INVESTIGATION

The HIST1 Locus Escapes Reprogramming in Cloned Bovine Embryos

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ABSTRACT

Epigenetic reprogramming is necessary in somatic cell nuclear transfer (SCNT) embryos in order to erase the differentiation-associated epigenetic marks of donor cells. However, such epigenetic memories often persist throughout the course of clonal development, thus decreasing cloning efficiency. Here, we explored reprogramming-refractory regions in bovine SCNT blastocyst transcriptomes. We observed that histone genes residing in the 1.5 Mb spanning the cow HIST1 cluster were coordinately downregulated in SCNT blastocysts. In contrast, both the nonhistone genes of this cluster, and histone genes elsewhere remained unaffected. This indicated that the downregulation was specific to HIST1 histone genes. We found that, after trichostatin A treatment, HIST1 histone genes were derepressed, and DNA methylation at their promoters was decreased to the level of in vitro fertilization embryos. Therefore, our results indicate that the reduced expression of HIST1 histone genes is a consequence of poor epigenetic reprogramming in SCNT blastocysts.

KEYWORDS

RNA-seq SCNT HIST1 cluster reprogramming epigenetics HDAC inhibitor DNA methylation

Successful cloning by somatic cell nuclear transfer (SCNT) depends on accurate reprogramming processes in which the differentiated state of the donor cell genome drifts to a totipotent, embryonic ground state (Kang et al. 2003). A variety of developmental defects and, consequently, low clonal viability are attributed to incomplete and poor genomic reprogramming in SCNT embryos (Amano et al. 2001; Eggan et al. 2001; Hill et al. 2000; Lanza et al. 2000; Ono et al. 2001). Several efforts have been dedicated to understand the physiogenetics of SCNT embryos, and to tackle the associated problem of low cloning efficiency. In this context, the most direct way to analyze SCNT embryos is to study their whole genome expression profiles. We recently performed RNA-seq in single cow blastocysts, and reported a set of differentially expressed genes (DEGs) between in vitro fertilization (IVF)-derived and SCNT blastocysts (Min et al. 2015). Additionally, we also reported features unique to SCNT blastocysts, including consistently aberrant expression of genes that function in either trophoderm development or epigenetic modification. As an extension to the study, here we aimed to identify poorly reprogrammed regions where densely packed DEGs are coordinately regulated, and discovered a reprogramming-refractory locus. To the best of our knowledge, this is the first study to report a reprogramming-resistant megabase-sized genomic stretch in SCNT embryos.

MATERIALS AND METHODS

In this paper, we processed and analyzed preexisting cow blastocyst transcriptomes data obtained from our recent RNA-seq experiment (Min et al. 2015). Therefore, all raw data and related procedures, including in vitro fertilization and culture of bovine oocytes, somatic cell nuclear transfer, isolation and amplification of the whole transcripts, library construction for RNA-seq, differential gene expression analysis, real-time PCR validation, and Circos plot generation were described in the previous report in detail.

In vitro fertilization of bovine oocytes

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Livestock Research Institute of Korea. The protocol was approved by the Committee on the Ethics of Animal Experiments of...
the Korea Research Institute of Bioscience and Biotechnology. We obtained bovine ovaries from a local slaughterhouse (Daejon-Ojung SH, Korea), and cumulus-oocyte complexes (COC) were extracted from follicles. Oocyte maturation and IVF were performed as previously described (Park et al. 2007). Blastocysts at midexpanded stage were produced after 7–8 d of IVF (Kwon et al. 2015) and used in downstream experiments. Inseminated oocytes were in vitro cultured (IVC) for 20 hr in IVC medium (3 mg/ml BSA in CR1aa), and the embryos were moved to IVC medium containing 25 nM trichostatin A (TSA), and cultured for another 20 hr. Finally, the embryos were transferred back into IVC medium, and cultured to the blastocyst stage.

**Somatic cell nuclear transfer**

For somatic cell nuclear transfer, bovine oocytes were enucleated, and somatic cells were injected under the zona pellucida using a micromanipulator (Leitz, Wetzlar, Germany). The injected donor and the oocyte were electrofused using an Electro Cell Manipulator 2001 (BTX). The fused eggs were activated and incubated until blastocysts were generated. Ear skin fibroblasts obtained from an adult female cow were passaged three times, and used as donor cells (Kwon et al. 2015). Cumulus cells obtained from COC during denudation were cultured and used as donors on the following day. We counted the cell number of each SCNT blastocyst by Hoechst staining, and picked out only healthy blastocysts comprising 60–80 blastomeres. To generate TSA-treated SCNT blastocysts, the activated oocytes were cultured in IVC medium supplemented with 25 nM TSA, and then removed to regular IVC medium 3.5 hr after. The embryos were further cultured to the blastocyst stage.

**RNA-seq of single bovine blastocysts and bioinformatic analyses**

Polyadenylated RNAs were isolated from each blastocyst using Dynabeads mRNA DIRECT kit (Invitrogen). cDNAs were amplified by the pico profiling method, and Illumina sequencing libraries were constructed using the amplified dsDNAs using TrueSeq DNA Sample Prep kit (Illumina). For bioinformatic analyses, TopHat-HTSeq-DESeq pipeline was followed. Raw read data from HiSeq2500 were aligned on *Bos taurus* UMD3.1 using TopHat, and gene expression levels were calculated using HTSeq followed by DESeq.

**qPCR validation of HIST1 histone gene expression**

For quantitative real-time PCR (qPCR) validation of *HIST1* histone gene expression, cDNAs were prepared from six male IVF or fSCNT blastocysts. One-twentieth volume of cDNA was mixed with 10 μl TOPreal qPCR 2X PreMIX (Enzynomics), and 10 μM of *HIST1* histone gene-specific primers. Quantitative PCR was performed in a 7500 Real-Time PCR System (Applied Biosystems). All experiments were performed in triplicate. The list of PCR primers is as follows: AACAAGCTGCTGGGCAAAGT and CGTTGTTTCCAATCTTGGTTC for *HIST1H2AJ*; CAAGCTGCTGGGTAAAGTCA and CGAAGTAATCCAGACTTCTA for *HIST1H2AG*; GCAAAGTCACCATCGCTCAG and CACTGAGATCTAGGTAGGTTCA for LOC618824.

**MSRE-PCR**

Genomic DNAs were extracted from 20 IVF or fSCNT blastocysts. For this, genomic DNAs were incubated in embryo lysis buffer (50 mM KCl, 2.5 mM MgCl₂, 0.1 μg/ml gelatin, 0.45% NP40, 0.45% Tween20, 10 mM Tris-Cl, and 200 μg/ml proteinase K) at 35°C for 1 hr followed by heat
inactivation of proteinase K at 85°C for 10 min. Whole lysates were reacted with 10 U of HpaII (NEB) or MspI (NEB) at 37°C overnight in 40 mM reaction volume (Yeo et al. 2007). Forty cycles of PCR was carried out on 1 μl digestion products using HIST1 histone-promoter-specific primers: TCAGAGGCTGCCAGGG and AAC TCGGGATCAAGTGGCAA for HIST1H1C; CACAGAGCAGGCAA CCAATCATCA and TGCTTCTGTTTAGCCAGGAAAGA for HIST1H1D; AAGAAGAAGGCAAAATGGG and CAGCCAG AGACAGGCCACT for HIST1H1E; TTCACGAACGTTCTCATGA TGCCCC and GAAAGGAGCAGCAGAAGGC for HIST1H2AG; GAGCTACTCGTGTACGTGTA and AAATGTGTTGAGAAA GAGTTCATGA for HIST1H2B; TTCAGAAGCAGATATTAGAT GCCC and GCAGGAGGACGGCAAGAAGA for HIST1H2BB; TACTTGGAGCTGGTGTACCTGG and AAGCGCTGACCATCA CATCTAG for HIST1H2BD; and ATTCACAAAGCCTGAC CNT and GTGTGGCTGACACTTTACTTGG for HIST1H2BN.

Data availability
Global expression data used in this study is available in our previous report (Min et al. 2015).

RESULTS
We recently reported RNA-seq data from single bovine blastocysts obtained by either IVF or SCNT. For the latter, we used two different donors, cumulus cells (cSCNT) and ear-skin fibroblasts (fSCNT) (Min et al. 2015). We collected an average of 12.7 million high-quality paired-end reads, and generated an expression profile dataset using TopHat and HTSeq, and Bos taurus UMD3.1 as reference genome. Analysis using DESeq revealed 630 and 1064 DEGs in cSCNT and fSCNT blastocysts, respectively, vs. IVF blastocysts. To identify poorly reprogrammed genomic regions where coordinately regulated DEGs are densely packed, we scanned the entire genome of SCNT blastocysts for erroneous domains by the sliding window analysis. Thirty-eight windows were detected to have six or more DEGs in single windows in the fSCNT blastocysts (Figure 1A). Of these, 13 were common to cSCNT blastocysts.

Among the DEG-rich loci, we were particularly interested in a locus where histone genes are bundled together. In both human (Albig et al. 1997) and mouse (Wang et al. 1996), about 50 histone genes heavily populate this locus, which is called the HIST1 cluster, and are transcriptionally regulated in a coordinated manner (Marzluff et al. 2002; Marzluff and Duronio 2002). The bovine HIST1 cluster on chromosome 23 resembles those of human and mouse. It spans about 1.5 Mb, and contains 47 histone and histone-like genes on Bos taurus UMD3.1 (Figure 1B). A heat map of genes on chromosome 23 unambiguously pinpointed downregulation at the HIST1 cluster in both cSCNT and fSCNT blastocysts (Figure 1C). When the expression of HIST1 histone genes was examined in donor fibroblasts, SCNT blastocysts appeared, overall, to be closer to donor cells than IVF blastocysts (Supplemental Material, Figure S1).
Detailed inspection of the transcriptomes revealed that individual histone genes residing in this cluster were, indeed, weakly expressed in both the fSCNT and cSCNT groups (Figure 2A). We further examined the expression of 47 HIST1 histone genes independently of 26 non-histone genes in the same cluster. We also examined 47 and 57 genes upstream and downstream of the cluster, respectively (Figure 2B). Interestingly, only the HIST1 histone genes were significantly downregulated in SCNT blastocysts, whereas genes of other subsets, including the non-histone genes within the HIST1 cluster, were not altered. This result agrees with the previous finding that histone genes in the human HIST1 cluster are coordinately regulated (Marzluff and Duronio 2002). By qPCR using cDNAs from freshly prepared IVF and fSCNT blastocysts, we confirmed that the HIST1H2AJ, HIST1H2AH, and LOC618824 (H2AI-like) genes in the HIST1 cluster were significantly downregulated in the SCNT blastocysts (Figure 2C). Furthermore, we found that bovine SCNT blastocysts also expressed genes, such as YYI, POU2F1, NPAT, and SLBP, known to be involved in coordinated regulation of HIST1 histone genes in human and mouse (Eliassen et al. 1998; Fletcher et al. 1987; Ma et al. 2000; Zhao et al. 2000; Whitfield et al. 2000). However, their expression in SCNT blastocysts was comparable (NPAT and SLBP) to, or even higher (YY1 and POU2F1) than, that in IVF blastocysts (Figure S2).

In contrary to HIST1 histones, histone genes such as H2AFL, H2AFY, and H2AFZ on other chromosomes (4, 7, and 6, respectively), and those in the HIST2-like cluster on chromosome 3 (HIST2H2AB, HIST2H2AC, HIST2H2BE, and HIST2H2BF) showed no difference in expression between the blastocyst groups (Figure 3A). When the HIST1 histone genes were compared with ~100 other histone genes residing elsewhere, their expression levels differed significantly between cSCNT and fSCNT (P = 8.34e-6, and 9.05e-14, respectively; Figure 3B). The sum of expression of all the histone and histone-like genes in the genome revealed that the fSCNT group expressed them at about 80% of what the IVF group did (Figure 3C). Notably, while the HIST1 histone gene expression took ~24% out of total histone gene expression in the IVF group, their contribution was only 8% in the fSCNT group, which was one-third (0.08/0.24) of the former. This indicates that the difference in total histone gene expression between the IVF and SCNT groups is derived largely from the difference in expression of the HIST1 histone genes.

Given the possibility that the HIST1 cluster is epigenetically regulated, we examined the epigenetic state of this region. First, DNA methylation at various HIST1 histone gene promoters was analyzed by methylation-sensitive restriction enzyme polymerase chain reaction (MSRE-PCR), using HpaII-digested genomic DNA from either IVF or fSCNT blastocysts as template. We found that most of the promoters (HIST1H1C, HIST1H1E, HIST1H2AG, HIST1H2BB, and HIST1H2BN) examined were more heavily methylated in the SCNT samples, whereas the rest were either similarly (HIST1H2B and HIST1H2BD) or less (HIST1H1D) methylated (Figure 4A). The results suggest that, in general, gene promoters at the HIST1 locus retain aberrant DNA methylation states in SCNT blastocysts. Next, we addressed whether this abnormal epigenetic state of the HIST1 cluster could be relieved by treatment with TSA, a histone deacetylase (HDAC) inhibitor. TSA positively influences animal cloning by promoting chromatin relaxation and increased global gene expression (Kishigami et al. 2006, 2007; Jee et al. 2012; Cui et al. 2011). RNA-seq was performed in TSA-treated IVF and fSCNT blastocysts. Differential expression analysis showed...
that TSA treatment upregulates a large number of genes in both blastocyst groups, as expected (Figure 4B). Pearson correlation analysis showed that correlation coefficient was the highest \( r = 0.998 \) between IVF and TSA-treated fSCNT groups, which was a substantial increase considering the relatively low coefficient \( r = 0.911 \) between them in the absence of TSA (Figure 4C). Examination of the genes in the HIST1 cluster revealed that, in contrast to the results shown in Figure 2B, the expression of histone and nonhistone genes within the HIST1 cluster did not significantly differ at \( P < 0.05 \) (Figure 4D). Compared with nonHIST1 histone genes, contrary to the result in Figure 2B, HIST1 histones showed increased expression in TSA-treated SCNT blastocysts (Figure 4E). Finally, we analyzed TSA-treated SCNT blastocysts for methylation state using MSRE-PCR. We observed that the methylation states of both SCNT and IVF blastocysts looked similar to each other at the HIST1 histone gene promoters after TSA treatment, suggesting that TSA might directly or indirectly induce demethylation of the HIST1 histone gene promoters (Figure 4F).

**DISCUSSION**

We found that histone genes in the HIST1 cluster were coordinately and epigenetically downregulated in SCNT blastocysts. Our findings suggest that, compared with IVF embryos, SCNT embryos have more repressive chromatin in the HIST1 locus, which hinders transcription factors approaching the region. Histone genes in the human and mouse HIST1 loci are known to be coregulated by YY1, POU2F1, NPAT, and SLBP, but these genes are found normally expressed in cow SCNT blastocysts (Figure S2). This implies that the presence of these regulators is not enough to derepress the HIST1 locus, and that the HIST1 region exists in a transcriptionally nonpermissive chromatin state that is highly resistant to reprogramming in SCNT embryos. This is supported by heavier methylation at the HIST1 histone gene promoters in SCNT (Figure 4A). The low expression of HIST1 histone genes in donor cells (Figure S1) leads to an assumption that the HIST1 histone gene expression anomaly shown in SCNT embryos might have originated in donor cell genomes. Figure 4, D–E shows that TSA can change the refractory chromatin, and relieve the HIST1 histone genes. This
gives a molecular mechanistic insight into the low expression of *HIST1* histone genes in SCNT embryos. The *HIST1* histone genes have lain under an inactive chromatin state in SCNT blastocysts, and were relieved by TSA, which removes repressive chromatin remodelers and recruits various transcription activators to the locus. In support of this view, DNA methylation at *HIST1* histone gene promoters was reduced to levels comparable to those in IVF embryos after TSA treatment (Figure 4F). Therefore, our findings indicate that TSA, through modulation of the epigenetic state of the *HIST1* locus in SCNT blastocysts, renders the histone genes transcriptionally competent, which is important for proper development of the embryo (see below).

The SCNT blastocyst group expressed lower levels of histone genes compared with the IVF group. This is attributed to the reduced expression of *HIST1* histone genes, rather than other histone genes (Figure 3C). The four core histone molecules, H2A, H2B, H3, and H4, comprise a nucleosome, maintaining a ratio of one H3–H4 tetramer, and two H2A–H2B dimers. Unequal histone levels, such as weighing toward a particular molecule, might induce cell cycle arrest in SCNT embryos (Ghule *et al.* 2014). Since ~100 histone-like genes with LOC-prefix names in the cow genome are, as yet, uncharacterized, we were unable to estimate the stoichiometric balance among the four histone molecules from the blastocyst transcriptomes. Therefore, whether the reduced histone expression causes a hazardous imbalance and leads to cell cycle arrest in SCNT embryos, remains to be ascertained. Another hypothesis is that, despite the reduced levels, SCNT blastocysts are able to maintain stable and proportionate histone mRNA levels and, consequently, equilibrate among them. Irrespective of these scenarios, the premise of successful clonal development is that all histone genes, which reside scattered on the chromosomes, are coordinately expressed against genome-wide reprogramming processes.

The effect of reduced *HIST1* histone gene expression on SCNT embryos is unresolved. It is known that histone synthesis is tightly coupled to that of DNA, and, likewise, inhibition of the latter during the replication phase of the cell cycle causes rapid decline in the former (Plumb *et al.* 1983; Sittman *et al.* 1983; Su *et al.* 2004; Ghule *et al.* 2014). Therefore, if the reduced transcription affects protein level, the diminished amount of histones might cause a deregulated cell cycle progression (*e.g.*, delayed S-phase entry) in blastomeres. Unlike normal embryonic cell cycle, which involves rapid successions of DNA replication without the intermittent G1 and G2 phases, that of SCNT embryos is a lengthy process that can be observed in typical differentiated somatic cells (Becker *et al.* 2006; Boiani *et al.* 2003; Balbach *et al.* 2012). Consequently, SCNT blastocysts frequently suffer from low cell counts. If this is true, the reduced expression of *HIST1* histone genes is indicative of poor genome reprogramming, which must be overcome for normal clonal development.

Numerous and variable transcriptomic dissimilarities occur unpredictably between SCNT embryos, indicating the stochastic nature of reprogramming processes. Nevertheless, some changes in these embryos are nonrandom and consistent, represented by those in the *HIST1* locus. In addition, we recently reported a 400-kb locus harboring zinc-finger (ZNF) protein family genes in chromosome 18 that were coordinately downregulated in fSCNT blastocysts, showing a feature of reprogramming-resistant regions (Min *et al.* 2015). Therefore, it is not entirely true that each SCNT embryo is unique in terms of its gene expression profile. To the best of our knowledge, this is the first study to report the identification of a reprogramming-resistant megabase-stretch in the genome of bovine SCNT embryos. Extensive efforts to discover such regions, which persistently escape reprogramming, might help establish a reprogramming error-prone genomic map in SCNT embryos. Such a map will shed light on the mechanisms underlying local and global reprogramming events, and help improve the efficiency of such events.

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