DNA methylation and histone modification patterns during the late embryonic and early postnatal development of chickens

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ABSTRACT Early mammalian embryonic cells have been proven to be essential for embryonic development and the health of neonates. A series of epigenetic reprogramming events, including DNA methylation and histone modifications, occur during early embryonic development. However, epigenetic marks in late embryos and neonates are not well understood, especially in avian species. To investigate the epigenetic patterns of developing embryos and posthatched chicks, embryos at embryonic day 5 (E5), E8, E11, E14, E17, and E20 and newly hatched chicks on day of life 1 (D1), D7, D14, D21 were collected. The levels of global DNA methylation and histone H3 at lysine 9 residue (H3K9) modifications were measured in samples of liver, jejunum, and breast skeletal muscles by Western blotting and immunofluorescence staining. According to our data, decreased levels of proliferating cell nuclear antigen expression were found in the liver and a V-shaped pattern of proliferating cell nuclear antigen expression was found in the jejunum. The level of proliferating cell nuclear antigen in muscle was relatively stable. Caspase 3 expression gradually decreased over time in liver, was stable in the jejunum, and increased in muscle. Levels of DNA methylation and H3K9 acetylation decreased in liver over time, while the pattern was N-shaped in jejunal tissue and W-shaped in pectoral muscles, and these changes were accompanied by dynamic changes of DNA methyltransferases, histone acetyltransferases 1, and histone deacetylase 2. Moreover, dimethylation, trimethylation, and acetylation of H3K9 were expressed in a time- and tissue-dependent manner. After birth, epigenetic marks were relatively stable and found at lower levels. These results indicate that spatiotemporal specific epigenetic alterations could be critical for the late development of chick embryos and neonates.

Key words: chicken embryo, jejunal development, histone acetylation, DNA methylation

INTRODUCTION

Complicated changes take place in cells during embryonic development. Abnormal alterations can result in disordered fetal development and metabolism, as well as the inhibition of postnatal growth (Picone et al., 2011; Spencer et al., 1994; Wu et al., 2004). For example, obesity is associated with impaired embryonic or fetal programming (Lau and Rogers, 2004). During embryogenesis and subsequent development, a series of epigenetic changes occur that do not alter the basic structure of DNA. Epigenetic modifications mediated by DNA methylation, histone modifications, and noncoding RNAs have been shown to be essential in developing embryos (Hyldig et al., 2011a; Jia et al., 2012; Li et al., 2011; Liu et al., 2013; Park et al., 2009; Strakovsky et al., 2011; Wienholds et al., 2005).

DNA methylation of genomic CpG dinucleotides plays a crucial role in regulating and maintaining gene expression and genomic imprinting. The process of methylation is catalyzed by three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b). A methyl group is transferred from S-adenosyl-methionine (SAM) to the 5′carbon position of a substrate cytosine residue (Altmann et al., 2012; Wallwork and Duerre, 1985; Waterland et al., 2006). DNMT3a and DNMT3b, de novo methyltransferases, are responsible for methylating nonmethylated DNA during embryogenesis and cell differentiation (Turek-Plewa and Jagodziński, 2005). Dynamic changes of DNA methylation in developing embryos have been observed. In mouse zygotes, active and passive methylation depletion of the paternal genome occurs in early embryonic development, which is followed by de novo methylation after blastula periods (Kafri et al., 1992). It has been shown that demethylation of the male pronucleus is completed within 4 hours after fertilization (Santos et al., 2002), and demethylation contributes to the differentiation of mouse primordial germ cells (Maatouk...
and Resnick, 2003). Moreover, a recent report indicated that oocytes exhibited a lower level of methylation in the zygote than in sperm of mouse gametes (Smith et al., 2012). A similar result in bovine embryos at the blastocyst stage was found, and DNMT3b expression is regarded as an indicator for changes in DNA methylation status (Dobbs et al., 2013). Limited DNA methylation reprogramming in normal sheep embryos is pivotal for the development of nuclear transferred embryos (Beaujean et al., 2004a).

Histone modifications, including acetylation, methylation, phosphorylation, and ubiquitination, are vital for the maintenance of chromatin structure and the regulation of gene transcription. During modifications, the replacement of several amino acid residues of histone N-terminal tails is catalyzed by enzymes, such as histone acetyltransferases (HATs) and deacetylases (HDACs) (Turner, 2000). Lysine 9 of histone H3 (H3K9), which can be acetylated or methylated (including monomethylated, dimethylated, and trimethylated), plays an important role in embryonic development (Segev et al., 2001; Turner, 2000). A previous study showed that the levels of histone deacetylases and acetyltransferases are variable when bovine germinal vesicle oocytes develop into the blastocyst stage (McGraw et al., 2003). Inadequate deacetylation of histones during oocyte meiosis causes aneuploidy and embryonic death in mice (Akiyama et al., 2006). However, disruption of DNA methylation and histone deacetylation removes the block to apoptosis in bovine 2-cell embryos (Carambula et al., 2009). A similar phenomenon has been observed in porcine embryos both for in vivo and in vitro studies (Park et al., 2012). The asymmetric distribution of H3K9 dimethylation has been found in human zygote pronuclei, and H3K9 demethylation is more likely to occur in intracytoplasmic sperm injection-derived embryos with a low morphological grade than in that of in vitro fertilization counterparts (Qiao et al., 2010).

Numerous investigations have focused on global epigenetic patterns at the early stage of embryonic development; however, few attempts have been made to explore changes during the late stage of embryonic development and during the postnatal period, especially in avian species. Additionally, changes of intestinal morphology in developing chicks, as well as muscular and hepatic morphology in chicken embryos, have been documented (Larson et al., 1970; Yokouchi, 2005). Although microRNA expression patterns in chicken embryos have been examined during the early stage of development (Darnell et al., 2006), the underlying epigenetic mechanism responsible for the subsequent embryonic and postnatal development of chickens has not been elucidated. In early chick embryos, arsenic exposure can induce oxidative stress in the neural tube by modulating DNA methylation (Han et al., 2011), which can be attenuated by choline (Song et al., 2012). A similar epigenetic effect has been observed in the cadmium-induced ventral body wall defect in the chick model (Doi et al., 2011). Interestingly, DNA methylation and histone modifications are involved in the pluripotency maintenance and differentiation process of chick embryonic germ cells (Jiao et al., 2013). These data suggest that epigenetic modifications may be critical for embryo development. In the present work, using enzyme-linked immunosorbent assays and Western blotting, we determined the global levels of DNA methylation and H3K9 acetylation, as well as the expression of related enzymes in chicken embryos in the later stages of incubation and in postnatal chickens from days 1 to 21. Additionally, we examined cell proliferation and apoptosis by immunoblotting against proliferating cell nuclear antigen (PCNA) and caspase 3. Furthermore, the distributed patterns of dimethylation, trimethylation, and acetylation of H3K9 were examined by immunofluorescence staining and confocal localization.

**MATERIALS AND METHODS**

This study was approved by the Beijing Administration Committee of Laboratory Animals, which is under the leadership of the Beijing Association for Science and Technology (Permit Number: SYXK, Beijing, 2007-0023). All experimental procedures in this study followed the guidelines of the China Agricultural University Animal Care and Use Committee.

**Animals**

Fertile eggs from 44-week-old Ross 308 broiler breeders were collected from the Zhuozhou broiler breeder farm at the China Agricultural University (Hebei, China). Eggs (n = 540) were incubated at 37.8°C with 70 to 80% humidity and intermittent rotations. Chicks had free access to feed and water and were housed in wire cages, each with eight birds. No fewer than 50 chick embryos and 30 posthatch chicks were randomly obtained per stage. A 23-h lighting program was employed. The chicks were sacrificed using pentobarbital anesthesia on embryonic days 5, 8, 11, 14, 17, and 20 (E5, E8, E11, E14, E17, and E20) and days 1, 7, 14, and 21 posthatch (D1, D7, D14, and D21). Samples of jejunum, pectoral muscles, and liver were collected and stored at −80°C until use.

**Tissue Protein Extraction**

Tissue samples from the liver, jejunum, and skeletal muscles were homogenized in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM protease inhibitor cocktail, and protein phosphatase inhibitor (Roche Applied Science, Rockford, IL) and sonicated for 15 s at 4°C. The homogenate was centrifuged at 12,000 × g for 15 min at 4°C to remove cellular debris. The concentration of protein was determined by the BCA assay (Applygen Technologies Inc., Beijing, China). Histones were
extracted using a Total Histone Extraction Kit (#OP-0006, Epigentek Group Inc., USA).

**Histological Examinations**

Jejunal sections were dissected from pre- and posthatch chicks (6 chicks at each time point, 2 sections for each chick), fixed in 4% (w/v) paraformaldehyde, and embedded in paraffin. Next, 5-µm slices were cut and paraffin removed by being immersed in xylene and rehydrated in graded ethanol solutions. Sections were stained by hematoxylin and eosin (**H&E**) and observed under a light microscope (Leica LB30T, Germany). The villus height and width as well as crypt depth were measured from eight randomly selected villi and associated crypts on each section by Image Pro Plus 5.1 (IPP5.1) software. Villus height was estimated by measuring the vertical distance from the villous tip to villous-crypt junction level, whereas villous width was estimated by measuring the horizontal distance from one side of the brush border membrane toward the other side. The vertical distance from the villous-crypt junction to the lower limit of the crypt was considered to represent the crypt depth (Peterson et al., 2008).

**Western Blotting Analysis**

Briefly, 40 µg of tissue lysates were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA) using a semi-dry electro-blotting system (Bio-Rad Laboratories). The membranes were blocked in a 5% skimmed milk solution at room temperature for 1 h, and then incubated with primary antibodies at 1:400 to 1:2,000 dilutions in 5% bovine serum albumin. After washing three times (each for 10 min) in TBS (0.025 M Tris-HCl (pH 7.6), 0.137 M NaCl) plus 1% milk and 0.1% Tween-20 (TBST), membranes were incubated with peroxidase conjugated secondary antibody (CoWin Biotech Co., Ltd., Beijing, China) diluted in TBST (1:2,000) at room temperature for 1 h, followed by washing in TBST for 10 min and in TBS three times (5 min each). Signals were detected with a Super Enhanced Chemiluminescence kit (Applygen Technologies Inc., Beijing, China) and films were scanned using a gel documentation system (Bio-Rad Laboratories). The quantification of band density was determined using Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Antibodies were used against DNMT3a (Abcam, CA, UK), DNMT3b (Santa Cruz, CA), HAT1 (Abcam), HDAC2 (Abcam), β-actin (Abcam), GAPDH (Abcam), H3K9Me3 (Abcam), H3K9Me2 (Abcam), H3 (Abcam), H3K9Ac (Millipore, MA), PCNA (Sigma, CA) and caspase 3 (Santa Cruz, CA).

**Global Methylation Assay**

Genomic DNA extracted from liver, jejunum, and skeletal muscle samples was extracted using a DNeasy Blood and Tissue Kit (69506, Qiagen, CA), and the DNA concentration was determined using an Eppendorf Biophotometer AG22331. DNA with an A260/280 nm ratio ranging from 1.8 to 2.0 was considered pure and used for further assays. The global level of DNA methylation was measured with the MethylFlash Methylated DNA Quantification Kit (Colorimetric, #P-1034, Epigentek Inc.), according to the manufacturer’s instructions. The amount and percentage of methylated DNA (5-mC) in the total DNA was calculated based on a standard curve. The amount of methylated polynucleotide containing 50% of 5-methylcytosine (5-mC) was used as a positive control, which was subtracted from each standard.

**Immunofluorescence Staining**

Jejunal and pectoral skeletal muscle sections (3-µm) were cut, dried, and fixed in 4% paraformaldehyde for 15 minutes. Non-specific antibody binding was blocked with 10% normal goat serum. Next, sections were incubated with H3K9Me3, H3K9Me2, and H3K9Ac antibodies at a 1:200 dilution, followed by secondary antibodies, including anti-mouse IgG (H+L) (F(ab’)2 fragment (Alexa Fluor® 488 Conjugate), #4408, 1:200, Cell Signaling Technology, USA), and anti-rabbit IgG (H+L) (F(ab’)2 fragment (Alexa Fluor® 488 Conjugate), #4412, 1:200, Cell Signaling Technology). Finally, sections were viewed with a Leica TCS SP5 confocal microscope at 400× magnification.

**Statistical Analysis**

The data was analyzed using one-way ANOVA by SPSS statistical software (SPSS for Windows, version 17.0, Chicago, IL, USA). After overall significance was determined, a Duncan post hoc analysis was performed for multiple comparisons. Results are presented as mean ± standard error of the mean (SEM). A *P*-value of <0.05 was used as the threshold for statistical significance.

**RESULTS**

In the jejunal samples, few intestinal follicles and columnar cells were found, and the intestinal villus was very short during the relatively early embryonic stage (E11). The villous height within the jejunum gradually increased in pre-hatch chicks (*P* < 0.001), but subsequently decreased at D1 (*P* < 0.001) and elevated sharply in posthatched chicks (*P* < 0.001). Crypt depth changed in a similar manner to villous height (*P* < 0.001), except an increase on D1 when compared to that on E20. Villous width at E14 was higher than at
E11 and lowest at E17, but it subsequently increased quickly, especially in neonatal chicks (P < 0.001). In addition, the number of enterocytes and columnar cells increased. However, the ratio of villus height to crypt depth initially rose to a peak at E17 from E11 and then decreased and stabilized at approximately 4.97 at D7 (Figure 1).

Levels of cell proliferation in the jejunum and the protein expression of PCNA in liver, jejunum, and skeletal muscles are shown in Figure 2. Before hatching, PCNA expression in the livers slowly decreased followed by an increase at D1, and then declined after hatching (P < 0.001). In embryos and chicks before D7, PCNA expression in the jejunum decreased, followed by increased levels of expression to D21 (P < 0.001). The PCNA level in muscles was low before E11, but sharply increased at E14 (P < 0.001) and was then maintained at a stable level.

Caspase 3 expression in the livers increased from E5 to E8 and then steadily decreased (P < 0.001) (Figure 3A, B). However, the level of caspase 3 in the jejunum increased from E11 to E17, decreased at E20 (P < 0.001) and was then stable until D7 followed by another increase (P < 0.001) (Figure 3C, D). Before D14, the fold change of caspase 3 expression in skeletal muscles increased from 1.0 to 11.0, but decreased to 5.0 at D21 (P < 0.001; Figure 3E, F).

To explore the temporospatial changes of DNA methylation, we first examined the global level of genomic methylation (Figure 4B, D, and F). The global methylation level in the jejunum (5-mC% >3.15%) was higher compared with that recorded in the liver and skeletal muscle (5-mC% <1.56%). The methylation level in liver rapidly decreased from E5 to E14 (P < 0.001) followed by a steady state level until D7; however, methylation increased at D14 until a decrease at D21 (P < 0.001; Figure 4B). The 5-mC% level in the jejunum sharply increased during development and peaked at E14, before decreasing rapidly to a minimum at E20, and finally increasing steadily until D21 (P < 0.05; Figure 4D). The 5-mC% level in muscle tissue was stable at E8 and E11 with a decrease at E14, but quickly reached a peak of 1.48 at E17, followed by a decrease to 0.64 at D7 and an increase to 1.0 (P < 0.05; Figure 4E).

Subsequently, the DNA methyltransferases, DNMT3a and DNMT3b, were detected. As shown in Figure 4A, C, and E, the levels of DNMT3a and DNMT3b expression in liver, jejunum, and skeletal muscles all decreased during embryo and chick
Figure 2. Protein expression of PCNA in chicken liver, jejunum, and muscle at different developmental stages. (A, C, E) Protein (50 μg of protein per lane) from liver, jejunum, and muscle were fractionated by SDS-PAGE, and the expression of PCNA was detected with an anti-PCNA antibody. (B, D, F) Fold change of PCNA levels were calculated from the first comparison of band density at various time points to the first time point followed by normalization of band density to β-actin or GAPDH expression. Means of different letters in the chart differ significantly (P < 0.05). P-values were calculated from the comparison of each mean at other time points to E11. Data are given as the mean ± SEM (n = 3).

Development as chicks matured. However, this result was not completely consistent with the global level of DNA methylation. The expression of DNMT3a in the liver and jejunum remained at a high level before E17, and continued to be expressed at a low level in livers, but DNMT3a levels were almost undetected in the jejunum after E17 (Figure 4A, C). DNMT3b expression in the jejunum was stable between E11 and D7, but was scarcely detected at either D14 or D21 (Figure 4C). However, the expression levels of DNMT3a and DNMT3b in muscles were maintained for longer than those of the liver and jejunum (Figure 4E). DNMT3a and DNMT3b expression was highest at E11 and D1, with little expression observed at D7 and D21.

Levels of DNA methylation were not perfectly parallel with the expression of DNA methyltransferases; therefore, we measured global levels of histone acetylation by immunoblotting. The fold changes of H3K9 acetylation in liver significantly decreased from 1.05 at E8 to 0.5 at E14. This was followed by an increase at E17, a reduction to 0.3 at D1, and escalated H3K9 acetylation to approximately 0.7 between D7 and D21 (P < 0.05; Figure 5B). The level of H3K9 acetylation in the jejunum increased during E11 to E17 and then decreased sharply at D1, followed by an increase at D14 (P < 0.05; Figure 5D). H3K9 acetylation in muscles exhibited similar changes as those observed for DNA methylation. Fold changes of H3K9 acetylation
Figure 3. Expression of caspase 3 and p-Akt in the liver, jejunum, and muscle of embryos and chicks. (A, C, E) Tissue lysates from liver, jejunum, and skeletal muscle were analyzed by Western blot with antibodies against caspase 3 and p-Akt at various time points. (B, D, F) Fold change of caspase 3 and P-Akt levels were calculated from the first comparison of band density at various time points to the first time point followed by normalization of band density to β-actin or GAPDH expression. Means of different letters in the chart differ significantly (P < 0.05). P-values were calculated from the comparison of each mean at other time points to E11. Data are given as the mean ± SEM (n=3).

remained stable from E8 to E14 and rapidly reached the maximum value of 2.7 at E17; however, H3K9 acetylation soon decreased rapidly to 0.0 at D7 and increased to 0.5 from D14 to D21 (P < 0.05; Figure 5F). To examine the underlying mechanism of histone modifications, histone deacetylase (HDAC) and histone acetyltransferase (HAT1) protein levels were measured by Western blotting (Figure 5A, C, E). HDACs in livers were highly expressed from E14 to D7 and D21, whereas HAT1 expression gradually decreased during the entire range of developmental and posthatch stages (Figure 5A). HDAC2 expression in the jejunum was high from E11 to E17, decreased to an undetectable level on D1 and then steadily decreased from D7 to D21. HATs were highly expressed during chick development, with the exception of a decrease at D21 (Figure 5C). HDACs in muscles were abundantly expressed on E8, E11, and E14, but were expressed at a low level from E17 to D7, with little expression on D14 and D21. HAT1 expression was rich from E8 to D7 (Figure 5E).

Histone H3K9 modification changes in the jejunum and skeletal muscles were different from those observed in the liver; the patterns of H3K9Ac distribution in the jejunum and skeletal muscle cells are shown in Figures 6–11, as well as the dimethylation or trimethylation patterns of H3K9, which play an important role in histone acetylation. Dimethylated, trimethylated, and acetylated forms of H4K9 were predominantly expressed in jejunal lamina propria and submucosa, whereas a homogeneous distribution was present along the muscle cell nucleus in skeletal muscles. Cells were H3K9Me2-positive in the jejunum on E11, D1, and D21, and a small number of H3K9Me2-positive cells were observed on E20 (Figure 6). The number of H3K9Me3- and H3K9Ac-positive cells in the jejunum
was the highest at E14, D1, D21 (Figure 7), and E11 and D1 (Figure 8), respectively, and the lowest were found on E20 and D14 (Figure 7), and D7 and D14 (Figure 8), respectively. There was no significant difference in levels of dimethylation, trimethylation, and acetylation of H3K9 among other time points. In skeletal muscles, the highest levels of H3K9Me2 and H3K9Me3 expression were observed at E8, E11 and E14, D20 (Figure 9), and E8 and E14 (Figure 10), respectively. Muscle cells had low expression levels of H3K9Me2 and H3K9Me3 at E17 and D14 (Figure 9), and E11, E20, D7, and D14 (Figure 10), respectively. As illustrated in Figure 11, the highest proportion of labeled acetylated H3K9 in muscles was observed at E8 and E11, and fewer H3K9Ac-positive cells were found on E11, D7, and D21.

**DISCUSSION**

The staging of chick gut development from embryos aged 2.5 to 10 days has been previously determined by Southwell (2006) (Southwell, 2006). Subsequently, intestinal looping morphogenesis at early stages of development in the chick embryo was demonstrated by Savin et al., (2011) (Savin et al., 2011). However, limited data are available that describe the complete morphological alterations of the small intestine during embryonic and neonatal chick development. In the present study, gradually increasing villous heights and crypt depths were observed in the jejunum, which could result from enhanced cellular differentiation or proliferation (Noah et al., 2011). A previous study showed that elevated
Figure 5. Expression of histone acetyltransferase (HAT), deacetylase (HDAC2), and H3K9 acetylation (H3K9Ac) in liver, jejunum, and muscle during chicken development. (A, C, E) Expression of HATs, HDACs and H3K9Ac was measured by Western blot, and β-Actin, GAPDH, and H3 were used as loading controls. Fold change of acetylated H3K9 was calculated from the first comparison of band density at various time points to the first time point followed by normalization of band density to β-actin or GAPDH expression. Means of different letters in the chart differ significantly (P < 0.05). P values were calculated from the comparison of each mean at various time points to E11. Data are given as the mean ± SEM (n = 3).

levels of cell proliferation in the caudal region of chicken embryos account for the differential growth in this region (Sanders et al., 1993). In particular, villus height and PCNA protein levels declined in neonatal birds after hatching compared to those determined in embryos at E20. Newly hatched chicks were transitarily fasted to adapt to exogenous feed and circumstances. Fasting in the immediate posthatch period could depress small intestinal enterocyte proliferation and villus development in chicks (Geyra et al., 2001; Sklan, 2001). In the present study, PCNA levels in the jejunum were maintained at stable levels before birth, but exhibited V-shaped changes in postnatal chicks and reached a nadir at D7 (Figure 2C, D). A previous study demonstrated a rapid transition from total jejunal epithelial cell proliferation to more than 80% of total proliferating cells in the crypts in chicks after 108 hours posthatch (Uni et al., 2000). Consequently, it appears that most enterocytes along the villus are no longer proliferating after 5 days posthatch. In liver tissue, PCNA levels decreased gradually in hatching embryos. Similar decreasing levels of PCNA-positive cells in chick livers...
from E4 to E8 were reported previously by Suksaweang et al., in 2004 (Suksaweang et al., 2004). At hatching, there were higher activities of some enzymes involved in lipogenesis, glycolysis, and glycogen metabolism to maintain energy utilization (Raheja et al., 1971). Thus, the rapid increase of PCNA protein levels in the newly hatched broilers may be essential for hepatocyte renewal and function. These data indicate that cell proliferation in the livers and jejunum of early chick embryos was prominent and tissue-specific. Conversely, the PCNA protein levels in pectoral muscles appeared to be steady, although levels were low during E8 and E11.
Figure 8. Expression patterns of H3K9Ac in chick jejunum during different developmental stages. Acetylated histone H3K9 was detected by immunofluorescence staining using the H3K9Ac antibody. The red arrow indicates the target protein, H3K9Ac, and the yellow arrow indicates the nucleus. Bars, 100 μm. Full color version is available online.

(Figures 2E and 3F). Incubation days 10 through 15 in chick embryo is a critical period for myofiber clusters to segregate and secondary myofibers to demarcate, and cell death is most pronounced in muscle at this stage (McClearn et al., 1995). Therefore, this period might partially account for the limited expression of PCNA in skeletal muscles at 11 days of incubation. Although there were no significant difference in muscles posthatch, PCNA expression declined after 7 days of incubation. PCNA’s mRNA levels of pectoral muscles of layer and broiler chicks were reduced from embryonic days 16, which was inconsistent with previous data (Al-Musawi et al., 2011). Furthermore, Pax7, an early marker for proliferating myoblasts, was prominently expressed in pectoralis muscle on day 1 posthatch and decreased further on days 3 and 6 (Halevy et al., 2004). In neonates, the feeding-induced stimulation of muscle protein synthesis and subsequent rapid growing protein mass in muscles are prominent (Davis and Fiorotto, 2009). These findings, combined with the rapid decline in number of satellite cells after 5 days posthatch (Halevy et al., 2001), suggest that the differentiation and growth of muscle during postnatal development of chicks play a major role compared with cell proliferation.

Apoptosis is another important process during embryo development and growth. The accumulation of caspase 3 mRNA accompanied by an induction of caspase 3-like activity can result in cellular fragmentation in a subset of human preimplantation embryos (Jurisicova et al., 2003). A recent study in sows suggested that the disturbance of the balance between apoptosis and cell proliferation resulted in the long-term presence of follicles in the granulosa and theca cells of cystic follicles (Sun et al., 2012). By approximately E8 in mice, which is a similar gestation period in chicks, the primary liver bud is generated (Zhao and Duncan, 2005). In chicken livers, differentiation starts whereas proliferation is restricted from E5 to E8 (Suksawang et al., 2004). Hence, the pro-apoptotic caspase 3 protein was markedly upregulated in liver at E8 compared with E5, and subsequently decreased moderately (Figure 3A, B). These data along with PCNA expression indicate that hypertrophy but not differentiation is predominant in more developed liver tissue. In addition, high levels of caspase 3 expression was found in the jejunum (Figure 3C, D), which suggested a strong renewal capacity in gut. Moreover, caspase 3 expression in breast skeletal muscle was increased until D14 but decreased at D21 (Figure 3E, F). Indeed, there is a rapid increase in the body weight of chickens after 14 days of life (Barbato, 1991). Thus, apoptosis in muscle showed significant levels of decline at D21, and these findings are partially consistent with the PCNA data. The inhibition of PCNA expression by RNA interference in cultured 18.5-day post-coital mouse ovaries did not enhance the apoptosis of oocytes but downregulated the expression of caspase 3 (Xu et al., 2011). Therefore, cell proliferation and apoptosis may exist simultaneously. Furthermore, this tissue specific expression pattern of caspase 3 is coincident with previous observations in mice (Correia-da-Silva et al., 2004; Xu et al., 2011).

DNA methylation is important for the regulation of cell apoptosis and embryonic development (Beaujean et al., 2004b; Chestnut et al., 2011; Hyldig et al., 2011b;
Liang et al., 2012; Takebayashi et al., 2007). In chicks, inhibition of transmethylation can disturb neurulation in early embryos (Afman et al., 2005). The data presented in Figure 4B, D, and F clearly exhibited variable and tissue-specific global genomic methylation patterns during chicken development. Similar dramatic differences in DNA methylation have been observed during early the embryonic development of mice, sheep, and bovines (Dobbs et al., 2013; Santos et al., 2002; Young and Beaujean, 2004). In fact, dynamic genomic methylation in avian animals during aging may contribute to modulating the development of specific organs (Gupta et al., 2006). Particularly, DNA methylation in liver at E5, E8, and E11, jejunum at E11, E14, E17, and D21, and muscles at E17, E20, D14, and D21 were higher than other stages, which was in accordance with levels of caspase 3 (Figure 3). This tissue-specific response is also present in dietary folate/methyl deficient rats (Pogribny et al., 2004). Increased levels of DNA methylation, as well as DNMT1 and DNMT3a expression, have been shown to promote apoptosis (Chestnut et al., 2011). Thus, DNA methylation may be associated with cell apoptosis during chick development. DNA methylation requires three members of the DNA methyltransferase (DNMT) family, namely DNMT1 (maintenance methyltransferase), DNMT3a, and DNMT3b (de novo methyltransferases). DNMT1 can sustain genomic methylation status by remethylating hemimethylated DNA after DNA replication, whereas DNMT3a and DNMT3b methylate nonmethylated DNA during embryogenesis and cellular differentiation (Adams, 1995; Chen and Li, 2004; Fang et al., 2012; Ponsuksili et al., 2012; Steine et al., 2011). In the present study, DNMT3a expression in the livers and jejunum was markedly upregulated in early embryos, in agreement with their global DNA methylation and PCNA status (Figure 4A, C). Thus, early high genomic methylation may contribute to the proliferation of hepatocytes and enterocytes. Conversely, DNMT3a, DNMT3b, and global DNA methylation frequency were significantly decreased at E20 and D1 (Figure 4C, D). The days immediately before and after hatching are a critical period for the development and survival of chickens, which is likely to be affected by circumstances (Geyra et al., 2001; Oviedo-Rondon et al., 2008; Uni and Ferket, 2004). Therefore, our data indicated that the perinatal period may be crucial for the reprogramming of DNA methylation in chicks. Moreover, the global DNA methylation levels in gut were markedly higher than those in livers and muscle. This may be due to the rapid renewal of cells in the intestine (van der Flier and Clevers, 2009), which results in more frequent opportunities for DNA modifications. Additionally, there were also opposite changes between

Figure 9. Expression patterns of H3K9Me2 in chick skeletal muscle during different developmental stages. Dimethylated histone H3K9 was detected by immunofluorescence staining using the H3K9Me2 antibody. The red arrow indicates the target protein, H3K9Me2, and the yellow arrow indicates the nucleus. Bars, 100 μm. Full color version is available online.
Figure 10. Expression patterns of H3K9Me3 in chick skeletal muscle during different developmental stages. Trimethylated histone H3K9 was detected by immunofluorescence staining using the H3K9Me3 antibody. The red arrow indicates the target protein, H3K9Me3, and the yellow arrow indicates the nucleus. Bars, 100 μm. Full color version is available online.

DNMT3a, DNMT3b protein levels, and DNA methylation levels in muscle at E17, D14, and D21. DNMT3b can induce site-specific DNA methylation (Suzuki et al., 2006). In ducks challenged with hepatitis virus type 1, site-specific methylation in a cluster of the differentiation 8A promoter region has been found (Xu et al., 2014). Moreover, DNA methylation and histone modification pathways are closely linked to each other (Cedar and Bergman, 2009). Indeed, it has been reported that dynamic DNA methylation and histone acetylation are involved in the differentiation of chick embryonic germ cells (Jiao et al., 2013). Therefore, a site-specific DNA methylation pattern or other epigenetic marks are likely to participate in the regulation of DNA methylation during chick embryo development.

Aberrant acetylation of histones (H3K9Ac) can result in abnormal phenotypes of cloned mice (Suzuki et al., 2008). Lower levels of totipotency of embryonic stem cells have been highly associated with low H3K9Ac levels, whereas high H3K9Ac levels induced by HDAC inhibitors enhance the activity of target genes, as well as the activity and pluripotency of the extracellular matrix (Hezroni et al., 2011). Aberrant gene expression in later developmental stages in mice has been associated with alterations in histone modifications at the blastocyst axin1Fα locus during the preimplantation period (Fernandez-Gonzalez et al., 2010). In this study, there was a higher protein level of HAT1 in samples of liver, jejunum, and muscle in pre-hatch embryos compared with those of postnatal birds, and these were correlated with the abundance of H3K9Ac. These changes were in line with PCNA expression, which indicates histone acetylation may contribute to cell proliferation. Similar alterations among H3K9Ac levels and DNA methylation patterns suggest that both DNA methylation and histone H3 acetylation might be involved in regulating proliferation or apoptosis during chick development. Furthermore, our results demonstrated contrasting changes between DNA methylation and histone acetylation, which also occurs in chick embryonic germ cells (Jiao et al., 2013). In rats with intrauterine growth retardation, increased levels of histone acetylation and decreased DNA methylation were observed (Ke et al., 2006; MacLennan et al., 2004). In addition, cloned blastocysts of cattle showed inconsistent alterations of H3K9 hyperacetylation and DNA hypomethylation (Jafarpour et al., 2011). Furthermore, our data are consistent with previously demonstrated tissue- and time-specific DNA methylation and H3 acetylation patterns (Hyldig et al., 2011a; Pogribny et al., 2004).

Given that epigenetic modifications were asymmetric in jejunal and breast skeletal muscle samples from embryos and neonates, it is vital to understand the precise expressed patterns of acetylated H3K9 and
other modifications, such as dimethylated and trimethylated H3K9. Dimethylation, trimethylation, and acetylation at H3K9 were expressed differentially both temporally and spatially. Moreover, H3K9Me2, H3K9Me3, and H3K9Ac were located mainly at the base of the crypts, the apical membrane of the villus, and the lamina propria in the jejunum (Figs. 6–8). Therefore, H3K9Me2, H3K9Me3, and H3K9Ac are highly expressed in sites where stem cell populations are rich (Bjerkes and Cheng, 1981; Hua et al., 2012). Most importantly, epigenetic methylation and acetylation contribute to the regulation of embryonic stem cell differentiation (Lohmann et al., 2010; Markowetz et al., 2010). A high proportion of labeled H3K9Me2 was seen at E11, E14, D1, and D21 and a low percentage at E17, E20, D7, and D14 in jejunum, which was partially consistent with the results of H3K9Me3 and H3K9Ac levels. The present work, along with the above data, showed that levels of DNA methylation and H3K9 acetylation were high at E11, E14, E17, D14, and D21 in jejunal tissues, which strongly indicates that DNA methylation and histone modifications play a synergic role in intestinal development. In contrast, H3K9Me2, H3K9Me3, and H3K9Ac were highly expressed in muscles during hatching, with only low levels of expression observed in chicks after hatching, in line with global levels of DNA methylation and H3K9 acetylation. These results imply that both the methylation and acetylation of H3K9 regulate embryonic development. Notably, a study in zebrafish has shown that interactions between DNMT3 and the H3K9 methyltransferase, G9a, as well as the specific DNMT-histone methyltransferase networks can silence critical regulators of cell fate in a tissue-specific manner (Rai et al., 2010).

In summary, DNA methylation and H3K9 acetylation underwent dynamic reprogramming during embryonic and postnatal development of chicks in a time- and tissue-dependent manner, and these findings were associated with changes in PCNA and caspase 3 expression. These changes could be due to the altered protein expression of DNMTs, HAT1, and HDAC2. This is the first report regarding epigenetic reprogramming in chick embryos and early neonatal chickens. These epigenetic markers in chicken embryos not only provide novel mechanistic information about organogenesis, but also offer preliminary clues to elucidate the epigenetic effects of early nutrition or environmental factors on embryonic development and offspring health.

**Authors’ contributions**

Yuming Guo conceived, designed the experiments, and revised manuscript. Changwu Li performed the experiments and wrote the manuscript. Shuangshuang Guo helped with sample collection, measurement of
DNA methylation, and revision of the manuscript. Ming Zhang helped to do Western blotting. Jing Gao helped with revising the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

REFERENCES


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