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The Epigenetics of Adult (Somatic) Stem Cells

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Abstract

While genetic studies have provided a wealth of information about health and disease, there is a growing awareness that individual characteristics are also determined by factors other than genetic sequences. These “epigenetic” changes broadly encompass the influence of the environment on gene regulation and expression and in a more narrow sense, describe the mechanisms controlling DNA methylation, histone modification and genetic imprinting. In this review, we focus on the epigenetic mechanisms that regulate adult (somatic) stem cell differentiation, beginning with the metabolic pathways and factors regulating chromatin structure and DNA methylation and the molecular biological tools that are currently available to study these processes. The role of these epigenetic mechanisms in manipulating adult stem cells is followed by a discussion of the challenges and opportunities facing this emerging field.

I. INTRODUCTION: SCOPE AND INTENT OF REVIEW

Historically, discovery activities in the biological and medical sciences have placed equal emphasis on the “nature” and “nurture” components of individual phenomenon. However, with the advent of molecular biological methods, the majority of investigations have focused almost exclusively on the genetic basis of disease and health. Although these studies provided a wealth of information, the pendulum of science has begun to swing back and there is a resurging interest in the contribution of the nurturing environment to gene regulation and expression. Recently, the term “epigenetic” has been used to broadly encompass this growing field of investigation. In a stricter sense, the scope of epigenetics is restricted to mechanisms involving DNA methylation, histone acetylation, and genetic imprinting. We have set out to review recent studies emphasizing the role of epigenetic mechanisms in regulating the development and commitment of eukaryotic stem cells. We are by no means the first (or the last) to do so. Since 2000, the number of reviews published annually on “epigenetic” and “stem cell” has increased steadily from 1 (2000) to 46 (2006). We acknowledge these excellent summaries of the field; however, we have noted that the majority focus their attention on the contribution of epigenetic

mechanisms to embryonic and cancer stem cells. In the current review, we will devote our attention to the epigenetics of somatic (adult) stem cells, which has received less attention. Emphasis is placed on the recent primary literature with speculation regarding future directions and opportunities facing this emerging field.

II. METABOLIC PATHWAYS AND MECHANISMS OF REGULATION

Cellular memory is regulated by epigenetic mechanisms that ensure heritable characteristics of cells, and functional differences between cell types, without changing DNA sequence. The epigenetic mechanisms alter chromatin (DNA and associated proteins) in ways that change the availability of genes to transcription factors required for their expression. These alterations are attributed to DNA methyltransferases, which add a methyl group to cytosine in the dinucleotide CpG and Polycomb group (PcG) and associated proteins, which modify histones.

The basic building block of chromatin is the nucleosome, which is based on an octamer of histone proteins. The octamer is formed by an H3-H4 tetramer surrounded by an H2A-H2B dimer. Histones have dual functions in the nucleus as important structural components and as regulators of gene expression. Histone tails protrude out of the nucleosome and undergo several posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, glycosylation, biotinylation, and carbonylation.¹ These modifications can exist in multiple combinations and together comprise what is being referred to as the “histone code.”² Histone modifications can distinguish euchromatin from heterochromatin and influence associations of proteins and protein complexes that regulate gene transcription or repression by altering the availability of genes to transcription factors. Several families of proteins have been identified that modify histones.

Histone acetylation is typically associated with a transcriptionally permissive state and occurs on lysines -5, -9, and -13 of histone 2A, and lysines -5, -12, -15, and -20 of Histone 2B. Histone H3 is acetylated on lysines -9, -14, -18, -23, and -24, and histone H4 is acetylated on lysines -5, -8, -12, and -127. Histone acetyl transferases (HATs) add acetyl groups to histone tails and, to date, three superfamilies have been identified, namely, GNAT (Gcn5-related N-acetyltransferase), p300/CBP and MYST (MOZ, Ybf2/Sas3, and Sas2 and TIP60).³

Histone deacetylation is catalyzed by histone deacetylases (HDACs) and is associated with a closed chromatin structure and gene repression. Acetyl groups are removed by three classes of HDACs, namely, class I (HDAC1, HDAC2, HDAC3 and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10), and class III consisting of the human sirtuin enzymes, of which there are seven (SIRT1–7). Class I HDACs have been reported to localize to the nucleus, whereas class II HDACs move between the nucleus and cytoplasm. SIRT proteins localize to various organelles depending on their function.⁴

Histone methyltransferases (HMTs) methylate histones at lysine residues and are associated with both gene activation and repression, depending on which lysine is methylated and whether it is mono-, di-, or trimethylated. These enzymes are characterized by a conserved SET [su (var)39, enhancer of zeste, trithorax] domain. Four subgroups of SET domain proteins have been identified based on homology, namely, SUV1, SUV2, SUV3, and RIZ.⁵

The complexity of the histone code is further illustrated by the effects of sumoylation and ubiquitylation on histone methylation and acetylation. Sumoylation of histones acts as a negative regulator of histone acetylation and ubiquitylation, resulting in repression of transcription.⁶ There is evidence that sumoylation also acts as a bridge between histone modification and DNA methylation. The methyl-CpG-binding protein MBD1 recognizes sites of DNA methylation and mediates chromatin structure changes leading to gene silencing.^{7,8}

Sumoylation of MBD1 modulates methylation of histone H3,⁹ providing a direct link between sumoylation, histone methylation, and DNA methylation. Histones H2A and H2B were among the first proteins found to be reversibly modified by ubiquitin.^{10,11} Monoubiquitylation of H2B is a prerequisite for methylation of histone H3¹² and is found at transcriptionally active chromatin.¹³ However, both the addition and removal of ubiquitin on H2B regulates transcriptional activation,^{14–16} suggesting that the dynamics of H2B ubiquitylation is the central feature of this modification. Ubiquitin modification of H2A leads to transcriptional repression and the E3 ligase responsible for the addition of ubiquitin to histone H2A contains proteins identified as Polycomb group proteins, which mediate gene silencing.¹⁷

Polycomb Group (PcG) proteins are a unique set of developmental regulators originally characterized in *Drosophila* as repressors of homeotic genes.^{18–23} Homeotic genes, which regulate segment identity in the developing embryo, are initially repressed by transcriptional repressors that bind DNA. PcG proteins are subsequently recruited to the site and form Polycomb repressive complexes (PRCs), which further modify chromatin to maintain gene silencing.^{22,24–26} For example, PRC2, a polycomb repressive complex required for embryonic development in mammals, catalyzes the methylation of histone H3 lysine-27 (H3K27), an activity required for PRC2-mediated gene silencing.^{27–31} EZH2, a component of PRC2, is a histone methyltransferase that carries out the methylation of lysine 27 of histone H3.^{32,33} Methylation of H3K27 recruits additional repressive complexes such as PRC1, which adds an additional level of repression that is propagated.^{27,28,34,35}

The DNA molecule can also be modified at the 5' position of cytosine rings present in CG dinucleotide sequences by addition of a methyl group.³⁶ Patterns of cytosine methylation are distinct for each cell type and confer cell type identity.³⁷ DNA methylation patterns are known to be established during development and subsequently maintained by the maintenance DNA methyltransferases. For a long time it was held that the established methylation pattern was reliably maintained for the life of the organism and was also irreversible. Feng, Y.-Q., Desprat, R., Fu, H., Olivier, E., Lin, C.M., Lobell, A., Gowda, S.N., Aladjem, M.I., Bouhasira, E.E., 2006. DNA methylation supports intrinsic epigenetic memory in mammalian cells. *PLOS Genetics* 2, 0461-0470.[2006] have suggested that methylation supports intrinsic memory in mammalian cells and is the primary mark contributing to long-term gene silencing and repression of viruses, transposons, and transgenes. Recent data, however, has suggested that DNA methylation is a reversible signal and can change in response to environmental and other signals.³⁸

DNA methylation patterns are closely linked to chromatin structure. Unmethylated DNA is typically associated with an active chromatin configuration, whereas methylated DNA is associated with inactive chromatin. The traditional view, or model, has maintained that cytosine methylation precedes chromatin structure and that DNA methylation attracts methylated cytosine binding proteins, which in turn recruit repressor complexes.^{39,40} The repressor complexes contain histone deacetylases, which further contribute to a repressive chromatin state. From this perspective, cytosine methylation is the primary epigenetic marker responsible for repressive chromatin structure. Recently, an alternative to this model has been described in which the state of or lysine 26 of histone H1, functions as part of Polycomb repressive complexes 2 and 3. The histone modifications catalyzed by EZH2 recruit chromatin determines, or influences, DNA methylation or demethylation.³⁷ Both scenarios may have implications for how DNA methylation patterns can be altered and how to approach demethylation in vitro to restore potential and improve reprogramming to a totipotent state after SCNT.

DNA methyltransferases (DNMTs) catalyze de novo and maintenance DNA methylation. These enzymes catalyze the transfer of a methyl group from the methyl donor S-adenosyl-

methionine (SAM) onto the 5' position of the cytosine ring found in CpG dinucleotides. Not all CpGs are methylated and patterns are tissue and time specific. Five enzymes have been identified, namely, DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L. DNMTs 2 and 3L lack enzymatic function due to loss of an amino-terminal regulatory domain in DNMT2 and the catalytic domain in DNMT3L.⁴¹ DNMT1 is regarded as a maintenance methyltransferase and recognizes hemimethylated DNA. DNMT1 localizes to the replication fork⁴² where it associates with PCNA (the replication processivity protein),⁴³ and association with the hemimethylated sequence has been localized to the N-terminal domain.⁴⁴ DNMTs 3a and 3b are classified as de novo methyltransferases; they bind to both hemimethylated and unmethylated CpG sites and add methyl groups to previously unmethylated cytosines. De novo methylation occurs extensively during early development and, once the methylation patterns are established, appear to be faithfully maintained for the life of the dividing cell. In contrast, if the cell were to exit the cell cycle, the methylation pattern would be maintained since demethylation is thermodynamically unfavorable.³⁶ Recent data, however, have suggested that a clear distinction between maintenance and de novo methylation does not exist and that all three methyltransferases exist in a multiprotein complex⁴⁵ and work together to maintain methylation patterns.⁴⁶ DNMT3L has been reported to participate in de novo methylation of retrotransposons⁴⁷ and establishing maternal imprints.⁴⁸

Adding complexity to this system, Vire' et al.⁴⁹ have demonstrated that EZH2, a histone methyltransferase that methylates lysine 27 of histone H3 ponents of more elaborate repressive complexes resulting in gene silencing. Interestingly, Vire et al.⁴⁹ have further shown that EZH2 interacts with DNMTs1, 3a, and 3b in vitro and in vivo, and that appropriate repression requires both EZH2 and DNMTs. Furthermore, appropriate recruitment of DNMTs absolutely requires EZH2. Moreover, reducing activity of DNMTs results in derepression of EZH2 target genes, and if EZH2 is down regulated, demethylation and derepression results. This suggests DNA methylation is dynamic and for some genes, silencing is driven by DNA binding and associated proteins.

II. TOOLS FOR STUDY

Sophisticated molecular biological methods account for many of the advances relating to epigenetics (Table 1). Until recently, the detection of methylated cytosine nucleotides relied on the use of the methylation inhibitory compound 5 aza-cytadine and methylation-sensitive restriction endonucleases. To perform these latter assays, genomic DNA was digested in parallel with enzymes that could cut a specific 4–8 bp fragment either independent or dependent on the phosphorylation status of a single CpG. Subsequent Southern blot analysis examined the digestion pattern within a single gene of interest. Limitations facing this method included its inability to evaluate all possible CpG sequences and its low throughput. Direct bisulfite sequencing is capable of examining the methylation status of all CpG elements within any gene; however, it is not an efficient high-throughput method. Genome microarrays have been developed that allow the evaluation of CpG methylation status across thousands of regulatory elements in a high-throughput manner. These tools are more widely available through commercial vendors with proven quality assurance and control.

Histone protein modification (acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation) has become a science in and of itself. There is a growing interest in the development of pharmacologic agents modulating histone acetylase and deacetylase activities. In addition to well-studied histone deacetylase inhibitors such as the trichostatin A and valproic acid, studies are now able to use new compounds such as MS-275,⁵⁰ pivaloyloxymethyl butyrate,^{51,52} and resveretrol, a polyphenolic compound found at high concentrations in functional foods. Antibody reagents are now commercially available to epitopes associated with modified histone residues. The tools have been used for immunoblots and

immunoprecipitations of total cell protein extracts. Such studies can identify histone associated proteins that change as a function of epigenetic inputs.

Antibodies to modified histone and other proteins have been used in chromatin immunoprecipitation (ChIP) assays to monitor the epigenetic modulation of gene regulatory regions. The traditional ChIP assay relies on PCR methods to detect the cross-linking of a protein of interest to a specific genomic DNA regulatory element following DNA fragmentation and antibody immunoprecipitation. Alternatively, this ChIP assay can be used in tandem with a genomic microarray (ChIP-on-chip assay) to evaluate targets in a high-throughput manner. Recently, the ChIP-on-chip method has been used productively to evaluate the repressor function of Polycomb proteins on developmental genes in embryonic stem cell. The circular-chromosome-conformation-capture (4-C) method provides a novel variation on the technology underlying the ChIP assay. Chromatin proteins are cross-linked to DNA and digested to completion with a restriction endonuclease. The isolated DNA fragments are incubated with ligase for an extended period (one week) to promote circularization of protein–cross-linked DNA fragments, followed by amplification with primers for a gene of interest. The resulting PCR fragments of varying size are then subcloned into a library, sequenced, and the novel genes identified. This approach has been used successfully to document the interchromosomal and intrachromosomal interaction of the maternally inherited H19 imprinting control region.

Additional molecular tools can be applied. Fluorescent in situ hybridization (FISH) analysis has been used to colocalize the expression of modified histone proteins to chromosomal translocation sites associated with imprinted genes or sites of X inactivation.⁵³ Multiple studies have applied global transcriptomic methods to epigenetic questions using stem cell cultures and whole animal models. There are likely to be similar studies appearing using both proteomic and metabolomic methods.

III. EPIGENETIC MECHANISMS AND MANIPULATION OF ADULT (SOMATIC) STEM

Although the role of epigenetic mechanisms regulating adult stem cell differentiation has garnered greater attention recently, the literature spans a number of decades. Studies exploring the role of DNA methylation in fibroblast cell lines (3T3, CH310T1/2)⁵⁴ paved the way for the discovery of the “master regulatory” factor MyoD^{55,56} that revolutionized the transcriptional field. To systematically address recent developments, this section will explore findings related to specific adult stem cell lineages.

A. Hematopoietic Stem Cells (HSCs)

The HSCs are the best characterized adult stem cells with respect to their epigenetic regulation, consistent with the fact that HSCs have been used clinically for over four decades. Interest in epigenetic modification of HSC differentiation can be traced to studies documenting the ability of 5' aza-cytidine⁵⁷ and butyrate to promote demethylation of fetal globin promoters. Consequently, these agents have been used to treat patients suffering from hemoglobinopathies such as beta thalassemia and sickle cell disease.^{57,58} Ex vivo, the histone deacetylase inhibitors (trichostatin A, sodium butyrate, valproic acid) have been found to reactivate fetal hemoglobin expression in HSC cultures^{59–61} The phenomenon can be potentiated by the addition of c-kit ligand in the presence of sodium butyrate.⁵⁹ The addition of granulocyte-macrophage colony stimulating factor (GM-CSF) alone likewise induced fetal hemoglobin switching, although to a lesser degree,⁵⁹ consistent with the observation that GM-CSF induces methylation of the p15 gene in an HDAC- and DNMT-dependent manner.⁶²

In vitro, HSCs require both hematopoietic cytokines and adhesion molecules, provided in vivo by bone marrow–derived mesenchymal stem cells (MSCs), to proliferate and differentiate. The addition of DNA methylation modifiers (5 aza-cytadine) or HDAC inhibitors (trichostatin A) directly to HSCs promoted their expansion in culture^{63–66} however, this has been associated with apoptosis.⁶⁷ In contrast, when these agents were added to cocultures of HSCs and MSCs together, the CD34+ HSC population was enhanced, suggesting that MSCs may provide survival factors that balance the apoptotic effects of the epigenetic agents.⁶⁷ A number of epigenetic genes have been implicated in the process of HSC renewal and differentiation. These include Hmgb3,⁶⁸ polycomb group genes,^{69,70} and Xist.⁷¹

B. Mesodermal Lineages

Taylor and Jones⁵⁴ first demonstrated that murine embryonic fibroblasts treated with 5 aza-cytadine were induced to undergo differentiation along the myocyte, adipocyte, and osteoblast pathways. Induction by 5 aza-cytadine has been used to promote the differentiation of ASCs and MSCs along the myocyte and cardiomyocyte pathways.^{72–76} Investigators have continued to pursue the role of CpG methylation in the context of adipogenesis using primary adipose-derived stem cells (ASCs) as well as MSCs.^{77–79} Analysis of the CpG methylation pattern of promoter elements in ASCs finds that while adipocyte-associated genes (lipoprotein lipase, leptin, peroxisome proliferator activated receptor gamma) were hypomethylated,⁷⁹ those of endothelial-associated genes (CD31, CD 144) remained stably methylated,⁷⁷ suggesting that ASC differentiation along the endothelial pathway is limited. Comparisons between ASC and MSC cell lines have indicated that CpG methylation regulates adipogenic events.⁷⁸

The HDAC inhibitors have been found to promote myocyte growth in vitro and in vivo. When trichostatin A was given to wild-type mice, their myocytes' size increased in a follistatin-dependent manner.⁸⁰ Similar findings have been reported in dystrophic mice, where HDAC inhibitors enhanced myofiber size and muscle function.⁸¹ This suggests a potential therapeutic role for HDAC inhibitors targeting adult stem cells or lineage-specific progenitors. In contrast, the HDAC inhibitors valproic acid and trichostatin A blocked adipogenesis in murine cell lines (3T3-L1) and primary human preadipocytes in a CAAT/enhancer-binding protein alpha–dependent manner.⁸² Whether such epigenetic-related actions are associated with chromatin organization, as suggested by studies on adipocytelated liposarcomas tumors, remains to be determined.⁸³

In general, adipogenesis and osteogenesis display an inverse relationship;⁸⁴ agents that promote differentiation along one pathway often do so at the expense of the other.⁸⁵ Consistent with this is the observation that the HDAC inhibitors valproic acid and trichostatin A promote osteogenesis by human MSCs in a bone morphogenetic protein–dependent manner in vitro.^{86,87} These findings suggest that HDAC inhibitors could enhance regeneration in bone, but further in vitro and in vivo studies will be necessary.⁸⁶ Although the mechanisms accounting for osteogenic promotion are unknown, several possibilities merit further consideration. One is the role of the polycomb genes, since their epigenetic regulation has been associated with skeleton formation in vivo.⁸⁸ A second is the role of histone arginine methylation, which has recently been identified as an epigenetic mechanism at work in murine embryos.⁸⁹ A third is the role of posttranscriptional regulators, such as microRNAs and their interactive RNA-binding proteins.^{90,91} Animals deficient in the expression of the Sam68 RNA-binding protein display a phenotype characterized by decreased bone marrow adipogenesis and protection against bone loss associated with aging.⁹¹ In vitro, fibroblasts with reduced expression of Sma68 increased their expression of the osteoblast-associated osteocalcin gene.⁹¹ These latter findings are consistent with the inverse relationship hypothesis regulating adipogenesis and osteogenesis in the bone marrow microenvironment and all merit further investigation with respect to the epigenetic regulation of adult stem cell fate.

C. Endodermal and Ectodermal Lineages

Bone marrow–derived MSCs have been reported to express markers consistent with beta-islet cell differentiation (insulin, glucagon, PDX-1) following in vitro exposure to the HDAC inhibitor trichostatin A in the presence of glucagon.⁹² Likewise, the presence of trichostatin A in combination with hepatocyte growth factor, fibroblast growth factor-4, and other agents, was found to induce hepatocyte biochemical markers in human MSCs.⁹³ Although these studies suggest that epigenetic mechanisms can be used to maximize the pluripotentiality of bone marrow–derived adult stem cells, such findings need to be reproduced and further validated using in vivo models. Others have used trichostatin A to manipulate the differentiation of liver-derived stellate cells, preventing myofibrotic biomarker expression.^{94, 95} Again, although this raises the possibility of exploiting epigenetic regulators for the treatment of fibrotic disease in the liver, in vivo studies are needed.

In the central nervous system, the histone deacetylase inhibitor valproic acid has been found to induce differentiation neural stem cells derived from the hippocampus.⁹⁶ Neuronal differentiation is accompanied by expression of the transcription factor Neuro D and inhibition of both the astrocyte and oligodendrocyte pathways.⁹⁶ Similar findings have been made with other HDAC inhibitors in vitro and in vivo, suggesting that these agents may have potential utility for the treatment of spinal muscular atrophy.^{97,98} In studies of both MSCs and ASCs in vitro, this same agent has been used in combination with other factors to induce neuronal biomarker expression.^{99,100} It is hypothesized that micro-RNAs, which modulate posttranscriptional events, may be downstream effectors of both HATs and HDACs.⁹⁰ The tissue-specific expression of these short RNAs may coordinate lineage commitment by adult stem cells in the brain and elsewhere in an epigenetic manner.⁹⁰

IV. LESSONS FROM ASSISTED REPRODUCTION: IMPACT OF CULTURE ON THE EPIGENOME OF STEM CELL-BASED THERAPEUTICS

Similar to stem cells planned for therapeutic purposes, the methods of assisted reproduction technology (ART) require culture. Embryo culture is an environment that can be regarded as less than optimal. As a result, perturbations can be introduced that may be detrimental to differentiation and development. In light of growing concerns about epigenetic disturbances associated with ART, there is clearly a need to understand adult stem cell epigenetic events and the impact of culture on these processes. What follows is a brief review of observed consequences associated with embryo culture.

A. Methylation Reprogramming Defects

In mouse, the maternal and paternal genomes are demethylated after fertilization by different mechanisms. Drastic differences in methylation reprogramming and development have been observed in embryos fertilized in vitro and cultured. These differences are likely due to an environment (e.g., culture) that is suboptimal that impacts epigenetic reprogramming. It has been demonstrated in mice and ruminants that isolation, manipulation, and culture of gametes and early embryos can disrupt methylation, resulting in phenotypic defects.^{101,102} Disruptions in establishing and maintaining appropriate methylation patterns may also contribute to issues associated with assisted reproductive technology (ART). Children conceived with ART have been reported to have unusually high incidences of rare imprinting-related diseases, including Beckwith-Wiedemann and Angelman syndromes. These diseases are associated with abnormal hypomethylation of typically methylated maternal alleles.^{103,104}

Similarly, somatic cell nuclear transfer (SCNT, or cloning) disrupts epigenetic reprogramming events and the consequences are more frequent and amplified. Methylcytosine labeling of cloned bovine embryos has demonstrated incomplete or delayed demethylation of the donor

genome,^{48,105} likely resulting in a mixture of normally and abnormally methylated sequences. Several reports have described consequences of disrupted epigenetic reprogramming associated with SCNT including aberrant gene expression in preimplantation embryos,¹⁰⁶ placentas, and livers of neonatal cloned mice,¹⁰⁷ improper reactivation of genes required for cell lineage differentiation,¹⁰⁸ and aberrant expression of imprinted genes in cloned mouse blastocysts.¹⁰⁹ Thus, the developmental failures and longer-term defects associated with cloning are associated with improper epigenetic reprogramming. The contribution of culture conditions to aberrant reprogramming in clones has not been fully assessed.

B. Embryo Culture Results in Shifts in Energy Metabolism

In vivo, the early cleavage-stage, relatively metabolically quiescent embryo uses pyruvate/lactate, but not glucose, as a sole energy source. In contrast, blastocysts are highly metabolically active and use glucose as a sole energy source.¹¹⁰ Culture results in a stress response that is manifested by changes in gene expression (e.g., expression of heat-shock genes), apoptosis, and metabolism. For example, mouse blastocysts that develop in vivo convert 40–50% of the glucose to lactate, whereas blastocysts that develop in vitro from the morula stage convert ~100% of the glucose to lactate. Early cleavage-stage embryos also exhibit an increase in glycolysis in response to culture that is associated with reduced implantation and development following embryo transfer. Inclusion of amino acids in the medium prevents this metabolic change and restores the embryo's ability to implant and develop following transfer.¹¹¹

The changes in metabolism that occur as a response to culture conditions may be coupled either directly or indirectly to changes in gene expression. Both Whitten's medium and KSOM support development of one-cell mouse embryos to the blastocyst stage. The major difference between these media is that KSOM has a reduced sodium chloride concentration relative to that in Whitten's medium. When comparing gene expression in cultured embryos to in vivo–derived embryos, it has been shown that the level of expression of transcripts encoding several growth factors and their receptors is significantly reduced in embryos cultured in Whitten's medium, whereas no significant differences were observed when the embryos were cultured in KSOM that includes amino acids.¹¹² Of greater interest is that although culture of embryos to the blastocyst stage in KSOM maintains the appropriate maternal monoallelic expression of the imprinted *H19* gene, *H19* expression is essentially biallelic following culture in Whitten's medium.¹¹³ Moreover, the loss of imprinting was associated with the loss of methylation on the paternal, repressed allele in the differentially methylated region (DMR);¹¹³ DNA methylation of the paternal allele in this region is strongly linked with the repression of the paternal *H19* allele.¹¹⁴

The loss of imprinting that occurs during culture under certain conditions, as well as global changes in gene expression, could have long-term consequences on development. In fact, a growing body of evidence documents that culture of bovine and ovine embryos to the blastocyst stage prior to embryo transfer results in higher incidences of fetal and perinatal loss.^{115–117} In the bovine, these abnormalities are not observed if the embryos are first transferred to the oviducts of sheep and then transferred at the blastocyst stage to the reproductive tracts of recipient heifers.¹¹⁵ Thus, the abnormalities are likely attributable to embryo culture. Interestingly, large offspring derived following culture of sheep preimplantation embryos exhibit lower levels of expression of maternally expressed imprinted *Igf2r* gene.¹⁰² This demethylation provides a molecular basis for the reduced *Igf2r* expression, since, in contrast to other imprinted genes, methylation of the *Igf2r* gene is associated with expression via a complicated mechanism that entails expression of an antisense transcript.^{118,119} It should be noted that in mice, reduced *Igf2r* expression also leads to increased size.¹²⁰ Thus, embryo culture can result in perturbations that manifest in the offspring and it is likely that epigenetic changes underlie long-term effects. Steele et al.¹²¹ have recently surveyed components of

common embryo culture media and have learned that several components, including those related to methyl metabolism, are present in nonphysiological concentrations. For example, vitamin B12 was either absent or present in up to 3000-fold to 5000-fold and vitamin B6 is present up to 160-fold to 500-fold the concentration reported in maternal serum or human follicular fluid. Similarly, folate and methionine were either absent or present in supraphysiological concentrations. Based on what is being realized with respect to ART, it is conceivable that suboptimal culture conditions, such as nonphysiological levels of methyl cycle-related components in culture media, could introduce novel epigenetic perturbations in adult stem cells during processes of culture and propagation.

V. POSSIBLE IN VITRO STRATEGIES TO MINIMIZE SPONTANEOUS DIFFERENTIATION IN CULTURE

A. DNA Methyltransferase Inhibitors

The classic DNA methyltransferase inhibitor is 5-azacytidine, a derivative of the nucleoside cytidine. The inhibitor was discovered more than 40 years ago¹²² and its demethylating activity was discovered subsequently due to its ability to influence cellular differentiation in vitro.¹²³ 5-azacytidine is a nucleoside inhibitor that can be incorporated into DNA and can be methylated by DNA methyltransferases. The DNA methyltransferase, however, becomes covalently trapped because the intermediate cannot be resolved, inactivating the enzyme. As the enzyme is depleted, genomic DNA is demethylated as a result of continued DNA replication. To become incorporated into DNA, 5-azacytidine must be modified to a deoxyribonucleoside triphosphate prior to incorporation into DNA. Prior to modification, 5-azacytidine can become incorporated into RNA, resulting in a variety of consequences including cytotoxicity. An analogue of 5-azacytidine, 5-aza-2'deoxyctidine (decitabine), does not require modification and is thought to be less cytotoxic, but still has severe cytotoxic effects.¹²⁴ For example, decitabine has been used in clinical trials and has shown promise for treatment of myeloid malignancies,^{125,126} but also has toxic effects including myelosuppression and neutropenic fever.¹²⁴ At least three reports have investigated the treatment of donor cells with 5-aza-2'deoxyctidine prior to nuclear transfer.^{127–129} Treatment at high levels (0.04 μ M or greater) resulted in demethylation to levels of in vitro embryos, yet blastocyst development rates were reduced or unchanged compared with controls. It is unclear whether the decrease in blastocyst development rates were due to cytotoxicity or demethylation of the genome.

Several nonnucleoside compounds also inhibit DNA methyltransferase activity, none of which have been examined in nuclear transfer models. Two compounds have been reasonably characterized, whereas compounds representing three subclasses are less understood. The two characterized compounds include (–)-epigallocatechin-3-gallate (EGCG), the key polyphenol in green tea,¹³⁰ and RG108, a molecule identified in an in silico screening assay.¹³¹ EGCG inhibits DNA methyltransferase activity in protein extracts and human cancer cell lines,¹³² and is thought to block the active site of DNMT1. Degradation of EGCG results in production of hydrogen peroxide, a strong oxidizing agent¹³³ that may result in cytotoxicity. In contrast, RG108 appears to have low toxicity and has been demonstrated to inhibit the catalytic activity of recombinant DNA methyltransferases. The inhibitory activity appears to be direct and specific for DNA methyltransferases.¹³¹ The additional classes of nonnucleoside inhibitors include the following: (i) 4-aminobenzoic acid derivatives, exemplified by Procaine,^{134,135} which is thought to bind to CpG-rich sequences, preventing access of DNMT to DNA, but high concentrations are thought to be required for effectiveness based on studies in cancer cell lines;¹³⁶ (ii) The psammaplins, which inhibit DNMT as well as histone deacetylase activity; and (iii) oligonucleotides.

B. Limiting the Methyl Group Supply

The availability of nutrients appears to play an important role in regulating DNA methylation. A growing body of evidence suggests environmental factors such as diet can influence DNA methylation. For example, prenatal feeding of a methyl-supplemented diet can increase the level of DNA methylation and phenotypic expression of genes in offspring. The coat color in mice is determined by agouti gene expression. This is determined by the DNA methylation status of the long terminal repeat of the agouti gene in the hair follicle. If this region is hypermethylated, the mouse is agouti in color whereas if the region is hypomethylated the mouse is yellow. When pregnant female mice were fed a methyl-supplemented diet enriched in zinc, methionine, betaine, choline, folate, and vitamin B12, there was an alteration in the methylation status of the agouti long terminal repeat and none of the pups had a yellow coat.¹³⁷ Thus, in utero exposure to nutrients can lead to epigenetic modifications of the genome in the offspring.

Folate, a water-soluble B vitamin, plays a significant role in DNA methylation status. The biochemical function of folate is to mediate the transfer of one-carbon moieties^{138–140} and thus has a central role in one-carbon metabolism. Animal studies have further shown that folate deficiency causes genomic and gene-specific hypomethylation in rat liver, and the degree appears to depend on the severity and duration of folate depletion.^{141,142} DNA hypomethylation also has been identified in lymphocytes of humans on low dietary folate and can be reversed by folate repletion.¹⁴³

Factors involved in one-carbon metabolism also likely play an important role in methylation status because they influence the supply of methyl groups and, therefore, the biochemical pathways of methylation processes. Methyl groups supplied from the “diet” (or culture environment) in the form of choline and methionine, or from the folate-dependent one-carbon pool, must be activated to S-adenosylmethionine (SAM) to serve as substrates in transmethylation reactions. Because S-adenosylhomocysteine (SAH) is a product of transmethylation reactions and a potent inhibitor of SAM-dependent methyltransferases, the ratio of SAM/SAH is an important index of transmethylation potential.^{144,145} When the ratio is high, methylation potential is high; when the ratio is low, methylation potential is low. It is well known that folate depletion alone is a sufficient perturbing force to diminish SAM pools. This leads to an increase in cellular levels of SAH because the equilibrium of the SAH-homocysteine interconversion actually favors SAH synthesis. Therefore, when homocysteine metabolism is inhibited (as in folate deficiency), cellular SAH levels will be increased. Increased SAH inhibits methyltransferase activity and consequently DNA methylation reactions.

The cytosolic enzyme glycine-N-methyltransferase (GNMT) functions to optimize transmethylation reactions by regulating the SAM:SAH ratio. When methyl groups are abundant and SAM levels are elevated, GNMT disposes of the excess methyl groups by forming sarcosine from glycine. SAM also reduces the supply of methyl groups originating from the one-carbon pool by inhibiting 5,10-methylenetetrahydrofolate reductase (MTHFR),^{146,147} the enzyme responsible for the synthesis of 5-methyltetrahydrofolate (5-methyl-THF), the folate coenzyme that donates its methyl group to homocysteine to form methionine. Because 5-methyl-THF also binds to GNMT and inhibits its activity,^{148,149} a decrease in 5-methyl-THF levels due to inhibition of MTHFR by SAM results in an increase in the activity of GNMT. Factors that activate GNMT lead to down regulation of methyltransferases, including DNA methyltransferases. To date, there have been no reports testing the hypothesis that activation of GNMT in cells in culture result in reduced DNA methylation; however, a few reports have described dietary manipulation of GNMT in rodents.^{150–152} For example, diets supplemented with vitamin A and its derivatives (e.g., all-trans retinoic acid, ATRA) result in increased GNMT activity and hypomethylated DNA in rat hepatocytes, while

decreases in dietary folate, choline, betaine, and vitamins B6 and B12 also result in decreased DNA methylation in specific tissues. Thus, alterations in the availability of single-carbon metabolism components (e.g., methyl donors) and adjusting the nutrient availability of methyl donors represent possible in vitro approaches to demethylate DNA and restore cell differentiation potential.

VI. FUTURE DIRECTIONS

Much remains to be done in the field of adult stem cell epigenetics despite the fact that the epigenetics of embryonic and cancer stem cells have received greater attention to date. Indeed, it is likely that adult stem cells exhibit unique epigenetic features. The following questions merit further investigation:

1. Can culture conditions be used to direct or reprogram the epigenome in adult stem cells to improve their utility for tissue regeneration and repair?
2. What other epigenetic targets, in addition to histones, chromatin proteins, and CpG islands, should be evaluated in adult stem cells?
3. Can diet be used to positively modify the epigenome of adult stem cells in the living organism? If so, can this approach be used effectively as a preventive medical therapy?
4. What elements of the adult stem cell epigenome are reversible? Can exposure to an environmental toxin, such as cigarette smoke, be reversed in adult stem cells prior to transformation into cancer stem cells? If so, how?

These are just some of the more obvious directions for research into the epigenetics of adult stem cells. New technologies and discoveries will point investigators to further, more probing questions in the future.

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TABLE 1
Epigenetic Methods

DNA methylation—bisulfite sequencing, restriction enzyme isoforms, genome microarrays
Histone acetylation/methylation/phosphorylation/ubiquitylation/sumoylation immunoblot, immunoprecipitation, pharmacologic agents, targeted gene expression
Chromatin conformation—chromatin immunoprecipitation (ChIP) assay, DNase hypersensitivity assay, in vivo footprinting
Circular chromosome conformation capture (4-C)
Immunohistochemical—FISH
Global expression—transcriptomics, proteomics, metabolomics