การศึกษาดีเอ็นเอเมทิลเลชั่นในเซลล์สืบพันธุ์และเซลล์ร่างกายของโคเพศผู้:
ความแตกต่างระหว่างชนิดของเซลล์และอิทธิพลของการเพาะเลี้ยงเซลล์ภายนอกร่างกาย

นางสาวนวเพ็ญ ภูติกนิษฐ์

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THE STUDY OF DNA METHYLATION PROFILES OF BULL GERM CELLS AND SOMATIC CELLS: LINEAGE DIFFERENCE AND EFFECT OF IN VITRO CELL CULTURE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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Thesis Title	THE STUDY OF DNA METHYLATION PROFILES OF BULL GERM CELLS AND SOMATIC CELLS: LINEAGE DIFFERENCE AND EFFECT OF IN VITRO CELL CULTURE
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นวเพ็ญ ภูติกนิษฐ์ : การศึกษาดีเอ็นเอเมทิลเลชั่นในเซลล์สืบพันธุ์และเซลล์ร่างกายของโคเพศผู้: ความแตกต่างระหว่างชนิดของเซลล์และอิทธิพลของการเพาะเลี้ยงเซลล์ภายนอกร่างกาย. (THE STUDY OF DNA METHYLATION PROFILES OF BULL GERM CELLS AND SOMATIC CELLS: LINEAGE DIFFERENCE AND EFFECT OF IN VITRO CELL CULTURE) อ. ที่ปรึกษา :ศ.น.สพ.ดร.มงคล เตชะกำพุ, อ.ที่ปรึกษาร่วม : รศ.น.สพ.ดร.ชัยณรงค์ โลหชิต, Prof. Dr.Michael D'Occhio. 69 หน้า.

ทำการศึกษาลักษณะของดีเอ็นเอเมทิลเลชั่นในเนื้อเยื่อชนิดต่าง ๆ ของพ่อโคพันธุ์โฮลไตน์จำนวน 3 ตัว อายุระหว่าง 2-3 ปี ด้วยเทคนิคพีซีอาร์ที่ใช้ไพรเมอร์ขนาดสั้นซึ่งได้รับการพัฒนาขึ้นค้นมาเพื่อการศึกษาในพืชที่มีชื่อว่า แอมพลิไฟล์ เมทิลเลชั่น โพลิมอร์ฟิซึม พีซีอาร์ ร่วมกับการใช้เอนไซม์ย่อยดีเอ็นเอชนิดที่ไวต่อเมทิลเลชั่น การศึกษาที่ 1 เป็นการศึกษาความแตกต่างของลักษณะดีเอ็นเอเมทิลเลชั่นระหว่างเซลล์สืบพันธุ์กับเซลล์ร่างกาย โดยเก็บตัวอย่างดีเอ็นเอจากตัวอสุจิ เม็ดเลือดขาว และเซลล์เนื้อเยื่อเกี่ยวพันเพาะเลี้ยงพาสซาจที่หนึ่ง ทำการย่อยดีเอ็นเอด้วยเอนไซม์ *Hpa*II จากนั้นนำตัวอย่างดีเอ็นเอทั้งที่ยังไม่ได้ย่อยและย่อยแล้วไปผ่านกระบวนการพีซีอาร์ แยกผลิตภัณฑ์พีซีอาร์ที่ได้บนเจลโพลีอะคริลาไมด์และย้อมด้วยซิลเวอร์ไนเตรต อ่านผลโดยการสังเกตการปรากฏของมาร์กเกอร์ 3 ชนิด คือ มาร์กเกอร์ที่ทนต่อการถูกย่อยด้วยเอนไซม์ มาร์กเกอร์ที่ไวต่อการย่อย และมาร์กเกอร์ที่พบได้เฉพาะในตัวอย่างดีเอ็นเอที่ผ่านการย่อยแล้ว ผลจากการใช้ไพรเมอร์ 27 ชิ้น สามารถผลิตมาร์กเกอร์ได้ประมาณ 1,000 มาร์กเกอร์จากตัวอย่างดีเอ็นเอของโคแต่ละตัว ลักษณะของมาร์กเกอร์ที่ได้จากโคเพศผู้ทั้งสามตัวคล้ายคลึงกัน มาร์กเกอร์ส่วนใหญ่เป็นมาร์กเกอร์ชนิดที่ทนต่อการย่อย เม็ดเลือดขาวมีมาร์กเกอร์ชนิดทนต่อการย่อยสูงที่สุด ($p<0.05$) และอสุจิมีมาร์กเกอร์ชนิดไวต่อการย่อยสูงที่สุด ($p<0.05$) เซลล์เนื้อเยื่อเกี่ยวพันมีมาร์กเกอร์ที่พบได้เฉพาะในตัวอย่างที่ผ่านการย่อยแล้วมากที่สุด ($p<0.05$) จากการศึกษานี้สรุปได้ว่า ณ ตำแหน่งการย่อยจำเพาะของเอนไซม์ *Hpa*II ในดีเอ็นเอของเซลล์สืบพันธุ์มีระดับเมทิลเลชั่นน้อยกว่าเซลล์ร่างกาย และเซลล์ร่างกายที่แตกต่างกันมีลักษณะของดีเอ็นเอเมทิลเลชั่นที่ต่างกันด้วย โดยเซลล์เม็ดเลือดขาวมีปริมาณดีเอ็นเอเมทิลเลชั่นในตำแหน่งที่ศึกษามากที่สุด ส่วนเซลล์เนื้อเยื่อเกี่ยวพันมีแนวโน้มที่จะมีลักษณะโครงสร้างของดีเอ็นเอแตกต่างออกไป การศึกษาที่ 2 เป็นการศึกษาผลของการเพาะเลี้ยงเซลล์เนื้อเยื่อเกี่ยวพันที่ได้จากใบหูโคภายนอกร่างกายต่อลักษณะของดีเอ็นเอเมทิลเลชั่น ทำการเพาะเลี้ยงเนื้อเยื่อใบหูจนถึงพาสซาจที่ 30 เก็บตัวอย่างดีเอ็นเอจากพาสซาจเลขคี่ แบ่งตัวอย่างออกเป็น 3 กลุ่มคือ กลุ่มพาสซาจระยะแรก กลุ่มพาสซาจระยะกลาง และพาสซาจระยะท้าย นำตัวอย่างผ่านกระบวนการพีซีอาร์ ผลปรากฏว่าไม่พบการเปลี่ยนแปลงของดีเอ็นเอเมทิลเลชั่นในตัวอย่างแต่ละกลุ่มเมื่อทำการตรวจสอบมาร์กเกอร์ประมาณ 1,500 มาร์กเกอร์ สรุปได้ว่าการเพาะเลี้ยงเซลล์เนื้อเยื่อเกี่ยวพันที่ได้จากใบหูภายนอกร่างกายโดยใช้วิธีการเพาะเลี้ยงเซลล์โดยทั่วไปติดต่อกันเป็นเวลา 5 เดือนไม่ส่งผลกระทบต่อลักษณะดีเอ็นเอเมทิลเลชั่น ผลสรุปรวมของวิทยานิพนธ์ฉบับนี้คือ 1) สามารถปรับใช้เทคนิคแอมพลิไฟล์ เมทิลเลชั่น โพลิมอร์ฟิซึม พีซีอาร์เพื่อการศึกษาดีเอ็นเอเมทิลเลชั่นในดีเอ็นเอของสัตว์เลี้ยงลูกด้วยนมได้ 2) เซลล์สืบพันธุ์กับเซลล์ร่างกายมีลักษณะของดีเอ็นเอเมทิลเลชั่นในตำแหน่งที่ศึกษาแตกต่างกัน โดยเซลล์ร่างกายมีปริมาณดีเอ็นเอเมทิลเลชั่นมากกว่าเซลล์สืบพันธุ์ และเซลล์ร่างกายที่สามารถพัฒนาเป็นเซลล์ชนิดอื่นได้มีแนวโน้มที่จะมีโครงสร้างของดีเอ็นเอที่แตกต่างออกไป และ 3) สภาพการเพาะเลี้ยงเซลล์ภายนอกร่างกายที่ใช้ในการศึกษาครั้งนี้ไม่มีผลต่อการเปลี่ยนแปลงรูปแบบของดีเอ็นเอเมทิลเลชั่นของเซลล์เนื้อเยื่อเกี่ยวพันจากใบหูที่ถูกเพาะเลี้ยงติดต่อกันจนถึงพาสซาจที่ 30

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....นวเพ็ญ ภูติกนิษฐ์.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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NAWAPEN PHUTIKANIT: THE STUDY OF DNA METHYLATION PROFILES OF BULL GERM CELLS AND SOMATIC CELLS: LINEAGE DIFFERENCE AND EFFECT OF IN VITRO CELL CULTURE. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., Doctorat de 3e cycle THESIS COADVISORS: ASSOC.PROF. CHAINARONG LOHACHIT, D.V.M., Dr. Med. Vet. , PROF. MICHAEL D'OCCHIO, B.Sc., Ph.D. 69 pp.

The DNA methylation profiles of various tissues collected from three Holstein bulls aged between 2-3 years old were evaluated by the arbitrarily-primed PCR technique originally designed for plant DNA research called Amplified methylation polymorphisms (AMPs)-PCR in combination with a methylation-sensitive restriction enzyme. Experiment 1 was aimed to study the difference of DNA methylation patterns between germ cells and somatic cells in bulls. DNA was extracted from sperm, white blood cells and fibroblast cell culture passage number 1, and was digested with *Hpa*II enzyme. The genomic and digested DNA samples were subjected for AMPs-PCR. The PCR products were separated on polyacrylamide gel and stained with silver nitrate. The result evaluation based on the presence-absence of three types of marker: digestion resistant-, digestion sensitive- and digestion dependent marker. From twenty-seven sets of primer, approximately 1,000 markers could be scored in each bull. The samples from all bulls showed a similar but not identical pattern. Most of the markers were digestion-resistant markers signifying that both germ cells and somatic cells are generally methylated at the *Hpa*II sites. Leukocytes had the highest percentage of digestion resistant markers ($p<0.05$), whereas sperm cells showed a highest percentage of digestion sensitive markers ($p<0.05$). Fibroblast cells yielded the highest percentage of digestion dependent markers ($p<0.05$). The results showed that germ cells have less methylation than somatic cells. There are different methylation patterns among somatic cell types: leukocyte DNA is more methylated than fibroblast cells and partial differentiated somatic cells such as fibroblasts may have a different chromatin structure. Experiment 2 was aimed to study the effect of long-term cell culture on the DNA methylation profile of cultured fibroblast cells. The ear fibroblast cells were cultured in a standard culture protocol using basic culture medium supplemented by fetal calf serum and broad spectrum antibiotics until passage number 30. The cells from odd number passages were collected for DNA extraction. The digestion of DNA was carried out with *Hpa*II enzyme. The samples were categorized into 3 groups: early-, medium- and late passages. The AMPs-PCR revealed that there was no alteration at the approximately 1,500 DNA methylation locations among groups. This can be concluded that the culture condition applied in this experiment did not affect the DNA methylation content in ear fibroblast cells culture maintained continuously for 5 months under the conventional cell culture conditions. Overall conclusions of this study are 1) the AMPs-PCR technique could be applied in the study of mammalian DNA methylation; 2) DNA methylation profiles of germ cells and somatic cells are different. At the *Hpa*II sites investigated, somatic cells have more methylation content than germ cells and partial differentiated somatic cell lineage tends to have different genomic structure and 3) *in vitro* cell culture condition used in this study did not affect the DNA methylation profiles of fibroblast cells cultured up to passage number 30.

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CHAPTER I

INTRODUCTION

The development of all living organisms depends on the genetic information stored in double-stranded DNA packed inside a cell nucleus. Once the fertilization takes place, one totipotent cell is formed and subsequently cleaves, develop and differentiate into many cell types to make up the complete body of a multi-cellular organism. Basically, every single cell has the same genomic sequence and the differentiation into specific cell lineages relies on dissimilar yet accurate gene expression. Epigenetic marks, such as DNA methylation, histone modification, and DNA binding proteins are thought to be an important mechanism to direct gene function without altering the DNA sequence (for review see Li, 2002; Jaenisch and Bird, 2003).

Among all epigenetic modifications found in plants and animals, DNA methylation is widely studied and well documented in many species (Jones and Takai, 2001; Martienssen and Colot, 2001). Methylation of DNA occurs mainly on the 5'-cytosine residues located adjacent to the guanine bases, known as the CpG dinucleotides. The formation of 5'-methylcytosines (5-mC) requires a catalytic process by a family of enzymes called DNA methyltransferase (Dnmt) (for review see Wilkins, 2005). DNA methylation, working in combination with other substances such as DNA-binding proteins, is considered to regulate gene expression by transcriptional repression. This epigenetic mark has been proposed to involve in many major events: imprinted genes, X-chromosome inactivation, tissue-specific gene expression and inactivation of intragenomic parasites (Reik et al., 2003). Moreover, its establishment and maintenance obviously plays a crucial role in normal embryo development (Li, 2002) and global genetic stability. There were reports of abnormal DNA methylation patterns in case of congenital anomalies (Delaval et al., 2006) and cancers (Baylin et al., 2001; Lin et al., 2001; Matsuzaki et al., 2005). Most interestingly, DNA methylation has been claimed

to be a major obstacle in the successful production of cloned animals derived from somatic cell nuclear transfer (SCNT) (for review see Latham, 2005).

The main impediment of SCNT experiments nowadays is a low number of healthy offspring that can survive to adulthood. Most of cloned embryos died *in utero*, while some developed to term but perished at or shortly after birth (Cibelli et al., 2002; Tsunoda and Kato, 2002). Gene expression in tissue samples collected from cloned embryos and fetuses showed signs of aberrancy (Wrenzycki et al. 2001; Humpherys et al., 2002; Li et al., 2005), reflecting the failure of the transcription regulation process. In relation to this finding, DNA methylation has stepped into a limelight for the reason that it holds the key to the success of embryo development after fertilization (Reik et al., 2001). Indeed, many studies have confirmed that DNA of cloned embryos/fetuses shows different and deviated methylation profiles compared to those of embryos/fetuses derived from normal fertilization.

The causes of abnormal methylation profiles in cloned animals are elusive. The idea that ooplasm fails to completely reprogram a transferred somatic cell nucleus is proved to be possible. The initial nuclear reprogramming process as seen in normal fertilization, such as nuclear envelop breakdown and chromosome condensation, has been reported in somatic cell nuclei after transfer (Latham, 2005), but further process seems to be missing. Although some of single-copy sequences are successfully reprogrammed, but repeat sequences are still highly methylated comparable to those in donor cells (Kang et al., 2002) Therefore, it is plausible that some sets of genes required for normal embryonic development are repressed by methylation at their promoter or regulatory region in cloned embryos, resulting in abnormal phenotypes or even the death of the embryos.

Why an ooplasm can reprogram a sperm cell but not a somatic cell? The knowledge of the different DNA methylation patterns between sperm cells and somatic cells has been aware of and was confirmed by many studies (Sturm and Tayler, 1981; Ponzetto-Zimmerman and Wolgemuth, 1984; Shiota et al., 2002; Li et al., 2006). Somatic

cell lineage possesses high methylation content (Reik and Dean, 2001), owing to the fact that only specific sets of gene necessary for the maintenance of a particular cell type is active while the other sets are turn off. While mature sperm cells, which although are regarded as inactive cells, contain less 5-mC content. There is evidence indicates a lower degree of methylation at satellite DNA sequences, as well as demethylation of some CpG islands in male germ cells contribute to this finding (Sturm and Tayler, 1981; Feinstein et al., 1985; Shiota et al., 2002). Therefore, one could hypothesize that incomplete reprogramming may occur at the multiple-copy or repetitive DNA sequences of somatic cell donor nucleus, which are heavily methylated and may be resistant to the demethylation substances stored in ooplasm.

According to this rationale, some researchers attempted to reduce the methylation content in somatic cells prior to transfer by applying a DNA methyltransferase inhibitor agent in donor cells. The chemical successfully reduced the methylation in somatic cells but, unfortunately, blastocyst formation rates were lower than those of untreated cells (Enright et al., 2003). Thus, it is likely that the amount of DNA methylation in donor cells might not be the major impediment. In concert with a report showing that *Xenopus* oocyte selectively demethylated specific locations of the *Oct4* gene promoter in mouse thymus nucleus and this activity was dependent on the existence of other methylated sites in the genome (Simonsson and Gurdon, 2004). Hence, the locations of the methylation might be more crucial to the success of nuclear reprogramming. The investigation of the global locations of DNA methylation between two cell lineages could provide more information on this topic.

Although it is possible that incomplete reprogramming of donor nucleus leads to the failure of SCNT, but the effect of cloning process itself cannot be neglected. There are many factors involving in the whole SCNT process: donor cells and recipient oocytes preparation, electro-fusion and *in vitro* culture of reconstructed oocytes. Amongst these factors, *in vitro* culture process has been extensively studied and shows a high tendency to affect the quality of cloned embryos. Synthetic culture media and serum supplementation used for the culture of preimplantation embryos were reported to be associated with

deregulation of many imprinted genes responsible for fetal development (Doherty et al., 2000; Khosla et al., 2001; Fernandez-Gonzalez et al., 2004). Thus, some fetuses encountered the *in vitro* culture at preimplantation stage developed phenotypic anomalies as often seen in Large Offspring Syndrome (LOS) (for review see Young et al., 1998).

According to this notion, the somatic donor cells cultured *in vitro* before transfer should also be affected. When compared with cultured embryos, the cells are maintained and propagated *in vitro* for a longer period. Therefore, the side effects from artificial environment stress and cell-aging process should be more severe. Should cultured cells harboring abnormal epigenetic pattern be used as donor nucleus, together with the evidence that recipient oocytes may fail to reset the fault epigenetic profile back to normal status, cloned embryos would eventually bare these flaws in their genome.

There are a number of experiments carried out to investigate the effects of the *in vitro* culture on the quality of donor cells; however, the results are still controversial. *In vitro* culture of donor cells is somewhat necessary, as non-passage cells were reported to have less ability to produce cloned embryos (Dinnyes et al., 2001). On the other hands, some researchers found detrimental effect of long-term culture of fibroblast cells on the quality of cloned embryos (Roh et al., 2000; Li et al., 2003; Jang et al., 2004) while the others reported no correlation between the duration of *in vitro* culture of donor cells and the outcome of cloning (Bhuiyan et al., 2004; Tian et al., 2003). This doubtful circumstance leads to the preference of early-passage cells to be used as donor nuclei for SCNT.

In-depth investigation of the effect of long-term culture on the quality of somatic cells is essential. DNA methylation is one factor reported to be affected by prolonged *in vitro* culture and cell aging process. Abnormal methylation pattern could initiate pathological conditions if the alterations occur at or near specific genes or their regulatory elements (Richardson, 2003) as well as at the locations containing parasitic DNA elements. Study in senescent human fibroblasts cultured *in vitro* revealed the loss

of methylation at satellite sequences, leading to chromosome instability (Suzuki et al., 2002). In animal cell culture, however, this field of knowledge is less investigated.

According to the rationale mentioned above, this dissertation had been set up to explore the genome-wide DNA methylation profile of mammalian cells by using a methylation sensitive enzyme in combination with a PCR-based technique, developed by researchers from the University of Queensland, Brisbane, Australia, in order to uncover epigenetic status of mammalian cells of different lineages. Moreover, this powerful technique was used as a tool to investigate any alteration of DNA methylation after long-term culture of somatic cells. The information gained from this experiment would support the basic knowledge of the DNA methylation profile in mammalian cells and would alert awareness of researchers working with cells and/or tissue cultured *in vitro* about the biological and environmental factors that might affect their experiments.

CHAPTER II

LITERATURE REVIEW

1. Epigenetic modification of the DNA

Multi-cellular organisms compose of many complex body structures. Various types of cells arise, precisely differentiate into various kinds of tissue and rearrange into assorted functional organs. Surprisingly, each of every cell in the body in fact has originated from just only one cell: a fertilized ovum. When paternal genome from a spermatozoon joins with maternal genome from an ovum, one totipotent cell springs into life and it starts divining to finally form the complete body of a living organism.

Basically, from the notion mentioned above, every cell in a body contains the same genetic information in its DNA and the differentiation of cells occurs without any alteration in the DNA sequence. Generally, cells exhibit different nature because they possess different regulatory proteins, reflecting the dissimilarity of gene expression among cell types. Therefore, the mechanism responsible for cell differentiation is the regulation of gene expression.

Genes can be regulated in many levels: from the complicate processes controlling protein activities to the common global mechanism aimed to prevent transcription. The DNA structure plays a crucial role in transcriptional initiation in both prokaryotes and eukaryotes by controlling the transcriptional regulator binding process. Generally, densely packed genome structure, as seen in heterochromatin regions, prevents transcriptional regulators to come into contact with the specific DNA regulatory sequence, hence the transcription is repressed, while DNA in diffuse form facilitates gene expression by allowing the transcriptional regulatory proteins to bind with the DNA and turn the genes on (Twyman, 1998).

Many factors influence DNA structure: stage of the cell cycle is one example. During cell division process, chromatin is most condense at the metaphase stage and becomes more diffuse at the interphase stage. Moreover, modifications of DNA component itself can also alter the architecture of chromatin and affect transcriptional property by facilitate or hinder regulatory protein binding (Luo and Dean, 1999). These modifications are heritable and some of them are reversible depending on intrinsic and extrinsic factors. This special mechanism is known as epigenetic modifications.

Epigenetic investigation is becoming more and more extensive both in prokaryotic and eukaryotic kingdoms. Plants and vertebrates accumulate many epigenetic marks in their genome so their gene regulation machinery is highly complex. Among various modifications of the chromatin, DNA methylation is widely studied and becomes well documented in many species (Jones and Takai, 2001; Martienssen and Colot, 2001).

2. DNA methylation

2.1) Definition of DNA methylation

DNA methylation is a reversible chemical modification of genomic DNA. Catalyzed by a special enzyme family, a methyl group is transferred from s-adenosylmethionine (SAM) to the carbon-5 position of the cytosine ring (figure 1); forming the 5-methylcytosine (5-mC) (Dolinoy et al., 2007). DNA methylation in mammals locates mainly at the palindromic sequence 5’-CpG-3’ or so called CpG dinucleotides, while DNA methylation in plants can also be found at the 5’-CpNpG-3’ or 5’-CpNpN-3’ (N stands for any nucleotide base) (Chan et al., 2005).

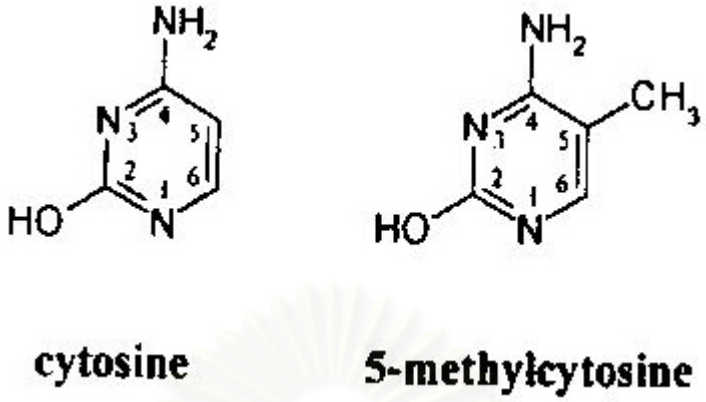

Figure 1. Illustration showing the cytosine and 5-methycytosine structures (Modified from Goto and Monk, 1998)

2.2) Maintenance of DNA methylation

In mammalian genome, more than 70% of CpG dinucleotides are methylated (Ehrlich et al., 1982) and this characteristic has been maintained through many cell divisions by enzymatic mechanism. The enzyme family known to responsible for the heritable property of this epigenetic mark is the DNA (cytosine-5') methyltransferases (Dnmts).

The most abundant enzyme of this family found in mammalian cells is the DNA (cytosine-5') methyltransferase1 (Dnmt1) (Robertson et al., 1999). The activity of the enzyme is to recognize the hemi-methylated DNA strand; generally occur after DNA replication and the daughter strand is unmethylated, then add the methyl group to it in order to retain the methylation profile in the new set of genome. According to its activity, researchers name this enzyme as the maintenance DNA methyltransferase. Mutant mice lacking the DNA coding regions of this enzyme displayed a hypomethylation pattern in their genome, resulting in growth retardation, abnormal fetal formation (Trasler et al., 1996) and died *in utero* (Li et al., 1992).

Other members of the Dnmts enzyme family are the *de novo* DNA methyltransferases, known as Dnmt3a and Dnmt3b. These enzymes are capable of catalyzing methyl group into unmethylated as well as hemi-methylated DNA (Chen et al., 2003) and play a crucial role in embryogenesis. The expression of both enzymes could be detected in mouse embryos from embryonic day (E) 7.5 onward (Okano et al., 1999)

The absence of gene expression for Dnmt3s also causes abnormalities in fetus. Heterozygous *Dnmt3a*$^{+/-}$ and *Dnmt3b*$^{+/-}$ mice were phenotypically normal and fertile. Homozygous mutants with *Dnmt3a*$^{-/-}$, though could develop to term and were born alive, died shortly afterward. In contrast, homozygous *Dnmt3b*$^{-/-}$ mice died *in utero* (Okano et al., 1999).

Furthermore, from the screening of genome database, the *Dnmt3L* (DNA methytransferase3-like) gene has been identified. The gene contains domains related to the *Dnmt3a* and *Dnmt3b*, but its protein lacks the enzymatic property (Meehan, 2003). However, many research works demonstrated that this protein was essential for germ cell development in mice (Bourc'his et al., 2001; Bourc'his and Bestor, 2004; Webster et al., 2005; La Salle et al., 2007)

There is another putative methyltransferase enzyme called Dnmt2, but its function and activity concerning DNA methylation is still unclear (Wilkins, 2005).

2.3) Roles of DNA methylation

Generally, DNA methylation plays an important role in controlling gene expression. Two models of gene regulating mechanisms of DNA methylation have been proposed. First hypothesis is that the DNA methylation blocks the transcriptional regulating proteins from binding to cognate DNA sequences by changing the chromatin structure to an inaccessible form (Lewis and Bird, 1991; Li, 2002). The second hypothesis proposes that DNA methylation attracts DNA methylation binding proteins (MBPs), which will co-operate with other repressor substances to interfere the binding of

transcriptional regulating factors at the gene promoter (Figure 2) (Richardson and Yung, 1999; Newell-Price et al., 2000).

However, DNA methylation machinery does not control every gene expression pathway in the body. Results from molecular studies showed evidence that DNA methylation mainly involves in 4 mechanisms: genomic imprinting, X-chromosome inactivation, tissue-specific gene expression and silencing of retrotransposable elements (Reik et al., 2003).

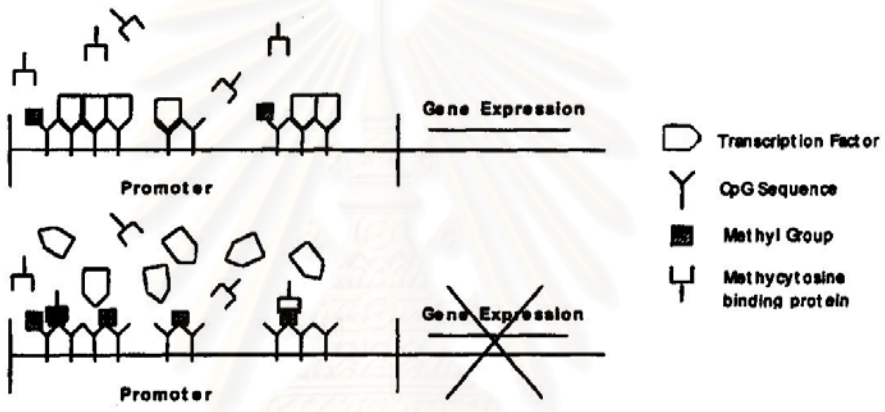

Figure 2. Illustration showing the mechanism of DNA methylation regulating gene expression by accumulating methylcytosine binding protein to the location of gene promoter (Richardson and Yung, 1999)

2.3.1) DNA methylation and genomic imprinting

Genomic imprinting is characterized by the mono-allelic expression of genes depending on the parental origin. This procedure is extremely crucial for normal embryo development. Imprinting is germ line specific (Tucker et al., 1996). The establishment and maintenance of the imprinting profile depend on the activity of Dnmt3a enzyme in combination with *Dnmt3L* protein (Kaneda et al., 2004). Aberrancy in expression of imprinted genes causes severe congenital defects in human (Jiang et al., 2004; Delaval et al., 2006).

Results from molecular investigation revealed that DNA methylation is the main mechanism of gene imprinting (Smith et al., 2003; Bruce et al., 2008). During the gametogenesis, the epigenetic marks of the primordial germ cells (PGCs) inherited from sperm and ovum are erased and a new methylation profile is set up in a specific manner according to sex of the individual (Arney et al., 2001). For example, *H19* allele is methylated in male gametes (Davis et al., 2000), whereas the insulin-like growth factor-II receptor (*igf2r*) allele is methylated in female gametes (Lucifero et al., 2004). Results from bisulfite sequencing detecting DNA methylation in germ cells revealed that timing of imprinting in the male and gemale gametes are different. Male germ cells acquire imprinting pattern prenatally, while female gametes complete the imprinting process postnatally (for review see Trasler, 2006)

2.3.2) DNA methylation and X-chromosome inactivation

X-chromosome inactivation is a dosage compensation strategy to equalize the activities of the X-chromosome between females who have two X-chromosomes and males who have only one X-chromosome. In female somatic cells, one X-chromosome becomes transcriptionally inactive (Xi). Xi genome structure is densely packed to form heterochromatin and most of the X-linked genes are repressed. This inactivation occurs in a random fashion between paternal- and maternal-derived X-chromosome. Once one X-chromosome is chosen to become inert, the inactivation process is irreversible and maintained through mitosis. This phenomenon leads to phenotypic variation in an individual female for cells express different X-linked genes grow and accumulate in patch (i.e. tortoiseshell coat color in cats) (Twyman, 1998).

The inactivation of X-chromosome consists of 4 steps: counting of the X-chromosomes, initiation, spreading and maintenance of inactivation. The counting step ensures that in one cell there is only one active X-chromosome while the other X-chromosome(s) is inactivated. By the action of this step, abnormal cells containing more than 2 X-chromosome are still alive and express normal level of X-linked genes (Twyman, 1998).

The initiation step occurs under supervision of the X inactivation center (Xic). This domain carries *Xist* gene, which is necessary for the initiation of inactivation process. The expression of *Xist* gene is controlled by DNA methylation. Nevertheless, the initiation of X-chromosome inactivation does not depend on the level of DNA methytransferase enzyme (Panning and Jaenisch, 1996; Sado et al., 2004).

2.3.3) DNA methylation and tissue-specific gene expression

In term of genome-wide DNA methylation pattern, the relationship between methylation profiles and cell types was uncovered by the digestion of genomic DNA of various tissue types with endonuclease enzymes. The results from the digestion suggested that DNA methylation profile is tissue-specific. Different tissue types from one individual or individuals from the same genetic background showed different DNA methylation patterns (Kaput and Sneider, 1979), while same tissue type from different individuals displayed similar DNA methylation profiles (Grunau et al., 2000).

When specific sets of genes in various tissues were evaluated, the interpretation of DNA methylation profile of gene regulatory regions was far more complicated. Since most of CpG islands are unmethylated regardless to the expression status of genes located downstream, thus methylation might not be the primary regulatory mechanism and other regulatory mechanism(s), such as transcriptional factors, should be involved. However, from the studies in mice and human, there are special locations in the genome known as tissue-specific differentially methylated regions (TDMRs) and they were reported to be associated with CpG island 5'-promoter regions, which influence the expression of genes located downstream (Song et al., 2005; Kitamura et al., 2007). Therefore, it is possible that a part of tissue-specific gene expression mechanisms depends on the action of DNA methylation located at the TDMRs.

DNA methylation also exhibits lineage-specific characteristic. There was obvious evidence indicating that DNA methylation is the primary mechanism to control the germ cell specific gene expression. The study in primordial germ cells (PGCs) in mice revealed

the expression of genes in postmigratory PGCs and its association with demethylation of CpG islands tentatively controlling their expression (Maatouuk et al., 2006). Moreover, expression of some unique germ cell specific genes was reported to rely on the DNA methylation status at the promoter. For example, the promoter and the first exon of the MAGE-A1 gene were heavily methylated in normal human somatic cells but were hypomethylated in testis tissue (De Smet et al., 1999).

2.3.4) DNA methylation and transposable elements

Nearly half of mammalian genome consists of transposable elements in form of interspersed repeats (Deininger and Batzer, 2002). These elements have a special property of being able to move from place to place in the genome. These elements can be categorized into 3 types according to the mechanism of transposition: DNA transposons, autonomous retrotransposons and non-autonomous retrotransposons.

DNA transposons are abundance in bacteria but can also be found in higher organisms, such as insects and human. They move in a “cut and paste” fashion: cutting themselves from one place and re-insert into a new place. These elements encode enzymes necessary for DNA strand breakage and recombination (Kazazian Jr., 2004). Transposition in some eukaryotes usually occurs next to the original site, so called regional reintegration (Twyman R.M., 1998)

Retrotransposons, on the other hands, move in a “copy and paste” way. They can be transcribed into RNA and then reverse transcribed into DNA at the new location. There are two sub-categories of retrotransposons: autonomous and non-autonomous retrotransposons (Kazazian Jr., 2004).

Autonomous retrotransposons have similar properties to retroviruses. They have open reading frames (ORFs) encoding proteins necessary for their transposition, i.e. reverse transcriptase enzyme to transcribe cDNA from their RNA form and endonuclease enzyme to digest host DNA for reintegration. There are two classes of autonomous

retrotransposons; characterized by the compositions of elements: Long terminal repeats (LTRs) and non-LTRs elements.

LTRs elements comprise of structures very similar to retroviruses except domains encoding envelop genes and viral capsule (Deininger and Batzer, 2002). They have long-terminal repeats at both ends, serving as transcriptional promoter and terminators. Examples of LTRs are Human endogenous retrovirus (HERV) found in humans and Intracisternal A particle (IAP) family in mice.

Non-LTRs elements consist of a 5'-untranslated region (5'UTR) harboring an internal promoter for RNA polymerase enzymes. They have two open reading frames (ORFs): one for producing a nucleic binding protein and another one for encoding enzymes necessary for transposition. At the end of the elements there is a 3'UTR and a poly(A) tail (Kazazian Jr., 2004). The most notable Non-LTRs in human is long interspersed nucleotide elements-1 or LINE-1 or L-1, comprising approximately 20% of human and mouse genome.

Non-autonomous retrotransposons utilize substrates from autonomous retrotransposons and also from host cells for transposition. Their elements have an internal promoter encoding RNA polymerase III and a poly(A) tail at the 3' end of the elements. This category of retroelements is also known as short interspersed nucleotide elements (SINEs) for their small size (approximately 80-400 bp) (Kazazian Jr., 2004). The examples of SINEs in mammalian genomes are *Alu* elements in humans and rodent B1 in mice.

Active transposable elements can cause genomic instability. Structural modification and/or undesirable element insertion occurs inside genes or gene regulatory regions (insertion mutation) will interfere gene activity, leading to diseases and abnormalities. Recent information demonstrates that DNA methylation is one pathway to deactivate these elements (Kazazian Jr., 2004). Indeed, repetitive sequences in somatic cells are heavily methylated (Yang et al., 2004) and demethylation and reactivation of

them was reported in many tumor cells (Neuhausen et al., 2006; Patthamadirok et al. 2007; Lin et al., 2001). However, some of these sequences, such as IAP and LINE-1, were hypomethylated in male germ cells (Yamagata et al., 2007). This discrimination between germ cells and somatic cells might contribute to the difference in total DNA methylation content between them (Sturm and Tayler, 1981; Ponzetto-Zimmerman and Wolgemuth, 1984; Shiota et al., 2002; Li et al., 2006). The explanation for this finding was proposed by Yamakata et al. (2007) that hypomethylation status of germ cell repetitive sequences might be involved in germ cell specific gene expression and also in the formation of unique germ cell chromatin structure.

2.4) DNA methylation dynamics during animal development

DNA methylation is a reversible modification mechanism. The genomic profile of methylation changes periodically, providing appropriate gene expression at a specific time of development. The major change of global DNA methylation takes place just after fertilization in order to prepare paternal and maternal genome for an important combination to produce a new life. This process is called genomic reprogramming.

Epigenetic reprogramming in embryo somatic cells is species-specific. In bovine, rat and human, paternal genome inside sperm nuclei undergoes active demethylation. This process occurs in a few hours after fertilization and before the first round of DNA replication (Oswald et al., 2000). While maternal genome becomes passive demethylated every time the cleavage takes place, resulting in hypomethylated embryos (Rougier et al., 1998). Most of the DNA methylation has been removed from embryonic DNA except the area of imprinted genes (Oswald et al., 2000). This step is speculated to benefit a new life by erasing all abnormal epigenetic marks inherited from parent genomes. Nonetheless, parental genome demethylation does not exist in sheep (Beaujean et al., 2004; Young and Beaujean, 2004) and rabbit embryogenesis (Shi et al., 2004), and in pig the maternal genome stays hypermethylated through the blastocyst stage (Fulka et al., 2006).

After the old epigenetic marks are removed, then a new set of marks will be added into embryos. This step is call *de novo* methylation. Catalyzed by Dnmt3a and Dnmt3b enzymes, new methyl groups are arranged into embryonic genome according to the fate of cells and maintained through cell division by the action of Dnmt1.

In bovine, this step takes place in 8- to 16-cell embryos (Santos et al., 2003), while in rat it occurs when embryos reach blastocyst stage (Santos et al., 2002). Remethylation of embryos occurs in a lineage-specific manner, resulting in a hypermentylation characteristic in the inner cell mass (ICM) while trophectoderm is hypomethylated (Santos et al., 2002). In pig embryo, though the demethylation process does not apparently occur but ICM of pig embryos become more methylated than that of trophoblast when blastocyst embryos contain 200-300 cells (Fulka et al., 2006).

The overall process of DNA methylation dynamic during early stages of embryogenesis is summarized in Figure 3.

On the other hands, epigenetic reprogramming of germ cell lineage is distinct. From investigation carried out in murine embryos, the reprogramming of primordial germ cells (PGCs) takes place when PGCs enter the gonadal ridges (approximately at embryonic day (E) 11.5-12.5) (Hajkova et al., 2002). Global demethylation of PGCs also includes imprinted genes and a new set of methylation is set up in a special fashion exclusively for germ cell production, and genomic imprinting is renewed according to sex of individual. Therefore, DNA methylation of germ cells is different from somatic cells, both in number and location, and this distinct characteristic is hypothesized to play an important role in the failure of somatic cell nuclear transfer technology.

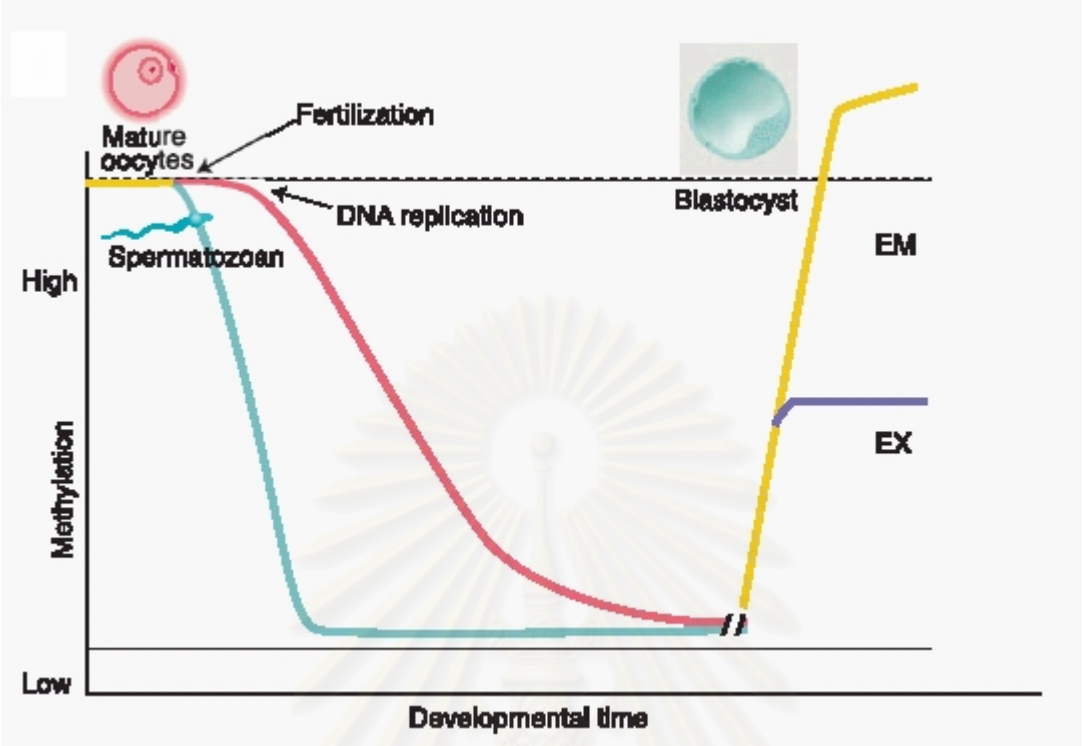

Figure 3. Diagram demonstrating the DNA methylation dynamic in preimplantation embryos. Paternal genome (blue line) becomes demethylated earlier than maternal genome (pink line). The embryos are hypomethylated at blastocyst stage and become re-methylated with different methylation contents between embryonic (EM, yellow line) and extraembryonic (EX, purple line) cell lineages. (Source: Reik et al., 2001)

3. Contribution of DNA methylation in modern technologies

3.1) DNA methylation and *In vitro* cell/tissue culture

Since the discovery of method to outgrow cells from pieces of tissue at the beginning of twentieth century, tissue or cell culture has been one of the most popular techniques universally employed in many experiments. *In vitro* cell culture provides a great opportunity for scientists to observe cellular functions in normal and abnormal cell populations without interferences from nearby tissues, as well as allow us to study effects of various factors upon cell morphology, physiology and molecular biology. Cell and tissue culture can be used as a model for disease investigation. Moreover, many biological and pharmaceutical products have been developed and tested base on cell culture system before applying in laboratory animal models (Figure 4).

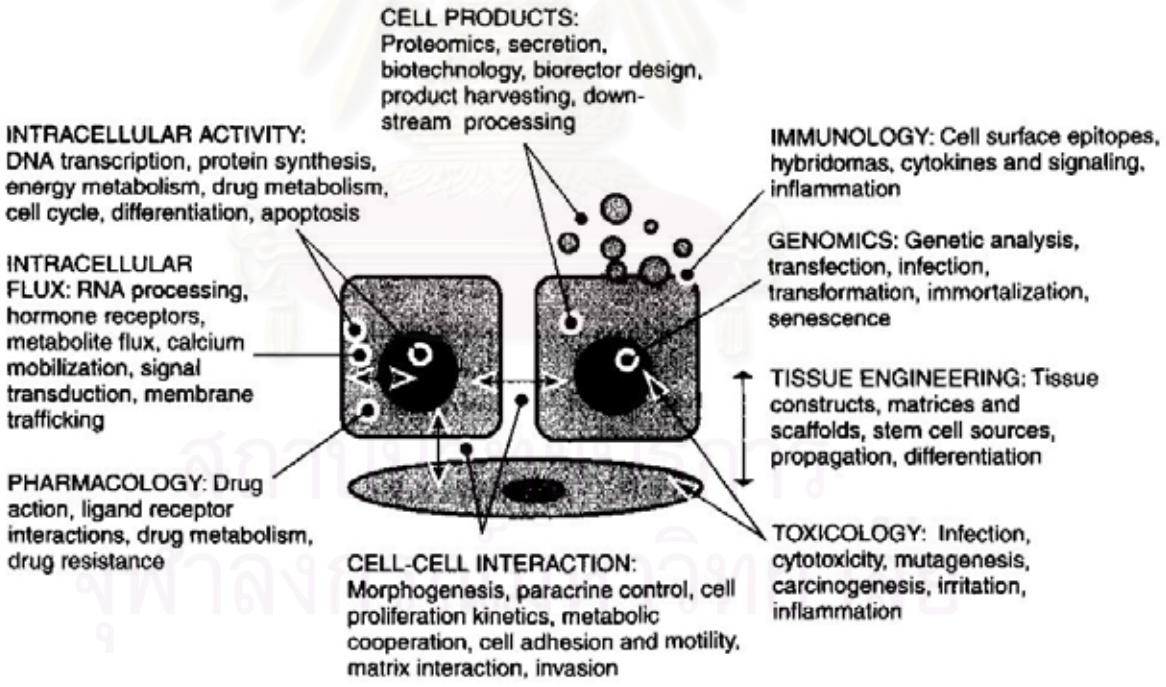

Figure 4. Applications of cell and tissue culture (Freshney, 2005)

Since cells are propagated and maintained in an artificial environment, every component directly comes into contact with cells possibly affects them in one way or another. Recently, many studies concerning gene expression have been done in cell culture models, so the effect of culture conditions on epigenetic signature of cultured cells has been aware of, in case it would alter gene expression and distort the results. Indeed, there are reports of epigenetic changes possibly resulted from *in vitro* culture of cells and embryos.

One of the basic components is culture medium. Generally, Eagle's minimal essential medium (MEM) or Dulbecco's modified of MEM (DMEM) is the medium of choice, but some cells and tissues, such as embryos, require special media to fulfill their complex cellular functions (Freshney, 2005). There was evidence suggested that culture medium could affect gene expression of cultured cells or tissues. Doherty et al. (2000) reported the observation of abnormal expression of imprinted gene *H19* in preimplantation mouse embryos cultured in different media. They also found loss-of-methylation at the CpG dinucleotides located upstream to the imprinting control region in embryos cultured in Whitten's medium but not in KSOM containing amino acids.

In some culture systems, serum has been included in culture medium as a source of growth factors. Moreover, serum helps cells to adhere onto culture vessel surface and it can stop the action of trypsin (Freshney, 2005). However, serum supplementation has been claimed to cause epigenetic alterations in embryos and embryonic stem cells cultured *in vitro*. Khosla et al. (2001) reported that mouse embryos cultured in the presence of serum showed aberrant expression of imprinted genes *H19, igf2* and *grb10*. Moreover, they also detected the gain-of-methylation at the differentially methylated region located upstream of imprinted gene *H19* in maternal allele in embryos cultured in serum supplemented medium. On the other hands, serum supplementation in medium used for long-term culture of embryonic stem (ES) cells was likely to alter the epigenetic pattern of many imprinted genes (Thompson et al., 2001). However, the exact pathway or mechanism of serum induced DNA methylation changes is still unknown.

Though not necessary, but some laboratories add antibiotics in culture media to reduce chance of contamination. However, some antibiotics used in the process of transgenic cells selection were reported to cause DNA methylation changes in cultured plant cells (Schmitt et al., 1997). Nevertheless, there is no report of such evidence in vertebrate cell culture system, possibly because of fewer antibiotics used owing to the fear of mycoplasma subinfection (Freshney, 2005).

Apart from the culture media and supplements that were reported to cause epigenetic changes and subsequently lead to abnormal gene expression, the culture technique itself can also affect cell quality by either direct or indirect way. The culture conditions, i.e. temperature, humidity, CO_2 and O_2 concentration in gas phase, can directly influence the cells to modify their activities in order to survive *in vitro*. For instance, human neuroblastoma cell lines cultured in a hypoxic condition (1% O_2) exhibited transcriptional changes when compared with cell lines cultured in a conventional condition with 5% CO_2/95% air (Fredlund et al., 2008). Bovine mammary epithelial cells cultured under high temperature (42°C) showed evidence of thermal stress-induced gene expression: genes associated with heat stress and protein repair were upregulated, while genes associated with biosynthesis, morphogenesis and metabolism were downregulated (Collier et al., 2006). However, epigenetic pattern of affected cell lines has never been reported.

One property of most cells is they cannot be maintained in culture vessels forever. Cultured cells can multiply *in vitro* for a period of time then they will stop growing and die. This phenomenon is called replicative senescence (Hensler and Pereira-Smith, 1995). Senescent cells lose their proliferative ability and express many genes associated with cell aging. Moreover, many experiments revealed that senescent cells contained abnormal DNA methylation. For example, senescent human fibroblasts lost their DNA methylation at the satellite sequences and their genome became unstable (Suzuki et al., 2002). To begin with, there were reports on the decrease in methylated cytosine residuals when cells were continuously maintained *in vitro* (Wilson and Jones, 1983; Matsumura et al., 1989), as well as the level of Dmnt enzymes (Kautiainen and Jones, 1986). This evidence

suggested the failure of DNA methylation maintenance process in cell culture, leading to chromosomal instability and irregular gene expression when cells become senile.

The culture duration before cells become senescent cannot be exactly calculated since it depends on many factors (Freshney, 2005). However, from an extensive experiment in human fibroblast culture, the most important factor is the seeding density. Low seeding density leads to multiple cell population doublings in a single passage and shortens the life span of cells (Balin et al., 2002). Therefore, the association between seeding density and cell senescence is one of the indirect effects of culture condition on the DNA methylation changes in cells maintained *in vitro*. This factor could interfere any experiment using cell lines as a tool to investigate cellular function and so on, as well as the SCNT technique that uses cell line as donor nuclei. Nonetheless, the effects of long-term culture on the ability of somatic cells to support cloned embryos to term were still on debate. Some research groups encountered low cloning efficiency when late passage cells were used as donor nuclei (Roh et al., 2000; Li et al., 2003; Jang et al., 2004), while the others reported no such evidence occurred (Bhuiyan et al., 2004; Tian et al., 2003).

According to the information reviewed earlier, most of the studies in DNA methylation change in cell and/or tissue culture targeted at imprinted genes or famous repetitive sequences. However, investigation of the genome-wide DNA methylation pattern might yield more information of the effect of *in vitro* culture on cells and/or tissue as a whole, and could possibly answer some questions that could not be done by exploring only a part of the whole genome.

3.2) DNA methylation and somatic cell nuclear transfer

Failure or incomplete of DNA methylation reprogramming in transferred nucleus has been claimed to be the major obstacle in SCNT in many species (Bourc'his et al., 2001; Cezar et al., 2003; Cho et al., 2007; Han et al., 2003; Humpherys et al., 2001; Kang et al., 2001; Kang et al., 2002; Kremenskoy et al., 2006; Shi and Haaf, 2002; Shi et al., 2004). DNA methylation patterns in preimplantation cloned embryos were reported to be

different from that of *in vivo*- and *in vitro*- produced counterparts; yet resembled the profiles of somatic cells used as donor nuclei (Han et al., 2003). From the notion that, generally, the parental DNA methylation markings, except at the imprinting sites, are erased during embryogenesis and *de novo* methylation takes place to set up a new profile of methylation for a new life. But in case of SCNT, the resemblance of DNA methylation patterns between somatic cell donor and cloned embryos signify the absence or incomplete genome-wide demethylation during early embryonic development.

Investigation in details revealed that complete reprogramming of transferred somatic cell nucleus indeed occurred at single-copy sequences, but repeat sequences are still highly methylated comparable to those in donor cells (Bourc'his et al., 2001; Kang et al., 2002). This evidence was supported by the difference of DNA methylation content between germ cells and somatic cells. Since male germ cells were reported to contain less methylation at the satellite sequences when compared with that of somatic cells, one could hypothesize that reprogramming factors stored in ooplasm, though is enough to demethylate paternal genome, might not be sufficient to totally reprogram the heavily methylated regions in somatic cell nuclei. To test this hypothesis, donor cells were pretreated with a DNA methyltransferase inhibitor agent: 5-aza-2'-deoxycytidine (5-aza-dC), in order to reduce the amount of DNA methylation before transfer. However, the results were negative. Enright et al. (2003), as well as Jones et al. (2001), reported a decrease in blastocyst formation rates when 5-aza-dC treated cells were used as donor nuclei. In this case it was difficult to interpret the results because the agent possible harmed the cells and reduced the cloning efficiency.

From this evidence, one could assume that the locations of methylation might be more crucial to the survival of cloned animal and that particular locations might lie within the repetitive nuclear elements, owing to the hypomethylation status of these elements in male germ cell (Pages and Roizes, 1982; Yamagata et al., 2007). Though the functions of repetitive sequences during embryogenesis are not yet clarified, but they might hold a key to complete the processes of epigenetic reprogramming in embryo development.

CHAPTER III

MATERIALS AND METHODS

1. Animals

Tissue, cells and semen samples were collected from three crossbred Holstein bulls, aged between 2 to 3 years old. Each bull was housed in an individual pen, fed with corn stem and concentrated feed twice a day. Water is provided *ad libitum*.

2. Collection of ear tissue and fibroblast cell culture from ear tissue explants

A piece of ear tissue was collected from an individual bull by a sterile technique. The lower border of the bull's ear was shaved and decontaminated with 70% alcohol and betadine solution. The incision site was marked by a curved forceps and a small piece of ear tissue (size approximately 4x10 mm) was cut out by a clean surgical blade. The wound was immediately taken care of according to the fresh wound dressing procedure. Then, the ear tissue sample from each individual was transferred into a clean plastic tube containing sterile Dulbecco's Modified Eagle Medium (DMEM, Gibco®, USA) solution supplemented with 2% v/v Fetal calf serum (FCS, Gibco®, USA) as a transfer medium. The tissue samples were kept at ambient temperature until they arrived at the laboratory.

Immediately after the ear tissue arrived at the laboratory, the tissue was washed with DMEM containing antibiotics (20,000 IU Penicillin + 20 mg Streptomycin and 5 mg Gentamicin). Remaining hair stumps were removed by shaving with a sterile surgical blade. After that, the tissue was cut into small pieces (2x2 mm), placed in 10x10 mm tissue culture dish (Falcon®, USA; 5-7 pieces/dish) and a small amount of culture medium (DMEM + 20% v/v FCS + 20,000 IU Penicillin + 20 mg Streptomycin + 5 mg Gentamicin) was added just to cover the bottom surface of the dish.

The culture was performed at 37°C in a humidified condition of 5% CO_2 in air and was examined every 2 days for cell outgrowth. The replacement of fresh culture

medium was carried out every 48-72 hrs. Once the outgrowth cells reached 90% confluent, they were sub-passaged according to the basic cell culture procedure. Briefly, the culture medium was discarded and outgrowth cells were washed twice with sterile Phosphate Buffer Saline (PBS). Trypsin-EDTA solution (0.25% w/v) was used to detach tissue pieces and cells from the surface of culture dish. After short incubation in the incubator, the explants and cells were dispersed from the dish surface by tapping the dish vigorously. The explants were separated from the cell suspension, washed with DMEM and replated into a new culture dish, while the cells were cultured in a new culture flask and were referred to as passage number 1 (P1).

P1 cells were cultured until they reached 90-100% confluent. One part of the culture was collected for DNA extraction, and another part was sub-passaged and cultured *in vitro* until the cell line reached P 30.

3. *In vitro* culture of ear fibroblast cells

Fibroblast cells were cultured in either tissue culture flasks (Corning®, USA) or dishes (Nunc®, Denmark) with DMEM supplemented with 10% FCS and antibiotics (20,000 IU Penicillin + 20 mg Streptomycin + 5 mg Gentamicin) until they reached 90% confluent then they were sub-cultured. Briefly, culture medium was removed from the culture flasks and the cells were washed with sterile PBS twice. Then the cells were treated with 0.25% trypsin-EDTA to detach them from culture surface. After cells were shaken free from culture surface, fresh culture medium with 10% FCS was added to stop the action of trypsin and cell suspension was replated in the ratio of 1:2 (one original flask was divided into 2 new flasks). The number of passage increased with time of trypsinization. Cells were cultured continuously until they reached passage number 30. The cultures of odd number passage were collected for DNA extraction.

4. Collection of whole blood and buffy coat preparation

Five milliliters of whole blood were drawn from the middle coccygeal vessels of each individual bull and kept in a clean 1.5 ml microtube containing EDTA as an anticoagulant.

Whole blood samples were centrifuged at 2,500 rpm for 10 min to precipitate the red and white blood cells from blood plasma. A thin film of white blood cells, known as buffy coat, was collected using micropipette and submitted for DNA extraction.

5. Semen collection

Fresh semen samples were collected from the bulls by artificial vagina. Semen sample from each individual bull was kept in a clean 15ml centrifuge tube (Corning®, USA) containing a small amount of penicillin powder, chilled and transferred to the laboratory for DNA extraction.

6. DNA extraction procedure

The DNA from cultured cells and leukocytes was extracted using commercial DNA extraction kit (QIAamp® DNA mini kit). The preparation of samples and the extraction procedure were carried out according to the handbook supplied by the manufacturer. Each sample was extracted twice to serve as replicates. The DNA was eluted from the extraction column with T0.1E solution (10 mM Tris-Cl, 0.1 mM EDTA).

The DNA extraction from sperm was carried out according to the procedure contributed by Ames B.N. from Children's Hospital Oakland Research Institute (available online at http://www.bio.com/protocolstools/protocol.jhtml?id=p9044) Briefly, a small aliquot of semen (50-100 μl) was used in each extraction and the extraction was carried out twice to serve as replicates. The semen samples were centrifuged at high speed for 1 minute to remove seminal plasma and the sperm pellet was resuspended in lysis buffer

(1% v/v Triton X-100 + 1 mM Deferoxamine mesylate + 5 mM $MgCl_2$ + 0.32 M Sucrose + 10 mM Tris), which would digest other cells contaminated in sperm pellet. Then sperm DNA was released from protamines by high salt solution and DTT. Absolute ethanol was used to precipitate DNA from the solution. The DNA pellet was finally resuspended with T0.1E buffer and kept at -20°C until the experiment began.

Before the beginning of the experiment, the qualification and quantification of the DNA samples was carried out by agarose gel electrophoresis alongside with 1kb ladder (GeneRuler™, Fermentas, USA). The concentration of the genomic DNA samples for the experiments was adjusted to 10-20 ng/μl and the samples was kept at 4°C.

7. Restriction enzyme digestion of the genomic DNA

A methylation-sensitive enzyme, *Hpa*II (Invitrogen®, Hong Kong), was used in this experiment. The digestion solution consisted of sterile de-ionized water and buffer solution plus BSA provided with the enzyme by the manufacturer. The amount of enzyme used to digest the genomic DNA, time and temperature applied to the digestion reaction was in accordant with the recommendation provided with the product.

Digested DNA samples were ethanol precipitated and separated from digestion buffer by centrifugation. DNA pellet was resuspended with sterile de-ionized water and kept at 4°C.

8. Amplified methylation polymorphisms (AMPs)-PCR

The PCR reaction consisted of DNA sample (genomic or digested DNA), *Taq* polymerase enzyme (AmpliTaq® Stoffel fragment, Applied Biosystems, USA), 10 mM dNTPs mix (Invitrogen®, Hong Kong), 10 μM oligonucleotide primers (Invitrogen Custom Primers, Hong Kong), Dimethyl sulphoxide (DMSO, AnalaR®, England), PCR buffer (10 mM Tris + 10 mM KCl + 5 mM $MgCl_2$) and de-ionized water.

The PCR reaction was started at 94°C for 2 min (hot start) and each cycle was as followed: 94°C 30 sec, 57°C 1 min, 56°C 1 min, 55°C 1 min, 54°C 1 min, 53°C 1 min. The cycle was repeated for 30 times plus a final extension at 72°C for 5 min. The PCR reactions were kept at 4°C until they were subjected to acrylamide gel electrophoresis.

In this experiment, thirty sets of oligonucleotide primer were used. Each primer contained 10 base pairs: four of which were *Hpa*II recognition sequence (5'-CCGG-3') and the other six bases were randomly designed. Names and sequences of all the primers are listed in Table 1.

Table 1 Names and sequences of oligonucleotide primers

No.	Name	Sequences	No.	Name	Sequences
1	C-08	TGGACCGGTG	16	AE-11	AAGACCGGGA
2	D-20	ACCCGGTCAC	17	AF-16	TCCCGGTGAG
3	F-17	AACCCGGGAA	18	AJ-15	GAATCCGGCA
4	F-18	TTCCCGGGTT	19	AK-18	ACCCGGAAAC
5	I-08	TTTGCCCGGT	20	AM-09	TGCCGGTTCA
6	J-01	CCCGGCATAA	21	AN-14	AGCCGGGTAA
7	J-14	CACCCGGATG	22	AQ-16	CCCGGAAGAG
8	M-17	TCAGTCCGGG	23	AR-18	CTACCGGCAC
9	N-09	TGCCGGCTTG	24	AT-10	ACCTCCGGTC
10	P-05	CCCCGGTAAC	25	AV-14	CTCCGGATCA
11	V-15	CAGTGCCGGT	26	AY-03	TTTCCGGGAG
12	V-17	ACCGGCTTGT	27	BB-09	AGGCCGGTCA
13	W-03	GTCCGGAGTG	28	BB-18	CAACCGGTCT
14	W-15	ACACCGGAAC	29	BF-13	CCGCCGGTAA
15	AB-16	CCCGGATGGT	30	BG-17	TCCGGGACTC

9. Polyacrylamide gel electrophoresis and silver staining

The polyacrylamide gel electrophoresis was carried out using the sequencing gel apparatus (BIO-RAD®, USA). The outer glass plate of the apparatus was treated with bind silane so the 4% polyacrylamide gel, consisted of 40% v/v of 19:1 acrylamide/bis solution (BIO-RAD®, USA) + urea (AnalaR®, England) + TBE + Ammonium persulfite (BIO-RAD®, USA) + TEMED (BIO-RAD®, USA), would attach to the plate and not peel off during the staining process.

The PCR reactions were mixed with loading dye (0.05% w/v Bromophenol blue + 0.05% w/v Xylene cyanol + 98% v/v Formamide + 10 mM EDTA), denatured at 95 °C for 5 min, and immediately placed on ice to maintain the denatured DNA structure. Eight microliters of the reaction solution were loaded into each well of the sequencing gel and electrophoresis procedure was carried out at fix power (110 Watts) for 2 hr.

When the electrophoresis was finished, the outer glass plate was separated from the gel apparatus and the gel was fixed in 7.5% acetic acid for at least 20 min. The fixative was washed away by de-ionized water and silver staining (0.2% w/v Silver nitrate (AnalaR®, England) + 0.6% v/v formaldehyde (AnalaR®, England)) was carried out on a rocking platform for at least 40 min. Then the gel was again washed with de-ionized water to remove access silver stain, and then was submerged into chilled developer (Sodium carbonate anhydrous (AnalaR®, England) + Sodium Thiosulfate (AnalaR®, England) + Formaldehyde (AnalaR®, England)) until the DNA bands became visible. When the bands were clearly seen and before the background turned dark, the gel was fixed again in 7.5% acetic acid solution for 15 min to stop the color developing process. The last step of the staining was to wash the fixed gel with de-ionized water to remove acid and air-dried overnight.

10. Markers and gel scoring

The comparison of markers was made between genomic and *Hpa*II digested templates by the classification of PCR markers appearing on the silver stained gel. Generally, three types of markers will be presented:

1. Digestion-resistant marker (R marker)

This marker appears both in the genomic and digested samples, referring that at the particular site of the genome recognized by the primer is methylated. So the *Hpa*II enzyme could not cleave the genomic DNA template and the primer could produce the amplicon from both templates.

2. Digestion-sensitive marker (S marker)

This marker appears only in the genomic template, but disappears in the digested template, referring that at the particular site of the genome is unmethylated. So the DNA had been cut at the *Hpa*II recognition site and the primer failed to produce the amplicon from the digested template.

3. Digestion-dependent marker (D marker)

This marker appears only in the digested template. The appearance of this kind of marker is still under investigation, yet we could speculate that the genome structure might play an important role in preventing the primer to get access to the recognition sites. Somehow this structure is sensitive to the *Hpa*II enzyme so the primer can bind to the recognition sites only in the digested template.

Scoring of the markers was based on the presence-absence manner when multiple samples were compared.

11. Experimental design and statistical analysis

There were two experiments including in this dissertation.

Experiment 1 The study of the DNA methylation profiles of somatic cells and germ cells of bulls

The hypothesis of this experiment was that the somatic cells and germ cells have different DNA methylation patterns, both in number and location. Comparing PCR amplicons produced from AMPs technique between genomic and emzyme-digested templates could identify these differences.

Samples used in this experiment were as followed:

1. DNA samples from leukocytes, representing the fully differentiated somatic cell lineage.
2. DNA samples from ear fibroblast cell culture passage number 1 (P1), representing the partial differentiated somatic cell lineage.
3. DNA samples from mature spermatozoa, representing the germ cell lineage.

The AMPs-PCR was performed using samples from all three individual bulls. The numbers of markers in each type from all primers in this experiment were reported in percentage individually and were pooled together to provide an overall picture of the DNA methylation profile of cattle genome. The difference of marker types in somatic cells and germ cells was calculated by Chi-square test using SAS program. The effect of individual animal was also reported. The difference was defined as statistically significant when $p \leq 0.05$.

Experiment 2 The study of the effect of long-term culture of fibroblasts on DNA methylation profile

The hypothesis of the experiment was that, in spite of basic culture media and procedure used worldwide for *in vitro* culture of cell lines, long-term culture of cells might cause changes or alteration of the chromatin, especially at the epigenetic level.

In this experiment, 3 sets of DNA sample collected from fibroblast cell cultured *in vitro* were tested for any polymorphism. The samples used in this experiment were as followed:

1. DNA samples from early passage cells

These DNA samples were harvested from fibroblast cell culture P3, P5 and P7

2. DNA samples from medium passage cells

These DNA samples were harvested from fibroblast cell culture P13, P15 and P17.

3. DNA samples from late passage cells

These DNA samples were harvested from fibroblast cell culture P23, P25 and P27.

The results of the AMPs-PCR of this experiment was analyzed based on the profiles of DNA methylation found in each set of samples alongside with the possible cause(s) of changes or alterations of the epigenetic status as shown in Table 2. If any polymorphism was evident, the frequency of it between different passage stages would be reported in percentage and analyzed using Chi-square test to identify whether culture duration has any effect on epigenetic status of the cells. The difference was defined as statistically significant when $p \leq 0.05$.

Table 2 Possible causes and factors contributing to the alterations of DNA methylation in long-term culture cell line.

Possible causes of polymorphisms	Marker model			
	Genomic		Digested	
	Early passage	Later passage	Early passage	Later passage
Loss of methylation	+	+	+	-
Gain of methylation	+	+	-	+
Gain of PCR inhibitor	+	-	+	+
Loss of DNA or primer binding site	+	-	+	-
Gain of PCR inhibitor and methylation	+	-	-	+
Loss of unmethylated DNA	+	-	-	-
Deletion of PCR inhibitor	-	+	+	+
Loss of PCR inhibitor and methylation	-	+	+	-
Loss of PCR inhibitor and gain of methylation	-	+	-	+
Loss of PCR inhibitor at the unmethylated DNA	-	+	-	-
Loss of DNA or methylation	-	-	+	-

+ = marker is present, - = marker is absent

CHAPTER IV

RESULTS

4.1) DNA methylation profiles of somatic cells and germ cells of bulls

In this experiment, three individual bulls (Bull KP, Bull SC and Bull NW) were included for whole genome scanning of DNA methylation profiles in somatic and germ cell lineage using Amplified Methylation Polymorphisms PCR (AMPs-PCR). From thirty sets of oligonucleotide primer, only twenty-seven primers gave a clear profile and could be scored. Three primers yielded very low number of markers and the profile was very faint.

The AMPs profiles of cell samples from 3 bulls showed a similar, but not identical pattern (Figure 5-6). In each individual, approximately 1,000 amplicons were produced from 27 sets of primer. Some good primers could detect up to 60-70 *Hpa*II locations in one genome set, but generally most primers produced approximately 30-40 amplicons. Since no bull effect was found, the markers of each cell types from all bulls were pooled together. The overall results were presented in percentage and shown in Table 3.

The majority (more than 90%) of amplicons found in every cell lineage were digestion-resistant (R) markers (figure 7). Comparison made within the same cell lineage showed that leukocytes had a significant higher number of R marker when compared with fibroblast cells (94.8% vs 92.3%, $p<0.05$), while fibroblast cells contained more digestion-dependent (D) markers (figure 9) (5.1% vs 3.0%, $p<0.05$). The digestion-sensitive (S) markers (figure 8) between the two cell types were similar (2.2% in leukocytes vs 2.6% in fibroblast).

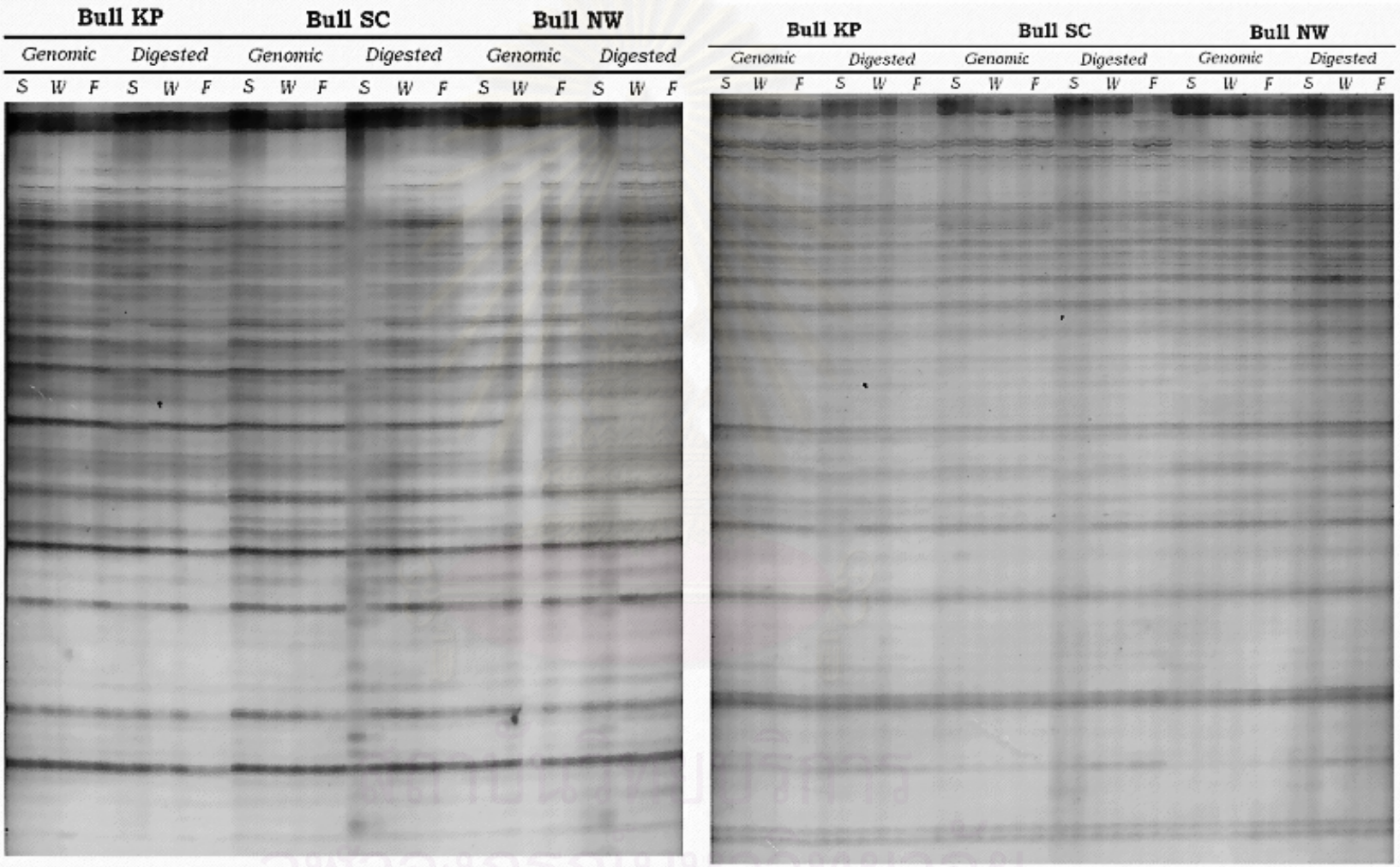

Figure 5. Examples of AMPs-PCR profile of DNA methylation in the three cell types; S = Sperm; W = White blood cell; F = Fibroblast

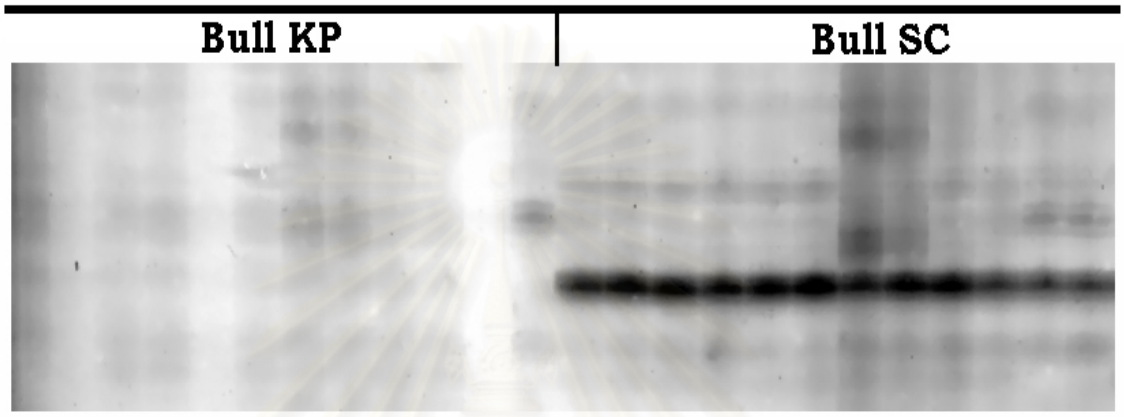

Figure 6. Example of individual variation in AMPs-PCR profile between two bulls

Sperm DNA, representing germ cell lineage, also had high percentage of R marker. Most surprisingly, it was higher than that of fibroblast cells (93.4% vs 92.3%) though there was not statistically significant. Moreover, germ cells had significantly high S markers when compared with somatic cells (3.6% vs 2.2% in leukocytes and 2.6% in fibroblast, $p<0.05$). The D markers in sperm DNA were similar with that in leukocyte DNA (3.0% vs3.0%) but less than that in fibroblast DNA (3.0% vs 5.1%, $p<0.05$).

Table 3 Percentage of markers found in each cell type

	R marker	S marker	D marker
Sperm DNA	93.4[a] (2981/3193)	3.6[d] (115/3193)	3.0[f] (97/3193)
Leukocyte DNA	94.8[b] (3016/3182)	2.2[e] (70/3182)	3.0[f] (96/3182)
Fibroblast DNA	92.3[ac] (3000/3251)	2.6[e] (86/3251)	5.1[g] (165/3251)

Number in parenthesis () = number of marker/number of total marker

Different letters within column specify statistic significant ($p<0.05$)

Although the difference of DNA methylation among cell types was significant when overall data was pooled together, yet there was individual dependent. For R markers, bull KP showed no significant difference of this particular marker among the three cell types, while bull SC and NW had a significantly higher degree of R markers in leukocytes when compared with fibroblast DNA (Table 4).

For S marker, only bull NW showed a significant higher percentage of this marker in sperm DNA when compared with leukocyte DNA, while no significant different was evident in the other two bulls (Table 5).

For D marker, significant different was found in all bull between fibroblast DNA and the other two cell types (Table 6).

Table 4 Percentage of R marker in cell samples from three bulls

	Bull KP	Bull SC	Bull NW
Sperm DNA	94.4 (994/1053)	92.9[ab] (997/1073)	92.8[c] (990/1067)
Leukocyte DNA	95.2 (1004/1054)	94.2[a] (1012/1074)	94.9[d] (1000/1054)
Fibroblast DNA	93.3 (1000/1072)	91.3[b] (1006/1102)	92.3[c] (994/1077)

Number in parenthesis () = number of marker/number of total marker

Different letters within column specify statistic significant ($p<0.05$)

Table 5 Percentage of S marker in cell samples from three bulls

	Bull KP	Bull SC	Bull NW
Sperm DNA	3.2 (34/1053)	4.1 (44/1073)	3.5[a] (37/1067)
Leukocyte DNA	1.9 (20/1054)	2.7 (29/1074)	2.0[b] (21/1054)
Fibroblast DNA	2.2 (24/1072)	3.2 (35/1102)	2.6[a] (27/1077)

Number in parenthesis () = number of marker/number of total marker

Different letters within column specify statistic significant ($p<0.05$)

Table 6 Percentage of D marker in cell samples from three bulls

	Bull KP	Bull SC	Bull NW
Sperm DNA	2.4[a] (25/1053)	3.0[c] (32/1073)	3.7[e] (40/1067)
Leukocyte DNA	2.8[a] (30/1054)	3.1[c] (33/1074)	3.1[e] (33/1054)
Fibroblast DNA	4.5[b] (48/1072)	5.5[d] (61/1102)	5.2[f] (56/1077)

Number in parenthesis () = number of marker/number of total marker

Different letters within column specify statistic significant ($p<0.05$)

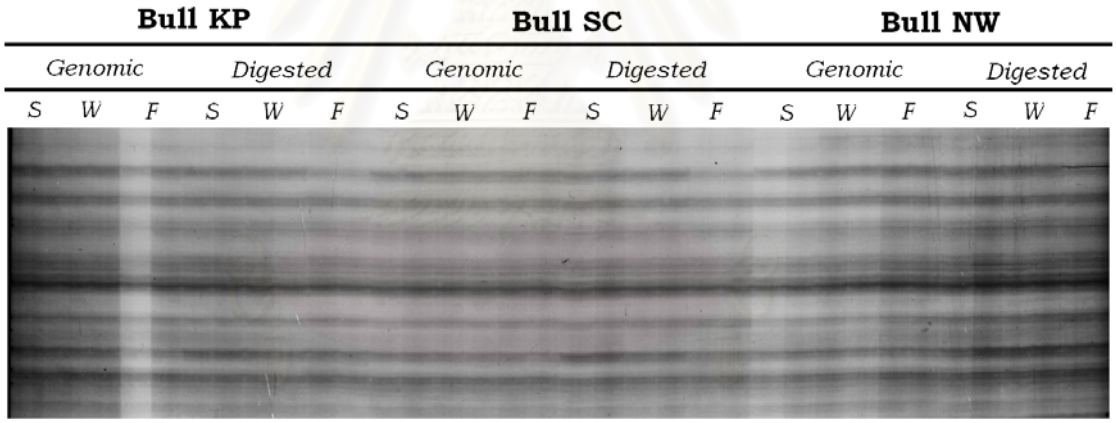

Figure 7. Example of the digestion resistant (R) marker generated by primer AR-18; S = Sperm; W = White blood cell, F = Fibroblast

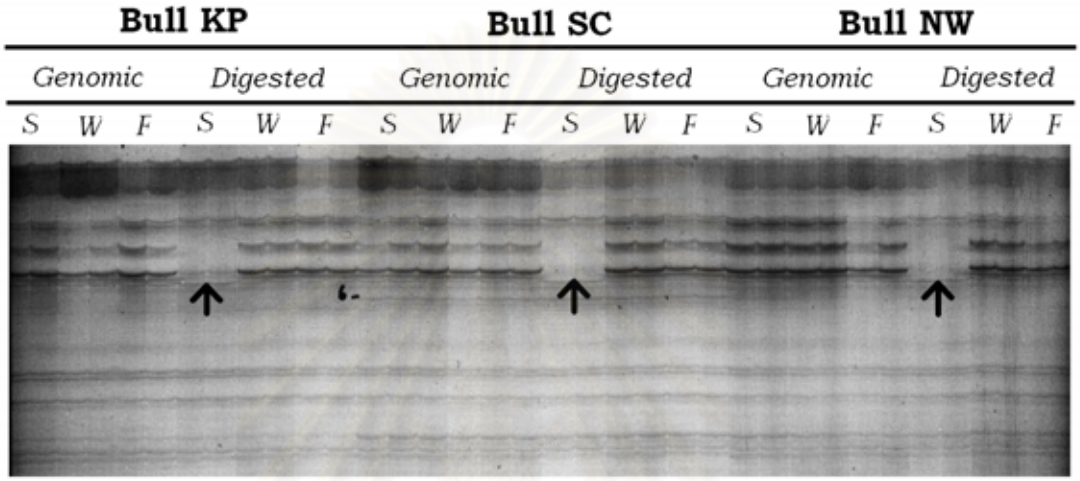

Figure 8. Example of the digestion sensitive (S) marker generated by primer BG-17; S = Sperm; W = White blood cell, F = Fibroblast; black arrows indicate the absent marker found in digested sperm DNA templates in all three bulls.

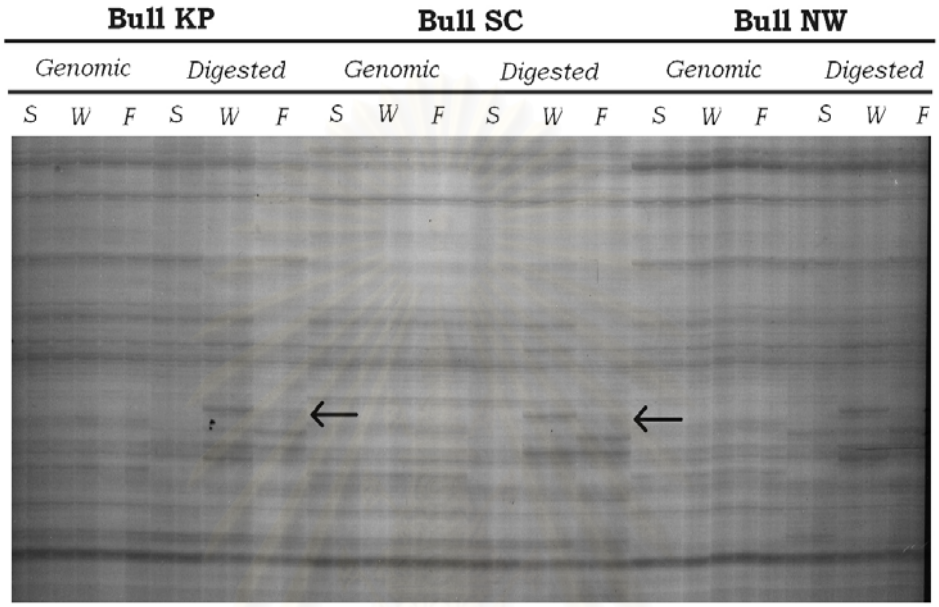

Figure 9. Example of the digestion dependent (D) marker generated by primer C-08; S = Sperm; W = White blood cell; F = Fibroblast; black arrows indicate the markers found in the digested W and F DNA templates in Bull KP and Bull SC

Further investigation was carried out to retrive more information of an amplicon. One distinct polymorphic marker between leukocyte and sperm DNA was selected. A small piece of amplicon-embedded gel slab was cut out of the dried polyacrylamide gel, washed with sterile deionized water to remove any trace of fixative and then soaked with TE buffer. The reaction was left at 52°C for 1 hr and incubated at 37°C overnight. Then the elution buffer was used as a templete for re-amplification PCR to increase yield of recovered DNA to be enough for DNA sequencing.

The sequences of the selected amplicon consisted of 586 bp, which was in accordant with the band shown on 1%agarose gel electrophoresis carried out before submitting the sample for sequencing (figure 10).

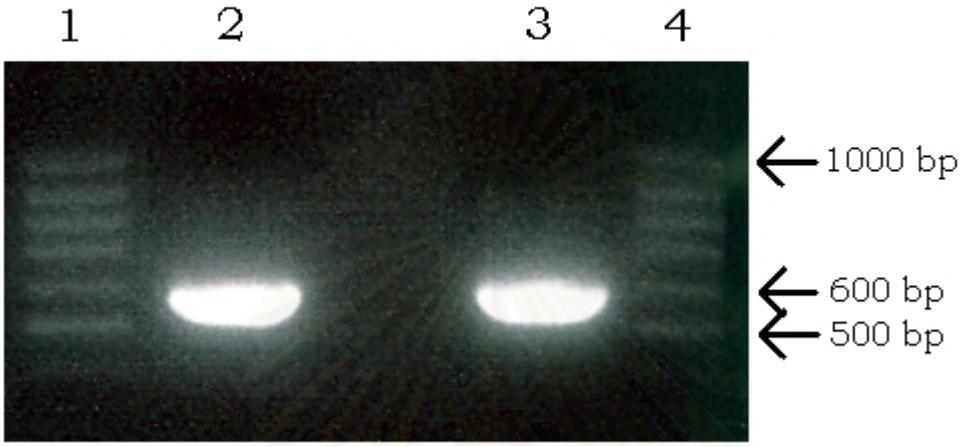

Figure 10. PCR product of the re-amplification of recovered DNA from the selected amplicon: lane 1 and 4 were 100 bp ladders; lane 2 and 3 were PCR products. Size of the product was estimated to be between 500 and 600 bp.

Result from automate DNA sequencer were as followed:

GTGATTTGGTCGGTCGCGCGCTTTCCGAGAAGTTCCGCACCAAATCCTGAGAAACCC
CCAAATCTGCGAAACTGCTTCTGGGTGATTCCCGGGATGACCTGCTCTCTCCCCTGTA
CCCCTGACCTGAGATTCCCAAGCACAAACAGGGTCCTCTGTGCATCAGGATGTCTAC
GCTAGGGCAAGCACTGAATGCTGGCTTGTGAAAAGTGCAATATGCTACAGCTCTAGC
TGTGGCACTACTATACTTGCCTGCAACAACGGTATGCTTGTTGGGCATACTCTGACCG
GGGAAGACTAACACTCCTGGCTGGTGTTCCCAGACTTTGCCTCGGCCCAGACCTTCTG
TTCAGACATCCTATGAATATGTGAAAGAGGACCCTTTGCTCTTTATAGGCGTGCGTCC
CTCTTTCATGGGGCAGGGGCCTGCCTCTCCACTGGGGCCCTTTTTCTAAAGAGGGAAG
AGGACAGACTAACAAGGCACCATGTGAAGTGCTTGCCAGGCACCATCTCACTTAGTC
TTCGCTGTCTCCGTGAGGAGGGGCAGCTACCATCGCCTGCATTTGCTAAATGCCATAC
CGAGGGAAAA

This contig was aligned with bovine genome database using NCBI BLAST program. A part of this sequence (284 bp out of 586 bp) matched with the *Bos Taurus* chromosome 13 genomic contig (ref|NW_001493115.1|Bt13_WGA1623_3) with 93% identity. The lower section of the sequence also matched with a short interspersed nucleotide element on the chromosome. There was no other genomic structure reported tolocated near by.

4.2) DNA methylation profile of fibroblast cell line cultured *in vitro*

In this experiment, a fibroblast cell line was established from ear tissue sample collected from Bull SC. The cell line was maintained *in vitro* continuously from P1 to P30 without any alteration in cell morphology when examined under an inverted stereomicroscope (Figure 11).

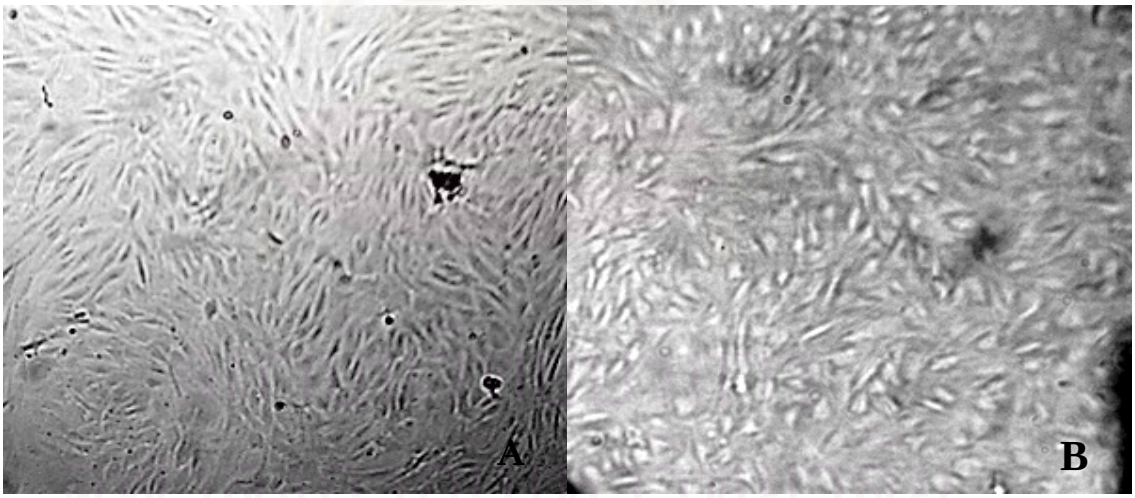

Figure 11. Morphology of fibroblast cell culture passage number 2 (A) and passage number 29 (B). The cells still maintained their spindle-like shape, which is the characteristic of fibroblast cell morphology.

AMPs-PCR was carried out to investigate any alteration in DNA methylation profile of cells exposed to *in vitro* culture condition in various time lengths. Thirty sets of oligonucleotide primer were tested and results from twenty-eight primers could be scored. The other two primers gave poor profiles.

From twenty-eight primers, one thousand six hundred and ninety amplicons were generated. Despite the fibroblast cells were maintained in an artificial condition for more than 5 months to reach passage number 30, no change in DNA methylation pattern was observed (figure 12-13).

When compared with the results from experiment 1, the numbers of each type of marker found in this experiment were different from the first experiment. The R markers of fibroblast cells reduced from 91.3% in the previous experiment to 86%. The S and D markers increased from 3.2% and 5.5% to 4.4% and 9.6% respectively.

The AMPs-PCR profiles between two experiments were compared in order to investigate the reproducibility of amplicoms produced by the same primer using AMPs technique. As seen in Figure 14-16, the profile patterns between two experiments were similar.

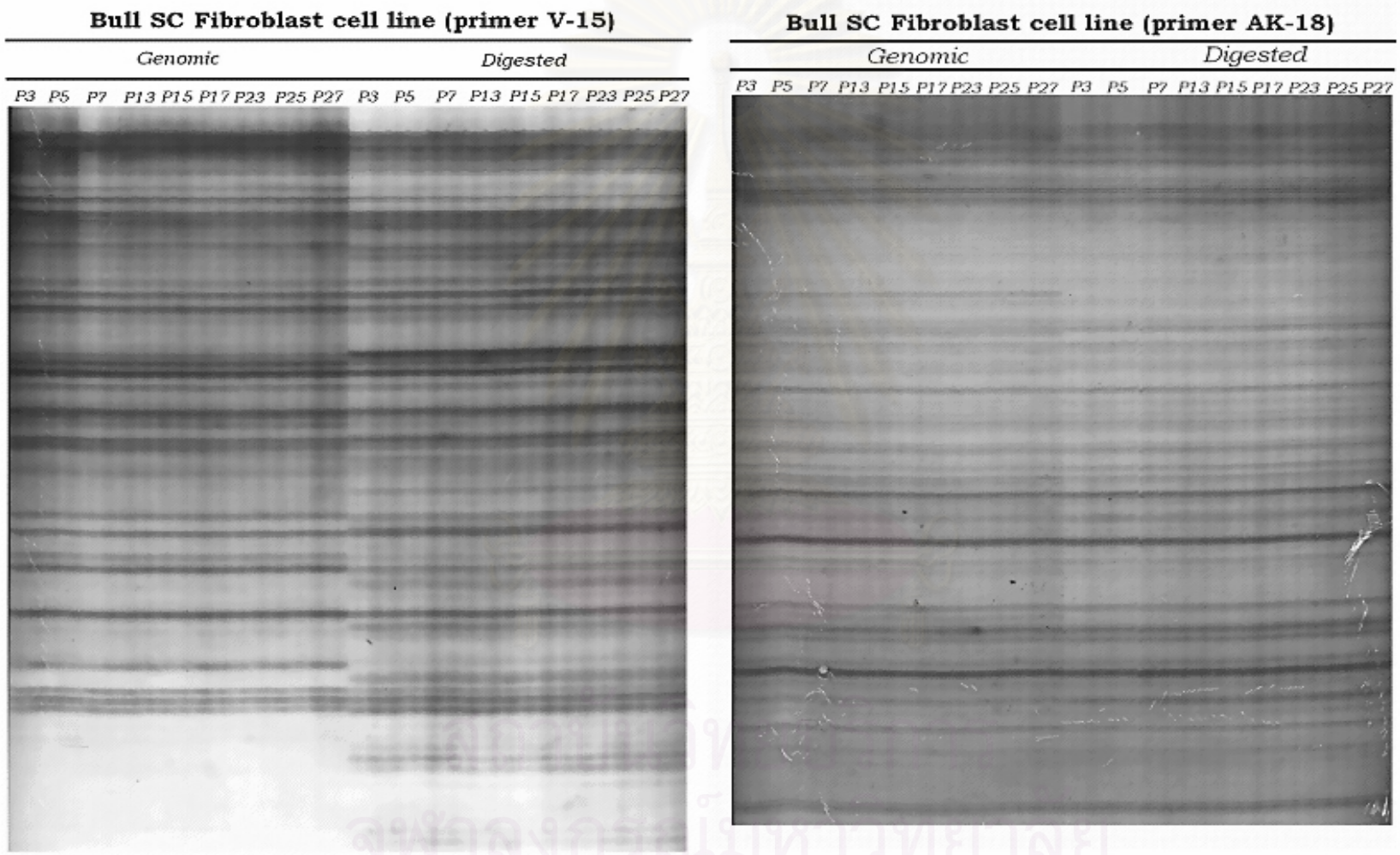

Figure 12. Examples of AMPs-PCR profiles of fibroblast cell DNA samples collected from various culture durations; P = Passage number

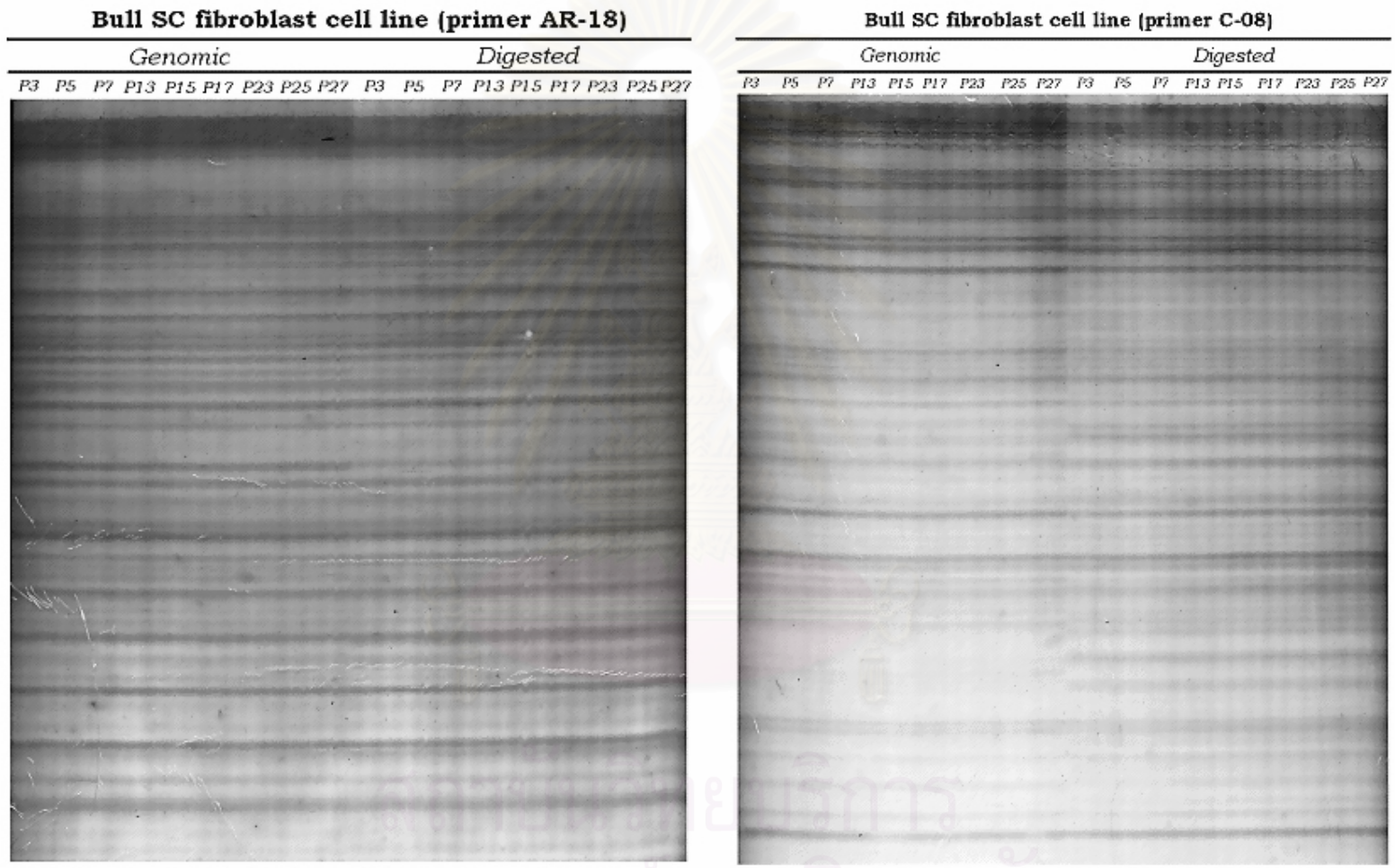

Figure 13. Examples of AMPs-PCR profiles of fibroblast cell DNA samples collected from various culture durations; P = Passage number

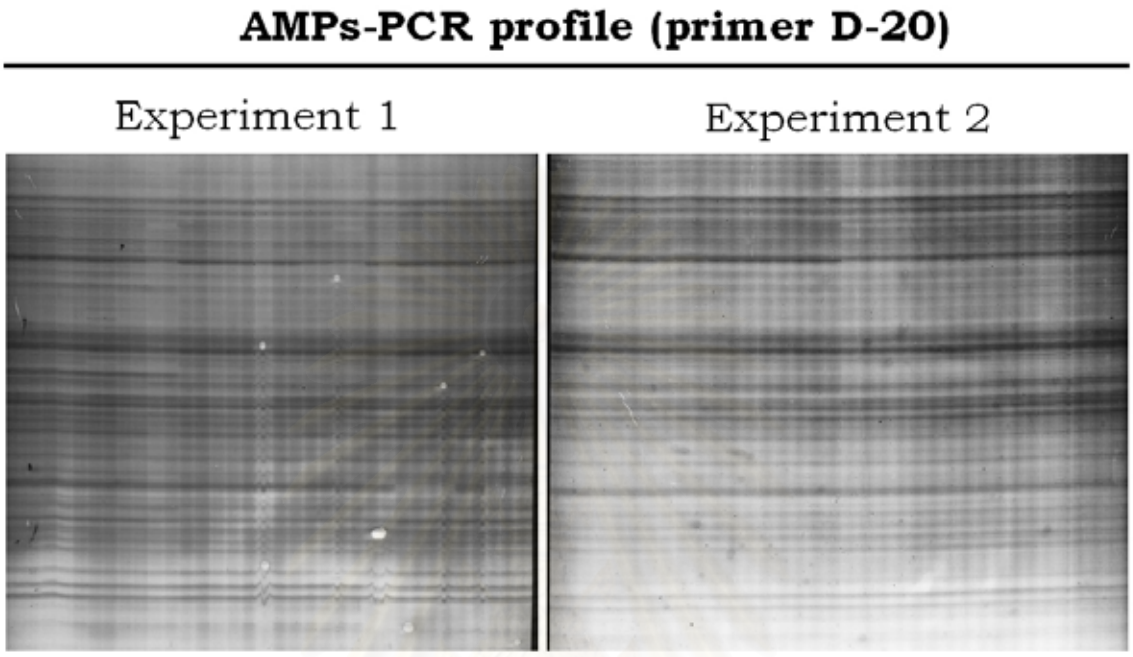

Figure 14. Similar AMPs-PCR profiles generated by primer D-20 between two experiments

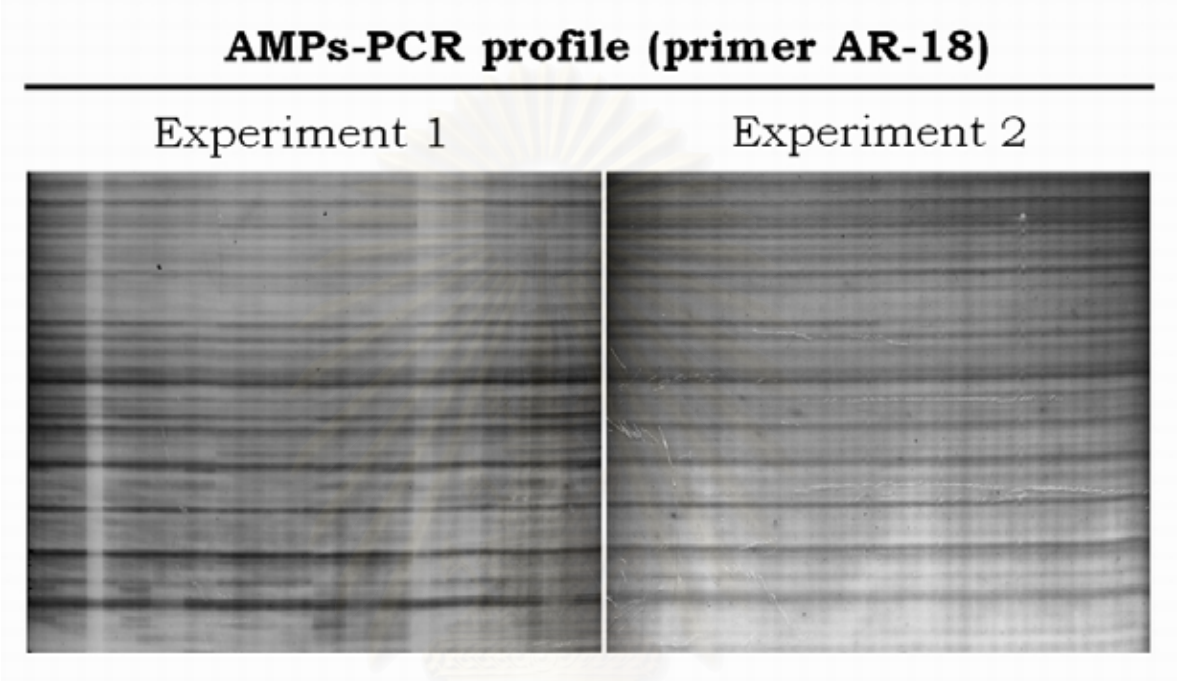

Figure 15. Similar AMPs-PCR profiles generated by primer AR-18 between two experiments

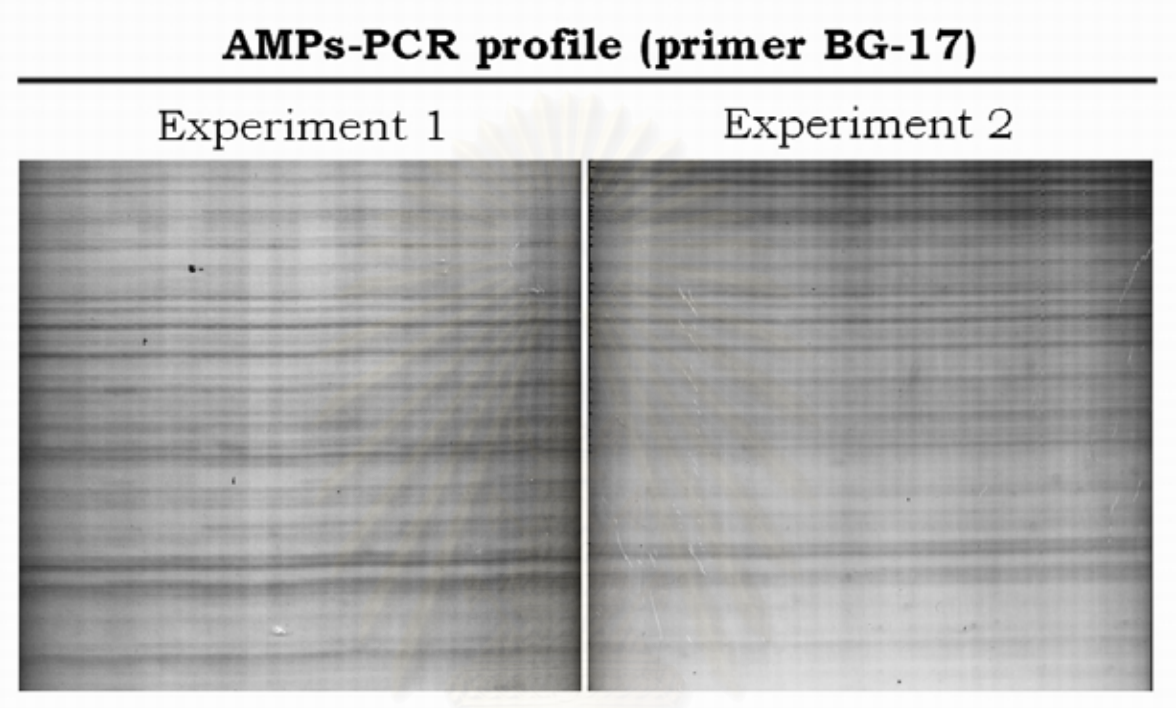

Figure 16. Similar AMPs-PCR profiles generated by primer BG-17 between two experiment

CHAPTER V

DISCUSSION

5.1) Investigation of the whole genome DNA methylation pattern using Amplified Methylation Polymorphisms (AMPs) technique in bull samples

AMPs technique has been developed by Associate Professor Dr. Bernard Carroll and his colleagues from the University of Queensland, Australia, to examine the genetic of sugarcane (Harrison, 2002). Since then, this technique has also been applied and used to study DNA methylation profiles of other plants and also mammalian genomes (Waldon et al., 2002; Webster et al., 2005).

The AMPs technique bases on the production of PCR products from the genomic template digested with methylation-sensitive restriction enzyme (Waldon et al., 2002). In these experiments, we used *Hpa*II enzyme to digest the unmethylated 5'-CCGG-3' palindromic sequences which distribute diffusely in mammalian genome. The most notable advantage of this technique is that one primer can reveal many genomic locations. Our results showed that each primer could produce approximately 30-40 amplicons on a silver-stained polyacrylamide gel, and from the total of 30 primers, we could investigate more than 1,000 sites in the bull genome. If radio-labelled dNTPs should be used in the AMPs-PCR and the autoradiography was applied for amplicon detection on the gel, it would possible to gain approximately 60-80 markers from just one good primer (preliminary study, data not shown).

Anyhow, this powerful technique also has some weak points. For example, the false positive results from the incomplete digestion of the genomic DNA template and the reproducibility of the AMPs profiles. However, these problems can be solved. The completeness of enzymatic digestion can be achieved by conducting the digestion reaction according to the recommendations given by the enzyme production company.

In these experiments, we adjusted the concentration of the genomic DNA template to be minimal, just enough for all PCR sessions for the particular study, by diluting DNA samples with T0.1E buffer to make the concentration of 10-20 ng/μl. This step could reduce the contamination of any substance that could interfere with the enzyme digestion of the genomic template. Then the digestion was carried out according to the enzyme supplier recommendations. The digested DNA was washed and precipitated from the digestion reactions as soon as the digestion time was up to prevent further non-specific cleavage or star effect. The digested DNA samples were checked for their quality by electrophoresis using 1% agarose gel alongside with their genomic DNA counterparts. The characteristic of DNA bands between genomic and digested samples seen on the agarose gel was not very different and this was later confirmed by the results of AMPs-PCR, showing that most of the *Hpa*II sites in bull genome were heavily methylated.

The reproducibility of the AMPs-PCR profiles depends on many factors, i.e. the concentration of DNA template and the efficiency of polymerase enzyme. Waldron et al., (2002) reported the similar PCR profiles generated from different concentration of DNA templates, ranging from 10-500 ng. In these experiment, we adjusted the concentration of DNA templates to be 10-20 ng/μl, which also yielded reproducible profiles. Moreover, we used a special polymerase enzyme: Stoffel fragment, which is claimed to be more effective than general *Taq* polymerase enzyme. Stoffel fragment tolerates many rounds of thermal change during PCR session and it has optimal activity over a broad range of magnesium-ion concentrations. There were reports showing that using Stoffel fragment could produce reproducible results (Rawadi et al., 1995; Waldron et al., 2002). In our case, we found that the AMPs-PCR profiles generated by the same primer in experiment 1 and 2 were similar; hence we could say that the condition of AMPs technique we applied in these experiments was optimum.

5.2) DNA methylation profiles of somatic cells and germ cells in bulls

Our results showed that cattle genome is highly conserved for the AMPs-PCR profiles, generated from custom-designed primers randomly bind to DNA without any knowledge of genomic template sequences, were similar in all three bulls, though not identical due to individual variations. From twenty-seven sets of primer containing *Hpa*II regconition sites, we could generate approximately 1,000 amplicons from each individual, or average 30-40 amplicons per primer. The numbers of amplicon produced in this experiment were lower than the report from Waldron et al., (2002). They gained approximately 70-100 markers per PCR, but they used radio-labelled dNTPs in their PCR and used autoradiography for marker investigation, which is more sensitive than silver staining used in our experiment.

We found that DNA methylation profiles differ between cell lineages. As expected, the terminally differentiated somatic cell lineage, i.e. leukocytes, contains a significantly high level of DNA methylation at the *Hpa*II sites when compared with germ cells. We also found that sperm DNA showed a significantly higher percentage of digestion sensitive markers in its profile when compared with somatic cells. These results were in accordance with other studies. Sturm and Taylor (1981) examined the distribution of methylated cytosine in the DNA of somatic and germline cells from cattle and found that thymus genome was heavily methylated when compared with sperm DNA. While Oakes et al., (2007) reported an exclusive hypomethylation status of testis tissue when compared with somatic cells. The hypomethylation status of sperm cells might be associate with the special genome structure of germ cell lineage designed for meiosis division, and possibly be involved in specific gene expression essential for early embryonic development after fertilization (Yamagata et al., 2007)

There was enough evidence showing that the difference in DNA methylation between germ cells and somatic cells occurs mainly at the satellite sequences. Experiment carried out in calf DNA showed that satellite DNA sequences of calf thymus were hypermethylated while those of sperm was hypomethylated (Pages and Roizes, 1982).

Similar findings were discovered when mouse DNA from various tissue types was investigated (Ponzetto-Zimmerman and Wolgemuth, 1984; Feinstein et al., 1985; Yamagata et al., 2007). Further investigation of a polymorphic amplicon between sperm and leukocyte DNA in our study confirmed this finding. Our result also showed that the particular site was a part of a short interspersed nucleotide element (SINE) in bovine chromosome 13. This site was methylated in leukocytes but unmethylated in sperm DNA. Primate sperm showed hypomethylation at many enzyme recognition sites including *Hpa*II locations in *Alu* sequences, which belong to SINEs family (Rubin et al., 1994). A protein extracted from human sperm nuclei showed ability to prevent methylation process *in vitro* and might play an important role in inhibiting *Alu* element methylation in human sperm *in vivo* (Chesnokov and Schmid, 1995). However, not all satellite sequences in sperm are hypomethylated. Oakes et al., (2007) reported that some long terminal repeats (LTRs) are unmethylated in somatic tissue but all are methylated in testis. Therefore, the location of methylation in germ cells is possibly more crucial on the success of epigenetic reprogramming during early phase of embryogenesis. More polymorphic amplicons must be investigated to gain more information of the locations of diverse methylation between the two cell types.

Among three cell lineages tested in our experiment, fibroblast cells, representing partial differentiated somatic cell lineage, possessed a significantly low amount of DNA methylation in their genome. The explanation of this finding can be made on the basis of cellular properties. Since fibroblast cells are versatile, thus their genome must be in an active stage, ready to transform into any specific cell type when properly induced. Unlike the fully differentiated somatic cells and also mature spermatozoa, which are inactive and require only a small number of genes at work to maintain their specific cellular functions, while the other genomic elements are suppressed by DNA methylation and other mechanisms to prevent undesirable gene expression that might lead to fatal abnormalities to cells and organs (Reik and Dean, 2001).

Furthermore, fibroblast cells also showed the highest percentage of digestion-dependent markers. The formation of this type of marker from AMPs-PCR is still elusive, but one could hypothesize that the secondary structure of the genomic DNA might play a role in this. Complex structure of the genome might prevent primers to get access to their recognition sites. Should the enzymatic digestion capable of removing this secondary architecture, then primers could bind to the DNA and generate amplicons only from the digested DNA template. This difference in genome structures might be due to the difference in the amount of DNA methylation between fibroblast cells and other cell types, for the methylation influences genome structure (Lewis and Bird, 1991). Nonetheless, This explanation is only a hypothesis, unless the mechanism of digestion dependent marker generation is well documented.

In conclusion of this experiment, Different cell lineage contained different level of DNA methylation at various *Hpa*II locations throughout the genome. Germ cells possess less methylation when compared with somatic cells, possibly at the repetitive elements. Partial differentiated somatic cells showed a tendency to have a different genomic architecture, which might associate with their genomic flexibility. Further investigation of polymorphic markers among cell lineages is essential for more detail of differential methylation sites. Other methylation-sensitive restriction endonucleases and more sets of primers can be used to gain more markers from different locations in the genome.

5.3) DNA methylation profiles of fibroblast cell line cultured *in vitro*

The AMPs-PCR profiles generated from fibroblast cell DNA collected from cell cultures maintained in the *in vitro* condition for various time length showed no alteration of the DNA methylation pattern at 1,500 *Hpa*II locations genome-wide. In our experiment, the fibroblast cell line was maintained in conventional cell culture condition supplemented with fetal calf serum and broad spectrum antibiotics for 5 months. This could be implied that the maintenance mechanism of methylation profile in fibroblast cell culture worked perfectly well throughout the culture period.

Nevertheless, our result was not in agreement with other reports. *In vitro* culture of living cells has been reported to cause methylation changes, both in term of genome-wide and specific location alterations. For specific location change, the site of imprinted genes shows a high tendency to be affected by *in vitro* manipulations. Many cases of humans and animals born from *in vitro* embryo production exhibited sign of abnormal imprinted gene expression, leading to abnormal phenotypes, i.e. Large Offspring Syndrome. Different culture conditions were reported to cause methylation changes at imprinted genes in embryos. Doherty et al. (2000) showed that different culture media caused methylation changes in the hypothesized imprinting gene control domains. While serum supplement in the culture medium was reported to influence the expression of some important imprinted genes; such as *igf2* and *H19* genes, in preimplantation mouse embryos (Khosla et al., 2001).

Not only *in vitro* cultured embryos suffered from epigenetic changes, somatic cells and embryonic stem cells cultured in artificial environment also faced the same fate. Thompson et al. (2001) reported the methylation changes at the differentially methylated regions of imprinted genes in mouse embryonic stem cell culture, possibly responsible for the reduction of cloning efficiency when the cells were used for SCNT. In somatic cell culture, the level of global DNA methylation decreased when cell passage number increased (Wilson and Jones, 1983; Matsumura et al., 1989). This evidence suggests that the formation and maintenance of DNA methylation in these cells were impaired.

As mentioned earlier, the formation and maintenance of DNA methylation depend on the activity of methytransferase enzyme family. Though this mechanism is well documented in early stages of animal development, but the maintenance of methylation profiles in adult cells is less investigated. Results from the study in mouse indicated that Dnmt1 was the most abundant enzyme in adult cells and tissues. In vivo, this enzyme is drawn to the replication site of DNA where the unmethylated CpG dinucleotides on the newly formed daughter strand are its targets (Yoder et al., 1997). There are some proteins involve in this catalytic process, such as proliferating cell nuclear antigen (PCNA) protein

that accumulates Dnmt1 enzyme at the replication fork during S1 phase of the cell cycle (Chuang et al., 1997; Yoder et al., 1997; Schermelleh et al., 2007).

Dmnt3s family also plays an important role in maintaining DNA methylation status in somatic cells, especially at the repetitive sequences. These locations are heavily methylated and require a rapid catalyzation to maintain the hypermethylation in the daughter strand during DNA replication. Therefore, the combination of Dnmt1 and Dnmt3s enzyme can accelerate the process and prevent the activation of the repetitive elements during cell division (Liang et al., 2002).

However, the ability of Dnmt enzymes to maintain the level of DNA methylation in cells might be impaired when cells are propagated in the artificial condition. One possible cause reported by Kautiainen and Jones (1986) was the decrease in Dnmt enzyme level in cultured cells, but the cause of reduction was not specified.

In this recent experiment, however, we detected no alteration in DNA methylation at approximately 1,600 *Hpa*II sites in the genome of cattle fibroblast cells cultured *in vitro* for 27 passages. Therefore we could possibly imply that the activity of Dnmt enzymes in cells was optimum during the culture duration and the methylation profile had been faithfully copied through each and every round of mitotic cell division. However, there might be some alteration sites that could not be detected by our method. Since the *Hpa*II recognition sites are abundant in the mammalian genome, but in our experiment, we used only thirty sets of primer, which might not be enough to investigate all the *Hpa*II locations in the whole genome. Moreover, there are other methylation locations outside the *Hpa*II sites that could not be evaluated by our present experiment. Further investigation with more sets of primer or by using other methylation-sensitive restriction enzymes containing different recognition sites would provide a better understanding and more complete picture of global methylation changes in cell culture.

From a preliminary study done earlier in Australia, bull ear fibroblast cell culture showed alterations in methylation profiles, as well as exhibited phenotypic changes when cells were culture for more than 20 passages (data not shown). In that case, DNA methylation changes might be due to many factors: animal effect from individual variation, different culture conditions or even cell senescence. These factors must be clarified before making comparison between the two experiments. Unfortunately, we did not have defined information of the cell origin and culture condition of the cells used in our preliminary study. However, according to our results, cells from Passage number (P)30 were morphologically healthy and showed no sign of senescence (i.e. slow growth rate and abnormal cell morphology). Therefore, we could say that culture condition we used in this experiment did not affect the DNA methylation of approximately 1,600 *Hpa*II locations in bull fibroblast cells cultured continuously for approximately 5 months.

Although there was no alteration in DNA methylation profiles between different culture duration in this experiment, yet we did notice some changes in marker numbers found in Bull SC fibroblast cell P3 when compared with that in fibroblast cell P1 in experiment 1. Fibroblast cell P3 and so on contained less R- markers and gained more S- and D-markers. This result was a surprise, but somehow explainable. Changes in DNA methylation pattern in cell culture might be due to the adaptation process of cells themselves to survive in an artificial environment. This phenomenon was also found in human embryonic stem cell culture. The embryonic stem cells exhibited DNA methylation changes during the early period of culture and that new methylation profile was maintained in the later passages (Allegrucci et al., 2007). Our finding was in agreement with this report.

However, inter-essay variations cannot be overlooked. Though most part of the profiles generated by the same primer was reproducible between the two experiments, yet there were some markers that appeared in experiment 1 but disappeared in experiment 2, or *vice versa*. These variations could be due to genuine changes in DNA methylation and/or genome structure between the two passages or possibly due to different PCR sessions carried out at a different time. Direct comparison between AMPs-PCR profiles

generated from fibroblast cell P1 and P3 in the same PCR session can provide a better understanding in this aspect.

In conclusion, this experiment showed that DNA methylation pattern of fibroblast cells culture *in vitro* might be altered during the early culture period, but was maintained in the later passages. Fibroblast cells cultured *in vitro* under a conventional mammalian cell culture procedure supplemented with fetal calf serum and broad spectrum antibiotics up to 30 passages showed no sign of cell senescence and the DNA methylation at 1,600 *Hpa*II sites investigated in this study was faithfully maintained.

5.4) Limitations and future steps

As mentioned earlier, the results in these experiments were from only one methylation-sensitive restriction enzyme (*Hpa*II) and thirty sets of primer. When compared with the total locations of *Hpa*II recognition sites (5'-CCGG-3'), only 1,000-1,600 markers we obtained were just a small fraction in the whole bull genome. This recent study was just a beginning. We proved that the AMPs technique originally designed for plant genome could be applied in the study of mammalian DNA and the condition of the AMPs technique we used in this study was suitable and reproducible. Therefore, we can continue our investigation by generating and testing new primers, or we can change from *Hpa*II to other methylation-sensitive enzymes to gain more information of the location of methylation in mammalian genome.

From this recent study, we were able to recover DNA fragments from a silver-stained marker in dried polyacrylamide gel and the quality of recovered DNA was good enough for DNA sequencing. This will allow us to study the polymorphic markers found between samples in details. The information gained from this kind of investigation would possibly lead to novel genes or nuclear elements in the future.

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