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Global DNA methylation: role, status and genome-wide approaches to study epigenetic mark in cloned embryos

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Abstract: Somatic cell nuclear transfer (SCNT) technique has been proving its worth for more than two decades now as over 20 different species have been successfully cloned. SCNT protocol for cloning is well established but efficiency in terms of live birth rate is still low. Epigenetic abnormality following nuclear reprogramming is considered as the main culprit behind its low efficiency. DNA methylation is one of the most important epigenetic modifications that directly or indirectly regulate gene expression pattern, development and genome stability. Embryos produced through SCNT are found to express abnormal DNA methylation profile in comparison with *in vivo* or *in vitro* produced embryos. In order to improve DNA methylation profile in cloned embryos, a complete database of whole genome is required to find out specific faulty targets. Many techniques including low throughput and high throughput approach has been used to profile DNA methylation pattern in bovine embryos throughout the developmental stages. In the present review, we have compiled the overall status of global DNA methylation, the effect of aberrant DNA methylation on development and evolution in methodologies used for profiling global DNA methylome in cloned embryos.

Keywords: DNA methylation; embryos; epigenetics; nuclear reprogramming; SCNT; somatic cell nuclear transfer

Introduction

The field of embryology gained emphasis in scientific research with the success of the somatic cell nuclear transfer (SCNT) technique. With the birth of cloned sheep "Dolly" in 1997 by the SCNT method [1], researchers became more interested in exploring the molecular events involved in reprogramming of reconstructed embryos throughout the developmental stages. The somatic cell nuclear transfer technique, which is based on converting a differentiated somatic cell to a totipotent state, depends upon reprogramming of epigenetic marks of the donor nuclei by the cytoplasmic factors of the enucleated oocyte. To date, more than 20 mammalian species have been cloned, using a different type of somatic cells, for multiple applications, including agriculture, biomedical industry, disease modelling, therapeutic cloning, bio-pharming, conservation and restoration of endangered species and xenotransplantation, demonstrating the usefulness of this procedure [2]. The diagrammatic procedure of somatic cell nuclear transfer (SCNT) is represented in figure 1.

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Despite several successful cloning of numerous animal species, it has not been implemented on a large scale, majorly due to the very low live birth rate with cloned embryos. In comparison with the live birth rate obtained with bovine embryos produced by *in vitro* fertilization (IVF), i.e., over 40%, the live birth rate obtained with cloned embryos is <5% [3-5]. The overall success rate of cloning has been reported to be as low as 0.9% to 6% in terms of cloned offspring born [6]. This procedure of SCNT has upraised many practical and relevant concerns, such as increased abortion rates, high incidence of abnormalities such as large offspring syndrome, severe placental deficiency, respiratory problems, prolonged gestation, short life span and perinatal death [7-10].

Although the exact aspects contributing to the low efficiency of cloning are still indistinct, aberrant epigenetic reprogramming is one possible reason behind its low success rate. Several factors affect epigenetic modification, which includes quality of recipient oocyte in terms of its cytoplasmic volume with factors necessary for reprogramming donor nucleus [11-13], the properties (origin, quality and plasticity) of donor somatic cell nucleus [14, 15] and skill-based variations (technical and biological) in different steps of cloning procedure [16]. The success of cloning in reference to proper development and viability of embryo needs complete deletion of expression profile responsible for the differentiated state of somatic nucleus followed by reprogramming of house-keeping and tissue-specific genes for embryonic development [17].

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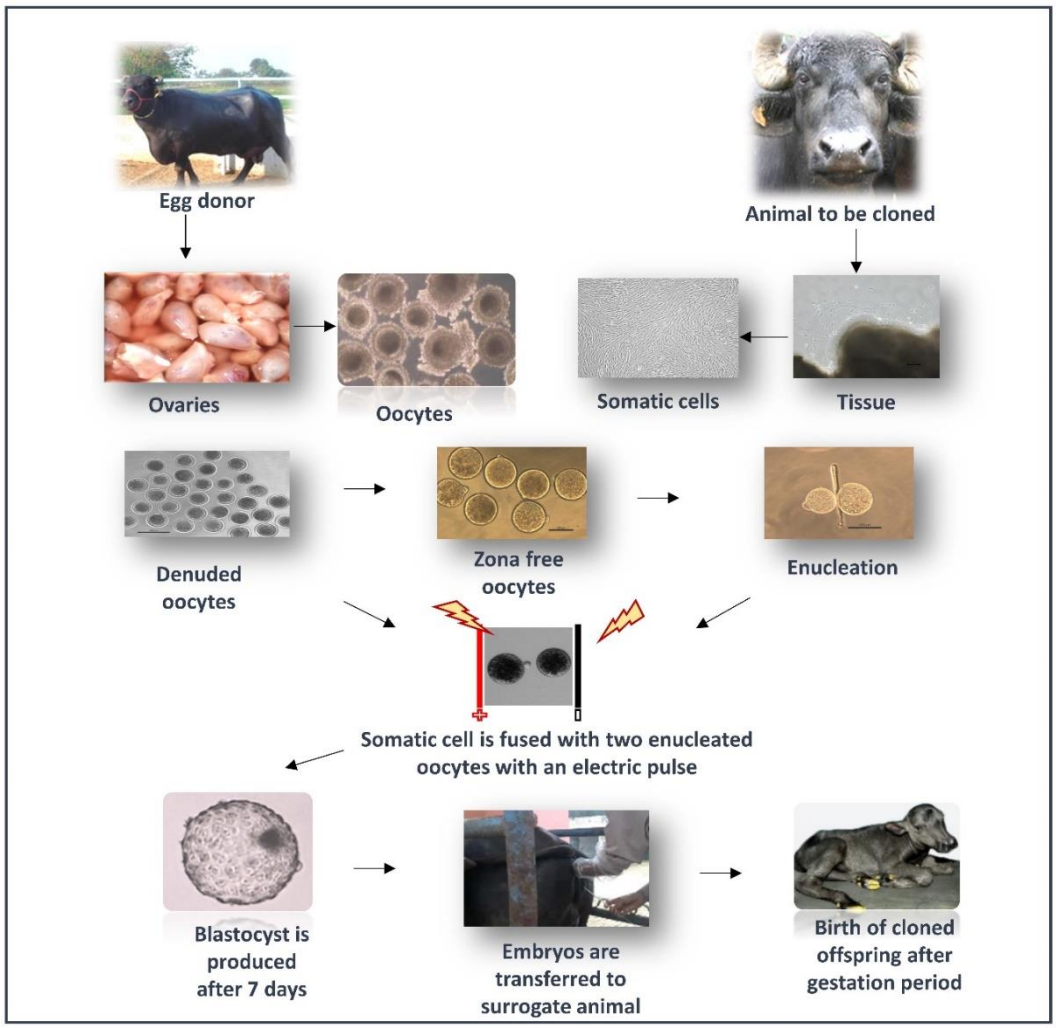

Figure 1: Schematic diagram of Somatic Cell Nuclear Transfer technique to produce cloned embryos in mammals.

Thus, it is apparent that epigenetics plays a crucial role in animal cloning through the SCNT technique as even with the idea of genomic equivalence, epigenetic modifications regulate the expression profile of the estimated 10,000 to 12,000 genes through various factors under different physiological conditions. Major epigenetic processes involve DNA methylation, chromatin remodelling, histone modifications and regulation of gene expression by noncoding RNAs [18-20]. DNA methylation and histone tail modifications play an important role during nuclear reprogramming as they alter chromatin condensation and DNA accessibility to regulate key cellular processes such as DNA replication and repair, gene transcription, and cell cycle progression, etc., alterations which lead to developmental failures of cloned embryos [21, 22]. DNA demethylation holds an important place as far as reprogramming of SCNT embryos is concerned because it is the initial step that occurs following nuclear reprogramming [23-25]. DNA methylation is necessary for transcription of octamer-binding transcription factor 4, which is a critical factor for pluripotency; thus, it certifies initiation and maintenance of early embryonic development after successful cloning [26, 27]. Therefore, aberrant DNA methylation may cause abnormal gene expression during the initial stages of development, ultimately leading to an embryo/ fetal developmental failure [23, 28-31]. Thus, this review aims at a brief discussion on DNA methylation and its role and pattern during reprogramming mechanisms in the growth and development of cloned embryos after successful SCNT procedure, including genome-wide approaches to study this modification in the genome.

DNA methylation: an important epigenetic mark

In the genome of mammals, DNA methylation is an important epigenetic modification as it is associated with transcriptional silencing and regulation of genes without altering the actual sequence of the DNA [32, 33]. This modification occurs at cytosine bases by the addition of a methyl group at 5^{th} carbon to generate 5-methylcytosine (5mC) in the DNA. This 5mC modification is majorly found in guanine- and cytosine dinucleotide rich sequences known as CpG islands in the genome of a differentiated somatic cell. About 60 to 90% of CpGs are found to be methylated in the mammalian genome [34, 35]. However, in a recent finding, mammalian genomes have shown the presence of adenosine methylation, but the physiological consequence of this remains unclear [36]. This chemical modification is carried by a class of enzymes called DNA methyltransferases (DNMTs) that catalyse the transfer of a methyl group from a methyl donor S-adenosyl-methionine to the 5^{th} carbon of cytosine base in DNA [37]. These methyltransferases have a specific target in DNA.

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Similarly, demethylation of DNA also involves a specific pathway and enzymes, i.e., ten-eleven translocation (TET1-3) proteins that convert 5mC to 5-hydroxymethylcytosine (5hmC) and further converts it to 5-formylcytosine and 5-carboxylcytosine [38, 39]. Finally, oxidised products are removed via DNA repair mechanism or through cell division [40]. This category of DNMTs has two classes of conserved enzymes, i.e., DNMT1 is maintenance DNMT and DNMT3A, DNMT3B and DNMT3L are *de novo* DNMTs [41]. DNMT1, along with UHRF1, is responsible for maintaining and ensuring the inheritance of methylation patterns of DNA after replication [42, 43]. Hemi-methylated DNA is a preferred substrate for DNMT1 [44]. For de novo DNMTs, both hemi-methylated and unmethylated DNA act as substrate, where DNMT3A and DNMT3B are able to regulate methylation-dependent silencing of genes during early embryonic development and cellular differentiation. DNMT3L has no catalytic role of its own but helps DNMT3A and DNMT3B to bind with DNA and promote their activity [45, 46]. Recently, DNMT3C has been recognised as a novel class of DNMTs family, which is an evolutionary duplicate of DNMT3B in rodents, happens to perform methylation of DNA in retrotransposons during the development of male germ cell [47]. According to structural chemistry, all the DNMTs have an analogous multidomain structure, including a variable N-terminal containing the regulatory domain and C-terminal managing catalytic methyltransferase domain [43]. DNMT1 and DNMT3 differ from each other in their regulatory N-terminal stipulating their different role in the genome [48].

DNA methylation is generally found at the promoters and enhancers of inactive genes, at repetitive elements, and within transcribed gene bodies. Its presence at promoters is dynamically linked to gene activity, suggesting that it could directly influence gene expression patterns and cellular identity [37]. It is extremely important for proper development as it controls a variety of biological functions, including long-term silencing of genes [49], X-chromosome inactivation in mammalian females [50, 51], genome imprinting [52, 53] and repression of repetitive and carcinogenic elements for genome stability [54, 55].

Dynamic pattern of DNA methylation during developmental stages of cloned embryos

SCNT technique is dependent upon a single genome of a differentiated somatic cell, unlike the *in vivo* or *in vitro* fertilization method. The nucleus of a somatic cell is being transferred to the cytoplasm of the enucleated oocyte (micromanipulator-based SCNT); a somatic cell is fused with an enucleated oocyte followed by electrical or chemical activation of reconstructed embryos to develop into a zygote. Inside the cytoplasm of demi-oocyte, somatic DNA is reprogrammed by various cytoplasmic factors such that imported nuclei get synchronized with oocyte [56]. Successful synchronization leads to chromatin-remodelling throughout the genome that causes coordinated spatial and temporal expression of important genes for totipotency. To date, it has not been clear regarding the exact factors responsible for reprogramming, but nuclear Mitosis or Meiosis Promoter Factor (MPF) is being demonstrated to assist reprogramming after cloning [57]. The level of MPF is kept high in oocytes at metaphase II (MII) and is being activated by the cytostatic factor (CSF) until oocyte activation [57-60]. MPF phosphorylates many cellular proteins for completing Meiosis II in reconstructed embryos after fusion/activation procedure in SCNT for chromatin remodelling and embryonic activation [61].

The process of nuclear reprogramming has an important relationship with global DNA methylation as it is required in repetitive disassembly and reassembly of chromatin of somatic DNA during the cloning procedure. The time span for epigenetic reprogramming to occur just after nuclear transfer should be short and specific for different species [62]. Initiation of embryonic transcription begins at 2-cell stage in the mouse, 4-cell stage in the pig and various from 8-cell to 16-cell stage in case of cattle, buffalo, sheep and human [21, 63-65].

Cloned embryos exhibit different kinetics of DNA demethylation and re-methylation than *in vivo*/ *in vitro* fertilized embryos. There are very few reports on the status of DNA methylation in SCNT embryos. Santos *et al*. employed immunofluorescence, using an antibody against 5-methyl cytosine, to examine DNA methylation in IVF and SCNT bovine embryos [25]. They reported that DNA methylation was characteristically reduced between the 2- and 4-cell stages, with *de novo* methylation occurring after the 8-cell stage. SCNT embryos stained more intensely for DNA methylation in all the stages than IVF controls, suggesting abnormal DNA methylation profile in cloned embryos. Similar results were reported by Suteevun *et al*., who examined the relationship between embryonic development and global DNA methylation in SCNT and IVF swamp buffalo embryos throughout the developmental stages using double immunostaining and quantification of the emission intensities using confocal microscopy [66]. They discovered an aberrant methylation pattern in early pre-implantation stage SCNT embryos. The relative level of DNA methylation decreased from the 2-cell stage until the 8-cell stage, then its level began to increase from the morula stage and became maximum at blastocyst stage in comparison to IVF counterparts. The highly heterogenic nature of DNA methylation was observed in cloned embryos. Chen *et al*. investigated the methylation status of a satellite sequence and the promoter region of a single-copy gene using bisulfite-sequencing technology in normal and cloned rabbit embryos [67]. During normal rabbit embryo development, both sequences maintained hypermethylation status until the 8- to 16-cell stage when progressive demethylation took place. In porcine, cloned and IVF embryos showed strong DNA methylation (both 5-mC and 5-hmC) from 2-cell stage to 8-cell stage with an abrupt and significant decrease in DNA methylation in morula stage and further increase at blastocyst stage [68].

Aberrant reprogramming in SCNT embryo

In developmental biology, a ground-breaking success of cloning in mammals challenged and changed the dogma of irreversibility of the differentiated state of a somatic cell [1, 69, 70]. Although, even after twenty years, the percentage of live birth rate obtained with cloned embryos is <5% [3-5]. Aberrant nuclear reprogramming is one of the most notorious events responsible for the failure of cloning,

leading to the faulty epigenetic configuration related to genotypic and phenotypic changes during embryonic development [23, 28, 29, 62]. When a somatic cell fuse with an enucleated oocyte, a series of molecular and chromatin remodelling events take place that is considered rapid, leads to the persistence of some epigenetic marks of differentiated somatic nuclei and loss of genome imprints [71]. The intrinsic resistance possessed by a somatic nucleus curbs the genome to undergo a complete de-differentiated state, thus retaining the memory of the genomic state of the donor cell [56, 72]. In some studies, it has been claimed that the higher the number of cell cycles, the higher will be the chances of proper remodelling of chromatin leading to successful embryonic genome activation (EGA) and development [73, 74]. The number of cell cycles required to respond to the microenvironment of the oocyte's cytoplasm to undergo chromatin repositioning and a balanced transcription of embryonic genes is species-specific. Like in cattle, three rounds of mitotic cell division occur for the genome of blastomeres to undergo transcription, whereas transcription occurs after a single cell cycle in mice [75].

DNA methylation profile of a somatic cell undergoing a dramatic cycle of demethylation followed by re-methylation in a reconstructed cloned embryo is a profound factor that decides the success or failure of further embryonic development. The effect of DNA methylation level in the genome of cloned embryos can be understood through a diagrammatic representation mentioned in figure 2. Various previous studies provide evidence for the presence of hypermethylated DNA in cloned embryos that are associated with incomplete reprogramming, loss of imprinted gene expression and sometimes leads to pathological abnormalities in cloned animals [9, 10, 76-79]. In transcriptional analyses, embryos produced by SCNT have shown anomalous gene expression in comparison with embryos produced by *in vivo* or *in vitro* fertilization (IVF) or any other assisted reproductive technique [80-84]. A classic example of aberrant gene expression in cloned embryos and animals is atypical X-chromosome inactivation that is caused by the abnormal expression pattern of *XIST* transcript, an ncRNA and some heat shock proteins that directly affect the embryonic and fetal development [85-88]. Cao *et al*. demonstrated the transcriptomic profile of SCNT embryos produced from Sertoli cells in the mouse, showing that abnormality in transcription occurred at the one-cell stage, which continued throughout all the developmental stages [74].

Likewise, cloned mouse embryos showed much lower loss of DNA methylation than that of IVF embryos and methylation patterns of former embryos were found to be more related to donor fibroblast cells than a zygote. Maps of genome-wide DNA methylation distinctly showed promoter regions of genes and repetitive elements to express different dynamics of DNA methylation than IVF counterparts [89].

Also, in bovine, cloned embryos have shown abnormal global DNA methylation profiles in satellite DNA. A report on the comparison between cloned and in vivo produced embryos in bovine found that repeat sequence of α-satellite I was hypermethylated in cloned blastocysts and fetuses than their counterparts [90]. In regards to the DNA methylation pattern of satellite I repeat sequence in the inner cell mass (ICM) and trophectoderm (TE) of cloned and *in vivo*– produced embryos, a significant difference was observed among the two categories of embryos [91]. Similarly, Zhang *et al*. showed a hypermethylated state of satellite I and α–satellite sequences in SCNT blastocysts than that of IVF blastocysts [92]. Developmental abnormalities were observed in cloned embryos due to hypermethylation of the Bov-B LINE sequence, which is

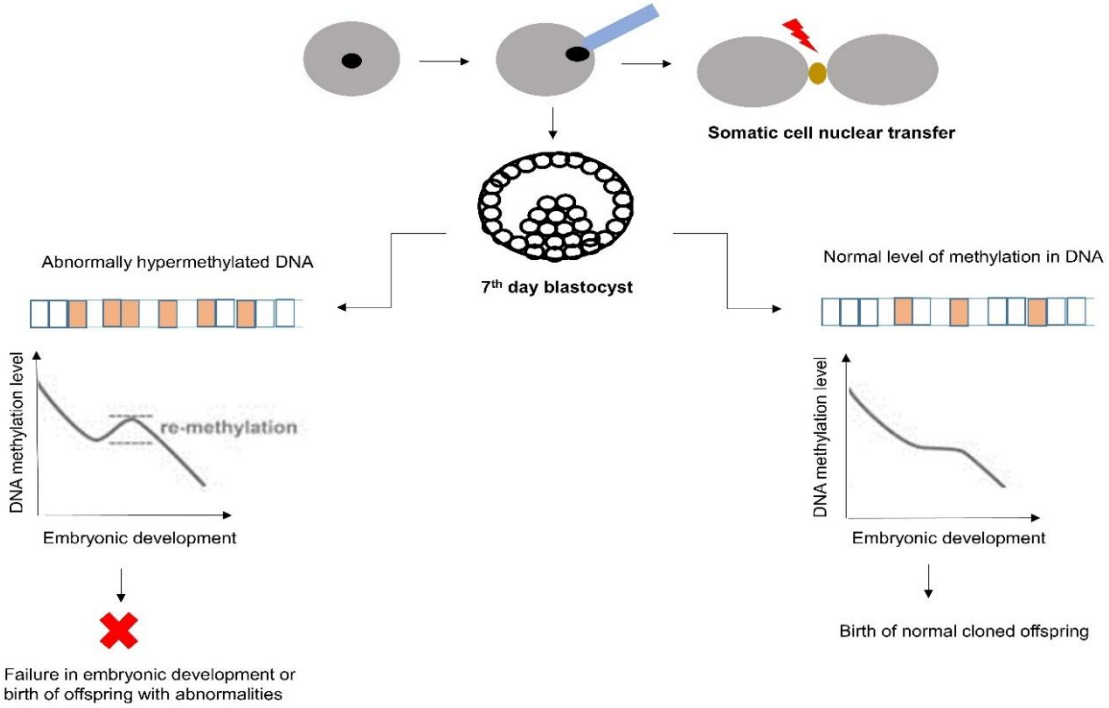

Figure 2: Diagrammatic representation of effect of DNA methylation level in embryonic development of cloned animal.

different from *in vivo*/ *in vitro* produced embryos. The pattern of DNA methylation was maintained in cloned embryos as that of donor nuclei throughout the developmental stages in bovine [93]. Dean *et al*. demonstrated that active DNA demethylation occurred only up to the one-cell stage in bovine cloned embryos, followed by *de novo* remethylation leading to the hypermethylation of the morula and blastocyst stage, which represents a different fate than observed in normal embryos [62]. Also, the methylation profile of cloned embryos showed resemblance with differentiated donor fibroblast cells that proves aberrant epigenetic reprogramming in cloned embryos. Gene expression pattern was found to be faulty in nuclear transfer (NT) embryos compared with *in vitro* production (IVP) embryos in reference to overall up- or downregulation of specific genes that control physiological functions. Abnormal DNA methylation is hypothesized as a primary reason for aberrant epigenetic remodelling, leading to large offspring syndrome [94]. Methylation patterns of chromosomes are also different between cloned and normal embryos in bovine as reconstructed embryos do not show a discrete pattern of methylation in parental-chromosome after demethylation. As compared to normal embryos, the cloned embryos exhibited an undermethylated state of euchromatin in the morula stage and a hypermethylated state of centromeric heterochromatin in the blastocyte stage [23]. The promoter of a single-copy gene (*SP-A*) and a satellite DNA (Rsat IIE) showed an aberrant pattern of DNA methylation in SCNT embryos than IVF counterparts in the rabbit when examined through bisulfite sequencing. While the *SP-A* gene promoter exhibited normal demethylation and remethylation event, the satellite DNA sequence of cloned blastocyst was found to remain methylated as that of donor cell through all the developmental stages [67]. All observations mentioned above thus justifies that due to hypermethylation in cloned embryos, gene expression pattern become aberrant and persist throughout the developmental stages and leads to abnormalities in cloned fetal tissues [95, 96].

Effect of aberrant DNA methylation on differentially methylated regions (DMRs), genomic imprinting and developmental consequences in cloned embryos

The *cis*-acting regions of the mammalian genome that exhibit differently methylated CpG rich domains, part of which are associated with regulation of gene expression and genome imprinting, are referred to as differentially methylated regions (DMRs). Identifying these DMRs provides inclusive evidence that different tissues express different epigenetic patterns in mammals [97]. For example, cancerous tissue exhibits abnormal methylation as that compared with normal tissue [98]. DNA methylation is crucial for cellular differentiation, proliferation and genome imprinting that signifies dynamic changes during the course of development [99-101]. The importance of DNA methylation in epigenetic reprogramming of SCNT embryos has been emphasized in a study including a siRNA-directed knockout of the *DNMT1* gene in cloned embryos. The rate of development of blastocysts significantly increased as reprogramming efficiency got improved due to appropriate DNA demethylation in cloned embryos with *DNMT1* knockdown [102].

Genome imprinting is an important mechanism that ensures mono-allelic gene expression, i.e., either paternal or maternal allele of a specific gene will express while other allele being epigenetically masked by DNA methylation and maintained throughout the development. Imprinted genes play fundamental role in embryonic and fetal growth and development [103, 104], placental functions/ anomalies [105] and postnatal survival [106, 107]. Imprinted genes are regulated by differential DNA methylation at CpG rich domains called imprinting control regions (ICRs). Methylation of ICR regulates regions-specific downstream mechanisms like the binding of an insulator protein or expression of non-coding RNAs that leads to mono-allelic expression of genes by methylation depended on repression. Genome imprinting is introduced at the time of gamete differentiation and maintained throughout the development and thus acts as a complex but essential process in a mammalian cell. A classic example of the importance of gene imprinting is the regulation of Igf2-H19 gene locus in mice that encode IGF2 growth factor and tumor-suppressive non-coding RNA precursor of several microRNAs. DMR present between the coding regions of *Igf2* and *H19* genes is unmethylated on the maternal allele and methylated on the paternal allele under the normal imprinting process. The expression of both these genes is being regulated by 3'-distal enhancer and CTCF insulator protein. Methylation of paternal DMR of the *Igf2* gene allows activation of *Igf2* promoter by distal enhancer while maternal DMR of the same gene is unmethylated; thus, CTCF insulator protein binding leads to inactivation of maternal *Igf2* promoter. Thus, the expression of the paternal allele of the *Igf2* gene along with a transcription of the maternal allele of the *H19* gene represents an example of a balanced expression pattern under gene imprinting [108-110].

Genome imprinting is more prone to abnormal epigenetic changes with respect to dysregulation of DMRs/ ICRs that may lead to physiological or morphological [111, 112] anomalies in the case of SCNT embryo production [107, 113, 114]. While Smith *et al*. reported only a single imprinted gene expressing differentially between NT and AI embryos reflecting proper reprogramming in NT embryos [115]. Other reports show a significant difference in the expression of imprinted genes in cloned embryos when compared with IVF counterparts due to improper activation or inactivation of important genes that diverts cloned embryos towards discrepancies after embryonic reprogramming [85, 116, 117]. In bovine cloned embryos, an abnormal change in expression of imprinted genes due to aberrant DNA methylation after reprogramming in blastomere may lead to trophectoderm cells (TE) that results in placental and fetal membrane disorders [118, 119]. Lucifero and co-workers observed aberrant DNA methylation status at imprinted SNRPN locus by using bisulfite sequencing in SCNT bovine embryos on Day 17 after embryo reconstruction in comparison to *in vivo* and *in vitro* produced embryos [120]. DMRs of *SNRPN* imprinted gene was found to be hypomethylated and expressed in a bi-allelic manner in all the tissues of day 17 and day 40 cloned bovine fetuses [121]. Similarly, both paternally expressed imprinted genes *NDN* (Necidin) and *XIST* were

found to be abnormally expressed in bovine cloned embryos in comparison to *in vitro* produced embryos [122]. Dindot *et al*. demonstrated aberrant genome imprinting at *XIST* locus in cloned bovine fetuses with the proper expression pattern of insulin-like growth factor 2 (*IGF2*; paternally expressed) and gene trap locus 2 (*GTL2*; maternally expressed) in the fetus as well as placental tissue [123]. *H19* imprinted gene was found to show bi-allelic expression in cloned bovine calves with stillbirth, which indicates faulty genome imprinting leads to abnormal development [124]. Likewise, Shen *et al*. also reported aberrant expression profile of *IGF2*, *H19* and *XIST* imprinted genes in five deceased bovine cloned calves [125]. A direct relationship was established between demethylation of the paternal allele of the *H19* gene with reduced growth and development in cattle cloned embryos. DMR of *H19* was found to be hypomethylated that leads to bi-allelic expression of the *H19* imprinting gene [126]. Abnormalities in the expression pattern of *H19* imprinted gene and DNA methylation profile were also reported in aborted cloned fetuses in porcine [127]. Deceased cloned bovine calves were reported to express abnormal expression profile of *IGF2*, *IGF2R*, and *H19* imprinted genes in their organs. In comparison to which, these imprinted genes were found to be expressed normally in tissues of surviving calves except for the expression of the *IGF2* gene in skeletal muscle [128]. Moore and co-workers demonstrated an aberrant expression profile of IGF family genes in day 7 cloned embryos and conceptuses from day 25 in bovine [129].

Similarly, tissue from kidneys of cloned calves in cattle that died immediately after birth showed abnormal expression of imprinting genes of the IGF family [130]. Some other imprinted genes such as *PEG3* (paternally expressed gene 3), *MAOA* (mono amine oxidase; X-chromosome expressed), *XIST*, and *PEG* were found to be aberrantly expressed in four aborted cloned calves in bovine [131]. Six imprinted genes, including *H19*, *XIST*, *IGF2R*, *SNRPN*, *PEG3*, and *IGF2*, were also reported to show deviation from normal expression patterns in placental tissues of deceased cloned calves in bovine in comparison with live cloned calves and the calves produced by conventional methods [132]. Thus, it is very clear that abnormal expression of imprinting genes may cause direct or indirect complications in cloned blastocysts and calves; and this could be one reason for high neonatal mortality in cloned animals.

Genome-scale methodologies to study global DNA methylation

Numerous genome-wide methods have been developed over the last decade for studying DNA methylation patterns and their role in understanding disease, cellular differentiation and development [133-136]. Most of these techniques were standardized and performed on different cell lines under *in vitro* conditions, thus provides limited information regarding DNA methylation status for *in vivo* systems. Monk *et al*. first developed a technique for isolating DNA in a low amount from mouse sperm, oocyte and early embryos and subjected it to genomic DNA methylation analysis by the end-labelling method [137]. This study provided relative quantification of global DNA methylation status of sperms becoming demethylated early after fertilization, oocytes exhibiting hypomethylated pattern. Dean *et al*. reported global DNA methylation status of in vivo produced and SCNT embryos in bovine, rat and pig by using indirect immunofluorescence method, which is one of the low throughput techniques [62]. Similarly, immunostaining using antibodies against 5-methylcytosine (5-mc)/ 5-hydroxymethylcytosine (5hmC) has been used to determine global DNA methylation status of *in vivo*/ *in vitro* derived and cloned embryos in bovine [23, 25, 66, 92], porcine [68], sheep [28] and mouse [21, 138, 139]. With advancements in technology, high throughput methods are being used to profile the global DNA methylation status of vertebrate embryos to produce a high-efficiency database of the whole genome [92, 93, 115, 140-145].

Genome-wide study of DNA methylation of a mammalian system is essentially based on local techniques like polymerase chain reaction (PCR), bisulfite modification of DNA, immunoprecipitation of DNA (viz. MeDIP, ChIP), restriction enzyme and mass spectroscopy-based methods, which on further merging with profiling technologies such as DNA microarrays or next-generation sequencing (NGS) give rise to high throughput results (Table 1).

Methylation-sensitive restriction enzyme-based approaches

With the discovery of restriction enzymes, many techniques were developed in the field of molecular biology that exploit their property of producing specific cleavage in the DNA sequence. The study of DNA methylation in the genome of vertebrates by using restriction enzyme was made possible by Bird and Southern [146]. Methylation-sensitive restriction enzymes that can recognize and discriminate between unmethylated and methylated DNA sequences to produce cleavage at unmethylated regions have been widely used in various experimental studies. Restriction enzymes *Hpa*II, *Ava*I, *Hha*I and *Hae*II were used to study the DNA methylation pattern of rDNA in erythrocytes of *Xenopus laevis* [146]. Popular methylation-sensitive enzymes such as HpaII and NotI recognize and cleaves unmethylated DNA sequences, whereas McrBC and MspI enzyme act on methylated DNA sequences. Hatada *et al*. established a method to assess global DNA methylation pattern in positional information by using methylation-sensitive enzymes, which is being referred to as restriction landmark genomic scanning (RLGS) [147]. Using enzymes that cleave at unmethylated recognition sites (CpGs islands) followed by end-labelling and 2-dimensional gel run, RLGS represents maps with unmethylated sites only [148]. RLGS method has been used in hundreds of studies as it can detect >1000 CpGs islands in a single experiment. This technique was used to find aberrant methylation of CpGs islands in around 98 primary tumors related to humans [149]; this proved to be the first genome-wide study of global DNA methylation altering CpGs islands in the case of cancer. Even with its accountable potential, this technique has a limitation with respect to the struggle involved in comparing individual spots to specific DNA sequences. Methylation-sensitive restriction-digestion was combined with the southern blotting technique to assess loci-specific DNA methylation in the genome of *Xenopus laevis*. It was found that the

Table 1: Summary of genome-scale methodologies to study global DNA methylation.

Sl. No.	Methodology	Techniques/platform used	Key findings	References
1	Methylation-sensitive restriction enzyme-based approaches	Restriction landmark genomic scanning (RLGS), Methyl-Sensitive Cut Counting (MSCC)	Provides a resolution up to single-nucleotide but limited to investigate the methylation status of DNA sequence by a particular methylation-sensitive restriction enzyme	[147, 154]
2	Microarray-based approaches	Methylation-Specific Oligonucleotide Microarray (MSO)	Microarray alone or combined with other methods has provided a platform for profiling genome-wide DNA methylation for many species and over a wide range of samples.	[155, 156]
3	Affinity-based approaches	Antibody against methylcytosine (MeDIP), Affinity chromatography based on methyl-CpG binding domain (MBD)	MeDIP-Seq gives better resolution as compared to MeDIP-chip but single base-pair resolution still cannot be expected from this approach as even with methylation of a single cytosine nucleotide present in DNA sequence, immunoprecipitation will occur for that fragment	[157-160]
4	Sequencing based approaches	Bisulphite sequencing, Roche/454 Genome Sequencer FLX system, Ilumina/ Solexa GAIIx platform, Support Oligonucleotide Ligation Detection (SOLiD) system, Pacific Biosciences/ PacBio, Complete Genomics, Ion Torrent/ Life Technologies, GridION/ Oxford Nanopore	High throughput instruments and reduction in cost proved themselves as highly suitable for profiling DNA methylation in mammalian genome over a large scale	[161-165]

genome is constitutively hypermethylated at Satellite I sequence (high copy number) and 5S rRNA genes of the oocyte in comparison to genes with a single-copy number that expresses a locus-specific methylation status [150]. Restriction enzymes have been used in combination with microarrays in the form of CpGs island microarrays to establish genome-wide DNA methylation profiles in human breast cancer cell lines [151], lung cancer, colon cancer and pancreatic cancer cell lines [152]. Also, direct sequencing is being combined with restriction enzymes to demonstrate global DNA methylation patterns in the human brain [153]. A next-generation sequencing strategy based on methylation-sensitive restriction enzymes known as Methyl-Sensitive Cut Counting (MSCC) was used by Ball *et al*. in human B- lymphocytes [154]. This technique generated genome-wide but non-targeted data for around 1.4 million HpaII restriction sites in B-lymphocytes DNA and reported gene-body methylation as a consistent occurrence in highly expressed genes in the human genome. Although this technique provides a resolution up to single-nucleotide, it is limited to investigate the methylation status of DNA sequence by a particular methylation-sensitive restriction enzyme.

Microarray-based approaches

The revolution in the area of functional genomics gained momentum with the development of microarrays as it provides information specific to more than a single locus on one array. This method is based on either hybridization or enzymatic specificity. It provided a platform for DNA methylome profiling as array-based DNA methylation analysis was first done in a human breast cancer cell line [155] and four other human cancer cell lines [156] on the basis of hybridization. This hybridization-based method is known as Methylation-Specific Oligonucleotide Microarray (MSO), which involves sodium bisulfite-modification of DNA followed by PCR amplification of the genomic region of interest and hybridization to the custom microarray. This method does not provide a single-base pair resolution and potentially reduces the sequence complexity making it difficult to design unique probes for genome-wide studies. Another approach for the microarray-based study is to exploit DNA polymerase enzyme specificity to detect methylation is Illumina HumanMethylation27 BeadChip (Illumina, Inc.). This method is able to provide single-base pair resolution as it involves designing separate probes for each CpGs site with methylated as well as the unmethylated version on microarray followed by primer extension using fluorescent-labelled nucleotides [166].

Microarray has been used along with three major techniques (viz. bisulfite conversion, Methylation-sensitive restriction enzyme digestion, immunoprecipitation technique) for profiling DNA methylation status in the mammalian genome. DNA microarray-based on bisulfite conversion has already been discussed above and a few literatures are available based on this technique [167-169]. A limitation accompanies this method that unique probes designing is difficult for microarray. Methylation-sensitive restriction enzyme digestion, along with microarray technique, was first used to profile genome-wide DNA methylation in *Arabidopsis thaliana*. It involved fragmentation of DNA by methyl-sensitive restriction endonuclease followed by separation of DNA segments based on size and hybridized on microarrays [170]. This method has proven to be sensitive and provides larger and better genomic coverage with single-base resolution than the bisulfite conversion method [171-173]. Its only drawback is that the region of DNA with restriction sites can be analyzed and used to profile the whole genome. Microarray, based on the immunoprecipitation technique, was first used by Zhang *et al*. for DNA methylation profiling in *Arabidopsis* [174]. This work became a milestone for profiling global methylation patterns in human normal and cancerous cell

lines by using methylated DNA immunoprecipitation (MeDIP) and methylcytosine immunoprecipitation (mCIP), respectively. The basic idea behind this is to immunoprecipitate the methylated parts of genomic DNA with an anti-methylcytosine monoclonal antibody followed by hybridization of immunoprecipitated DNA fragments on a microarray against the input/total fraction [157, 158]. However, immunoprecipitation-based microarray does not provide more than 100 base pairs resolution, but it is further less sequence bias than that of bisulfite conversion or methyl sensitive restriction digestion approaches. Genome-wide DNA methylation profile was established for tissues isolated from phenotypically normal cloned pigs and pigs bred conventionally using Affymetrix Porcine expression array along with modified methylation-specific digital karyotyping (MMSDK) and Solexa sequencing technology [141]. Similarly, Ispada *et al*. recently profiled the global DNA methylation status of bovine embryos with different developmental kinetics in order to identify different pathways and genomic regions being influenced by this kinetics by using EmbryoGENE DNA methylation microarray [145]. Overall, microarray alone or combined with other methods has provided a platform for profiling genome-wide DNA methylation for many species and over a wide range of samples.

Affinity-based approaches

The affinity-based method involves the use of an antibody against methylcytosine (MeDIP) [157, 158] or using affinity chromatography based on the methyl-CpG binding domain (MBD) [175] to precipitate methylated regions of genomic DNA. MeDIP technique of immunoprecipitating methylated DNA, when used in combination with microarray, is referred to as the MeDIP- chip approach. The principle behind this technique is being explained in an earlier section. MeDip-chip was intensively used to profile the DNA methylation state of 11,201 proximal promoters in mESCs in the mouse. It was reported that most of the genes involved in general cellular functions were unmethylated, whereas genes related to differentiation were highly methylated. It was also observed that low CpGs content at the promoters is more likely to be methylated than high CpGs content [176]. Kiefer used MeDIP-chip to identify methylated loci in the genome of bovine clones and non-clone animals to demonstrate the effect of the SCNT method on the genome [177]. Phenotypically normal cattle clones were compared to clones with pathological conditions having the same genotypes to study the role of epigenetic factors in perinatal mortality by using the MeDIP-chip technique. It was found that the adult clones did not show major phenotypic and epigenetic abnormalities in the liver in comparison with deceased clones showing that genotype does not completely affect phenotype [159]. In a recent report, the MeDIP-chip method was used to profile global DNA methylation profiles in sperm, oocytes and different developmental stages of preimplantation embryos of humans. From the results of this study, it can be concluded that there is a sharp decline in the methylation level of CpGs islands just after fertilization, which goes on increasing from morula stage to blastomeres in ICM with re-establishment of methylation of CpGs islands in TE cells of blastocysts [178]. MeDIP-chip approach is able to provide whole-genome coverage data, but it has many drawbacks, including low resolution of microarray, needs prior knowledge of designing probes, cross-hybridization and its high cost [179]. Performing a high-density microarray for whole-genome coverage with reasonable resolution needs 10-20 arrays that increase its cost and complexity. Thus, a new combination of techniques was used to overcome these issues.

The MeDIP-Seq approach involves a pool-down of immunoprecipitated methylated DNA sequences using an antibody against methylcytosine followed by the alignment of purified DNA fragment sequences with the reference genome. This technique is able to discriminate even among highly similar sequences, which was not possible by the MeDIP-chip method. This technique provides region-specific information of DNA sequences that are methylated in promoter and gene-body and thus makes data analysis easier and also lowers the cost per sample for studying genome-wide methylation in any tissue or cell [160, 180]. Zhao *et al*. demonstrated MeDIP-Seq protocol on mESC culture with as low as 1ng of genomic DNA by using antibodies against 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) and observed consistent results for DNA methylation patterns in sample DNA [181]. This study provides proof of the feasibility and efficiency of this technique in profiling genome-wide DNA methylation status with a minute DNA sample, and it can be used to examine DNA samples from oocytes, embryos, and human biopsies. MeDIP-Seq has been used in generating a high-resolution DNA methylation profile of rat liver. It was observed that simple repeats were found to be hypermethylated, promoter regions of both references, as well as expressed genes, were hypo-methylated and exon hypermethylation was common in both kinds of genes. Also, hypermethylations were more dominant in coding/ partially coding exon regions than non-coding exons regions [182].

Genome-wide DNA methylation profile was established in placental tissue derived from SCNT and control cattle using MeDIP-Seq. It was found that the gene body is highly methylated while promoter regions were hypomethylated. A negative correlation was found between DNA methylation levels and gene expression levels around transcription start sites (TSS). While genes with the highest expression showed the lowest DNA methylation levels, moderately expressed genes showed the highest DNA methylations [183]. Huang *et al*. generated genome-wide DNA methylation maps for longissimus dorsi muscle (LDM) samples isolated from fetal and adult stage of elite native cattle breed Qinchuan by using MeDIP-Seq [184]. DNA methylation data were correlated with mRNA as well as miRNA and revealed a negative correlation between expression of high-read genes and methylation levels at different developmental stages of muscle tissues. This technique has also been implied to study abnormality in deceased cloned piglets compared with normal cloned piglets with respect to the DNA methylation status of the whole-genome. This study is being the first one reported for studying aberrant methylation patterns in cloned animals. It was reported that abnormal cloned piglets suffered more hypomethylation than hypermethylation in comparison

with normal cloned piglets. But hypermethylations were found at CpGs islands in the genome of abnormal cloned piglets. Around 1711 differentially expressed genes were detected between the groups. MAPK signalling pathway, hypertrophic cardiomyopathy pathway, and the imprinted gene *PLAGL1* were found to be different between the groups and were found responsible for abnormal phenotype [185]. Although MeDIP-Seq gives better resolution than MeDIP-chip, the single base-pair resolution cannot be expected from this approach, as even with methylation of a single cytosine nucleotide present in DNA sequence; immunoprecipitation will occur for that fragment [186].

Another approach has been developed to get methylated DNA by exploiting the high binding affinity of the methyl-binding domain (MBD). This technique is referred to as methylated CpG island recovery assay (MIRA) as it uses MBD2/MBD3L1 complex to specifically bind to a double-strand DNA sequence, which contains methylated CpGs. MIRA can detect methylated CpGs sites even if it is a single CpGs in a target DNA sequence. This technique is currently being commercialized by many reputed companies, including Life Technologies (Invitrogen). It has been used in various studies related to human cancerous cell lines [187] and human B lymphocytes [188]. Recently, Maldonado *et al*. used MIRA combined with next-generation sequencing (MIRA-Seq) to generate SNPs database and methylation profiles in the DNA samples extracted from cattle tissues having different feed efficiencies [189]. They identified 12,836,763 meSNPs from genomic samples of 1000 cattle bulls that could be used to determine epigenetic polymorphism that causes phenotypic variations in bovine.

Sequencing based approaches

With the advent of evolution in technologies for profiling genome-wide DNA methylation in the mammalian genome, maximum coverage data in a single run has been made easier and efficient to achieve by the next-generation sequencing (NGS) platform. Automated Sanger sequencing method, which is also referred to as "first-generation sequencing" gave a nudge to next-generation sequencing for performing a variety of experiments based on typical protocol including the type of strategy, template and library preparation, sequencing, sequence alignment with reference genome followed by bioinformatics analysis of raw data generated. The first platform for NGS was introduced as Roche/454 Genome Sequencer FLX system that was based on pyrophosphate detection and sequencing by synthesis with the help of fluorescent substrate to detect signals during sequencing. This system provides up to 500 base length for about one million reads per single run. Then came the Illumina/Solexa GAIIx platform with the key feature of performing bridge PCR for real-time sequencing that differs from the Roche platform. This system can generate high throughput data with 100 nucleotide long sequence read length at its best as longer read length can cause a signal decay. Under the second/ next-generation sequencing platform, Life Technologies introduced a Support Oligonucleotide Ligation Detection (SOLiD) system, based on sequencing by ligation principle, including a cycle of hybridization and ligation of fluorescent probes. This system is among the most widely accepted platforms giving high throughput efficiency with a short read length of 50 bases and a low error rate. With the introduction of third-generation sequencing technologies, much more efficient, high throughput and long read length providing systems are being introduced under the name of Pacific Biosciences/ PacBio, Complete Genomics, and Ion Torrent/ Life Technologies. GridION/ Oxford Nanopore is recently introduced under fourth-generation sequencing based on nanopore exonuclease sequencing promising to provide a long read length. NGS technology has many advantages over other techniques, including single base-pair resolution, less background noise, and genome-wide coverage that increase the authenticity of data generated.

Maxam and Gilbert developed the first method to directly sequence genomic DNA by exploiting hydrazine (N_2H_4) that show less reactivity with methylated and high reactivity with unmethylated cytosine in sequencing reaction leading to the formation of cytosine bands whose intensity is related to the level of methylation [190]. But with the development of 'bisulfite sequencing', profiling of DNA methylation in the mammalian genome became easier and more accurate [161]. Throughout the last decade, profiling DNA methylation using the sequencing method underwent a major change to give rise to these approaches viz. bisulfite-sequencing, reduced representation bisulfite sequencing, paired-end sequencing of methylated and unmethylated genomic domains (like MeDIP-Seq). Bisulfite sequencing involves the use of any sequencing method on bisulfite-treated DNA. With conventional approaches, bisulfite sequencing has been used for DNA methylation profiling with a single base-pair resolution for a specific area of the genome. The first conventional approach is referred to as bisulfite PCR sequencing, which aims at the high-throughput analysis of methylation directly from bisulfite sequenced PCR products along with normalization of values due to incomplete conversion. Direct sequencing of 122 bisulfite PCR products was amplified from human lymphocytes, lymphomas and leukaemia, covering 25 genes in a single run, facilitates more than a thousand sequences for each PCR fragment [162]. Similarly, Eckhardt *et al*. used this approach to establish a DNA methylation profile of 3 human chromosomes in 12 different tissues showing no changes in the methylation profile of tissues depending upon age [191]. Also, a 2 million CpGs sites methylation data was created in this study revealing new patterns, distribution, ontogenetic stability and evolutionary conservation of DNA methylation.

There is another method for studying methylation of the targeted region of the genome is reduced representation bisulfite sequencing (RRBS). This method involves restriction digestion of genomic DNA and selection of fragmented DNA based on size (500-600bp). These selected fragments are then linked with adapters followed by bisulfite conversion, PCR amplification, cloning and finally, leads to high throughput sequencing [163]. With RRBS, a large fraction of genomic DNA (up to 5 mb) can be analysed over many cell types and tissues that make it a better strategy than the directed PCR method. It also needs a comparatively smaller number of sequencing reads required for achieving better sequencing quality for

quantitative analysis of the methylation profile of the target sequence. RRBS also reduces the size of the reference genome because of the less complexity of the target sequence. Chan *et al*. generated genome-wide DNA methylation profiles from donor fibroblasts and SCNT mouse embryos with single base-pair resolution using the RRBS method [89]. It was found that SCNT embryos have DNA methylation pattern was more closely related to donor fibroblasts than that of the paternal genome after fertilization and the demethylation process is inefficient after the SCNT procedure. Genome-wide DNA methylation profiles are recently established in bovine sperm, in vivo developed oocyte and preimplantation embryos using RRBS. It was demonstrated that the 8-cell stage of developing embryo completes major demethylation. This study also provided insights into various differentially methylated regions (DMRs) in sperms, oocytes and *in vivo* produced embryos [144]. However, this method has a limitation related to its efficiency as this cannot be applied to tissue samples because of heterogeneity; thus, it is difficult to produce deeper sequence coverage with this method.

A complete DNA methylation map of the mammalian genome covering all CpGs sites with the single base-pair resolution has become feasible with whole-genome bisulfite sequencing (WGBS). The first report on the use of this method was published in profiling DNA methylation status in cloned and *in vitro* fertilized embryos in rabbits to evaluate comparison among both types of embryos [67]. Bisulfite sequencing involves bisulfite conversion of genomic DNA followed by sequencing with Solexa sequencing technology to measure cytosine methylation within the targeted genome. Compared with traditional sequencing approaches, this platform provides much high throughput per single run and offers much more precise methylation measurements even with repetitive sequences in the genomic DNA. Zhang *et al*. used bisulfite sequencing to demonstrate hypermethylation of DNA in satellite I and α –satellite sequences in SCNT blastocysts than that of IVF blastocysts that indicate an abnormality in cloned embryos [92]. Three retrotransposons (*L1_BT*, *BovB*, and *ERV1-1-I_BT*) and *Satellite I* repetitive sequence were sequenced by bisulfite sequencing method in preimplantation embryos in bovine. It was found that *Satellite I* and *L1_BT* showed significantly reduced methylation at the blastocyst stage while *BovB* and *ERV1-1-I_BT* showed no difference [192]. Likewise, genome-wide DNA methylation profile at each developmental stage was established by using WGBS in bovine sperm, immature oocytes, *in vivo* and *in vitro* matured oocytes and *in vivo* derived embryos at 2-, 4-, 8-, and 16-cell stages. Sperms and oocytes showed methylated DMRs in intergenic regions of the non-coding part of the genome, suggesting its importance in gamete specification. Methylation pattern of DMRs between *in vivo* and *in vitro* matured oocytes indicates the effect of environmental conditions on epigenetics. Mitochondrial DNA showed almost no methylation. Also, major demethylation of DNA was found to be completed by the 8-cell stage [191]. Koike and co-workers identified genome-scale DNA methylation errors using post bisulfite sequencing method in sperm of cloned mouse [165]. It was reported that there was a significantly high number of differentially methylated CpG sites (P=0.0045 and P=0.0116) in the sperm genome of the cloned mouse. This result indicates that DNA methylation errors resulting from embryo cloning are being transmitted to the sperm genome of offspring by escaping the germline reprogramming barrier.

Another high-resolution approach is MeDIP-Seq that can be used to study genome-scale DNA methylations in the mammalian genome. The working principle behind MeDIP is already being described in the previous section. When the high throughput sequencing platform collaborates with the MeDIP method, it becomes methylated DNA immunoprecipitation sequencing (MeDIP-Seq). This strategy has introduced the concept of short-read sequencing for methylome analysis. Earlier major studies were done with MeDIP-Seq to determine methylation profiles of epigenetic marks like histone modifications and protein-DNA interactions on high throughput platforms [193-196]. Down *et al*. estimated absolute methylation using MeDIP-Seq by developing a cross-platform algorithm named Batman that showed about 90% coverage of CpG sites within CpG islands, promoters' regions, exon and introns, whereas 60% CpGs sites coverage in the human genome [186]. MeDIP-Sequencing has been applied for producing DNA methylation maps in rat liver [182], bovine muscle tissue [184], bovine placenta [183] and muscle tissue of cloned pigs [185], which has already been discussed in an earlier section. MeDIP-Seq is beneficial as it reduces read length, but computational processing has to be much more advanced for high efficiency. Although several approaches for profiling DNA methylation have been described in this review, no single technique can provide a whole picture of the methylation status of the mammalian genome alone. Techniques are being used in combinations to produce comprehensive genome-wide DNA methylation maps. Next-generation sequencing has made its way among different methods available due to its ability to generate an immense amount of data with high efficiency and availability of sophisticated bioinformatics tools and statistical skills. With improvements in sequencing chemistry, high throughput instruments, and cost reduction, NGS technologies have proven themselves highly suitable for profiling DNA methylation in the mammalian genome on a large scale.

Conclusions

The somatic cell nuclear transfer (SCNT) technique has been improved since 1997 in the context of protocols, tools and efficiency. Different types of somatic cells have been used as donor cells in cloning to produce good quality preimplantation embryos in various mammals. Even so, the live birth rate of cloned embryos is still less than observed with *in vivo* or *in vitro* produced embryos across all the species. However, with best efforts to increase the efficiency of cloning have fallen short as epigenetic barriers are still not entirely understood. Understanding epigenetics behind the nuclear reprogramming in SCNT embryos is very important and our knowledge for the same is progressively increasing since the birth of "Dolly," the sheep. DNA methylation is one of the critical epigenetic marks in the reversal of the differentiated state of donor somatic cells in cloning. Establishing a genome-wide methylation profile of cloned embryos at different

developmental stages would facilitate a better understanding of chromatin modifications, DNA sequence composition, gene expression, and a reference base in embryonic studies. Genome-wide studies of DNA methylation in cloned embryos will also help in determining the crosstalk between DNA methylation and other epigenetic modifications that together maintain the genetic integrity of SCNT-derived embryos towards normalcy. To date, most of the DNA methylation studies in cloned embryos has been done using low throughput techniques (viz. immunofluorescence, bisulfite PCR, small-scale microarrays). Still, limited literature is available on the use of high throughput techniques (viz. chip-based microarrays, MeDIP-sequencing, whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), etc.) to compare DNA methylation profiles with *in vivo*/*in vitro* produced embryos. There is an immediate need to explore complete DNA methylation profiles of cloned embryos at different developmental stages in bovine with nanoscale techniques to establish a comprehensive database that can be exploited to increase cloning efficiency much higher than the present rate. In summary, there is no single bullet in terms of epigenetic modifiers to improve the efficiency of the cloning process; thus, a complete and exact status of the genome of reconstructs is required to be known so that specific modifier can be used to target a specific genomic site for successful cloning. The purpose of this review is to summarize the available knowledge and data in regards to the status and role of global DNA methylation during the embryonic development of cloned embryos in mammals.

Declarations

Author Contribution All persons designated as authors qualify for authorship. S.M. wrote the review and A.H. prepared the final draft of the manuscript.

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