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DNA methylation restricts spontaneous multi-lineage differentiation of mesenchymal progenitor cells, but is stable during growth factor-induced terminal differentiation

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ABSTRACT

The progressive restriction of differentiation potential from pluripotent embryonic stem cells, via multipotent progenitor cells to terminally differentiated, mature somatic cells, involves step-wise changes in transcription patterns that are tightly controlled by the coordinated action of key transcription factors and changes in epigenetic modifications. While previous studies have demonstrated tissue-specific differences in DNA methylation patterns that might function in lineage restriction, it is unclear at what exact developmental stage these differences arise. Here, we have studied whether terminal, multi-lineage differentiation of C2C12 myoblasts is accompanied by lineage-specific changes in DNA methylation patterns. Using bisulfite sequencing and genome-wide methylated DNA- and chromatin immunoprecipitation-on-chip techniques we show that in these cells, in general, myogenic genes are enriched for RNA polymerase II and hypomethylated, whereas osteogenic genes show lower polymerase occupancy and are hypermethylated. Removal of DNA methylation marks by 5-azacytidine (5AC) treatment alters the myogenic lineage commitment of these cells and induces spontaneous osteogenic and adipogenic differentiation. This is accompanied by upregulation of key lineage-specific transcription factors. We subsequently analyzed genome-wide changes in DNA methylation and polymerase II occupancy during BMP2-induced osteogenesis. Our data indicate that BMP2 is able to induce the transcriptional program underlying osteogenesis without changing the methylation status of the genome. We conclude that DNA methylation primes C2C12 cells for myogenesis and prevents spontaneous osteogenesis, but still permits induction of the osteogenic transcriptional program upon BMP2 stimulation. Based on these results, we propose that cell type-specific DNA methylation patterns are established prior to terminal differentiation of adult progenitor cells.

1. Introduction

The generation of distinct populations of specialized cells from a single embryonic stem cell (ESC) is characterized by a progressive restriction of differentiation potential. ESCs are pluripotent and first differentiate into a variety of multipotent adult stem/progenitor cells with a differentiation potential that is limited to specific cell types. Subsequent lineage commitment gives rise to transit amplifying cells that undergo a series of cell divisions, thereby stably maintaining their lineage characteristics, before terminal differentiation into a specialized cell takes place. These processes involve a tightly controlled, coordinated activation and repression of specific subsets of genes, which depend on the orchestrated action of key regulatory transcription factors, in combination with changes in epigenetic marks such as DNA methylation, histone modifications and chromatin remodeling [1,2]. These epigenetic marks regulate which regions in the genome are accessible for transcription and it has been hypothesized that they thereby contribute to lineage restriction, either by switching off multipotency-associated genes, or by repressing genes specific to other lineages [3].

Methylation of the 5′-position of cytosine in a CpG dinucleotide is a well-characterized epigenetic modification, which is passed on to daughter cells through so-called maintenance DNA methyltransferase (Dnmt) activity upon cell division [4]. This epigenetic mark was originally considered to mediate stable gene silencing [4], but it has

Abbreviations: 5A(d)C, 5-aza(deoxy)cytidine; BMP, bone morphogenetic protein; ChIP, chromatin immunoprecipitation; Dnmt, DNA methyltransferase; ESC, embryonic stem cell; GM, growth medium; MeDIP, methylated DNA immunoprecipitation; MSC, mesenchymal stem cell; Pol-II, RNA polymerase II

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recently been shown that the effect of promoter DNA methylation on gene expression strongly depends on its CpG density [5]. DNA methylation is essential for embryonic development [6,7] and mediates processes such as X chromosome inactivation [8], genomic imprinting [9] and silencing of parasitic elements [10].

The involvement of DNA methylation in restriction of developmental potential has been the focus of recent studies in which high-throughput strategies have been employed to generate and compare DNA methylation profiles of pluripotent ESCs, adult stem/progenitor cells and/or differentiated somatic cells. First of all, these studies have shown that pluripotency- and germ line-specific genes are hypermethylated in progenitor and differentiated somatic cells, while these are hypomethylated in ESCs, suggesting a role for DNA methylation in stable repression of genes required for maintenance of the unrestricted developmental potential of ESCs [5,11–13].

In addition, various of these studies, as well as several single-gene analyses, have identified regions that are differentially methylated in distinct cell types and might be associated with lineage-specific gene expression, suggesting that DNA methylation might also participate in restriction of the differentiation potential of progenitor cells [13–25]. A role for DNA methylation in lineage restriction is further supported by the profound effects of treatment with the DNA methylation inhibitor 5-aza(deoxy)cytidine (5A(d)C) on cellular phenotype [22,26,27]. For example, it has been shown that treatment of C3H10T1/2 fibroblasts with 5AC induces differentiation towards myogenic, adipogenic and chondrogenic lineages, suggesting that DNA demethylation reverts these cells to a less restricted state, from which new phenotypes can subsequently differentiate in the absence of external stimuli [27].

The aforementioned studies have shown that pluripotent ESCs show lower levels of promoter methylation than specialized somatic cells. However, it remains unclear at which stages during cellular development the observed potency- and cell type-related differences in DNA methylation patterns are formed. Studies on neuronal differentiation have indicated that methylation contributes to the conversion of ESCs to adult neuroprogenitors, but not to the subsequent terminal differentiation [13]. Studies addressing this issue for cells from other germ layers are, however, still limited [28–32]. Here, we have addressed late stage development of progenitor cells of mesodermal origin. To this end, we took advantage of the robust and homogeneous differentiation characteristics of the mouse C2C12 myoblast cell line as a model system to study changes in DNA methylation upon terminal differentiation into either bone or muscle cells. C2C12 cells were originally derived from regenerating muscle tissue [33] and are considered to represent the transit amplifying progenitor population that is derived from muscle satellite stem cells [34]. When cultured routinely, C2C12 cells terminally differentiate and fuse into multinucleated myotubes upon reaching confluence, which is preceded by upregulation of the key myogenic transcription factors *Myod1* and *Myog*. However, treatment of C2C12 cells with bone morphogenetic protein (BMP) 2 induces these cells to differentiate into osteoblasts, which involves the upregulation of key osteogenic transcription factors *Dlx5*, *Sp7* and *Runx2* [35–37], subsequently leading to the expression of late osteoblast marker genes, such as *Alpl* and *Bglap* [38,39].

We have previously observed differential expression of Dnmts during BMP2-induced osteogenic differentiation of C2C12 cells, suggesting remodeling of DNA methylation marks [38]. In the present study we have used a genome-wide parallel MeDIP (methylated DNA immunoprecipitation)- and Pol-II (RNA polymerase II) ChIP (chromatin IP)-on-chip approach, together with single-gene bisulfite sequencing analyses, to investigate whether lineage-specific changes in DNA methylation patterns underlie terminal, multi-lineage differentiation of C2C12 progenitor cells.

2. Materials and methods

2.1. Cell culture

Murine C2C12 myoblasts (American Type Culture Collection) were maintained at sub-confluent densities in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% newborn calf serum (NCS; Thermo Fisher Scientific, Waltham, MA), antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin: Sigma-Aldrich, St. Louis, MO), and 2 mM L-glutamine (Invitrogen), further designated as growth medium (GM), at 37 °C in a humidified atmosphere containing 7.5% CO_2. To study the effect of 5AC on differentiation, cells were plated at 1.5×10^3 cells per cm^2 in GM, treated with or without 10 μM 5AC (Sigma-Aldrich) in GM for 10 days and subsequently maintained on GM. Medium was replaced every 24 h for the first 4 days and every 3–4 days during the remaining culture period. For growth factor-induced differentiation studies, cells were plated at 2.5×10^4 cells per cm^2 in GM and grown for 24 h to sub-confluence. Subsequently, medium was replaced by DMEM containing 5% NCS in the presence or absence of 300 ng/ml recombinant human bone morphogenetic protein 2 (BMP2; R&D Systems, Minneapolis, MN). Medium was replaced every 3–4 days.

2.2. Characterization of cellular phenotypes

To study osteogenic differentiation, histochemical analysis of alkaline phosphatase (Alpl) activity was performed as described elsewhere [40]. Adipogenic differentiation was characterized by Oil Red O staining as described previously [41].

2.3. RNA isolation and real-time polymerase chain reaction (PCR)

RNA extraction, reverse transcription and real-time PCR were performed as described previously [42]. Primer sequences are presented in Table 1. Gene expression levels are expressed relative to the housekeeping gene *Rpl19*.

2.4. Bisulfite sequencing

Genomic DNA was isolated using the Wizard® genomic DNA purification kit (Promega, Madison, WI). A total of 700 ng of genomic DNA was converted with the EZ DNA methylation-gold kit (Zymo Research, Orange, CA) and amplified by touchdown PCR with primer sets designed using MethPrimer software [43]. Primer sequences are presented in Table 2. PCR mixtures contained 1× PCR buffer, 1× Q-solution, 1.5 mM $MgCl_2$, 1 unit Taq DNA polymerase (all from Qiagen, Valencia, CA), 0.4 mM of each dNTP (Fermentas, Burlington,

Table 1
Real-time PCR primer sequences.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
Acadl	GGACTTGCTCTCAACAGCAGTTAC	AGGGCCTGTGCAATTGGA
Alpl	GACTCGCCAACCCTTCACTG	CACCCCGCTATTCCAAACAG
Bglap	CCCTGAGTCTGACAAAGCC	CTGTGACATCCATACTTGCAG
Dlx5	CAGAACGCGCGGAGTTG	CCAGATTTTCACCTGTGTTTGC
Fabp4	GCGTGGAATTCGATGAAATCA	GGGCCCCGCCATCTAG
Itga6	TTCCTACCCCGACCTTGCT	GGCCGGGATCTGAAAATAGTG
Lpl	GCTGGCGTAGCAGGAAGTCT	CCAGCTGGATCCAAACCAGTA
Myod1	CGACACAGAACAGGGAACCC	GGCCACTCAAGGATCAGCTC
Myog	CCAGGAGATCATTTGCTCGC	GCACTCATGTCTCTCAAACGG
Pdia2	GAGCATTCAGCCCTGATGGT	CTCGGGAGCTAGTTCTTTGCA
Rassf3	GCCGTTACAGACAAGCTGAAGA	TGCACCTTAATGAAGCCAGTGT
Rpl19	CCAATGAAATCGCCAATGC	CCCATCCTTGATCAGCTTCCT
Sp7	TGCTCCGACCTCCTCAACTT	GGCCAGATGGAAGCTGTGA
Tnnc2	CGAGGATGGCAGCGGTACTA	CCTTCGCATCCTCTTTCATCTG
Usp15	CCAGATGGGAGATCAAAATGTCT	CGTCGCCATCTTTGAGAAGTC