. 2007. DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA repair 6: 923-935.

Ho, K., L. S. Yazan, N. Ismail, and M. Ismail. 2009. Apoptosis and cell cycle arrest of human colorectal cancer cell line HT-29 induced by vanillin. Cancer epidemiology 33: 155-160.

Holley, R. W., and J. A. Kiernan. 1968. "Contact inhibition" of cell division in 3T3 cells. Proceedings of the National Academy of Sciences of the United States of America 60: 300-304.

Huang, J. et al. 2014. RAG1/2 knockout pigs with severe combined immunodeficiency. Journal of immunology (Baltimore, Md. : 1950) 193: 1496-1503.

Ittner, L. M., and J. Gotz. 2007. Pronuclear injection for the production of transgenic mice. Nat. Protocols 2: 1206-1215.

Iyer, V. R. et al. 1999. The transcriptional program in the response of human fibroblasts to serum. Science (New York, N.Y.) 283: 83-87.

Jayathilaka, K. et al. 2008. A chemical compound that stimulates the human homologous recombination protein RAD51. Proceedings of the National Academy of Sciences of the United States of America 105: 15848-15853.

Jinek, M. et al. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.) 337: 816-821.

Kamimura, K., T. Suda, G. Zhang, and D. Liu. 2011. Advances in Gene Delivery Systems. Pharmaceutical medicine 25: 293-306.

Kues, W. A. et al. 2000. Cell cycle synchronization of porcine fetal fibroblasts: effects of serum deprivation and reversible cell cycle inhibitors. Biology of reproduction 62: 412-419.

Kurimasa, A. et al. 1999. Requirement for the kinase activity of human DNA-dependent protein kinase catalytic subunit in DNA strand break rejoining. Molecular and cellular biology 19: 3877-3884.

Kwon, D. N. et al. 2013. Production of biallelic CMP-Neu5Ac hydroxylase knock-out pigs. Scientific reports 3: 1981.

Lai, L. et al. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science (New York, N.Y.) 295: 1089-1092.

Lei, S. et al. 2016. Increased and prolonged human norovirus infection in RAG2/IL2RG deficient gnotobiotic pigs with severe combined immunodeficiency. Scientific reports 6: 25222.

Lirdprapamongkol, K. et al. 2005. Vanillin suppresses in vitro invasion and in vivo metastasis of mouse breast cancer cells. European Journal of Pharmaceutical Sciences 25: 57-65.

Lundin, C. et al. 2002. Different Roles for Nonhomologous End Joining and Homologous Recombination following Replication Arrest in Mammalian Cells. Molecular and cellular biology 22: 5869-5878.

Lutz, A. J. et al. 2013. Double knockout pigs deficient in N-glycolylneuraminic acid and galactose alpha-1,3-galactose reduce the humoral barrier to xenotransplantation. Xenotransplantation 20: 27-35.

M, J. et al. 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (New York, N.Y.).

Mali, P. et al. 2013a. CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. Nature biotechnology 31: 833-838.

Mali, P. et al. 2013b. RNA-guided human genome engineering via Cas9. Science (New York, N.Y.) 339: 823-826.

Mao, Z., M. Bozzella, A. Seluanov, and V. Gorbunova. 2008. Comparison of nonhomologous end joining and homologous recombination in human cells. DNA repair 7: 1765-1771.

Maruyama, T. et al. 2015. Inhibition of non-homologous end joining increases the efficiency of CRISPR/Cas9-mediated precise [TM: inserted] genome editing. Nature

biotechnology 33: 538-542.

McGregor, C. G. et al. 2012. Human CD55 expression blocks hyperacute rejection and restricts complement activation in Gal knockout cardiac xenografts. Transplantation 93: 686-692.

Medicine, N. L. o. 2011. Transfection. https://www.nlm.nih.gov/cgi/mesh/2011/MB_cgi?mode=&term=Transfection.

Menuel, S. et al. 2008. Synthesis and complexation ability of a novel bis- (guanidinium)-tetrakis-(beta-cyclodextrin) dendrimeric tetrapod as a potential gene delivery (DNA and siRNA) system. Study of cellular siRNA transfection. Bioconjugate chemistry 19: 2357-2362.

Miller, J. C. et al. 2011. A TALE nuclease architecture for efficient genome editing. Nature biotechnology 29: 143-148.

Miller, J. C. et al. 2005. Development of Zinc Finger Nucleases for Therapeutic Gene Correction of Sickle Cell Anemia. Mol Ther 11: S35-S35.

Mimitou, E. P., and L. S. Symington. 2009. Nucleases and helicases take center stage in homologous recombination. Trends in biochemical sciences 34: 264-272.

Mir, B., and J. A. Piedrahita. 2004. Nuclear localization signal and cell synchrony enhance gene targeting efficiency in primary fetal fibroblasts. Nucleic Acids Research 32: e25-e25.

Moore, J. K., and J. E. Haber. 1996. Cell cycle and genetic requirements of two pathways of nonhomologous end-joining repair of double-strand breaks in Saccharomyces cerevisiae. Molecular and cellular biology 16: 2164-2173.

Neumann, E., M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider. 1982. Gene transfer into mouse lyoma cells by electroporation in high electric fields. The EMBO journal 1: 841-845.

Nutley, B. P. et al. 2005. Preclinical pharmacokinetics and metabolism of a novel prototype DNA-PK inhibitor NU7026. British journal of cancer 93: 1011-1018.

Ogawa, T., X. Yu, A. Shinohara, and E. H. Egelman. 1993. Similarity of the yeast RAD51 filament to the bacterial RecA filament. Science (New York, N.Y.) 259: 1896-1899.

Onishi, A. et al. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science (New York, N.Y.) 289: 1188-1190.

Paffett, K. S., J. A. Clikeman, S. Palmer, and J. A. Nickoloff. 2005. Overexpression of Rad51 inhibits double-strand break-induced homologous recombination but does not affect gene conversion tract lengths. DNA repair 4: 687-698.

Panier, S., and S. J. Boulton. 2014. Double-strand break repair: 53BP1 comes into focus. Nature reviews. Molecular cell biology 15: 7-18.

Park, K. E. et al. 2016. Targeted Gene Knockin in Porcine Somatic Cells Using CRISPR/Cas Ribonucleoproteins. International journal of molecular sciences 17.

Pierce, A. J., P. Hu, M. Han, N. Ellis, and M. Jasin. 2001. Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. Genes & development 15: 3237-3242.

Polejaeva, I. A. et al. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407: 86-90.

Prather, R. S. et al. 1987. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biology of reproduction 37: 859-866.

Prather, R. S., M. Lorson, J. W. Ross, J. J. Whyte, and E. Walters. 2013. Genetically Engineered Pig Models for Human Diseases. Annual review of animal biosciences 1: 203-219.

Prather, R. S., M. M. Sims, and N. L. First. 1989. Nuclear transplantation in early pig embryos. Biology of reproduction 41: 414-418.

Ran, F. A. et al. 2013a. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154: 1380-1389.

Ran, F. A. et al. 2013b. Genome engineering using the CRISPR-Cas9 system. Nat. Protocols 8: 2281-2308.

Reece, J. B., and N. A. Campbell. 2011. Campbell biology. Benjamin Cummings / Pearson, Boston.

Rehbinder, C. et al. 1998. FELASA recommendations for the health monitoring of breeding colonies and experimental units of cats, dogs and pigs. Report of the Federation of European Laboratory Animal Science Associations (FELASA) Working Group on Animal Health. Laboratory animals 32: 1-17.

Riballo, E. et al. 2009. XLF-Cernunnos promotes DNA ligase IV–XRCC4 re-adenylation following ligation. Nucleic Acids Research 37: 482-492.

Rich, T., R. L. Allen, and A. H. Wyllie. 2000. Defying death after DNA damage. Nature 407: 777-783.

Rogers, C. S. et al. 2008. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science (New York, N.Y.) 321: 1837-1841.

Rothkamm, K., I. Kruger, L. H. Thompson, and M. Lobrich. 2003. Pathways of DNA double-strand break repair during the mammalian cell cycle. Molecular and cellular biology 23: 5706-5715.

Schwacha, A., and N. Kleckner. 1995. Identification of double Holliday junctions as intermediates in meiotic recombination. Cell 83: 783-791.

Sharei, A. et al. 2013. A vector-free microfluidic platform for intracellular delivery. Proceedings of the National Academy of Sciences 110: 2082-2087.

Singh, P., J. C. Schimenti, and E. Bolcun-Filas. 2015. A mouse geneticist's practical guide to CRISPR applications. Genetics 199: 1-15.

Smith, G. C., and S. P. Jackson. 1999. The DNA-dependent protein kinase. Genes & development 13: 916-934.

Song, J. et al. 2016. RS-1 enhances CRISPR/Cas9- and TALEN-mediated knock-in efficiency. Nat Commun 7.

Srivastava, M. et al. 2012. An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. Cell 151: 1474-1487.

Stoltz, D. A. et al. 2010. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Science translational medicine 2: 29ra31.

Sung, P. 1997. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. Genes & development 11: 1111-1121.

Sung, P., and D. L. Robberson. 1995. DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. Cell 82: 453-461.

Suzuki, S. et al. 2016. Generation and characterization of RAG2 knockout pigs as animal model for severe combined immunodeficiency. Veterinary immunology and immunopathology 178: 37-49.

Takata, M. et al. 1998. Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. The EMBO journal 17: 5497-5508.

Thomas, D. B., and C. A. Lingwood. 1975. A model of cell cycle control: effects of thymidine on synchronous cell cultures. Cell 5: 37-42.

Thomas, K. R., and M. R. Capecchi. 1987. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. Cell 51: 503-512.

Thompson, L. H., and D. Schild. 2001. Homologous recombinational repair of DNA ensures

mammalian chromosome stability. Mutation research 477: 131-153.

Urnov. 2005. Highly efficient endogenous human gene correction

using designed zinc-finger nucleases.

Urushihara, Y. et al. 2012. DNA-PK inhibition causes a low level of H2AX phosphorylation and homologous recombination repair in Medaka (Oryzias latipes) cells. Biochemical and biophysical research communications 429: 131-136.

Veuger, S. J., N. J. Curtin, C. J. Richardson, G. C. Smith, and B. W. Durkacz. 2003. Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. Cancer research 63: 6008-6015.

VodiČKa, P. et al. 2005. The Miniature Pig as an Animal Model in Biomedical Research. Annals of the New York Academy of Sciences 1049: 161-171.

Vouillot, L., A. Thelie, and N. Pollet. 2015. Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases. G3 (Bethesda, Md.) 5: 407-415.

Wang, H. et al. 2013. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153: 910-918.

Welsh, M. J., C. S. Rogers, D. A. Stoltz, D. K. Meyerholz, and R. S. Prather. 2009. Development of a porcine model of cystic fibrosis. Transactions of the American Clinical and Climatological Association 120: 149-162.

West, F. D. et al. 2010. Porcine induced pluripotent stem cells produce chimeric offspring. Stem cells and development 19: 1211-1220.

Whitt, M., L. Buonocore, and J. K. Rose. 2001. Liposome-mediated transfection. Current protocols in immunology / edited by John E. Coligan ... [et al.] Chapter 10: Unit 10.16.

Whitworth, K. M. et al. 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. Biology of reproduction 91: 78.

Whyte, J. J., and R. S. Prather. 2011. Genetic modifications of pigs for medicine and agriculture. Molecular reproduction and development 78: 879-891.

Willadsen, S. M. 1986. Nuclear transplantation in sheep embryos. Nature 320: 63-65.

Willmore, E. et al. 2004. A novel DNA-dependent protein kinase inhibitor, NU7026, potentiates the cytotoxicity of topoisomerase II poisons used in the treatment of leukemia. Blood 103: 4659-4665.

Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. S. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

Wolf, E. et al. 2000. Transgenic technology in farm animals--progress and perspectives. Experimental physiology 85: 615-625.

Yang, D. et al. 2014. Effective gene targeting in rabbits using RNA-guided Cas9 nucleases. Journal of Molecular Cell Biology 6: 97-99.

Yang, H., Q. Li, J. Fan, W. K. Holloman, and N. P. Pavletich. 2005. The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. Nature 433: 653-657.

Yang, H. et al. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154: 1370-1379.

Yano, K.-i. et al. 2008. Ku recruits XLF to DNA double-strand breaks. EMBO Reports 9: 91-96.

Yuan, Y. et al. 2014. Cell cycle synchronization of leukemia inhibitory factor (LIF)-dependent porcine-induced pluripotent stem cells and the generation of cloned embryos. Cell

Cycle 13: 1265-1276.

Zhu, H., and N. J. Gooderham. 2006. Mechanisms of Induction of Cell Cycle Arrest and Cell Death by Cryptolepine in Human Lung Adenocarcinoma A549 Cells. Toxicological Sciences 91: 132-139.

Zhu, Z., F. González, and D. Huangfu. 2014. The iCRISPR platform for rapid genome editing in human Pluripotent Stem Cells. Methods in enzymology 546: 215-250.