Original Article

DNA methylation patterns of imprinting centers for *H19*, *SNRPN*, and K*CNQ1OT1* in single-cell clones of human amniotic fluid mesenchymal stem cell

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Abstract

Objective: To test the hypothesis that human amniotic fluid mesenchymal stem cells contain a unique epigenetic signature in imprinting centers of *H19*, *SNRPN*, and K*CNQ1OT1* during *in vitro* cell culture.
Materials and Methods: By bisulfite genomic sequencing, we analyzed the imprinting centers of three imprinted genes (including *H19*, *SNRPN*, and *KCNQ1OT1*) in a total of six single-cell clones of human amniotic fluid mesenchymal stem cells at cell passages 7, 8, 9, and 10 during *in vitro* cell culture.
Results: The imprinting centers of H19 and KCNQ1OT1 showed hypermethylation at passage 7 in all single-cell clones of human amniotic fluid mesenchymal stem cells, and there was no significant change in DNA methylation patterns during in vitro cell culture. The imprinting centers of *SNRPN* showed variable methylation patterns at passage 7 in six single-cell clones, and DNA methylation patterns varied during in vitro cell culture from passages 8 to 10.
Conclusion: In conclusion, human amniotic fluid mesenchymal stem cells contain a unique epigenetic signature during *in vitro* cell culture. *H19* and *KCNQ1OT1* possessed a substantial degree of hypermethylation status, and variable DNA methylation patterns of *SNRPN* was observed during *in vitro* cell culture of human amniotic fluid mesenchymal stem cells. Our results urge further understanding of epigenetic status of human amniotic fluid mesenchymal stem cells before it is applied in cell replacement therapy.
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Keywords: amniotic fluid mesenchymal stem cell; DNA methylation; human imprinting gene; single-cell clones

Introduction

Mesenchymal stem cells with the potential to differentiate to adipocytes, chondrocytes, and osteocytes have been isolated from human amniotic fluid [1–4]. Amniotic fluid mesenchymal stem cells (AFMSCs) could be successfully induced to differentiate into various tissue types, such as skin, cartilage, cardiac tissue, nerve, muscle, bone, kidney, and endothelium [4–8]. As AFMSCs are able to differentiate into lineages representative of all three germ layers but do not form tumors when injected *in vivo* [4,9] and are less likely to raise ethical concerns of their generation, AFMSCs have many potential applications in regenerative medicine [4–6,8,10]. Tissue engineered products from mesenchymal amniocytes had been used for diaphragm reconstruction in lambs [11], trachea reconstruction in lambs [12], and sternum repair in rabbits [13].

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Recently, the potential applications of AFMSCs for cell-based therapy had also been demonstrated in a sheep model by using autologous stem cell transplantation in a sheep fetus [14]. These autologous cells derived from AFMSCs showed widespread organ migration in fetal tissues including liver, heart, placenta, membrane, umbilical cord, adrenal gland, and muscle. Although not proven in humans, the autologous human AFMSCs may offer an alternative means to ameliorate prenatal congenital disease [8] and could make it possible to have an autologous cell source for adult diseases, such as heart valve diseases [15]. However, considering the heterogeneity of the cells found in amniotic fluid, we have further isolated and characterized single-cell clones from amniotic fluid specimens. The clonal amniotic fluid-derived stem cells can express characteristics of both mesenchymal and neural stem cells [2].

A central assumption of Mendel's laws of inheritance is that genes originating from maternal and paternal genomes are equally expressed in the offspring; however, there are some exceptions [16]. In some disorders, such as Beckwith−Wiedemann syndrome (BWS), Prader−Willi syndrome, and Angelman syndrome (AS), the sex of the transmitting parent plays a role in the expression of the phenotype in his or her affected children. This led to the discovery of parental imprinted genes: a gene expressed only from the paternally inherited chromosome is maternally imprinted (the maternal allele is inactivated); similarly, a gene expressed only from the maternally inherited chromosome is paternally imprinted (the paternal allele is inactivated).

Progenies of assisted reproductive technologies are reported to be at higher risk for an altered imprinting status, leading to BWS [17,18] and AS [19,20]. In BWS, methylation abnormalities are found in two differentially methylated regions (DMR) that are located in 11q15: DMR1 of *H19* and DMR2 of *KCNQ10T1* [20]. Defect of the *SNRPN* imprinting control center/DMR in chromosome 15q11.2 is part of the pathogenesis of AS.

Disturbed expression of particular imprinted genes has been linked to fetal growth and development abnormalities as well as other human diseases [21]. Previous studies on sheep [22] and mouse embryos [23,24], and mouse stem cells [25] have shown that they are prone to epigenetic variation and lose normal imprinted gene expression when cultured *in vitro*. It has been reported that the DNA methylation patterns of human embryonic stem cells (hESCs) are distinct from those of other cells [26]. Importantly, abnormal CpG island methylation occurs during *in vitro* differentiation of hES. The more passages embryonic stem cells experienced, the more they appeared to differ from other cells of the same line that underwent fewer passages [27].

Given the observations between assisted reproductive technologies and human epigenetic disorders, we hypothesized that human AFMSCs might have unique epigenetic characters during *in vitro* cell culture. To fill the current knowledge gap on AFMSCs, herein we study the epigenetic characteristics of CpG islands of three imprinted genes (*H19*, *SNRPN*, and *KCNQ1OT1*) on human AFMSC, especially focusing on the differences among various single-cell clones and the effect of *in vitro* cell culture on epigenetic changes of imprinted genes on human AFMSCs. A better understanding of the epigenetic characteristics of AFMSCs and their epigenetic changes during *in vitro* cell culture will give us more information for future applications of these AFMSCs in disease therapy and tissue engineering.

Material and methods

Patient enrollment

We recruited patients who were given prenatal care and planning to deliver at the Department of Obstetrics and Gynecology, Chang Gung Memorial Hospital (CGMH), Taiwan. This study was approved by the Institute Review Board of CGMH (IRB #97-1341A3). Patients were enrolled only after written informed consents were obtained.

Specimen collection

Two amniotic fluid samples (AF20451744 and AF21251150) were obtained after amniocentesis in CGMH [3]. Amniocentesis for medical reasons was performed between 16 and 20 weeks of pregnancy, and a total of 20 mL of amniotic fluid was aspirated from the amniotic sac transabdominally under sono-guide. The fetal cells were cultured in Chang medium, and the adhering cells were used for chromosomal studies. The nonadhering cells suspended in Chang medium for the first 3 days were collected for stem cells isolation and culture [1].

Culture of single-cell clones of AFMSCs

AFMSCs were cultured in alpha-modified minimum essential medium (Hyclone, Logan, UT) supplemented with 20% fetal bovine serum (Hyclone) and 4 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN) and incubated at $37^{\circ}C$ in a humidified atmosphere with 5% CO_2. Isolation of single-cell clones of AFMSCs was performed using cloning cylinders (Sigma, St. Louis, MO) on cell colonies at the first 2 weeks of growth on plates. These single-cell clones of AFMSCs were successively expanded and confirmed to have mesenchymal stem cell surface antigen markers by flow cytometry and differentiation abilities into osteoblasts, chondrocytes, and adipocytes, as in our previous report [28]. In this study, AFMSCs were further characterized for the pluripotent markers of Oct4 (R&D Systems) and Nanog (R&D Systems) by flow cytometry.

Chromosome study of two amniotic fluid samples

The karyotypes of two amniotic fluid samples were analyzed using a standard G-banding technique.

Single-cell clones of AFMSC DNA extraction and bisulfite treatment of DNA

Genomic DNA from human AFMSC was extracted using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA)

according to the manufacturer's recommendations. The quality and quantity of DNA were analyzed by Nanodrop. Aliquots of 1 μg of DNA from AFMSCs underwent bisulfite conversion by EpiTect Bisulfite Kit (Qiagen).

Bisulfite PCR and sequencing

The eluted DNA samples (after bisulfite conversion) were amplified using polymerase chain reaction (PCR) primers specific for the DMRs of the imprinted genes being studied (Fig. 1), including *H19*, *SNRPN*, and *KCNQ1OT1*, as previously described [16]. The primers used for bisulfite sequencing are as follows:

*H19*_OuterF TTTTTGGTAGGTATAGAGTT
*H19*_OuterR AAACCATAACACTAAAACCC
*H19*_NestedF
TGTATAGTATATGGGTATTTTTGGAGGTTT
*H19*_NestedR
TCCTATAAATATCCTATTCCCAAATAACC
*SNRPN*_OuterF
GGTTTTTTTTTATTGTAATAGTGTTGTGGGG
*SNRPN*_OuterR
CTCCAAAACAAAAAACTTTAAAACCCAAATTC
*SNRPN*_NestedF
GGTTTTAGGGGTTTAGTAGTTTTTTTTTTTTGG
*SNRPN*_NestedR
CAATACTCCAAATCCTAAAAACTTAAAATATC
*KCNQ1OT1*_F TGTTGAGGAGTTTYGGGGAGGATTA
*KCNQ1OT1*_R CACCTCACACCCAACCAATACCTCAT

TOPO cloning of bisulfite PCR product and DNA sequencing

PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into competent cells. Individual clones from at least two independent reactions were selected for sequencing. The DNA sequence results were then analyzed for the methylation status of the CpG dinucleotide [16].

Analysis of different passages of AFMSC

We studied and tracked cells in four different passages: passages 7, 8, 9, and 10.

Results

Chromosome study of two amniotic fluid samples

The karyotypes of both amniotic fluid samples were normal female karyotypes (46,XX).

Culture and characterization of single-cell clones of AFMSCs

Six single-cell clones of AFMSCs from two amniotic fluid specimens were established. Three clones were from sample AF20451744 and three clones were from sample AF21251150. Besides the MSC surface markers that were
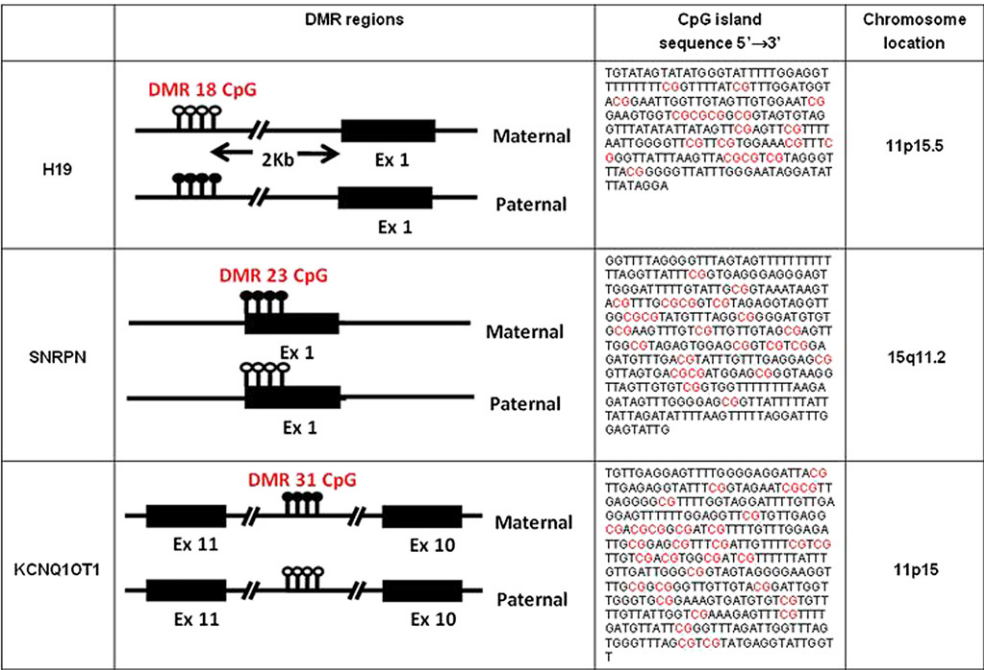

Fig. 1. Differentially methylated regions of *H19*, *SNRPN*, and *KCNQ1OT1*. DNA sequences of differentially methylated regions (DMRs) and CpG islands of the studied imprinted genes: *H19*, *SNRPN*, and *KCNQ1OT1* are shown. *H19* has 18 CpG sites in the DMR region, *SNRPN* has 23 CpG sites in the DMR region, and *KCNQ1OT1* has 31 CpG sites in the DMR region. The CpG sites are labeled in red.

expressed (data not shown), the six single-cell clones of human AFMSCs were also strongly positive for Oct4 and Nanog pluripotent markers (Fig. 2). The osteogenetic differentiation capacity was identified by von Kossa staining of all six single-cell clones of human AFMSCs (Fig. 3).

Epigenetic characteristics of CpG islands of imprinted gene H19 on six single-cell clones derived from human AFMSCs and the effect of in vitro cell culture on epigenetic changes of imprinted gene H19 on human AFMSCs.

In the six single-cell clones of human AFMSCs, the DNA methylation pattern shows a unique epigenetic signature of hypermethylation in CpG islands of the imprinted gene *H19*. In passage 7, the methylation rate was 79−98% in six single-cell clones of amniotic fluid stem cells (Table 1). Further studies on different passages (passages 8−10) showed no significant methylation changes during *in vitro* cell culture. We found that in passages 8−10 of six single-cell clones of AFMSCs during *in vitro* cell culture, almost all were

hypermethylated with methylation rates of 65−96% with only two exceptions (50% and 39%) (Table 1).

Epigenetic characteristics of CpG islands of imprinted gene SNRPN on six single-cell clones derived from human AFMSCs and the effect of in vitro cell culture on epigenetic changes of imprinted gene SNRPN on human AFMSCs.

In the six single-cell clones of human AFMSCs, the DNA methylation patterns showed variable methylation on CpG islands of the imprinted gene *SNRPN*. In passage 7, the methylation rate is 32−47% in six single-cell clones of amniotic fluid stem cell (Table 2). Further studies on different passages (passages 8−10) of six single-cell clones of amniotic fluid stem cells shows there were variable methylation pattern changes during *in vitro* cell culture with methylation rates of 21−88% (Table 2).

Epigenetic characteristics of CpG islands of imprinted gene KCNQ1OT1 in six single-cell clones derived from human AFMSCs and the effect of in vitro cell culture on
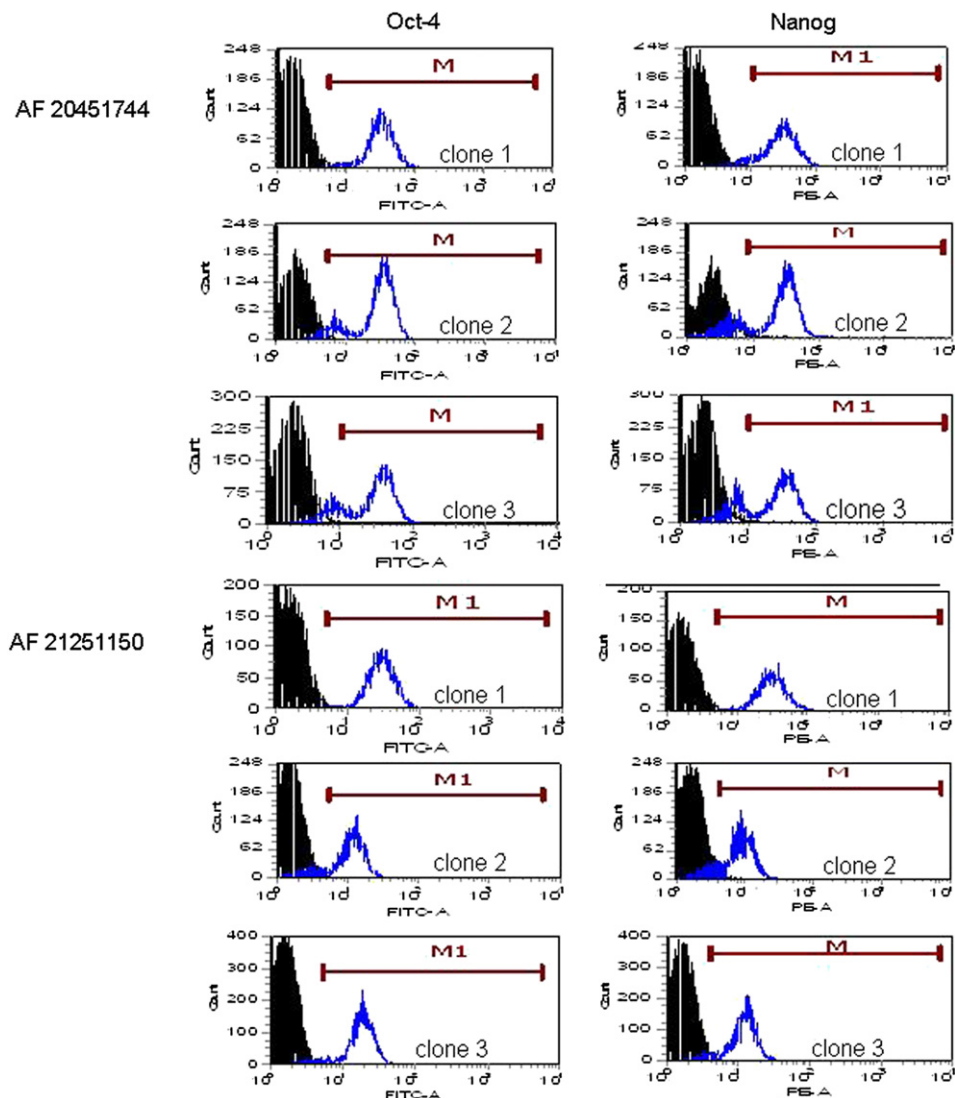

Fig. 2. Expression of Oct-4 and Nanog in single-cell clones of amniotic fluid mesenchymal stem cells. Each single-cell clone of amniotic fluid mesenchymal stem cell (MSCs) (total, six clones) are harvested, and the stem cell surface markers are characterized. Flow cytometry analyses revealed the expression of stem cell surface markers (both Oct-4 and Nanog) are strongly positive in all six clones of amniotic fluid MSCs.
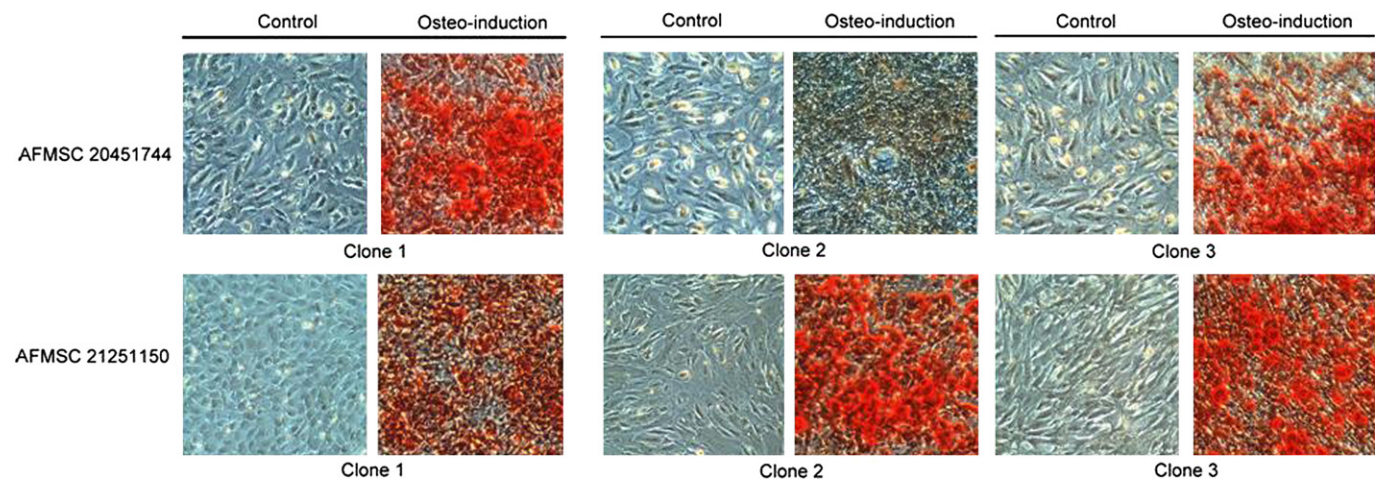
Fig. 3. Induction potential of single-cell clones of amniotic fluid mesenchymal stem cells. To demonstrate osteogenic differentiation in each single-cell clone of amniotic fluid MSCs, the mineralization of calcium accumulation in the cells was made visible by von Kossa staining. All six clones of amniotic fluid MSCs showed aggregates of calcium mineralization within the cells, and five of them have strongly positive signals.

epigenetic changes of imprinted gene KCNQ1OT1 on human AFMSCs.

In the six single-cell clones of human AFMSCs, the DNA methylation patterns all showed a unique epigenetic signature of hypermethylation in the CpG island of the imprinted gene *KCNQ1OT1* with only one exception. In passage 7, the methylation rates ranged from 96% to 99% in five single-cell clones of AFMSCs; only one clone had a significantly lower methylation rate of 39% (Table 3). Further studies on different passages (passages 8−10) show that there were no significant methylation pattern changes during *in vitro* cell culture. We found that during in vitro cell culture in passages 8−10 of six

single-cell clones of amniotic fluid stem cells, all of them were hypermethylated with methylation rates of 71−99% (Table 3).

Discussion

Stem cells can be derived from a variety of tissues during embryo, fetal, and adult development [29−32]. Recently, several studies have shown the possibility of deriving progenitors from amniotic fluid [1−4]. Amniotic fluid is the liquid surrounding the fetus, and the main component of amniotic fluid is fetal urine. The fetal cells from urinary tract, gastrointestinal tract, respiratory tract, skin, and other organs

Table 1
Methylation pattern on imprinting center of H19.

H19	● (%)	○ (%)
H19-C1P7	93	7
H19-C1P8	90	10
H19-C1P9	90	10
H19-C1P10	96	4
H19-C2P7	98	2
H19-C2P8	50	50
H19-C2P9	83	17
H19-C2P10	81	19
H19-C3P7	79	21
H19-C3P8	69	31
H19-C3P9	39	61
H19-C3P10	79	21
H19-C4P7	94	6
H19-C4P8	94	6
H19-C4P9	93	7
H19-C4P10	96	4
H19-C5P7	79	21
H19-C5P8	93	7
H19-C5P9	69	31
H19-C5P10	65	35
H19-C6P7	92	8
H19-C6P8	70	30
H19-C6P9	69	31
H19-C6P10	78	22

● = methylated; ○ = unmethylated; C = clone; P = passage.

Table 2
Methylation pattern on imprinting center of SNRPN.

SNRPN	● (%)	○ (%)
SNRPN-C1P7	32	68
SNRPN-C1P8	80	20
SNRPN-C1P9	66	34
SNRPN-C1P10	77	23
SNRPN-C2P7	36	64
SNRPN-C2P8	37	63
SNRPN-C2P9	21	79
SNRPN-C2P10	26	74
SNRPN-C3P7	34	66
SNRPN-C3P8	50	50
SNRPN-C3P9	88	12
SNRPN-C3P10	46	54
SNRPN-C4P7	46	54
SNRPN-C4P8	78	22
SNRPN-C4P9	33	67
SNRPN-C4P10	96	4
SNRPN-C5P7	47	53
SNRPN-C5P8	65	35
SNRPN-C5P9	82	18
SNRPN-C5P10	49	51
SNRPN-C6P7	32	68
SNRPN-C6P8	45	55
SNRPN-C6P9	52	48
SNRPN-C6P10	41	59

● = methylated; ○ = unmethylated; C = clone; P = passage.

Table 3
Methylation pattern on imprinting center of KCNQ1OT1.

KCNQ1OT1	● (%)	○ (%)
KCNQ1OT1-C1P7	39	61
KCNQ1OT1-C1P8	95	5
KCNQ1OT1-C1P9	96	4
KCNQ1OT1-C1P10	80	20
KCNQ1OT1-C2P7	96	4
KCNQ1OT1-C2P8	96	4
KCNQ1OT1-C2P9	97	3
KCNQ1OT1-C2P10	77	23
KCNQ1OT1-C3P7	97	3
KCNQ1OT1-C3P8	81	19
KCNQ1OT1-C3P9	97	3
KCNQ1OT1-C3P10	97	3
KCNQ1OT1-C4P7	98	2
KCNQ1OT1-C4P8	83	17
KCNQ1OT1-C4P9	81	19
KCNQ1OT1-C4P10	99	1
KCNQ1OT1-C5P7	99	1
KCNQ1OT1-C5P8	80	20
KCNQ1OT1-C5P9	75	25
KCNQ1OT1-C5P10	79	21
KCNQ1OT1-C6P7	97	3
KCNQ1OT1-C6P8	71	29
KCNQ1OT1-C6P9	82	18
KCNQ1OT1-C6P10	83	17

● = methylated; ○ = unmethylated; C = clone; P = passage.

can be found in amniotic fluid [33,34]. Amniocentesis is a widely accepted method of prenatal diagnosis, and the extra samples can be used to isolate AFMSCs, which has raised fewer ethical issues than other fetal stem cell sources, while still representing a good candidate for cell therapeutic applications and tissue engineering [1]. The potential use of AFMSCs for tissue regeneration were shown to be successful in animal models, including cartilage [35], smooth muscle [36], and cardiomyocyte cell sheet formation [37].

DNA methylation, which is responsible for gene silencing, is an epigenetic system that determines the specific characteristics of many cells, including stem cells. Until now, most studies have focused on the epigenetic changes on embryonic stem cells; however, the studies on DNA methylation patterns of human AFMSCs are limited. It had been reported that the DNA methylation patterns of hESCs are distinct from those in other cells, which may further our understanding of the mechanism behind stem cells' pluripotency [26]. Another report proposes gene-specific differences in the stability of imprinted loci in hESCs and disrupted DNA methylation as one potential mechanism [38]. Moreover, in vitro hESCs differentiation causes epigenetic abnormalities such as hypermethylation of CpG islands [27]. Recent studies suggest that genetic lesions and epigenetic instability including abnormal DNA methylation in cancer-related genes occur during long-term passage of hESCs [39]. The more passages the embryonic stem cells experienced, the more they appeared to differ from other cells of the same line that underwent fewer passages. This issue is important because alterations in DNA methylation have been associated with cancer and human genetic disorders [40]. Also, it is believed that the genome/

epigenome damage may elevate the risk for adverse health outcomes during various stages of life, contributing to problems with fertility and fetal development [41]. Because in vitro cell culture may change the epigenetic characteristics of the hESCs, it makes therapeutic cloning more challenging because of concerns that these epigenetic changes could affect human health, which can limit our applications of these in vitro cell culture cells in human therapies, cell transplantation, and tissue repair.

To our knowledge, this is the first study on the DNA methylation patterns on DMRs of imprinting genes (H19, SNRPN, and KCNQ1OT1) in human single-cell clones of AFMSCs during in vitro cell culture. In our study, we found that there is a hypermethylation pattern in the imprinting center of H19, and there are no significant changes in methylation patterns in most passages of AFMSCs during in vitro cell culture. In a previous report, abnormal hypermethylation within the IGF2/H19 imprinting center was found in monkey ESC lines [42]. In mouse multipotent germ line stem cells, the imprinting center of H19 is hypermethylated during in vitro cell culture [43]. In a review article on human ESC cells, variable epigenetic stability is observed [44]. In our study, there is variable methylation pattern on the imprinting center of SNRPN in single-cell clones of AFMSCs, and there are also variable changes in methylation patterns during in vitro cell culture. In a previous report, the SNURF/SNRPN imprinting centers are differentially methylated in monkey ESC lines [42]. Another report on methylation patterns of the Snurf/Snrpn cluster in mouse ES cells exhibited notable stability during in vitro manipulation [45]. In human ESCs, SNRPN is highly epigenetically stable [44]. In our study, there are altered methylation patterns (hypermethylation) at the imprinting center of KCNQ1OT1 in single-cell clones of AFMSCs, and there are no significant changes in methylation patterns in all passages during in vitro cell culture. In a previous report, KCNQ1OT1 is highly epigenetically stable in human ESCs [44]. Based on these findings, we know that DNA methylation patterns at the imprinting center of imprinting genes might be quite different in different source of stem cells and species.

Our study on the epigenetics of AFMSCs is an interesting initial look into what might be done for these stem cells that can potentially be applicable in therapies and tissue engineering. From the results of our study, we know about the altered DNA methylation pattern (hypermethylation) of H19 and KCNQ1OT1 of human single-cell clones of AFMSCs, which might cause therapeutic cloning to face enormous challenges. On the other hand, we found the variable epigenetic status of SNRPN in human AFMSCs during in vitro cell culture. These findings may have an impact on the suitability of AFMSCs for therapeutic use. Therefore, periodic monitoring of these cell lines may be required before they are used in in vivo applications.

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