Differences during the first lactation between cows cloned by somatic cell nuclear transfer and noncloned cows

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ABSTRACT

Lactation performance is dependent on both the genetic characteristics and the environmental conditions surrounding lactating cows. However, individual variations can still be observed within a given breed under similar environmental conditions. The role of the environment between birth and lactation could be better appreciated in cloned cows, which are presumed to be genetically identical, but differences in lactation performance between cloned and noncloned cows first need to be clearly evaluated. Conflicting results have been described in the literature, so our aim was to clarify this situation. Nine cloned Prim' Holstein cows were produced by the transfer of nuclei from a single fibroblast cell line after cell fusion with enucleated oocytes. The cloned cows and 9 noncloned counterparts were raised under similar conditions. Milk production and composition were recorded monthly from calving until 200 d in milk. At 67 d in milk, biopsies were sampled from the rear quarter of the udder, their mammary epithelial cell content was evaluated, and mammary cell renewal, RNA, and DNA were then analyzed in relevant samples. The results showed that milk production did not differ significantly between cloned and noncloned cows, but milk protein and fat contents were less variable in cloned cows. Furthermore, milk fat yield and contents were lower in cloned cows during early lactation. At around 67 DIM, milk fat and protein yields, as well as milk fat, protein, and lactose contents, were also lower in cloned cows. These lower yields could be linked to the higher apoptotic rate observed in cloned cows. Apoptosis is triggered by insulin-like factor growth binding protein 5 (IGFBP5) and plasminogen activator inhibitor (PAI), which both interact with *CSN1S2*. During

our experiments, *CSN1S2* transcript levels were lower in the mammary gland of cloned cows. The mammary cell apoptotic rate observed in cloned cows may have been related to the higher levels of DNA (cytosine-5)-methyltransferase 1 (DNMT1) transcripts, coding for products that maintain the epigenetic status of cells. We conclude, therefore, that milk production in cloned cows differs slightly from that of noncloned cows. These differences may be due, in part, to a higher incidence of subclinical mastitis. They were associated with differences in cell apoptosis and linked to variations in *DNMT1* mRNA. However, milk protein and fat contents were more similar among cloned cows than among noncloned cows.

Key words: epigenetics, DNA (cytosine-5)-methyltransferase 1 (DNMT1), milk somatic cell counts, subclinical mastitis

INTRODUCTION

Prim' Holstein cows have been highly selected for their milk production and account for 71.4% of French cows. They are highly sensitive to variations in livestock systems (Dahl and Petitclerc, 2003; Kelsey et al., 2003; Martens, 2013) and are therefore usually bred under tightly controlled livestock systems (Le Cozler et al., 2009) subject to limited environmental variations. Despite this selection, milk performance can differ, even in a controlled environment, and the heritability of different lactation performance criteria varies. For example, the average heritabilities of milk yield and milk fat yield are estimated at 0.33 and 0.62, respectively (Schneider and VanVleck, 1986), suggesting that bovine milk production and its variability depend on both genetic and environmental factors, which may have affected cows long before lactation. Genetic, and more recently genomic, selection combined with a better control of livestock systems throughout the animal's productive life have contributed to markedly improving

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milk production. Whereas genetic studies have pointed at numerous genetic polymorphisms associated with milk production, the epigenetic mechanisms underlying the influence of the environment on phenotypes remain largely unknown.

Healthy cloned cows obtained through nuclear transfer from a defined nuclear donor cell line (Liu et al., 2013) are presumed to display limited genetic differences (Hanada et al., 2005). However, several reports have described some phenotypic variability between clones from a given nuclear donor cell line (Yonai et al., 2005), most likely due to environmental influences on the cloned cows, and cloned cattle achieve reproductive performance similar to that of noncloned ones (Polejaeva et al., 2013).

The long-term objective of cloning large animals is to generate more uniformity in product outcomes, but cloning might also be expected to enable a superior product, as already discussed (FDA Consumer Magazine, 2003). Conflicting results have been also published regarding differences between the milk yields, milk compositions, and milk SCC (an indicator of inflammation) of cloned and noncloned cows (Pace et al., 2002; Walsh et al., 2003; Heyman et al., 2004; Norman et al., 2004). These differences can be explained by the fact that the cloned cows were bred in experimental farms whereas the noncloned cows were bred in dairy farms for commercial purposes (Yonai et al., 2005), by differences in lactation stages (DIM) or lactation rank, or by the different origin of nuclear cell donors (Pace et al., 2002; Yonai et al., 2005; Bui et al., 2009; Liu et al., 2013) and their initial epigenetic states (McLean et al., 2010).

Nevertheless, the differences observed between the milk of cloned and noncloned cows remain within the range of differences observed between breeds in different livestock systems. The US Food and Drug Administration (FDA Consumer Health Information, 2008) therefore concluded that “meat and milk from clones of cattle, swine, and goats, and the offspring of clones from any species traditionally consumed as food, are as safe to eat as food from conventionally bred animals.”

These differences in lactation performance between cloned and noncloned cows may be related to epigenetic modifications induced by the environment during embryonic life, fetal life, or the early stages of postnatal life, and are maintained throughout adult life. Differences between cloned cows may also occur because during embryonic life, transferred nuclei are placed within the cytoplasm of enucleated oocytes that have been harvested at the slaughterhouse and are all different. Furthermore, during fetal life, cloned embryos are implanted in different recipient cows. Finally, although every effort is made to ensure similar environmental

conditions for calves, some experience severe health problems during the early stages of postnatal life and need to be euthanized. These differences may alter the mammary epigenome normally found in this tissue, or in the different cell types that form the tissue, at different stages of development.

To shed more light on potential differences between the lactation phenotypes of cloned and noncloned cows, and on the potential genetic versus epigenetic effects of cloning, lactation performance has been compared during similar periods of the year between cloned and noncloned cows born during similar periods of the year and raised under similar conditions. In such a setting, 10 cloned cows studied at INRA (UE1298 Unité commune d'expérimentation animale, Jouy-en-Josas, France) displayed few differences in milk fat yield and milk yields, which were shown to vary less than in milk from noncloned cows (Heyman et al., 2007b). These cloned cows had been generated by nuclear transfer using 4 different cell lines.

To further that study and to restrict the genetic differences even more, we subsequently produced 9 cloned cows using a single skin fibroblast cell line (reference 5538) as the nuclear donor. This cell line was derived from a high-yielding cow. Nuclei from this cell line had been shown to efficiently support the *in vivo* development of somatic cell nuclear transfer (SCNT) embryos and to induce only a limited number of animals with large offspring syndrome (Bui et al., 2009). The growth and lactation performances of these 9 cloned cows were compared with those of 9 noncloned cows raised under similar livestock systems. The evaluation criteria were weight at birth, growth curves, BCS at calving, and lactation performance including SCC. At 67 DIM, a stage that is theoretically after the peak of lactation when mammary cell activity is stabilized (Capuco et al., 2003) and for which epithelial cell turnover has been already studied (Nguyen et al., 2014), these criteria also included mammary morphology, mammary contents in mRNA involved in milk protein and lactose synthesis, together with mRNA coding for DNA methyl transferase and DNA methylation levels of 3 CpG sites (CpG1, CpG2, and CpG3) located in the distal regulatory region of the *CSN1S1* gene (−10,276, −10,216, and −10,196 nucleotides from the transcription start site, respectively), which have been shown to vary in line with gene expression (Nguyen et al., 2014).

MATERIALS AND METHODS

Care and Use of Animals

All research on animals was carried out in compliance with the French laws and regulations and with

the principles and specific guidelines presented in the *Guidelines for the Care and Use of Agricultural Animals in Research and Teaching* (FASS, 2010). Before starting the study, the experimental design was submitted for approval by the Comité d'éthique en expérimentation animale (Jouy-en-Josas, France) Comité national de Réflexion éthique sur l'Expérimentation animale (France) 45 Ethics Committee (ref 12–160, registered on November 20, 2012).

Herd and Animals

All 18 Prim' Holstein cows (9 cloned cows and 9 noncloned cows) involved in this study were born on INRA's experimental farm (UE1298 Unité Commune d'Expérimentation Animale, Jouy-en-Josas, France). Noncloned cows born during a similar period of the year and with BW similar to those of the cloned cows were selected. Both cloned and noncloned cows were raised together under identical conditions until adulthood.

The noncloned cows were conceived after AI and were from 5 different sires. The clones were derived from somatic nuclear transfer (Vignon et al., 1998), using confluent cells at passage 8 of the previously described skin fibroblast 5538 line (Bui et al., 2009) as donor nuclei. Recipient oocytes originated from ovaries collected at the slaughterhouse from adult Prim' Holstein cows. They were matured in vitro and enucleated at 20 to 22 h postmaturation. They were then fused to nucleus donor cells (Bui et al., 2009). Only Day 7 blastocysts graded as 1 (according to the International Embryo Transfer Society grading system; IETS, 2008) were implanted into the uterine horn of recipient Prim' Holstein cows (2 embryos per recipient cow) that had been estrus-synchronized as previously described (Constant et al., 2006). To induce fetal maturation, a single dose of dexamethasone (20 mg) was injected 24 h before the Cesarean section, and neonatal care was assured in compliance with IETS guidelines.

All calves were fed colostrum (10% weight at birth) within the first 12 h of life, and then fed cow's milk for 1 wk. After this neonatal period, they received milk formula until 90 d of age. Calves were weaned when they reached a weight of 100 kg, about 1 wk later. The calves then received starter feed until the age of 6 mo, when they reached a weight of 200 to 220 kg. From that time onward, the cows were fed hay, straw, grass silage, concentrates (barley and soybean), and a full complement of minerals and vitamins.

Between parturition and mo 4 or 8 of lactation, 4 noncloned cows and 3 cloned cows, or 1 noncloned cow and 2 cloned cows, respectively, were fed the following total mixed diet, diet 1, distributed ad libitum, contain-

ing (on a DM basis) 56% corn silage, 12% rolled barley grain, 10% brewer's spent grain, 7% soybean, 3% Sandi-excel (food supplement, Sanders, Pontivy, France), 34% cane sugar molasses, 4% straw, 2% fescue hay, and 2% vitamin supplement. The TMR was formulated to meet their energy needs ($NE_L = 1.6$ Mcal/kg of DM) and to exceed the MP requirement (i.e., protein digestible in the intestine, under the French system = 112 g/kg of DM). Subsequent to this, these animals received diet 2 distributed ad libitum. Diet 2 contained (on a DM basis) 60% corn silage, 9.3% rolled barley grain, 7.3% brewer's spent grain, 11.6% soybean, 4.5% Sandiexcel (Sanders), 4.7% straw, and 2.6% vitamin supplement. Two noncloned cows and 3 cloned cows received diet 2 starting from the first 2 mo following parturition.

At the age of 18 mo, all the cows were synchronized with 3-mg doses of progesterone (Crestar Pack implant, Intervet, Beaucouzé, France) from d 1 to d 10, and an injection of cloprostenol on d 8 (0.25 mg, Estrumate, Intervet) and of pregnant mare serum gonadotropin (300 IU, Chronogest PMSG, Intervet) on d 10. One to 4 insemination procedures were required to obtain successful pregnancies in 8 cloned heifers and 7 noncloned heifers. This number was not statistically different between the cloned and noncloned heifers. Six cloned heifers delivered between the end of November and the end of December, and 2 cloned heifers delivered between the end of April and the end of May. Six noncloned heifers delivered between the end of November and the end of February, and 1 delivered at the beginning of June. Body condition score was evaluated twice, independently, by 2 technicians trained for this analysis, using BCS on a scale from 0 (very thin) to 5 (very fat) according to the protocols previously described (Remond et al., 1988). None of the cows was inseminated during this first lactation.

Milk Analyses

Daily milk production was evaluated once a month. Samples were collected separately from each cow and different samples taken during both morning and evening milking. Milk production (kg/d) from each udder quarter of each cow was also evaluated for 3 or 4 d before the biopsy, and milk samples were then collected a few minutes after the start of the milking protocol. Milk protein, fat, and lactose contents, as well as SCC, were evaluated by the Syndicat Interdépartemental de l'Élevage (SIE, Paris, France).

Mammary Biopsies

Biopsies (70 × 4 mm) were collected at around 67 DIM, before the afternoon milking as previously described (Boutinaud et al., 2013), under local anesthesia

using a subcutaneous injection of 3 mL of lignocaine hydrochloride (21.33 mg/mL of Xylovet; Ethical Agents Ltd., Auckland, New Zealand). After the surgical procedure, the cows received antibiotic therapy by means of intramuscular injections of 0.6 g of Naxcel (Pfizer, Paris, France) for 3 d. Biopsies were taken from the upper portion of the rear quarter of the mammary gland using a 70 × 4 mm instrument, as previously described (Farr et al., 1996). Connective and fat tissues were quickly dissected and the remaining tissue was cut into pieces. Tissue specimens for RNA and DNA analyses were frozen in liquid nitrogen and stored at -80°C until use, and those for histological analyses were fixed and processed as described below.

Histology

For histological investigations, mammary specimens were fixed in RCL2 (Excilone, Elancourt, France) for 24 h at 4°C . They were then dehydrated in 70% ethanol and embedded in paraffin according to standard histological protocols. Five-micrometer sections, lying at least 100 μm apart, were mounted on slides that were stained with hematoxylin-eosin-Safran (Sigma, Saint Quentin Fallavier, France) and then digitized using a NanoZoomer scanner (Hamamatsu Photonics, Hamamatsu City, Japan). This latter technique enabled observation of the entire section. Five sections per cow were processed. The epithelial tissue content of each biopsy was estimated from the area corresponding to cells surrounding the lumen organized in mono- or bilayers, as a percentage of the whole biopsy area. The areas were evaluated using both the Hamamatsu NanoZoomer Digital Pathology Virtual Slide Viewer and Image J software (Wayne Rasband; National Institutes of Health, Bethesda, MD).

Apoptotic Cell Detection in Mammary Tissue Sections

Determinations of the percentages of apoptotic cells in mammary gland biopsy sections were based on DNA fragmentation detection using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, as previously described (Boutin-aud et al., 2013). After TUNEL labeling, the sections were incubated for 3 min with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) at a concentration of 0.33 $\mu\text{g}/\text{mL}$. All slides were mounted with Vectashield (Valbiotech, Paris, France) and examined under fluorescence using a Nikon Eclipse E400 microscope (Nikon France, Le Pallet, France). The images were captured with a DXM 1200 digital still camera (Nikon France) and analyzed with ImageJ software (National Institutes

of Health). Eight microscopic fields (magnification = 200 $\times$; area = 0.14 mm^2 per microscopic field) were examined for each staining. The percentages of apoptotic cells in mammary tissue were each determined as a ratio between the TUNEL-labeled cells and DAPI-counterstained nuclei.

RNA Extraction

Total RNA was extracted using 1 mL of Trizol (Invitrogen, Cergy-Pontoise, France) per 100 mg of tissue. Integrity of RNA was determined based on an optical density ratio at 260:280 nm greater than 1.8, as previously described (Nguyen et al., 2014).

Reverse Transcription PCR

Reverse transcription (RT) assays were performed in 20 μL , on 2.5 μg of total RNA, in the presence (RT+) or absence (RT-) of Superscript III RT (SuperScript VILO cDNA synthesis kit, which includes random primers and RNaseOUT; Invitrogen, Cergy-Pontoise, France), as recommended by the manufacturer. Quantitative (q)PCR analyses were conducted in 25 μL on 1/100 to 1/1,000 dilutions of RT products using specific primers, as previously described (Table 1); that is, Absolute Blue qPCR SYBR Green ROX Mix (Thermo Fisher Scientific, Saint Aubin, France) and an ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Cyclophilin (CCPL) and ubiquitously expressed prefoldin-like chaperone (UXT) mRNA were analyzed as internal control genes (Bionaz and Looor, 2007); GAPDH mRNA levels were highly variable and not used as a standard. The geometric average of CCPL and UXT was therefore used for normalization. Relative gene expression using the comparative cycle threshold method ($2^{-\Delta\Delta\text{Ct}}$; Livak and Schmittgen, 2001), and fold differences between cloned and noncloned animals were evaluated for each transcript.

Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from mammary specimens (50 mg), as previously described (Sambrook and Russell, 2001). The purity and quantity of gDNA were checked on agarose and using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

Bisulfite Treatment, Amplification, and Pyrosequencing of the CSN1S1 Regulatory Region

Bisulfite pyrosequencing was performed to study the 3 CpG sites (CpG1, CpG2, and CpG3) located in the

Table 1. Sequence of primers used for real-time quantitative PCR analyses¹

mRNA	Reference	Primer
<i>CSN1S1</i>	Nguyen et al., 2014	For 5'-TGAGGATCAAGCCATGGAAGATAT-3' Rev 5'-TCAGTGTATTGTGTGCCTAGTGGAA-3'
<i>CSN2</i>	Sigl et al., 2014	For 5'-GTGAGGAACAGCAGCAAACA-3' Rev 5'-AGGGAAGGGCATTCTTTGT-3'
<i>CSN1S2</i>	Sigl et al., 2014	For 5'-AGCTCTCCACCAGTGAGGAA-3' Rev 5'-GCAAGGCGAATTTCTGGTAA-3'
<i>CSN3</i>	Sigl et al., 2014	For 5'-TGCAATGATGAAGAGTTTTTTCCTAG-3' Rev 5'-GATTGGGATATATTTGGCTATTTTGT-3'
<i>LALBA</i>	Sigl et al., 2014	For 5'-CTCTCTGCTCCTGGTAGGCAT-3' Rev 5'-GTGAGGGTTCTGGTCTGCTT
<i>ACACA</i>	Oliveira et al., 2014	For 5'-TGAAGAAGCAATGGATGAACC-3' Rev 5'-TTCAGACACGGAGCCAATAA-3'
<i>LPL</i>	Oliveira et al., 2014	For 5'-CTCAGGACTCCCGAAGACAC-3' Rev 5'-GTTTTGCTGCTGTGGTTGAA-3'
<i>FASN</i>	Bionaz and Loor, 2008	For 5'-ACCTCGTGAAGGCTGTGACTCA-3' Rev 5'-TGAGTCGAGGCCAAGGTCTGAA-3'
<i>SCD</i>	Oliveira et al., 2014	For 5'-TTATTCCGTTATGCCCTTGG-3' Rev 5'-TTGTCATAAGGGCGGTATCC-3'
<i>SREBF1</i>	Bionaz and Loor, 2008	For 5'-CCAGCTGACAGCTCCATTGA-3' Rev 5'-TGCGCGCCACAAGGA-3'
<i>DNMT1</i>	Wang et al., 2014	For 5'-TAGCAAACGGAGACCAGAAGAA-3' Rev 5'-TTGGTCTTGGACACTACGGCTAT-3'
<i>DNMT3A</i>	Bian et al., 2015	For 5'-AAGCCGCTGTTACCTCTTGT-3' Rev 5'-ATGTTTTTACTCATCTCGCTGTTGT-3'
<i>DNMT3B</i>	Bian et al., 2015	For 5'-GGCAAGTTCTCCGAGATTCCAG-3' Rev 5'-TGGTACATGGCCTTCCTGTAAGAGA-3'
<i>GAPDH</i>	Kadegowda et al., 2009	For 5'-TTGTCTCCTGCGACTTCAACA-3' Rev 5'-TCGTACCAGGAAATGAGCTTGAC-3'
<i>CCPL2</i>	Nguyen et al., 2014	For 5'-GGTGACTTCACACGCCATAATGGTA-3' Rev 5'-GGACAAGATGCCAGGACCTGTATG-3'
<i>UXT</i>	Kadegowda et al., 2009	For 5'-CAGCTGGCCAAATACCTTCAA-3' Rev 5'-GTGTCTGGGACCACTGTGTCAA-3'

¹The primer sequences were designed or selected from publications as indicated, but several were renamed so that the sequence of forward primers (For) is always identical to that of the mRNA and that of reverse primers (Rev) is always inverse and complementary.

distal regulatory region of the *CSN1S1* gene, as described previously (Nguyen et al., 2014). Briefly, 1 µg of the *Eco*RI-linearized gDNA was incubated in the dark in 0.5 mL of freshly prepared 5 M sodium bisulfite and 130 mM hydroquinone at 55°C for 4 h. Bisulfite-treated gDNA was then purified using the Wizard DNA clean-up system (cat. no. A7280, Promega France, Charbonnières-les-Bains, France), incubated in 0.3 M NaOH at 37°C for 20 min, and then ethanol precipitated overnight at -20°C in the presence of 2 M ammonium acetate. Precipitated DNA was recovered after 45 min at 14,000 × g and the pellet was dissolved in 20 µL of water. One microliter of bisulfite-modified gDNA was then amplified using a pair of primers, of which one was biotinylated to capture the antisense DNA strand for further pyrosequencing. The PCR reactions were carried out in 25-µL reactions containing 2 mM MgCl₂, 0.4 µM of each amplification primer, 0.4 mM dNTPs, and 2 IU of Platinum Taq DNA Polymerase (cat. No. 10966-034, Invitrogen/Thermo Fisher Scientific). The cycling conditions were as follows: initial denaturation at 95°C for 5 min; 45 cycles including: denaturation at

95°C for 30 s, annealing at 54°C for 30 s, elongation at 72°C for 30 s; and an extension step at 72°C for 5 min.

The antisense DNA strand was captured using Streptavidin Sepharose High Performance (cat. no. 17-5113-01, GE-Healthcare, Velizy-Villacoublay, France), hybridized with sequencing primers and run on a Pyrosequencer (PyroMarkQ24, Qiagen, Courtaboeuf, France). The percentage of DNA methylation at each CpG site was analyzed using the software supplied by Qiagen. The PCR and sequencing primers were designed using Methyl Primer Express v1.0 software and have been described previously (Nguyen et al., 2014)

Statistical Analyses

Longitudinal data analyses were applied to both milk production data and milk composition findings, using a linear mixed model with a random animal effect and a first-order autoregressive residual AR(1) correlation structure. These analyses were performed using the nlme package under R (Pinheiro et al., 2015). The variability of longitudinal data between cows within

a group (cloned or noncloned) was analyzed using a Fisher test. Principal components analysis (PCA) projects the data into a lower dimensional space, revealing the inherent data structure and providing a reduced dimensional representation of the original data. The amount of variation pertaining to the grouping of data was measured by the ratio of total inertia due to the differences between groups divided by the inertia computed in the PCA. Its significance was tested using a Monte-Carlo permutation test. The analyses were performed using the ade4 package (Dray and Dufour, 2007) in R software (Pinheiro et al., 2015). At 67 DIM, all differences between cloned cows and noncloned cows were analyzed using a Mann-Whitney V5.1.1 test (Siegel and Castellan, 1988), which is appropriate for small data sets.

RESULTS

Generation of Cloned Cows

After nuclear transfer, 110 embryos were obtained and transferred into 55 synchronized recipient mothers (2 embryos per mother). Fifteen fetuses developed to birth but 6 newborn calves died during the perinatal period from different pathological causes. Nine cloned calves were born from 7 cows. Cloned cow 1069 had a twin sister that died during delivery, cloned cow 1125 had a twin sister (1124) that was not included in the lactation study, cloned cows 1126 and 1127 were twins, and the other 5 cloned cows were singletons. Body weight at birth was higher in the cloned cows than in the noncloned cows (50.05 ± 2.6 kg and 41.96 ± 1.5 kg, respectively; Figure 1A).

Growth and BCS

For both cloned and noncloned cows ($n = 9$ in each group), a clear slope break in growth curves was observed in most cows at around 180 d of age, when the animals were moved from the nursery to outdoor barns and fed adult food (farm hay and commercial compound feed given partly in the milking parlor and partly in the manger) rather than second-stage milk (Figure 1C and 1D).

One cloned and 2 noncloned cows, which had the lowest BW at around d 180 and 240 (indicated by arrows in Figure 1C and 1D), did not become pregnant. The 8 cloned cows and 7 noncloned cows that developed a pregnancy had differed in terms of their BW at birth (48.3 ± 2.3 and 41.7 ± 1.5 kg, respectively; $P = 0.03$) and the slopes of their growth curves until mating, at around the age of 18 mo (Figure 1C and 1D; $P = 0.0003$), as did the initial 2 groups of 9 cows.

However, by 1 yr, differences in BW were no longer observed (314 ± 16 kg for cloned cows and 330 ± 13 kg for noncloned cows). During pregnancy, no differences in udder morphology or development were observed in the 8 cloned cows versus the 7 noncloned cows, according to the breed references (Primholstein.com, 2012), but the BCS at calving was lower in cloned cows than in noncloned cows (Figure 1B; $P = 0.025$).

Milk Production During 7 Months Postpartum

Milk production was recorded for 7 mo postpartum. A marked variability in milk production was observed among both cloned and noncloned cows (Figure 2, $n = 8$ for each group). At some time points between 100 and 150 DIM, sharp decreases in milk production were observed in some cloned (1069, 1072, and 1127) and noncloned (1068, 1074, 1103, and 1130) cows. However, throughout the first 210 DIM, milk production did not differ significantly between the 2 groups. Cow 5538, from which the cloned cows had been derived, had the highest milk production.

Milk protein yield was also highly variable among cows but did not differ between cloned and noncloned cows (Figure 3A and 3B). Cow 5538 was among those with the highest milk protein yield. Milk protein content did not differ between cloned and noncloned cows, but less variability was observed in cloned cows versus their noncloned counterparts (Figure 3C and 3D; $P = 0.087$).

Milk fat yield also varied considerably among cows, with no difference between cloned and noncloned cows during the first 210 DIM, although a difference at the intercept of the curves was observed (Figure 4A and 4B; $P < 0.0074$). Similarly, during the first days of lactation, the milk fat content was lower among cloned cows (Figure 4C and 4D; $P < 0.0001$), but when the milk fat contents were plotted, a difference in the slopes was subsequently observed, as they were less steep in noncloned cows (Figure 4, lower panel; $P < 0.0001$). These plots also revealed that the milk fat content was less variable among cloned cows (Figure 4C and 4D; $P = 0.004$).

Milk SCC

Somatic cell counts were evaluated in milk pooled from the 4 udder quarters of each cow ($n = 8$ for cloned cows and $n = 7$ for noncloned cows), about once a month from delivery until 270 or 290 DIM, depending on the cow. Somatic cell counts were also evaluated daily for 3 to 4 d before the biopsy was collected from each udder quarter. The SCC in 3 cloned cows and 4 noncloned cows were greater than 200×10^3 cells/

Figure 1. Cow weight as a function of age and BCS. (A) Differences in weight at birth of all cloned and noncloned cows ($P = 0.015$); (B) BCS at calving ($P = 0.025$). In A and B, the boxplots represent the median (inside the box), the 75th and 25th percentiles (upper and lower sides of the box), and the minimum and maximum values observed within 1.5-fold of the interquartile range and the minimal and maximal values observed (bars and circles at the end of whiskers, respectively). Significant differences are indicated by letters. (C and D) Differences in growth curves between cloned (panel C, $n = 9$) and noncloned (panel D, $n = 9$) cows, from which the fibroblast nuclear donor cell line was derived. Lower slopes are observed for cloned cows ($P = 0.0003$). Cows that displayed lower weight at around d 180 or 240 compared with other cows in the same group and that did not become pregnant (solid line and broken line, respectively) are indicated by arrows.

mL before the biopsy. The SCC value in one of the cloned cows was $1,477 \times 10^3$ cells/mL on the morning of the biopsy, but only in the fore right quarter of the udder, whereas the biopsy was collected from the back left quarter. In most cows (5/7 noncloned cows, and 6/8 cloned cows), SCC levels were around 100×10^3 cells/mL at the next monthly milk control. After the biopsy and until DIM 270 to 290, 6 cloned cows (the 3 with high SCC before the biopsy and 3 others) and 2 noncloned cows (among the 4 with high SCC before the biopsy) had SCC $>200 \times 10^3$ cells/mL at least once over the period. However, none of them displayed any clinical abnormalities affecting their general state of health or received antibiotic therapy (except for the treatment related to the biopsy). On average, during the first 6 to 8 mo of lactation, the SCC in milk from cloned cows (363×10^3 cells/mL), as well as the average ratio between milk samples with SCC $>200 \times 10^3$ cells/mL and the number of samples collected from each cow (0.40) both tended to be higher than those seen in noncloned cows (101×10^3 cells/mL, $P = 0.10$ and 0.40 versus 0.16; $P = 0.09$, respectively).

Milk Production Around Day 67 of Lactation

At around d 67 of lactation, milk production was recorded for only 7 cloned cows and 7 noncloned cows, as 1 milk sample was mislaid. This sample had been taken from a cow with a milk composition and milk yield over the first 7 mo of lactation within the range of the other cows. No significant differences in milk produc-

tion were observed between cloned and noncloned cows (Figure 5A). However, milk protein and milk fat yields were lower in cloned cows ($P = 0.05$ for both), whereas lactose yields did not differ (Figure 5B, 5C, and 5D, respectively). These differences in yield were related to the lower protein and fat contents in the milk and, surprisingly, also to lower lactose contents (Figures 5E, $P = 0.04$; Figure 5F, $P = 0.01$; and Figure 5G, $P = 0.02$, respectively).

When milk production and protein, fat, and lactose, as well as milk SCC, were subjected to PCA, the data segregated into 2 groups representing the cloned and noncloned cows (Supplemental Figure S1; <http://dx.doi.org/10.3168/jds.2015-10532>). The degree of variation pertaining to this segregation was 19% and significant, as shown by a Monte Carlo permutation test ($P = 0.007$, after 999 permutations).

When the cloned cow whose milk from the fore quarter of the mammary gland had a very high SCC at d 65 of lactation (2.3×10^6 cells/mL, just before the biopsy) was omitted from the analyses listed above (including PCA), the data from cloned and noncloned cows still segregated clearly (data not shown; ratio of inertia = 0.16, $P = 0.04$ after 999 permutation).

Mammary Biopsies

Biopsies were collected before afternoon milking on around d 67 of lactation. Sections ($5 \mu\text{m}$ and 70 nm) were observed using NanoZoomer technology and electron microscopy. However, 2 biopsies from cloned cows

Figure 2. Milk production (kg/d) as a function of DIM. Differences in milk production between cloned cows (A, $n = 8$) and noncloned cows (B, $n = 8$, including the nuclear donor cow, indicated by a bold line) were not significant.

and 1 biopsy from a noncloned cow did not correspond to epithelial tissue and were not analyzed further. The other biopsies (6 cloned and 6 noncloned cows) had an average content (68%) in mammary epithelial tissue that did not differ significantly between cloned and noncloned cows (Figure 6A). No differences in epithelial tissue morphology were observed between cloned and noncloned cows. During lactation, mammary cell renewal is dependent on both cell apoptosis and cell proliferation. At around 67 DIM, the percentage of

apoptotic cells detected by a TUNEL assay was 2.6-fold higher in cloned cows than in noncloned cows ($P < 0.001$; Figure 6B to 6F).

Mammary Gland Levels of mRNA Coding for Milk Components or for Enzymes Involved in Milk Synthesis

Total RNA was extracted from the 12 samples (from 6 cloned and 6 noncloned cows). One RNA sample was

Figure 3. Milk protein production throughout 200 DIM: (A) milk protein yield (kg/d) and (B) milk protein content (%); data from cloned cows (panels A and C, $n = 8$) and noncloned cows (panels B and D, $n = 8$, including the nuclear donor cow, indicated by a bold line) are depicted. The curve slopes describing milk protein yield and milk protein content (%) as a function of DIM did not differ between cloned and noncloned cows. However, milk protein content (%) varied less among cloned cows than noncloned cows, $P = 0.087$.

degraded in each group so that only 10 RNA samples (from 5 cloned and 5 noncloned cows) were analyzed by RT-qPCR. These RNA samples corresponded to cows whose milk parameters were representative of the 9 cows initially studied (similar significant differences in milk protein, fat, and lactose contents).

No significant differences were observed in the milk protein mRNA levels of *CSN1S1*, *CSN2*, and *CSN3*, *LALBA*, and milk fat synthesis enzyme mRNA levels of *SCD*, *ACACA*, *LPL*, *FASN*, or *SREBF*, although the average levels for each mRNA were always lower in cloned cows. The PCA data were not able to dem-

Figure 4. Milk fat production throughout 200 DIM. (A and B) Milk fat yield (kg/d) and (C and D) milk fat content (%) are depicted; data from cloned cows (panels A and C, $n = 8$) and noncloned cows (panels B and D, $n = 8$, including the nuclear donor cow, indicated by a bold line) are depicted. The curve slopes describing milk fat yield did not differ between cloned and noncloned cows but intercepts were lower for cloned cows. The curve slopes describing milk fat content (%) as a function of DIM differed ($P = 0.0001$) and intercepts were lower for cloned cows ($P = 0.0001$). Furthermore, milk fat content (%) varied less among cloned cows than among noncloned cows, $P = 0.004$.

onstrate any significant differences either. However, significant differences were observed in cloned cows (Figure 7A, $P = 0.05$) with respect to *CSN1S2* and

GAPDH mRNA. These mRNA levels were lower and higher, respectively, in cloned cows than in noncloned cows. The representation of all transcripts in each cow

Figure 5. Milk production at around 67 DIM. (A) Differences in milk yield (kg/d) and (B to G) differences in milk composition (g/kg) between cloned cows ($n = 8$) and noncloned cows ($n = 7$) were analyzed. The box plots represent the median (inside the box), the 75th and 25th percentiles (upper and lower sides of the box), and the minimum and maximum values observed (bars and circles at the end of whiskers, respectively). Milk production (kg/d, panel A) and milk lactose yield (kg/d, panel D) did not differ, whereas milk protein (kg/d, panel B) and milk fat yields (kg/d, panel C) were lower in cloned cows ($P = 0.05$). Milk protein, fat, and lactose contents (panels E to G) were lower in cloned cows ($P = 0.04$; $P = 0.01$; $P = 0.02$, respectively). Significant differences between cloned and noncloned cows are indicated by a letter and nonsignificant differences by NS.

clearly showed that one cloned cow behaved differently (Supplemental Figure S2; <http://dx.doi.org/10.3168/jds.2015-10532>), this being the animal mentioned above with a high SCC in milk from the fore quarter 2 d be-

fore the biopsy. However, when analyses of the results were run without this sample, differences in the mean values for *CSN1S2* and *GAPDH* mRNA were still observed between cloned and noncloned cows ($P = 0.06$).

Figure 6. Mammary epithelial cell contents and detection of apoptotic cells in biopsies. (A) Mammary epithelial cell content was evaluated as the percentage of mammary epithelial area in each biopsy. This did not differ between cloned cows ($n = 6$) and noncloned cows ($n = 6$). (B) The percentage of apoptotic cells was estimated using a terminal deoxy-nucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The box plots represent the median (inside the box), the 75th and 25th percentiles (upper and lower sides of the box), and the minimum and maximum values observed within 1.5-fold of the interquartile range and the minimum and maximum values observed (bars and circles at the end of whiskers, respectively). Mammary tissue sections from cloned cows (C and E) and noncloned cows (D and F) were stained simultaneously with the TUNEL assay (C to F) and 4',6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei (C and D). Representative images from 5 cloned cows (panels C and E) and 6 noncloned cows (panels D and F) are shown after DAPI staining (C and D) and TUNEL labeling (E and F). The percentage of apoptotic cells detected by the TUNEL assay was 2.6-fold higher in cloned cows than in noncloned cows ($P < 0.001$). Significant differences are indicated by a letter and nonsignificant differences by NS. Color version available online.

Methylation Profile Around a Distal Regulatory Region of the *CSN1S1* Gene and *DNMT1* mRNA Level in Total RNA

The methylation levels of 3 CpG (CpG1, CpG2, and CpG3) were evaluated by pyrosequencing after bisulfite conversion in the mammary gland biopsies collected

around d 67 of lactation. In 5 out of 7 noncloned cows, methylation levels varied between animals, with CpG1 being the least methylated and CpG3 the most methylated. Similar results were observed in 3 cloned cows, whereas in the other 3, almost no differences between the methylation levels of the 3 CpG were observed. Thus, no significant differences were observed between

Figure 7. Transcript analyses and DNA methylation profiles. (A) Ratio of relative levels of mRNA coding for milk proteins, milk fatty acid metabolism enzymes sterol regulatory element-binding transcription factor 1 (*SREBF1*) and DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*) in mammary total RNA, in cloned cows ($n = 5$) versus noncloned cows ($n = 5$). The levels of each transcript in total RNA were normalized using both ubiquitously expressed prefoldin-like chaperone (*UXT*) and cyclophilin (*CCPL*) transcript levels, which did not vary among samples. For each transcript, the ratio between normalized results observed for cloned cows and noncloned cows is shown. Significant differences are indicated by stars (2 stars for $P = 0.01$). (B to D) DNA methylation levels of 3 CpG located in a distal regulatory region of the *CSN1S1* gene, close to 2 signal transducer and activator of transcription 5 (*STAT5*) binding elements. These methylation levels were evaluated in mammary biopsies of 6 cloned cows and 7 noncloned cows. No significant differences were observed. The box plots represent the median (inside the box), the 75th and 25th percentiles (upper and lower sides of the box), and the minimum and maximum values observed within 1.5-fold of the interquartile range and the minimum and maximum values observed (bars and circles at the end of whiskers, respectively).

the average methylation levels of the 3 CpG in cloned cows (43.3, 47.3, and 48.1% for CpG1, 2, and 3, respectively) and noncloned cows (38.8, 46.3, and 50.7% for CpG1, 2, and 3, respectively; Figure 7, lower panel).

Maintenance of the methylation profile in a given tissue is related to the activity of DNA (cytosine-5)-methyltransferase 1 (**DNMT1**). Although this activity is not strictly linked to *DNMT1* mRNA levels in the tissue, a significantly higher or lower level of this mRNA might have an effect on the enzyme level and this is easy to evaluate. Indeed, a significantly higher level of *DNMT1* mRNA was observed in the mammary biopsies of cloned cows ($P = 0.05$; Figure 7 upper panel) and a slight difference was still observed when the cow that had a high SCC on the morning of the biopsy was not included in the analysis ($P = 0.1$).

DISCUSSION

The results described above were obtained in cows cloned from a single cell line. Cloning efficiency (14%), a higher BW at birth, and the number of newborn calves dying during the perinatal period from different pathologies (6/15 newborn calves) were well within the range of previous results obtained using the same cell line (Bui et al., 2009), as well as for other cell lines produced by the same laboratory (Heyman et al., 2007a).

By studying cloned cows that were all derived from a single cell line, we were able to build on data from previous studies and show that during the first 18 mo of life, there was a slower increase in BW among cloned cows compared with noncloned cows, so that by the age of 1 yr (at around puberty) this difference was no longer observed. Thereafter, reproductive performance was similar between the 2 groups, as previously described (Enright et al., 2002), but, at parturition, the BCS was lower in cloned cows. These results differed from those obtained on average among cows born from different cell lines. In surviving phenotypically normal cloned individuals, BW at 180 DIM has been reported to be higher compared with the controls (Bernard et al., 2015), with higher *Igf2* gene expression in the muscle (Yang et al., 2005) and a high variability of *Igf2* expression in some clones. This increase in BW was associated with a late onset of puberty (Heyman et al., 2007a; Wang et al., 2011). Other groups have also described a lack of difference in the average BCS at parturition or at 180 DIM (Bernard et al., 2015).

We were also able to show that milk production and milk protein yield or content did not differ between the 2 groups, as reported for cows cloned from different nuclear donor cells (Walsh et al., 2003; Tian et al., 2005; Bernard et al., 2015), even though higher protein

contents were reported in cloned cows derived from 1 of 4 nuclear donor cell lines (Heyman et al., 2007b).

During early lactation in our study, milk fat yield and milk fat content were lower in cloned cows, whereas, during the subsequent DIM, the slopes of milk fat yield curves did not differ and those of milk fat content curves were less steep. We therefore observed lower milk fat contents, but such variation may be strongly dependent on the cell line studied (Tian et al., 2005; Heyman et al., 2007b; Bernard et al., 2015). In our case, such differences could not be related to differences in raising conditions between cloned and noncloned animals, as mentioned in other studies (Walsh et al., 2003). The lower milk fat content during early lactation may be related to the lower BCS of the cloned cows, as described for noncloned cows (Roche et al., 2009). We also saw that the variability of protein and milk fat contents was more limited in cloned cows derived from a single cell line, presumed to be genetically identical. This extends previous results that showed less variability in milk yield only in one set of cloned cows (Heyman et al., 2007a). However, we cannot conclude that this remaining variability among cloned cows from a single cell line is due to the environment because cloned cows may display some slight genetic differences. This requires verification because genome defects leading to abnormal gene expression, chromosomal instability, and improper imprinting have been observed in such animals (McLean et al., 2010) and a study of the chromosomes of cloned cattle revealed that 2 out of 20 of them experience a significantly higher incidence (about 20%) of chromosomal abnormalities than the donor cell line (pseudodiploid, near-triploid, and tetraploid cells; Hanada et al., 2005).

Our results were consistent with the fact that milk fat yield and content were significantly lower in cloned cows versus noncloned cows at around 67 DIM. Interestingly, whereas no differences were observed in protein yields or contents throughout lactation, both levels were lower at around 67 DIM in the cloned cows. At this stage of lactation, lactose yield and milk lactose contents were also lower, which contrast with findings obtained elsewhere (Walsh et al., 2003). A low lactose concentration in milk has been reported to induce higher concentrations of other compounds (Stinnakre et al., 1994; Stacey et al., 1995). During the present study, despite moderate variations in lactose contents, it is interesting to note that such a relationship was not observed and that mechanisms other than local osmotic regulation might have been involved.

Low milk contents of proteins, fat, or lactose may be due to low milk contents of casein or mammary enzymes involved in milk synthesis. Interestingly, in

the mammary gland at around 67 DIM, no significant differences between cloned and noncloned cows were seen regarding the levels of accumulation of the transcripts coding for milk fat synthesis, lactose synthesis, or for 3 of the 4 different *CSN* transcripts. However, the lower level of *CSN1S2* transcripts coding for products corresponding to 12 to 16% of milk protein may have been linked to the lower protein production observed. As expected from the absence of variation in *CSN1S1* transcript levels, we did not see any differences in the DNA methylation profile of a regulatory region that has been linked to the expression of the corresponding *CSN1S1* gene (Nguyen et al., 2014). Unexpectedly, the accumulation level of *CSN1S2* was significantly lower in cloned cows. This was perhaps not surprising as regulation of this gene differs from that of other casein genes (Riley et al., 2010): it is expressed later during pregnancy than other *CSN* genes (Gao et al., 2013) and its promoter, at least in the mouse, is less potent in mammary cell culture (Kolb, 2002).

Although the levels of each milk-related transcript, except *CSN1S2*, did not differ significantly between the 2 groups of cows, their average values were lower in cloned cows and according to PCA, all the milk-related transcripts we analyzed segregated differently between cloned and noncloned cows ($P = 0.06$). The generally lower transcript level might be related to a global increase in methylation of the genome in cloned cows, which is likely to occur as the *DNMT1* transcript level rises. These results are similar to a recently published description of higher DNMT1 levels in the heart, liver, lung, kidney, and brain of cloned cows, and that also describe a relationship between high DNMT1 transcript levels and poorer milk quality (Wang et al., 2014).

The expression of *DNMT* genes, whose products can modify chromatin structure, has previously been described as being linked to the DNA methylation profile during development of the oocyte (O'Doherty et al., 2012) and mammary gland (Wang et al., 2014) in dairy cows. Taken together, our results show that animals born from the cell line we used had lactation phenotypes that differed slightly from those of animals born from other cell lines. These differences can be due to many biological factors, among which some epigenetic mechanisms may be involved.

At some time points in both groups, lower milk production was observed and linked to high milk SCC, which were frequently $>200 \times 10^3$ cells/mL. In noncloned cows, SCC were more frequently higher than the levels found in noncloned cows during a previous experiment run at another INRA experimental farm (Nguyen et al., 2014). These higher milk SCC might be related to a predisposition to subclinical mastitis, which are more frequent with SCC $>200 \times 10^3$ cells/

mL (Agabriel et al., 1997; Biggs, 2009). In turn, subclinical mastitis may be related to the lower milk production we observed during our study (mean value: 29 kg/d compared with 33 kg/d in the previous study) and could explain why the distal regulatory region we studied was more heavily methylated in biopsies sampled during our experiment compared with the previous study (Nguyen et al., 2014). High milk SCC are more common in cloned cows than in noncloned cows ($P = 0.09$). Such an increase in milk SCC was not always observed during other experiments (Norman et al., 2004). This discrepancy is not because the fibroblast cell line originated from a cow that was susceptible to mastitis. Indeed, the sires of noncloned cows had lower mastitis indices (Govignon-Gion et al., 2015) than the sire of the cow from which the nuclei originated. However, a higher incidence of inflammation in cloned cows might not be surprising, as the proportions of $\gamma\delta$ and WC1+ $\gamma\delta$ cells have already been shown to decline temporarily in cloned cows at an early stage of lactation or in young heifers, and blood neutrophil counts are higher (Tanaka et al., 2006; Heyman et al., 2007a). More frequent inflammation may also be related to an increase in the apoptotic cell rate found in the mammary tissue of cloned cows compared with noncloned cows. During our study, apoptotic cell rates reached intermediate values between those obtained in mammary tissue under the effect of feed restriction (Dessauge et al., 2011) and under once-daily milking (Boutinaud et al., 2013). This could be linked to the lower *CSN1S2* transcript levels we observed. Indeed, lower mammary *CSN1S2* levels may release potentially interacting proteins such as insulin factor growth binding protein 5 (IGFBP5) and plasminogen activator inhibitor (PAI), as previously described in rats (Tonner et al., 2000).

A reduction in cell viability has been linked to higher *DNMT1* transcript levels (Wang et al., 2014). The rise in DNMT1 levels that we observed might be related to an increase in mammary cell apoptosis, but may also improve maintenance of the DNA methylation profile after mammary cell renewal. The mammary cell renewal potential of cloned versus noncloned cows is currently under investigation.

CONCLUSIONS

Our results showed that milk composition differs slightly between cloned and noncloned cows. These results contrasted with those previously reported, perhaps because of the cell line we used. Cell line origin may therefore play a key role in the milk production of cloned cows. Throughout the 210 DIM period, we saw less variability in milk fat and milk protein contents among cloned cows than among noncloned cows, in

contrast to a similar variability in milk yields. Such a lower variability might be because all the cloned cows were derived from a single donor cell line and might therefore be genetically closer to each other than to noncloned cows. However, during our experiments, the differences between cloned and noncloned cows may also have been due in part to higher SCC, possibly related to a higher incidence of subclinical mastitis among the cloned cows, associated with differences in cell turnover and variations in DNMT1 mRNA.

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