CHAPTER II
LITERATURE REVIEW

2.1 Somatic cell nuclear transfer (SCNT)

The technique known as somatic cell nuclear transfer (SCNT) has great potential for cloning desirable livestock species phenotypes and genotypes. (Men et al., 2012; Polejaeva et al. 2000). The several crucial steps involved in SCNT including (1) genetic selection of donor cells (2) aspiration or bisection of a MII-arrested oocyte to remove metaphase chromosomes (Enucleation) (3) transfer of donor cell to an enucleated oocyte (Injection) and fused by an electrical pulse (Fusion) (4) activation of the reconstructed embryos (Activation) (5) embryo culture and transfer into recipients (Lai & Prather, 2003). According to Cooper et al. (2002), Lai et al. (2002), Perota et al. (2019), and Kemter et al. (2020), the procedure is technically challenging and requires high-cost production, making it less likely to be adopted by professionals in the field. Animal production by SCNT has been applied in agriculture and medicine, and the association with genome editing is both practical and commercial. Currently, porcine SCNT to generate cloned embryos is of great importance and has been used for basic research and biomedical science. Because its immunology, metabolism, physiology, pathology and organ size are highly similar to those of humans. Porcine is considered an excellent source for xenotransplantation. However, the extremely low cloning efficiency and low developmental rate, these associated with incomplete reprogramming in the resulting embryos have been still enormous problem in porcine SCNT.

2.2 Low efficiency of cloning underlies incomplete epigenetic reprogramming

"Epigenetics" was first named in 1942 by Waddington (Waddington, 2012), a Cold Spring Harbor summit in 2008 that the scientific community agreed on its definition: a stable heritable phenotype arising from chromosome modifications without changes to the DNA sequence is called an epigenetic trait (Berger et al., 2009). The SCNT is a potential technique that allows the reprogramming of terminally differentiated cells (adult cells) to the totipotent state (embryonic cells) (Gurdon and Wilmut, 2011). During embryonic development, totipotent embryos differentiate into

pluripotent stem cell and after that develop into differentiated involving DNA methylation, histone modification (histone methylation, histone acethylation), genomic imprinting and X chromosome inactivation (XCI) (Reik et al., 2003). The crucial significance in the development of SCNT embryos is these are inherited variations in gene expression without modification in genomic DNA sequences occur during the process from cells of the newly formed zygote start dividing to embryo (Niemann, 2016).

2.2.1 DNA methylation

DNA methylation (5-methylCytocine, 5-mC) is commonly linked to transcriptional silence and occurs at cytosine residues in the CpG sites of DNA sequence regions (Bird, 2002). In order to activate or inactivate particular genes in accordance with the necessities of organism growth and development, the genome goes through DNA methylation maintenance, which includes DNA demethylation and DNA remethylation (Li & Zhang, 2014). DNA methyltransferases (DNMTs) such as DNMT1, are required for de novo DNA methylation, while DNMT3 (DNMT3A and DNMT3B) is crucial for maintaining DNA methylation during embryogenesis (Chen and Zhang, 2019). Following DNA demethylation repair mechanism, ten-eleven translocation (Tet) activation-induced cytidine deaminase and DNA glycosylases are examples of oxidative DNA demethylation enzymes (Ito et al., 2010; Iqbal et al., 2011; Shen et al., 2013). There are several ways in which active DNA demethylation takes place throughout the early stages of embryonic development. (Wang et al., 2014), and when zygote enter to the blastocyst or later implantation stage, genomic DNA is remethylated (Reik et al., 2001; Yang et al., 2007). The genome of SCNT embryos also undergoes de- and re-methylation; the genomic DNA of somatic cells used as donor cells in the SCNT process is highly methylated, and DNA methylation reprogramming, particularly DNA demethylation, is crucial for proper embryonic development. However, these cause incomplete reprogramming when compared with normal embryos or embryos derived from fertilization (Bourc'his et al., 2001; Dean et al., 2001; Yang et al., 2007).

2.2.2 Histone modification

Histone modification is one of major elements that affects associate with chromatin structure and regulate gene expression (Sproul et al., 2005; Yi and Kim, 2018). In eukaryotic cells, the DNA molecule appears in a nucleoprotein complex that resembles chromatin. The basic component of chromatin, which is composed of two molecules of each of the histones H2A, H2B, H3, and H4 as well as H1 acting as

linker, is the nucleosome, which is made up of 147 base pairs of DNA encircled by an octamer

of core histones. Linker DNA, which are short segments, connect each nucleosome to the next (Kurumizaka and Kobayashi, 2019). The regulation of gene expression is influenced by chromatin accessibility, which is governed by chromatin remodeling factors and covalent modification, such as acetylation, methylation, and phosphorylation of amino acids in the histone tail (Kobayashi and Kurumizaka, 2019).

2.2.2.1 Histone acetylation

Histone acetyltransferase (HAT) and histone deacetylase (HDAC) are the enzymes that mediate histone acetylation; HAT causes the opening of chromatin to facilitate transcription factor binding and activates gene transcription, whereas HDAC regulates inactivation of gene (Sun et al., 2003). Histone acetylation, including that of histone H3, occurs during fertilization and permits the proper expression of genes involved in early embryonic development (Rybouchkin et al., 2006; Ziegler-Birling et al., 2016). Moreover, Histone deacetylation occurs one step of SCNT during the *in vitro* maturation (IVM) of porcine oocytes, and is catalyzed by Class IIb histone deacetylases (Endo et al., 2008; Endo et al., 2005; Ling et al., 2018). The acetylation level of global histones is highest in germinal vesicle (GV) oocytes and lowest in metaphase I (MI) and metaphase II (MII) oocytes (Endo et al., 2005; Akiyama et al., 2004; Zhou et al., 2017). Histone acetylation is eliminated by HDAC in the cytoplasm (Endo et al., 2008). During the SCNT step of injection, after the nuclear membrane breaks down, the donor cells injected into MII oocytes quickly lose their high levels of acetylation on histones (Endo et al., 2008). Histone acetylation markers, such as Lys9 acetylation of H3 (H3K9ac) at the ZGA stage and H3K14ac at the blastocyst stage, eventually decrease and disappear (Rybouchkin et al., 2006; Wee et al., 2006; Liu et al., 2012; Zhai et al., 2018). In addition, Sun et al. (2020) reported that HDAC in the cytoplasm still continues to eliminate the high levels H3K9ac of donor cell after injection. Broad-spectrum deacetylase inhibitors (HDACi) are used to preserve the acetylation of histones H4K8 and H3K9 in SCNT embryos (Wang et al., 2011; Chawalit 2012). The low cloning efficiency is the result of these disrupted histone modifications, which also impair chromatin accessibility and cause disordered expression of genes necessary for the normal development of cloned embryos (Liu et al., 2012; Xie et al., 2016; Zhai et al., 2018). Hence, one important factor influencing the development of cloned embryos is histone modification.

2.2.2.2 Histone methylation

Histone methylation mainly occurs on lysine and arginine, there are 3 types of methylation patterns: monomethylation (me), dimethylation (me2), and trimethylation (me3) (Izzo and Schneider, 2010). According to Liu et al. (2016)

and Ninova et al. (2019), The most common changes are trimethylation of H3 such as H3K4me3, H3K27me3 and H3K9me3. The H3K4me3 is linked to gene activation and is regulated by the Trithorax group (TrxG) complex, whereas H3K27me3 is mediated by the Polycomb group (PcG) proteins results in gene suppression and H3K9me3 catalyzed by suppressor histone lysine methyltransferase of variegation 39H1/2 (Suv39H1/2) and eliminated by lysine demethylase (Kdm)4. In addition, the depletion of trimethylation of H3, such as H3K4me3 and H3K9me3 improved gene expression patterns and increased blastocyst rates in porcine SCNT embryos (Zhang et al., 2018; Jeong et al., 2021).

2.3 Small molecules for enhancing the development of cloned embryos by ameliorating epigenetic reprogramming

2.3.1 2-(4-morpholinoanilino)-6-cyclohexylamino-purine-analogue (Reversine)

According to Chen et al. (2004), Reversine, the small molecule, induces the plasticity of C2C12 myoblasts at the single-cell level, and Reversine-treated cells gain the ability to differentiate into osteoblasts and adipocytes under lineage-specific inducing conditions. Hence, Reversine functions in cell reprogramming and regenerative medicine have been the focus of numerous investigations (Anastasia et al., 2006; Kim et al., 2007; Saraiya et al., 2010; Jung et al., 2011). The goal of Reversine is to reprogram somatic cells into multipotent, meaning that can differentiate into numerous cell types. Several investigations have shown that Reversine suppresses tumors in human cancer cells by causing apoptosis, cell cycle arrest, polyploidy and autophagy (Lu et al., 2016; Lu et al., 2012; Lee et al., 2012; Kuo et al., 2014). In the first report of Reversine treatment in bovine SCNT embryos, Yoisungnern et al. (2011) investigated the effect of Reversine treatment on the developmental potential of bovine cloned embryos. The result showed that the bovine SCNT embryos after fusion, the reconstructed embryos were cultured in activated and culture medium supplemented with different concentrations of Reversine (0, 1, 5 and 10 μM) and different duration times (0, 6, 12 and 18 h), the blastocyst formation rates of embryos treated with 1 μM Reversine for 6 h and 12 h were significantly higher than those of Reversine treated with 5, 10 μM and without Reversine ($P<0.05$). These results suggest

that Reversine under optimal condition could enhance development of bovine SCNT embryos. Reversine has been used to treat porcine SCNT embryos with different concentrations (0, 1, 5 and 10 μM) and durations (0, 6, 12, 18 and 24 h) in culture medium. The result showed porcine SCNT embryos treated with 5 μM Reversine for

12 h, the blastocyst formation rate was significantly higher than those without Reversine treatment (P<0.01) (Miyoshi et al., 2010). These results suggest that Reversine under optimal condition could enhance development of porcine cloned embryos. Although, the previous studies indicate that treatment with Reversine can improve the development of SCNT embryos, the underlying histone modification is still unclear.

2.3.2 Suberoylanilide hydroxamic acid (SAHA) or Vorinostat

A histone deacetylase inhibitor (HDACi), vorinostat, also known as SAHA, induces malignant cells to undergo growth arrest, death, or differentiation in both *in vitro* and *in vivo* settings (Roth et al., 2007). Hence, various studies have focused on the roles of SAHA in cell reprogramming and regenerative medicine. According to Wang et al. (2007), a certain kind of HDACi small molecules enhances the genomic reprogramming of somatic cells and positively changes epigenetic abnormalities by raising the levels of H3K9ac and H3K14ac. The HDACs are key enzymes in almost all tissues by the regulation of gene expression, but the expression level of various HDACs are different within different tissue types (Ruijter et al., 2003) and divided into five categories: class I (HDAC1-3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (SIRT1–7) and class IV (HDAC11) (Blackwell et al., 2008). Ono et al. (2010), reported that mouse SCNT embryos treated different concentration of SAHA (0.1, 1, 10 and 100 μM) for 6 h in culture medium. The results showed that when embryos were treated with 1 μM SAHA for 6 h, the blastocyst formation rate was significantly higher than that of embryos from other (P<0.01), suggesting that SAHA is an important HDACi small molecule to reprogram mouse SCNT embryos. Yoisungnern et al. (2012), investigated the effects of SAHA treatment on the development of cloned bovine embryos. The bovine SCNT embryos after fusion, the reconstructed embryos were cultured in activated and culture medium supplemented with different concentrations of SAHA (0, 0.1, 1 and 10 μM) and different duration times (0, 6, 12 and 18 h). The blastocyst rates of embryos treated with 1 μM SAHA for 6 h were significantly higher than those of SAHA-treated embryos (P<0.05), suggesting that SAHA supplemented in activated and culture medium could enhance development of bovine SCNT embryos. In additionally, Whitworth et al., (2015) investigated the effect of SAHA treatment (0, 1 and 10 μM for 14-16 h) in post-

fusion, activation and IVC of porcine embryos. The porcine SCNT embryos treated with 10 μM SAHA for 14-16 h had the highest level of the blastocyst formation rates, upregulated lysosome and successfully produced healthy piglets. However, the level of total cell number 1.0 μM SAHA were significantly higher than non-treatment group

(P<0.021). Sun et al. (2020), investigated the effect of SAHA on the epigenetic modification and preimplantation development of porcine cloned embryos. The mini pig fetal fibroblast was cultured in medium supplemented with different concentration of SAHA (0, 1.5, 3 and 6 μM) and different durations (0 to 96 h). After SCNT, the resulting porcine embryos were treated with 1.5 μM SAHA for 72 h, the blastocyst formation rate and the total cell number were significantly higher than that of embryos without SAHA treatment (P<0.05); Moreover, the H3K14ac levels in the treated SCNT blastocysts were close to that of IVF blastocysts. These results suggest that SAHA treated in fibroblast donor cells could improve histone acetylation and development of porcine SCNT embryos.

2.4 Beneficial application of cloning in pig

Pigs serve as an important agricultural resource and animal model in biomedical research (Prather et al., 2008).

2.4.1 Agricultural applications

Porcine cloned embryos, developed through techniques like SCNT for producing genetically engineered (GE), particularly in enhancing livestock productivity, improving disease resistance (Lee et al., 2020). The distribution of breeding livestock can be expanded genetic resources preserved with the use of cloning (Keefer, 2015).

2.4.1.1 Enhanced Livestock Traits

Cloning in pigs allows the replication of individuals with desirable traits, such as increased growth rates, lean meat production, or resistance to specific diseases. The strategies to produce embryos with selected genetic profiles, breeders can introduce specific advantageous traits into herds faster than through traditional breeding (Niemann and Lucas-Hahn, 2012).

2.4.1.2 Disease Resistance

Cloned embryos enable researchers to introduce genes that can help pigs resist common diseases that threaten livestock such as African swine fever (ASF). ASFV is a large, enveloped double-stranded DNA virus and the single member of the family Asfarviridae (Dixon et al., 2005). Warthogs act as a host to the virus as it causes a non-clinical and persistent infection. For instance, through gene

editing combined with cloning, scientists can engineer pigs to be more resistant to these pathogens, improving herd health and reducing the need for antibiotics.

2.4.1.3 Genetic Preservation

Since the outbreak of African swine fever in European and Southeast Asian worsened the status of local pig breeds (Sánchez-Vizcaíno et al., 2013; Nga et al., 2019). Cloning provides a means to preserve the genetics of valuable animals for biodiversity, sustainable food production, and heritage preservation, ensuring that genetic resources from elite breeding stock or endangered breeds are not lost (Ibtisham et al., 2017). Cloning embryos from high-performing pigs enables the continued replication of these valuable genetics, which can be particularly useful in breed improvement programs.

2.4.2 Biomedical Applications

Pigs have emerged as a critical model, due to physiological similarities between pigs and humans, initially as potential organ donors in xenotransplantation (Lu et al., 2020), which is the transfer of organs across other species such as pig to human (Montgomery et al., 2022; Kavarana et al 2022) and subsequently as models for studying human diseases (Aigner et al., 2010) or as bioreactors for hyperimmune sera production (Reynard et al., 2016). Although the CRISPR/Cas9 technique via zygote microinjection is becoming popular for single-gene editing due to its high efficiency (Lee et al., 2020), SCNT remains the preferred method for complex modifications, such as multiplexed knockouts (KO) and knock-ins (KI) (Niu et al., 2017; Fischer et al., 2016), where edited cells are used.