

## Research Article

# Search for Conditions to Detect Epigenetic Marks and Nuclear Proteins in Immunostaining of the Testis and Cartilage

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Received 8 January 2014; Accepted 10 February 2014; Published 19 March 2014

Academic Editor: Luigi F. Rodella

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The localization of nuclear proteins and modified histone tails changes during cell differentiation at the tissue as well as at the cellular level. Immunostaining in paraffin sections is the most powerful approach available to evaluate protein localization. Since nuclear proteins are sensitive to fixation, immunohistochemical conditions should be optimized in light of the particular antibodies and tissues employed. In this study, we searched for optimal conditions to detect histone modification at histone H3 lysine 9 (H3K9) and H3K9 methyltransferase G9a in the testis and cartilage in paraffin sections. In the testis, antigen retrieval (AR) was indispensable for detecting H3K9me1 and me3, G9a, and nuclear protein proliferating cell nuclear antigen (PCNA). With AR, shorter fixation times yielded better results for the detection of G9a and PCNA. Without AR, H3K9me2 and H3K9ac could be detected at shorter fixation times in primary spermatocytes of the testis. In contrast to the testis, all antibodies tested could detect their epitopes irrespective of AR application in the growth plate cartilage. Thus, conditions for the detection of epigenetic marks and nuclear proteins should be optimized in consideration of fixation time and AR application in different tissues and antibodies.

## 1. Introduction

Tissue-specific factors are expressed exclusively in certain groups of cells during cellular differentiation and cell fate determination. Genes activated during differentiation are maintained in a transcriptionally competent state in chromatin, whereas genes that are not activated in a given lineage are maintained in a silenced state. The transcriptionally competent state is characterized by an open chromatin locus, which is accessible to tissue-specific factors. In a silenced state, transcriptionally inactive condensed chromatin is formed [1–3]. Open or closed chromatin structures are characterized by the acetylation or methylation of histone tails, which are referred to as epigenetic marks, as well as

chromatin-modifying nuclear proteins [4–6]. The modifications of histone tails are regulated by histone modification enzymes. For example, there are four methylated states at lysine 9 of histone H3: non-, mono-, di-, and trimethylated H3K9. These methylated states are determined by the balance of methyltransferases and demethylases. The key histone methyltransferases is G9a, which is a member of the Suv39h subgroup of SET domain-containing molecules [7]. G9a is responsible for the modification of H3K9me1 and H3K9me2, and affects chromatin status, leading to gene expression [8]. Thus, elucidation of the localization and abundance of epigenetic marks and chromatin-modifying factors is essential for understanding the epigenetic regulation of cellular differentiation.

Immunohistochemistry is the most powerful approach available to evaluate protein localization *in vivo* and *in vitro*. However, immunostaining often results in inconsistent findings. An important parameter of immunostaining is tissue fixation [9]. Formaldehyde and paraformaldehyde are suitable fixatives for immunohistochemistry since they have marginal deteriorative effects on tissue that can be reversed after extensive washing with an appropriate buffer [10]. However, the duration of tissue fixation strongly affects staining results, including those of nuclear proteins. To circumvent this problem, sections are subjected to antigen retrieval (AR), a treatment performed before immunostaining. AR is believed to restore the antigen structure modified by formaldehyde [11, 12].

In this study, we investigated the optimal conditions for the detection of epigenetic marks and nuclear proteins in the testis and growth plate cartilage in paraffin sections. Both tissues contain morphologically distinct differentiating cells: spermatogonia and primary spermatocytes for the testis and proliferating and hypertrophic chondrocytes for the growth plate cartilage [13–15]. As epigenetic marks, we chose methylated histone H3 lysine 9 (H3K9me) 1, 2, and 3, which are related to repressive chromatin, and acetylated histone H3 lysine 9 (H3K9ac) as an activated mark. As a key histone methyltransferase in H3K9 modification, we chose G9a. We also assessed localization of a nuclear protein, proliferating cell nuclear antigen (PCNA), which exclusively resides in nucleus.

We evaluated the duration of tissue fixation and requirement for application of AR. In the testis, we found that AR was essential for the detection of H3K9me1 and 3, G9a, and PCNA. In contrast, all antibodies tested in this study were able to detect their epitopes irrespective of AR application in the growth plate cartilage, suggesting that the requirement for AR differs between tissues. Fixation time affected the detection of certain nuclear proteins in both tissues.

## 2. Materials and Methods

**2.1. Antibodies.** The following antibodies were used in this study: anti-H3K9me1, anti-H3K9me2, anti-H3K9me3, and anti-H3K9ac (mouse monoclonal; prepared by H. Kimura); anti-G9a (PP-A8620A-00, mouse monoclonal; Perseus Proteomics; Tokyo, Japan), anti-PCNA (2586, mouse monoclonal; Cell Signaling; Beverly, MA), and anti-mouse IgG (H+L)-Alexa488 (A11001, goat polyclonal; Invitrogen; Carlsbad, CA). The specificity and sensitivity of these antibodies have been thoroughly tested in previous studies [16].

**2.2. Animals and Tissue Fixation.** Testes were excised from 2-week-old C57BL6 mice and fixed immediately with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3 h, 8 h, or 23 h at 4°C. We fixed two testes per one fixation condition. After fixation, the testis tissue was embedded in paraffin. For the growth plate cartilage, entire E16.5 embryos were fixed and embedded in paraffin (fixation of whole body

group), or the humeri were excised from E16.5 embryos, fixed, and embedded (fixation of isolated humerus group). Fixation was performed in PBS with 4% PFA for 3 h, 8 h, or 23 h at 4°C. We fixed two whole embryos for 23 h fixation condition and one embryo each for 3 h and 8 h condition, whereas fixed one isolated long bone per each fixation condition. Fixed tissues and bodies were dehydrated with 50%, 70%, 80%, 90%, 100%, 100%, 100% EtOH and Tissue-Tek Tissue-Clear (Sakura Finetek; Tokyo, Japan) (60 min each step) at room temperature (RT). Dehydrated tissues and bodies were infused with paraffin for 120 min at 65°C, deaerated for 120 min at 65°C, and embedded in paraffin. Sections with a thickness of 5 µm were placed on FRONTIER-coated glass slides (Matsunami Glass; Osaka, Japan).

**2.3. Immunohistochemistry and Capturing Images.** Sections were deparaffinized with two xylene washes (10 min), two 100% EtOH washes, a 90% EtOH wash, a 70% EtOH wash, and three distilled water washes (5 min each step) at RT. For the application of AR, the sections were subjected to microwaving in 10 mM citric acid buffer (pH 6) for 20 min. During microwaving, the temperature of the buffer was maintained at 80°C. The sections were then incubated for 20 min at RT. In the presence or absence of AR application, the sections were treated with blocking solution (1.5% goat serum in PBS) for 60 min at RT and incubated overnight at 4°C with individual primary antibodies: anti-H3K9me1 (1:1), anti-H3K9me2 (1:200), anti-H3K9me3 (1:200), anti-H3K9ac (1:200), anti-G9a (1:100), or anti-PCNA (1:100). After washing, the sections were incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:100) and 4',6-diamidino-2-phenylindole (DAPI) (1:10, CS-2010-06; Cosmobio; Tokyo, Japan).

The fluorescent images were captured with an oil objective lens (60x) by using a laser-scanning confocal microscope (FV-1000; Olympus; Tokyo, Japan) and analyzed using FLUOVIEW software (Olympus). Images of confocal z-stacks were acquired at 0.5-µm intervals, and a stack of the 7 images was merged into a projection. In all panels, DAPI-stained, antibody-stained, and merged images are shown.

**2.4. Counting and Calculation of the Signal-Positive Cells.** In the testis, cells with high-intensity DAPI staining, suggesting high DNA content, were referred to as primary spermatocytes. Cells with low-intensity DAPI staining lying near the basement membrane of the seminiferous tubule were referred to as spermatogonia (see Supplemental Figure 1 in the Supplementary Material available online at <http://dx.doi.org/10.1155/2014/658293>). Positive cells were defined as cells whose DAPI- and each antibody-stained signal could be merged. We manually counted cells in two different fields of view (100 × 100 µm) that contained one seminiferous tubule per area and then calculated the ratios of positive cells to DAPI-stained cells. In the cartilage, positive cells and DAPI-stained cells were manually counted in a selected area (100 × 200 µm) containing the prehypertrophic and hypertrophic chondrocytes, and the ratios of positive cells to DAPI-stained cells were calculated.

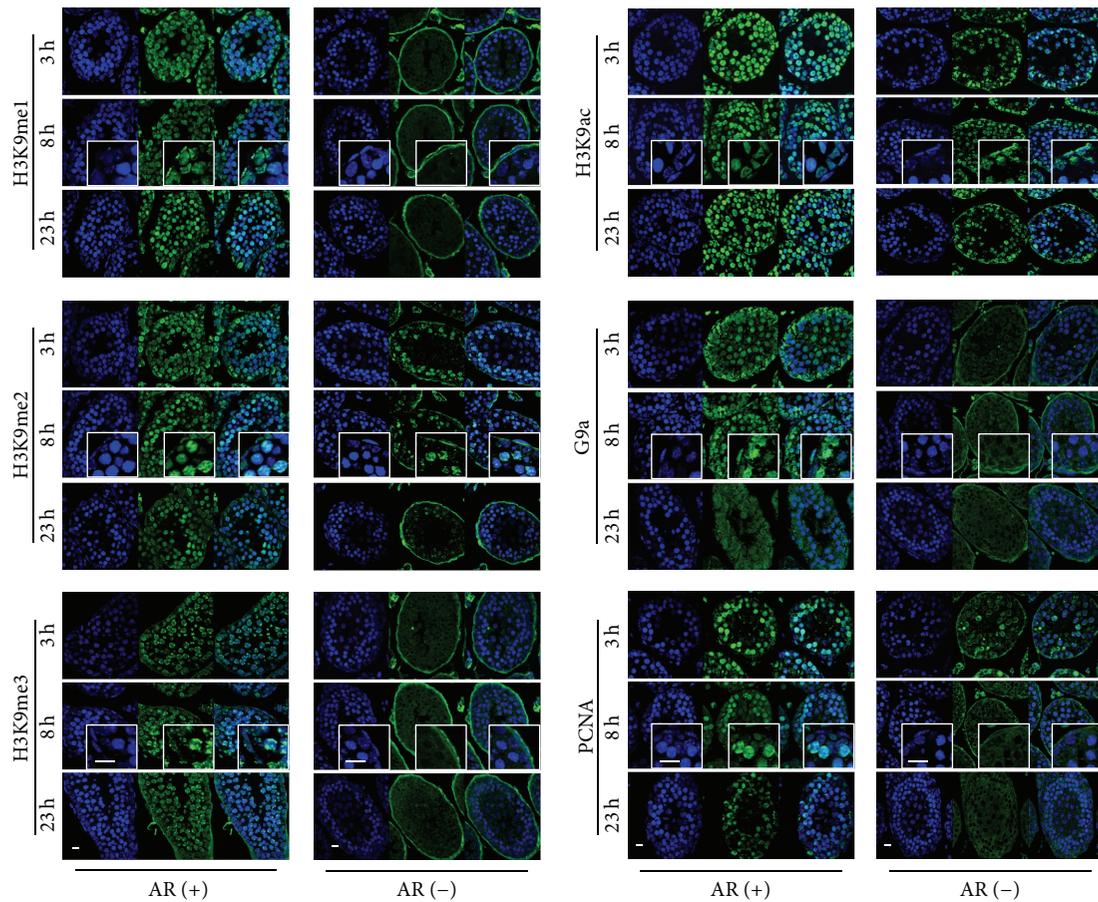


FIGURE 1: Localization of methylated H3K9, acetylated H3K9, G9a, and PCNA in the testis. Confocal images of immunohistochemical detection of H3K9me1, H3K9me2, H3K9me3, H3K9Ac, G9a, and proliferating cell nuclear antigen (PCNA) in the testis are shown. The testis was fixed in PFA for 3 h, 8 h, and 23 h fixation durations and was embedded in paraffin. Sections were treated with antigen retrieval (AR (+)) or without (AR (-)). All panels are shown as 4',6-diamidino-2-phenylindole (DAPI)-stained images (left, blue), antigen-stained images (middle, Alexa488, green), and merged images (right). Insets of higher magnification were shown in 8 h fixation group. The same experiments are performed twice in each condition and representative photos are shown. Scale bar = 10  $\mu$ m.

### 3. Results

Since nuclear proteins are primarily localized in the nucleus, where DAPI staining labels DNA, the results are displayed in three panels: DAPI-stained, antibody-stained, and merged (Figures 1 and 3). We counted the number of positive cells in which DAPI-stained and antibody-stained signals could be merged, and the ratios of positive cells to DAPI-stained cells are shown (Figures 2 and 4).

Cells in the premature testis are divided into two types: spermatogonia and primary spermatocytes. Spermatogonia are large precursor cells with low-intensity DAPI staining lying near the basement membrane. Primary spermatocytes have heterochromatic nuclei with high-intensity DAPI staining and are located between the basement membrane and the lumen of the tubule (Supplemental Figure 1). In the absence of AR application, signals corresponding to H3K9me1, H3K9me3, G9a, and PCNA were not detected in any nuclei in the spermatogonia and primary spermatocytes of the testis. The distinct staining around the peritubular

myoid cell layer, which surrounds the spermatogonia, seemed to be nonspecific since it was observed outside of the nuclei (see insets of 8 h fixation group in Figure 1). In primary spermatocytes, H3K9me2 was detected in the nuclei of all fixation groups, and the number of nuclei that were signal-positive for H3K9me2 was inversely correlated with the duration of fixation (Figures 1 and 2). In spermatogonia, H3K9me2 was barely detected in all fixation groups. On the other hand, H3K9ac was detected in many nuclei in all fixation groups in both the spermatogonia and primary spermatocytes of the testis (Figures 1 and 2).

AR application substantially improved the signal intensities and numbers of positive nuclei of all antibodies in the testis. H3K9me1, H3K9me2, H3K9me3, and H3K9ac were detected at all fixation times in both spermatogonia and primary spermatocytes when AR was applied (Figure 1). In accordance with previous reports [17–19], H3K9me1, H3K9me2, and H3K9ac were detected in regions of weak DAPI staining in the nucleus, while H3K9me3 was detected in DAPI-dense heterochromatic regions. The number of

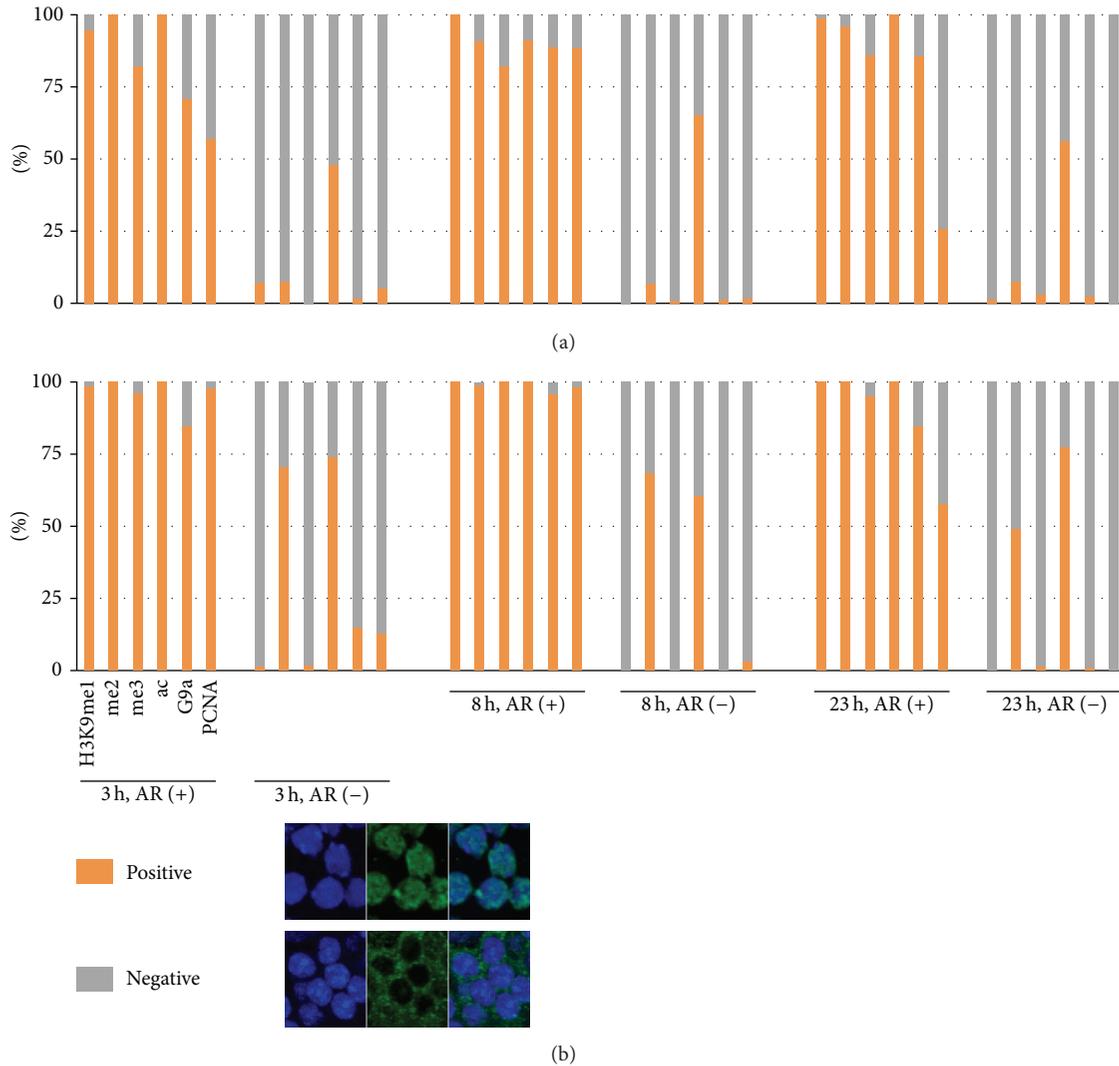


FIGURE 2: Ratios of antigen-positive and -negative cells under different conditions of fixation time and antigen retrieval for the detection of various nuclear antigens in the testis. The ratios of antigen-positive and -negative cells under each different condition are shown as percentage bar charts for spermatogonia (a) and primary spermatocytes (b). Orange: the proportions of positive cells per DAPI-stained cells, and gray: the proportions of negative cells per DAPI-stained cells. Antibodies used in this study, fixation time, and presence or absence of antigen retrieval ("AR+" and "AR-") are shown in the lower part of the figure. At the bottom, representative panels of positive and negative cells are shown.

signal-positive nuclei for PCNA was high in the 3 h fixation group and low in the 23 h group in both spermatogonia and primary spermatocytes (Figure 1). A higher intensity of G9a signals was observed in the 3 h fixation group compared with the 23 h group, whereas the number of signal-positive nuclei was similar in the three fixation groups.

We next examined protein localization in the embryonic growth plate cartilage. Whole E16.5 embryos (Figure 3(a)) and isolated long bones (humeri) from E16.5 embryos (Figure 3(b)) were fixed over three different durations and subjected to immunohistochemistry. All panels shown in Figure 3 are prehypertrophic and hypertrophic chondrocytes. In contrast to the testis, all antibodies tested were able to detect their epitopes in the growth plate cartilage irrespective of AR application and fixation conditions, in which fixation

was performed in whole embryos or isolated long bones. AR application significantly enhanced the signal intensity of certain epitopes, such as H3K9me1 and H3K9me3, in the 3-h fixation group (Figure 3). Interestingly, the 3 h fixation time negatively affected the signals corresponding to H3K9me1 and H3K9me3 compared with the 23 h fixation time (Figure 4). Fixation in whole embryos and isolated long bones gave rise to similar results.

#### 4. Discussion

In order to preserve morphological features, formalin fixation and paraffin embedding are the optimal procedures for morphological examination and immunohistology [9, 20]. However, formalin fixation leads to protein cross-linking,

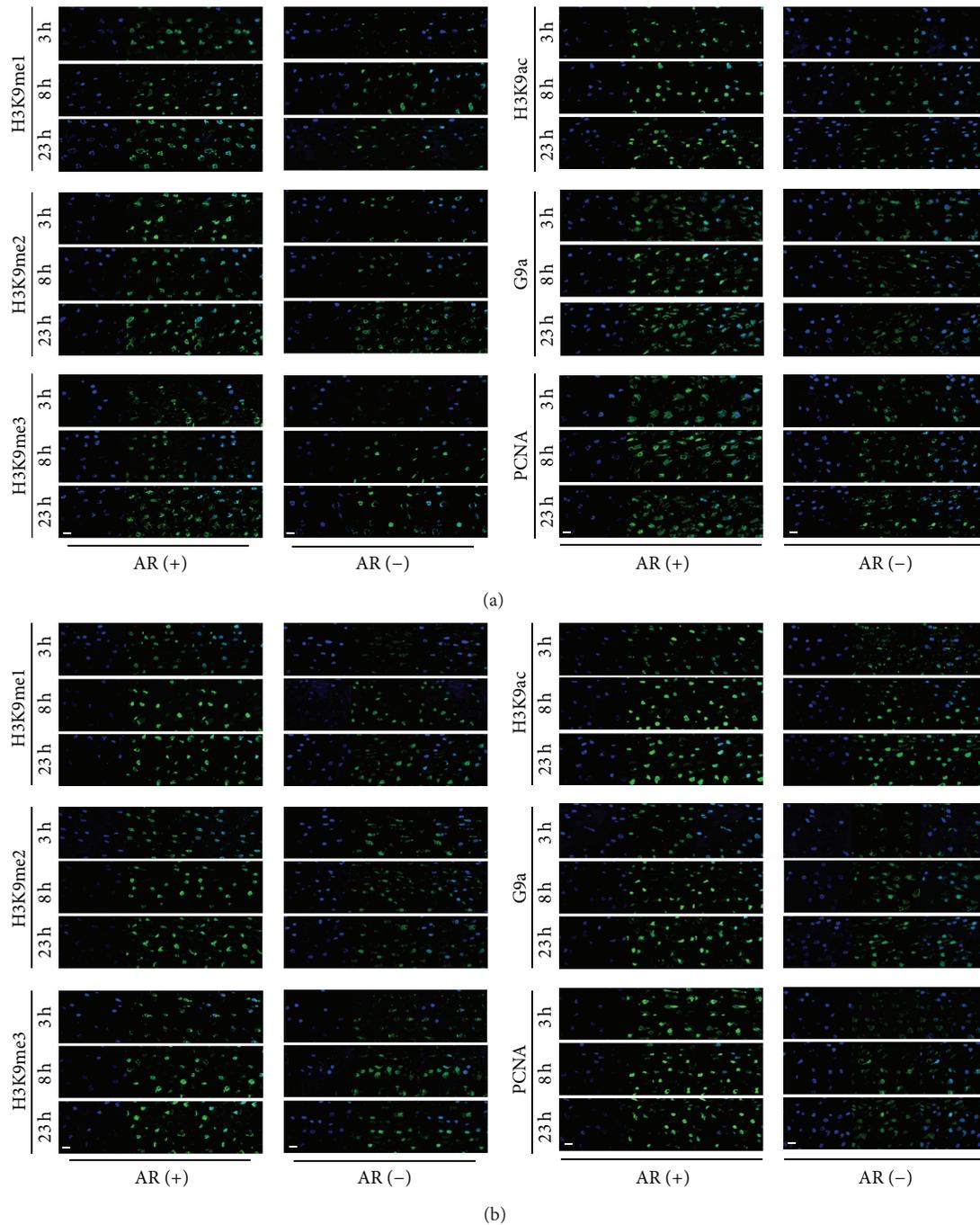


FIGURE 3: Localization of methylated H3K9, acetylated H3K9, G9a, and PCNA in chondrocytes at embryonic day 16.5. Confocal images of immunohistochemical detection of H3K9me1, H3K9me2, H3K9me3, H3K9Ac, G9a, and PCNA in chondrocytes at embryonic day 16.5 are shown. A whole embryo was fixed (a) or an isolated humerus was fixed (b), embedded in paraffin, and sectioned along the longitudinal plane of the humerus. Sections were treated with antigen retrieval (AR (+)) or without (AR (-)). Scale bar = 10  $\mu$ m.

which causes masking of antigenic epitopes. AR reverses this cross-linking, allowing the masked epitopes to be exposed to antibodies. Our study demonstrated that, in the testis, except for H3K9me2 and H3K9ac, intranuclear chromatin staining was hardly detected in the absence of AR. The non-specific staining of the nuclear envelope was often observed in H3K9me1, me3, and G9a, which may be caused by

inaccessibility of the antibodies (Figure 1). In the presence of AR, positive nuclear staining was observed with all antibodies. Therefore, AR is very effective for the detection of nuclear proteins in formalin-fixed tissues.

In the absence of AR application, a shorter fixation duration was favorable for the detection of H3K9me2 in primary spermatocytes, suggesting that longer fixation decreases

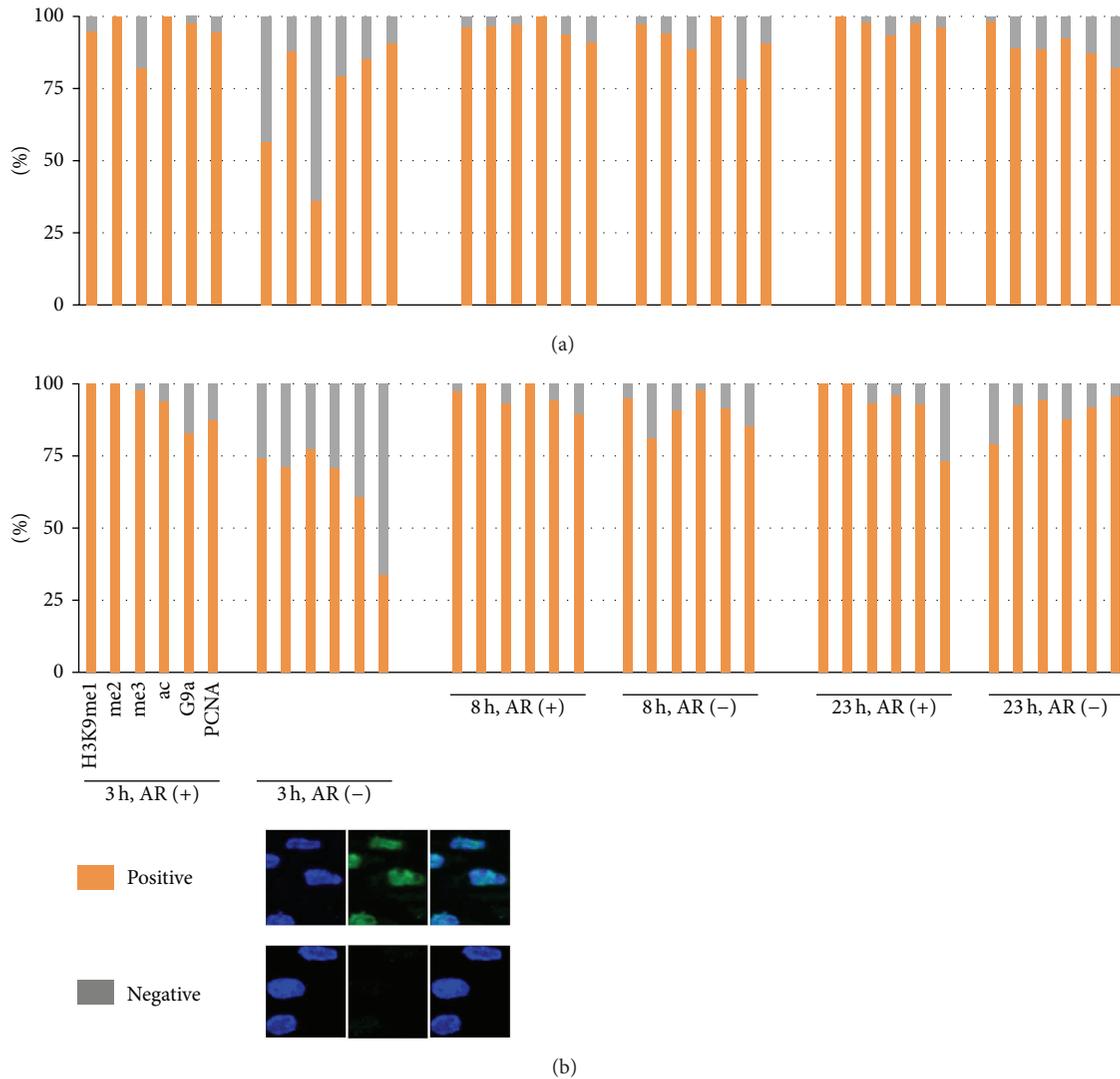


FIGURE 4: Ratios of antigen-positive and -negative cells under different conditions of fixation time and antigen retrieval for the detection of various nuclear antigens in the growth plate cartilage. The ratios of antigen-positive and -negative cells under each different condition are shown as percentage bar charts for fixation as a whole embryo (a) and fixation as an isolated humerus (b). Orange: the proportion of positive cells per DAPI-stained cells, and gray: the proportion of negative cells per DAPI-stained cells. Antibodies used in this study, fixation time, and presence or absence of antigen retrieval (“AR+” and “AR-”) are shown in the lower part of the figure.

epitope accessibility in these cells. In the presence of AR, longer fixation time led to inferior results for the detection of PCNA. Thus, fixation time should be adjusted for certain nuclear epitopes [21, 22].

Heat treatment of sections in the application of AR can cause tissue damage, particularly in the case of hard tissues such as bone, cartilage, and teeth, which can easily drop off from the slide glass after heating [10]. Thus, to preserve sections in their entirety, immunohistochemistry conditions without AR may be preferable for these tissue types. In the absence of AR, determining whether the antibody to be examined can target its epitope is a prerequisite.

An important finding of this study is that the effectiveness of AR differs between the two tissues, since the application of AR was indispensable for the detection of nuclear proteins in

the testis tissue, but not in cartilage. This difference may not be due to being less fixed in cartilage than the testis tissue, since fixation of cartilage in both whole embryos and isolated long bones gave rise to similar results. The finding here raises an intriguing question: what are the characteristic differences in the nucleus between the testis and cartilage with respect to antibody accessibility to nuclear epitopes? As shown in Figures 1, 3, and 4, in the absence of AR, antibodies directed against histone marks might not penetrate into the nuclei of testis cells, whereas they could penetrate into chondrocyte nuclei. This finding suggests that some structural or physical traits differ between testis and cartilage cell nuclei [5, 23]. Even in the testis, H3K9me2 could be detected without AR in primary spermatocytes, but not spermatogonia, suggesting that antibody accessibility differs between the two cell types.

Therefore, different characteristic features of the nuclei that determine antibody accessibility may exist in different stages of cell differentiation.

A recent study investigating reliable conditions for the detection of other epigenetic marks in the mouse retina revealed that although detection of the majority of the epigenetic marks tested was affected by the length of fixation, certain epitopes, such as H4K8ac, did not adhere to this general rule [24]. This study also pointed out the dependence of some epitopes on the length of AR time. The epitopes analyzed in our study may also depend on the length of AR time, which awaits further study for confirmation.

In conclusion, our results show that the application of AR is essential for the detection of most of the nuclear proteins and epigenetic marks tested in the testis but is nonessential for detection in embryonic cartilage. A shorter fixation time with AR is suitable for the testis tissue, whereas 8 h or 23 h fixation times, with or without AR, are suitable for the cartilage. Thus, conditions for the detection of epigenetic marks and nuclear proteins should be optimized in consideration of fixation time and AR application in different tissues and antibodies.

## Abbreviations

AR:	Antigen retrieval
H3K9me1:	Monomethylated lysine 9 at histone H3
H3K9me2:	Dimethylated lysine 9 at histone H3
H3K9me3:	Trimethylated lysine 9 at histone H3
H3K9ac:	Acetylated lysine 9 at histone H3
PFA:	Paraformaldehyde
PBS:	Phosphate buffered saline
PCNA:	Proliferating cell nuclear antigen.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgment

This work was supported by Grants to AN (22390344, 23659862, and 25670784) or KN (24592784) from the Ministry of Education, Science and Culture of Japan.

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