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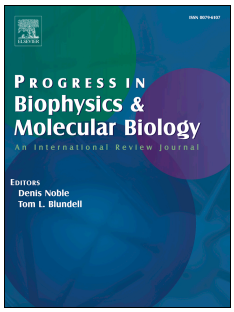

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The role of DNA demethylation in induction of stem cells

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Abstract

DNA methylation is an epigenetic factor, which plays important roles in embryo and many other diseases development. This factor determines gene expression, and when half of them have CpG islands, DNA methylation and its enzyme effectors have been under the vast studies. Whole genome DNA demethylation is a crucial step of embryogenesis and also cell fate determination in embryos. Therefore, demethylation agents were used as a tool for dedifferentiation and transdifferentiation. Although many of these efforts have been successful, but using this method gave us a vast spectral cell type which is confusing. In this article, we briefly reviewed DNA methylation, and its role in embryogenesis and gene expression. In addition to that, we introduce studies that used this action as a direct method in induction of stem cells and cell fate decision.

Introduction

It has been shown that, epigenetic factors determine transcriptome of cells. These factors determine cell functions and identities [1]. Cell fate determination is among the most significant aspects influenced by these factors [2]. DNA methylation occurring on cytosine in mammals is a major epigenetic factor, which its presence and absence may pose control over 50% of genes, especially developmental-related genes [3]. It has been hypothesized that; DNA demethylation is one of the most significant events in initiation of

embryo development after fertilization, which has been named as Zygote Gene Activation (ZGA) [4]. This procedure induces expression of many stemness-related genes. Recently, many studies showed role of DNA demethylation and other epigenetic factors in changing cell fate [2]. It has been proved that, DNA demethylation could change fate of specific somatic cells to another somatic identity in a process named as transdifferentiation [5]. This phenomenon can reprogram somatic cells directly to stem cells [6]. DNA methylation enzyme effectors can establish new fates in differentiation process of stem cells as well [7]. Although it is believed that, disruption of epigenome or reactivation of developmental process in demethylation process could be a potential answer to these phenomena, but related findings have shown that, different roles of DNA demethylation are very confusing to determine. Furthermore, the basic concept and application of this field have been hindered because of its reproducibility and cell fate heterogeneity.

DNA methylation and its role in cell fate decision

DNA methylation appears on fifth position of cytosine by DNA methyltransferase1 (Dnmt1), Dnmt3a, and DNMT3b in mammals. Substrates of these enzymes are single cytosine or CG (CpG) content of the genome [8, 9]. In general, DNA methylation is known for its role in repressing and condensing chromatin, but it plays a variety of other roles based on its position in chromosomes and genes [10-12]. DNA demethylation will lead to gene transcripts upregulation [13]. DNA hypermethylation causes downregulation of the gene in promoters or enhancers [14, 15]. This factor might interfere with direct protein interaction to the DNA sequences like promoters and enhancers [16]. There is also a strong evidence for cross talking between DNA methylation and histone modifications. In general, H3K4 methylation, H3K27 acetylation, and DNA demethylation have a robust correlation with upregulation, and establish of each other's in-situ. H3, H1 deacetylation, H3K36 methylation, Ubiquitination, and DNA methylation on Promoters have a firm relationship with each other and also gene silencing. It has also been hypothesized that body methylation gene's silenced antisense transcript, which aborts mRNA degradation or vice versa [17, 18].

Dnmts are the enzymes, which were responsible for DNA methylation. Dnmt1 is known for maintenance of DNA methylation in cell division by recognizing hemimethylated CpG in PCNA and methylation of them [19]. It has been shown that the passive demethylation of genome by inhibition of Dnmt1 induces hypomethylation and Concentration of Dnmt1 will rise in the cell cycle phase of S/G1, due to methylation of new synthesis DNA, and cell fate maintenance [20]; other Dnmt family members are Dnmt3a and b, which most of their roles are in de novo methylation and establishing new pattern [21], It is remarkable that expression of Dnmt3a and Dnmt3b in order to lose pervious patterns will downregulate before the differentiation [22].

One of the most significant roles and changes in DNA methylation content of cells are in embryogenesis and induction of stem cells. The genome will go under heavy whole genome DNA demethylation after fertilization. In this notion, Dnmt1, Dnmt3a, and Dnmt3b will not be effective on the nucleus [23] and relocate the cytosol causing DNA demethylation of parental genomes by destabilizing and dilution of DNA methylation after every cell division, which is called passive demethylation. Tet1, Tet2, and Tet3 enzyme are another mechanism for demethylation of the genome (active demethylation), which catalyzes methyl cytosine to intermediated compounds [23]. It has been shown that many of embryogenesis and organogenesis genes, which are crucial for preimplantation and development of the zygote, are controlled and upregulated by demethylation [24]. This wave of upregulation includes many genes, and demethylation inducing dedifferentiates many cells to stemness lineage artificially [25-27].

After pervious stages and in initiation of organogenesis, Dnmt3a, and Dnmt3b upregulate and present in the nucleus, and de novo wave of methylation occurs subsequently. This de novo wave establishes the right pattern of epigenome, in accordance with specific fate of cells during this time of events, the majority of embryonic genes will heavily methylated and silenced (Figure1) [28].

Demethylation studies methods

Many researchers devote their time to investigate the importance of DNA demethylation in embryo development and its role in gene expression. There are different approaches to study about DNA demethylation. Many studies have been performed based on chemical component, which targets DNMTs. The most famous one amongst these agents is azacitidine (AZA-c) [29], which is a cytosine analog. This component is studied in stem cell and cancer researches extensively. This agent has a nitrogen atom in the fifth position instead of carbon, so there is no extra valance for methyl group to bond, according to this change [30]. This substance cannot be methylated and DNMTs captured in this area and it will be degraded promptly [31], and this procedure dilutes the DNMTs concentration. There are some upside downs for this agent. First, this agent makes the chromosomes unstable, and induces double strand break and mutation. Demethylation leads to apoptosis and senescing in somatic cells, which in addition to the AZA effect, will be catastrophic [32]. Another disadvantage of this agent is related to Dnmts variants, as some of them are not catalytic and as a result they cannot be affected.

Another way to study about demethylation is by genome editing methods based on knock downing Dnmts, which is a great approach to study demethylation, but most of these methods are permanent for genome editing [33], so cannot be tolerated by cells and creatures. Using siRNA because of numerous variants of Dnmts and knock downing efficiency may not be as effective as it needs to be, although some great progress had been made for this problem [34, 35].

The final approach is using array and next generation sequencing between embryonic stem cells and somatic cells as a comparative and predictive approach for demethylation studies [36]. Although all above mentioned methods for methylation have their own advantages and disadvantages, and also new chemical components have been discovered with a lower side effect for cells which has not been tackled on differentiation and transdifferentiation studies. The most important substance of these components is 5-aza-2-deoxycytidine; this substance is more tolerable for cells and animals subjected to these kinds of studies [37]. Another approach for investigation the role of DNA demethylation in induction of stem cells and transdifferentiation is the over expression of Tet enzymes in cells. Therefore, some researchers have

been performed to obtain pluripotent cells and muscle differentiation [38]. Although all above mentioned Tet enzymes play a significant role in demethylation of embryonic stem cells, it has been proven that Tet2 enzyme is contributed in stem cells inducing [39].

DNA demethylation and induction of stem cells

These surveys use the fact that embryo-related genes are heavily methylated, and demethylation leads to reactivation of these genes, in early embryo development. As a result, somatic cells demethylation might dedifferentiate cells to embryonic stem cells.

It has also been shown that demethylation upregulates many embryo-related genes (Figure1), and they are not only including epiblast markers like *Nanog*, and *Pou5f1* [40,41,42,43], but also it will reactivate many genes related to Trophoblast lineage like *Cdx2*, *Emoes*, *Elf5*, *Fgfr* [42,44], and primitive endoderm lineage like *Gata*4 and 6 [45]. It is remarkable that genes like *Nanog*, *Cdx2*and *Gata*4 have transcription factors, which are upregulated when the right network will be established in cells. It is considerable that because of these genes' role establishing totipotent stem cells and developing the embryos, and also providing the development potential to downstream lineage for totipotent stem cells happened [46]. It has been shown that demethylation of somatic cells, reprograms these cells to pluripotent stem cells and Trophoblast stem cells [41, 44]. However, the totipotent phenotype, despite existence of numerous studies with these substances has not been observed and obtained.

Other important genes that are reactivated by demethylation are *Hox* cluster, which are essential for hematopoiesis and embryo development (table1). Induction of these genes by demethylation would result in hematopoietic production of cells and theirs progenies [45].

Some interesting genes have been expressed after demethylation which is considerable, including *Dnmt3l* [46], which are only expressed in embryonic cells. Other examples are genes related to telomerase activity and elongation. It is shown that the transcript of the hTERT increases after demethylation [47]. The

demethylation increases expression of *12qF1* and its cluster, which will influence chimer formation, and will cause survival in pluripotent stem cells [48] (Tbale1). It is notable that the other demethylation substances, such as RG108 and decitabine, have been administrated for inducing the stem cells, which lead to upregulation of *Nanog* and *Oct4* [49].

Role of DNA Demethylation in Differentiation and Transdifferentiation

Obviously, after fertilization and generation of totipotent stem cells (the cells which can generate embryonic and extra embryonic tissues) and their development to pluripotent (the cells which could generate all of three germ layers of cells including mesoderm, ectoderm, and endoderm) and multipotent cells (the cells which could rise to various cells from a defined germ layer), epigenetic pattern becomes more and more defined and restricted. It is notable that, in this process, stemness-related genes become strongly silenced (by DNA methylation and ubiquitination of histones) and other required genes will be activated [50, 51]. Eventually, when developmental stages come to an end, each type of cell has its own remarkable epigenetic pattern [52-56]. Significant role of epigenetics in differentiation can be elucidated by knock downing of epigenetic enzyme effectors before induction of differentiation. After knock downing of *Hdac 1or Dnmt*s genes and withdraw of LIF from ES cells (the factors which are used rationally in ESc culture and are the main factors for self renewal of stem cells and maintenance of their stemness) normally leading to differentiation of these cells in three-germ layer cells, these cells hold their identity and cannot undergo differentiation steps nor cell death. This result is replicated by induction of stem cells with different simulators of differentiation [57-61].

DNA demethylation is applied for transdifferentiation purposes in disruption of epigenetic pattern or enhancing transdifferentiation process including transdifferentiation of fibroblast to other types of cell like adipocytes, myocytes, cardiocyte, and chondrocyte [62-64]. Heterogeneity of obtained cells has been reported as one of the most important observations of these surveys. Different types of cell will be obtained after DNA demethylation at low concentration. This issue has hindered progress related to this subject in the literature. In this essence, many researchers use this substance as an initiator of

intermediates, and promote it using other factors like growth factors in order to obtain desired cell fate. However, heterogeneity of cell fate remains an enigmatic problem due to obtaining different cell types (Table2).

Controversy between transdifferentiation and dedifferentiation

DNA somatic genome demethylation reactivated many genes (Table1). We now know that DNA methylation is the main obstacle and determining factor for repressing the genes [65]. Although we mentioned that many genes related to embryogenesis and development undergo the DNA demethylation, but the other genes play pivotal roles in cell fate, and other processes regulate by this mechanism as well. This issue may raise conflict in determination of cell's fate. DNA demethylation induced many different cell types from source cell type in agreement with this notion, which raised the question that how cell type will be determined. There can be some explanation about this subject (Figure2), one of the explanations to this controversy is that DNA demethylation dedifferentiated cells to stemness, and also lack of maintain factors differentiates these stem cells or partially reprograms stem cells to somatic cells. As a proof, withdrawing LIF or feeder layer from stem cell's culture leads to differentiation of stem cells to other somatic cells [66]. Second hypothesis in cell fate studies by DNA demethylation is that researchers use supporting proteins for establishing a specific cell fate, as this reagent can activate many genes[41,44,47], but in some cases if the DNA demethylation reactivated receptors and growth factors, it can be participated to determining cell's fate directly. Supporting proteins can give an advantage to particular cell subpopulation and also increase their population, if DNA demethylation differentiates somatic cells to wide spectral of cell types, and this is another hypothesis. The last explanation is based on demethylation induction, embryogenic activation, and organogenesis genes, which have been inhibiting role on each other and based on differences, cell fate will be determined in order of loci demethylation (which seems to be random), and their conflicts (Figure2).

Effect of demethylation agents on *in-vivo* generation of stem cells

The use of commercial name vidaza or decitabine, or names for AZA-c and 5-AZA-2-deoxycitidne, are wide spread for patients' treatment and clinical researches [67]. The main areas are cancers in which this drug can be considered. Although we do not know the exact mechanisms, many cancers have been occurred due to disruptions in epigenetics patterns, which can be erased by these agents. As a treatment for the disease, consuming this drug has a considerable effect, which is an improvement of the target tissue. The most accessible examples of this effect can be observed in leukemia and increasing number of hematopoietic stem cells [68].

Although curing the cancers, and blood cancers, especially leads to death caused by low number of blood cell counts, rather than cancer and organ malfunction [69], using this drug improves the level of hematopoietic and blood cell numbers in the majority of cases, and it can be due to induction of hematopoietic stem cells from mesenchymal cells in bone marrow. Actually, using epigenetics drugs in order to induce stem cells in *in-vivo* tests has been done [60, 70].

Cancer Stem Cells and DNA Methylation

Cancer Stem Cells (CSCs) are cancer cells that are capable of unlimited self-renewal and differentiation [71]. CSCs are one of the major theories related to development and progression of cancer. The significant role of epigenetics in initiation of CSCs rises when almost 25% of patients with Acute Myeloid Leukemia (AML) have mutation in their *DNMT3A*. These mutations occur in catalectic part of this enzyme [72]. Although many studies supported role of demethylation in CSCs, other investigations have indicated significant roles of DNA hypermethylation. This phenomenon rises from role of DNA methylation in gene expression [73-77]. It has been demonstrated that, hypomethylation of oncogenes leads to upregulation of these genes, resulting in initiation of cancer progression. Accordingly, hypermethylation of tumor suppressors could result in uncontrollable growth of cells as examples for these phenomena are found in P53, P21, and RB cancer suppressor genes locus [78-81]. Wnt and Hedgehog pathways and its related genes are significant genes influenced by this factor. These pathways have crucial roles in differentiation and formation of stem cells in embryo. It has been shown that, hypermethylation of Wnt genes pathway

such as *WIF-1* and *SFRP-1* will result in abnormal upregulation followed by progression of cancers through suppression of corresponding pathway. Hg pathway has also been reported to suffer from aberrant DNA methylation pattern. It has been elucidated that, hypomethylation of Shh promoter is linked with development of various types of cancer. Also, it has been shown that, aberrant DNA methylation and other epigenetic factors can initiate development of CSCs and EMT phenotype. In this regard, many DNA methylation modulators such as azacitidine and decitabine have been suggested for cancer elimination, which could lead to silencing of cancer-related genes and a significant decrease in the tumorigenicity of CSCs in many cases [82-84].

Conclusion

DNA demethylation studies, because of its roles in cell fate determination and diseases such as auto-immune diseases and cancers are a curial step in cellular and developmental biology. DNA methylation is a remarkable step in embryonic stem cells induction. It is a roadblock to dedifferentiation research, and a part of the natural way for stem cells inducing in embryos. Although there are many studies on the direct effect of epigenetic reprogramming on induction stem cells, previous studies have focused on its effect as an enhancer of induction. This neglect could be a direct affection of heterogeneity of reprogrammed cells, even in single study. The most reasonable answer to this questionable mixture could be induction of stem cells and future differentiation of reprogrammed stem cells. The differentiation of reprogrammed cells could be due to used medium and the disrupted transcriptome. Another raising matter due to this research is induction of totipotent stem cells which has not been detected over 50 years of research. This subject can be explained by the degree of demethylation which reaches its maximum level in blastula stage and presumably affects cells in these stages as pluripotent stem cells and trophoblast lineage. As it has been seen, this process could be complex. But advantages of this field is efficiency of induced stem cells;

dedifferentiation and first source transdifferentiation to a wide spectral of cell types by a natural way which could eliminate many obstacles in stem cells field.

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Author Disclosure Statement

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Legends

Table 1: List of embryogenesis and organogenesis reactivation genes in demethylation studies by AZA.

Table 2: cell types list, which has been activated by DNA demethylation.

Figure1: Demonstration of dynamic factors in different cleavage stages. Green line refers to the methylation amount, blue is demonstrating transcriptome of zygote, and correlation of its timing versus methylation, and red line illustrates oocyte transcriptome degradation.

Figure2 demonstrated different predicted answers to dedifferentiating and transdifferentiation controversy. First pathways are explaining that demethylation dedifferentiates with another cell line randomly. The second way demonstrates a two-stage hypothesis, which at first is somatic cells reprogram to stem cells, and then they differences with another linage due to the absence of maintaining factors. Third way shows that maybe there is an internal conflict in transcription, and the outcomes are based on their concentration and network.

Table1.

genes	function	reference
Nanog	**Key transcription factor of pluripotent stem cells and totipotent stem cells**	**41**
Pou5f1	**Key transcription factor of pluripotent stem cells and**	**41,55**

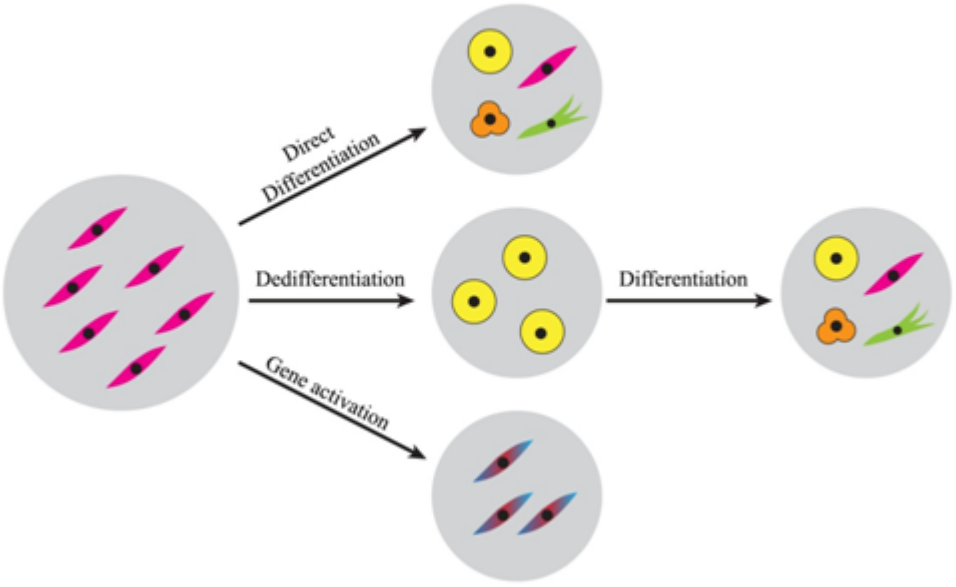
	totipotent stem cells	
Lifr	**Stem cells receptors**	**41**
Elf5	**transcription factor of trophoblast stem cells**	**44**
Cdx2	**Key transcription factor of trophoblast stem cells and totipotent stem cells**	**44**
Fgfr4	**Stem cells receptors**	**44**
Gata4	**Key transcription factor of pluripotent stem cells and totipotent stem cells**	**45**
Gata6	**Stem cells receptors**	**45**
Hox cluster	**transcription factor of hematopoietic stem cells**	**47**
C-kit	**Stem cells receptors**	**47**
Mir 146a	**A key miRNA in pluripotent network and regulation of OCT4**	**85**
F12q8	**A non coding RNA**	**63**

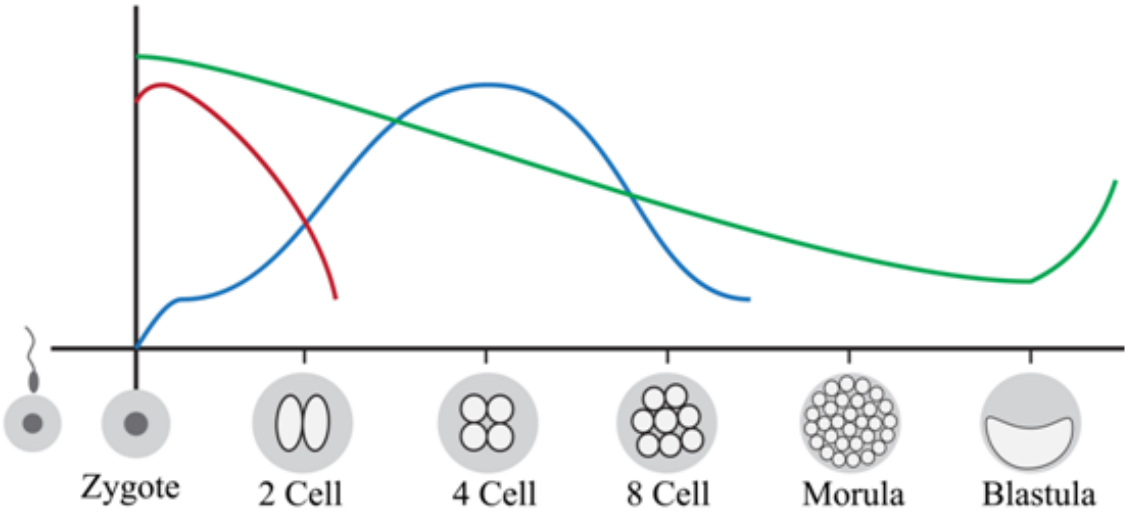
	related to pluripotent and chimer engrafting	
Dnmt3l	**A epigenetic enzyme in first step of per implantation**	**33**
hTERT	**Increasing telomeres**	**62**

Table2

First fate	Second fate	Differentiation agent	reference
fibroblast	**Pluripotent stem cell**	**AZA,AZA and TSA, AZA and LIF**	**41**
	Trophoblast stem cells(MEGA)	**AZA, AZA and FGF4**	**44**
Fibroblast and mesenchymal cell	**Hematopoietic stem cells**	**AZA, AZA and SCF**	**47**
fibroblast	**Adipocytes**	**AZA**	**65**
	Chondrocyte	**AZA**	**64,65**
	myocyte	**AZA**	**64,65**
	Cardiomyocyte	**AZA**	**66**
	Monocyte and	**AZA,AZA and SCF**	**47**

	lymphocyte





- Epigenetics factors have undeniable duties in Developmental process of the preimplantation embryo.
- These factors are determining factors in regulation of natural stem cells induction.
- Many researchers have been administrating the reprogramming of epigenetics as a stem cell induction method.
- This method might be an answer to many problematic issues in fields of dedifferentiation.