Oocyte-type linker histone B4 is required for transdifferentiation of somatic cells in vivo

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ABSTRACT The ability to reprogram in vivo a somatic cell after differentiation is quite limited. One of the most impressive examples of such a process is transdifferentiation of pigmented epithelial cells (PECs) to lens cells during lens regeneration in newts. However, very little is known of the molecular events that allow newt cells to transdifferentiate. Histone B4 is an oocyte-type linker histone that replaces the somatic-type linker histone H1 during reprogramming mediated by somatic cell nuclear transfer (SCNT). We found that B4 is expressed and required during transdifferentiation of PECs. Knocking down of B4 decreased proliferation and increased apoptosis, which resulted in considerably smaller lens. Furthermore, B4 knockdown altered gene expression of key genes of lens differentiation and nearly abolished expression of γ-crystallin. These data are the first to show expression of oocyte-type linker histone in somatic cells and its requirement in newt lens transdifferentiation and suggest that transdifferentiation in newts might share common strategies with reprogramming after SCNT.—Maki, N., Suetsugu-Maki, R., Sano, S., Nakamura, K., Nishimura, O., Tarui, H., Del Rio-Tsonis, K., Ohsumi, K., Agata, K., Tsonis, P. A. Oocyte-type linker histone B4 is required for transdifferentiation of somatic cells in vivo. FASEBJ. 24, 3462–3467 (2010). www.fasebj.org

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As stem or progenitor cells are diverted to differentiate toward a particular lineage, they lose their multipotentiality. At the endpoint, when they are terminally differentiated, cells are thought to be unable to revert to multipotent state. Contrary to this finding, the case of newt lens regeneration is a prime paradigm of transdifferentiation at work. After lens removal, the iris pigmented epithelial cells (PECs) dedifferentiate and then change their fate to become lens cells. Four to 5 d after lentectomy, the pigmented cells at the tip of the dorsal iris shed their pigments and start proliferating, thus losing their original tissue characteristics. Depigmented cells are observed initially around d 8. At 13 or 14 d after lentectomy, these depigmented PECs have formed a vesicle, which is still undifferentiated and expresses no lens-specific markers. The last step marks the onset of transdifferentiation and the formation of the lost lens. After d 14, the posterior cells of the vesicle elongate and start expressing lens markers. The vesicle grows and differentiates to lens, which by d 20 has a considerable size and normal morphology. It is important to state here that while the ventral iris PECs undergo some of the initial events, they fail to transdifferentiate to lens. Transdifferentiation of PECs has been directly demonstrated by clonal culture experiments (1, 2).

Since transdifferentiation should involve large-scale reprogramming, we hypothesized that histones, which are known to regulate gene expression and reprogramming (3, 4), would be good candidates to explore such possibility. In this study, we focused on linker histones. Four types of linker histone, i.e., somatic-, oocyte-, testis-, and erythrocyte-type linker histones, have been identified (5, 6). Oocyte-type linker histone, which is known to be expressed during oogenesis and early embryogenesis (7–12), is believed to play an important role in the reprogramming mediated by SCNT into oocyte. After SCNT somatic-type linker histone H1 is replaced by oocyte-type linker histone (13–15), which allows chromatin to be remodeled by an ATP-dependent chromatin remodeling factor (16) and causes genome-wide chromatin decondensation (17). Here we show that oocyte-type linker histone B4 is recruited specifically into nucleus and is necessary for lens transdifferentiation.

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MATERIALS AND METHODS

Cloning of newt B4 cDNA

Total RNA was extracted from *Cynops pyrrogaster* ovary using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the following primers: nucleostemin-qPCR-F, 5'-TGAGGCTCTAATCAACTCTGCCA-3'; nucleostemin-qPCR-R, 5'-CACTGCGGCCAAGAAAGAAACTGC-3'. The reaction was performed using a iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA), and the following primers, nucleostemin-qPCR-F, 5'-ATGTTTATCAGGAGCCTGGTCGGT-3'; nucleostemin-qPCR-R, 5'-AGATGGACGGACAC-GCCAGGGCATGACTGTAAGGT-3'. To quantitate the expression of each gene, Cq values were compared to a standard curve generated using a series of dilutions of cloned cDNAs. The amount of mRNA was normalized to that of ribosomal protein L27. Specific PCR amplifications were confirmed by melting curve analysis.

Vivo-morpholino

Vivo-morpholinos specialized to enter cells in living animals were purchased from Gene Tools (Philomath, OR, USA). After lentectomy, B4 vivo-morpholino (5'-AGCAGTCTCTCT-TAGAAGCCATTG3') or the control vivo-morpholino recommended by Gene Tools (5'-CTCTTACCTGATTAGAATTATA-3') was intraperitoneally injected at 12.5 μg/g body weight every day until different samples were collected.

Antibodies

Rabbit polyclonal antibody against newt B4 was raised against a mixture of two peptides, ALRKNDKRKAT and TDKD-SAKPTAKRGKK (see Supplemental Fig. 1A) and affinity-purified using the peptides (Scrumb Inc., Tokyo, Japan). The other primary antibodies used were histone H1 (V7013; Biomedia, South San Francisco, CA, USA), histone H3 (ab1791; Abcam, Cambridge, UK), and BrdU (MAB3510; Millipore, Billerica, MA, USA).

Immunohistochemistry

Eyeballs and ovaries were fixed with methanol-acetic acid solution (75% methanol and 25% acetic acid, v/v) at 4°C overnight, embedded in paraffin, and sectioned at 16 μm. After deparaffinization, sections were treated with permeable solution (0.05% Triton X-100, 0.05% saponin, and 2× SSC) for 1 h, rinsed with 2× SSC, and blocked in TSB buffer (0.1 M Tris-HCl, pH7.5; 0.15 M NaCl; and 0.5% blocking reagent) supplied with the TSA kit (Perkin Elmer, Waltham, MA, USA) for 1 h.

For detection of B4 and histone H1, the samples were incubated at 4°C overnight with a mixture of the primary antibodies, B4 antibody diluted 1:10 and histone H1 antibody diluted 1:100 with TSB buffer, and then incubated with the following secondary antibodies at room temperature for 90 min: Alexa Fluor 488-conjugated goat anti-rabbit IgG for B4 and Cy3-conjugated sheep anti-mouse IgG for histone H1. Nuclei were counterstained with Hoechst 33258. Images of stained tissue were taken using the BX-51 fluorescence microscope system (Olympus, Tokyo, Japan) equipped with a Cool SNAP cf2 camera (Photometrics, Tucson, AZ, USA). All images taken were saved in TIFF format. For measurement of signal intensity, the average signal intensity per pixel of B4, histone H1, and Hoechst33258 in each nucleus was measured using Metamorph 7.1 software (Molecular Devices, Sunnyvale, CA, USA) without any image processing.

For BrdU staining, the samples were incubated at 4°C overnight with BrdU antibody diluted 1:100 with TSB buffer and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG at room temperature for 90 min. To detect apoptotic cells, ApopTag Plus In Situ Apoptosis Fluorescence Detection kit (Millipore) was used according to the manufacturer’s instruction.

Western blot analysis

Tissues were homogenized in 10 vol of 70% PBS. Two volumes of 0.6N HCl (final 0.2 N) was added to the homog-
enatenate and kept on ice 30 min to extract histones. The supernatant was recovered after centrifugation at 10,000 g for 10 min at 4°C and dialyzed twice with 0.1 N acetic acid for 30 min and twice with milli Q water for 30 min, followed by overnight incubation at 4°C. The extracted proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane (0.2 μm pore size, Invitrogen). The B4 band was probed with anti-B4 antibody followed by alkaline phosphatase-conjugated anti-rabbit IgG (Abcam). The histone H3 band was probed with anti-histone H3 antibody followed by alkaline phosphatase-conjugated anti-rabbit IgG (Abcam). The bands were visualized by incubation with NBT/BCIP solution (Roche, Basel, Switzerland). Intensity of the detected bands was measured using ImageJ 1.40g software (U.S. National Institutes of Health, Bethesda, MD, USA). The amount of each histone in B4 morpholino-treated iris sample was calibrated using a standard curve generated by dilutions of control morpholino-treated iris sample. The amount of B4 protein was normalized to that of histone H3.

Animal study compliance

All animal care and use protocols were in compliance with the Animal Experiment Handbook at the Kobe Center for Developmental Biology (RIKEN Kobe) and the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

RESULTS

Expression of B4 during Newt lens regeneration

Initial immunohistochemical analysis using a Xenopus oocyte-type linker histone B4 antibody showed that antigens reacting with the antibody accumulate in nuclei of PECs during newt lens regeneration. This finding prompted us to clone a full-length B4 cDNA from two newt species, C. pyrrhogaster and N. viridescens (Supplemental Fig. 1). RT-PCR experiments clearly indicated that B4 is expressed during lens regeneration in both species (Fig. 1A). Antibody specific for newt B4 was raised using a mixture of newt B4 peptide sequences as antigens. Western blot analysis using this antibody also indicated that B4 is expressed during newt lens regeneration (Fig. 1B, C).

Nuclear recruitment of B4 during lens regeneration

Having established the presence of B4 during regeneration, we decided to follow expression of B4 and H1 throughout a period of 20 d after lentectomy. The results are shown in Fig. 2. In ovaries, B4 localizes in the germinal vesicle, while H1 localizes in nucleus of follicle cells (Fig. 2A). In intact iris (d 0), B4 is virtually absent, while H1 is present in PECs. On d 8, when dedifferentiation is ongoing, PECs are positive for both B4 and H1. Similar patterns were observed at d 12 (Fig. 2B). A clear pattern emerged when we quantitated the expression and plotted the ratio of B4 to H1 (see Materials and Methods). Starting at d 8, the ratio of B4 to H1 clearly increases in the dorsal iris vesicle. Then, this ratio reaches a peak at d 12 before lens differentiation begins. Finally, it declines by d 18, when the transdifferentiation process is completed and what continues is the growth of the lens (Fig. 2C). Such a peak of the ratio is not seen in the ventral iris.

B4 knockdown affects proliferation and apoptosis

These striking patterns of B4 recruitment prompted us to examine its role in more details. For this, we proceeded by knocking down expression of B4, employing vivo-morpholino technology (19). To assess whether B4 morpholino reduces expression of B4, we injected morpholino every day intraperitonealy for 10 d after lentectomy. Then, dorsal irises were collected, and linker and core histones were extracted with hydrochloric acid. The extracted histones were analyzed by Western blotting. The amount of B4 was calculated based on the intensity of the detected band and normalized with that of histone H3. In irises from newts treated with the B4 morpholino, the amount of B4 protein was decreased by nearly 50% when compared to the levels in irises from newts injected with control morpholino (Fig. 3A).
Following such a positive outcome of the morpholino treatment, we conducted a large-scale experiment by injecting morpholino and examining the regenerating lens for a period up to 20 d. We started seeing effects on lens differentiation and morphology after d 12, which correlated well with the expression and recruitment of B4. By d 20, we could conclude that the regenerated lens was considerably smaller in B4 morpholino-treated newts (Fig. 3B, E, F). On d 20, the lens size of B4 morpholino-injected newts was nearly half that of the control morpholino-injected newts (Fig. 3A). To investigate how B4 mediates such an effect on lens differentiation, we studied levels of cell proliferation and apoptosis in the regenerating lenses. BrdU was administered 24 h before fixation at different times, and its incorporation was analyzed by immunostaining. As expected, in control morpholino-injected newts the percentage of BrdU-positive cells was increased after 12 d. However, this percentage was significantly decreased in B4 morpholino-injected newts on d 16 and 20 (Fig. 3C, E). Next, Tunel staining was performed to examine whether lack of B4 leads to cell death. Indeed, it was shown that on d 20 significantly higher numbers of cells were undergoing apoptosis due to B4 morpholino treatment (Fig. 3D, F). Thus, so far our results clearly indicate an association between expression of B4 and lens transdifferentiation, which leads to a structurally normal lens. The reader should bear in mind that complete loss of B4 cannot result from the morpholino treatment; thus, even 50% decline in expression could elicit these results.

B4 is required for lens-specific gene expression during transdifferentiation

Despite the fact that the smaller lenses in B4 morpholino-injected newts can be attributed to an effect on proliferation and apoptosis, it is imperative to show whether expression of key genes known to regulate lens regeneration is affected by the B4 morpholino treatment. To examine this, we again injected newts for 20 d with B4 morpholino, collected only the regenerated lenses, isolated RNA, and examined the levels of several genes that are known to be structural and regulatory markers in lens differentiation and regeneration by qPCR. The expression of each gene was normalized with that encoding for ribosomal protein L27 so that we could account for the effect on lens size. Interestingly, we observed a dramatic down-regulation of γ-crystallin to 4% in regenerated lens from B4 morpholino-treated newts when compared to control. Likewise, we observed down-regulation of MafB and Pax-6, both known transcriptional factors that bind crystallin gene promoters (20, 21). On the contrary, we showed up-regulation of nucleostemin (Fig. 4). Nucleostemin, a nucleolar protein that has been found in stem cells and cancer cells (22), is expressed in PECs during dedifferentiation and disappears after lens differentiation (23). Thus, it seems that the lens in B4 morpholino-treated animals retains the status of an earlier stage. From the effect of B4 knockdown on gene expression, it is clear that B4 is required for lens-specific gene expression during transdifferentiation.
DISCUSSION

Transdifferentiation of PECs in newt lens regeneration is one of the most obvious examples of in vivo reprogramming of somatic cells. It is also known that nuclear reprogramming is induced artificially by somatic cell nuclear transfer (SCNT) into oocytes, where genome-wide chromatin decondensation is mediated by replacement of linker histone H1 with histone B4. We hypothesized that similar events to SCNT might occur during reprogramming of PECs to lens cells. To test this hypothesis, we first cloned and examined expression of B4 during the process of lens regeneration. Indeed, we show that B4 is expressed in newt somatic cells. This is the first time that B4 was found in somatic cells. All previous studies in many animals, including mice, zebrafish, frogs, and sea urchins, have shown that B4 is specific to oocyte and early embryo before the onset of zygotic gene expression (7–12).

In addition to showing expression of B4 in PECs, we have also demonstrated that B4 has a function during lens transdifferentiation. Knocking down B4 induces apoptosis and negatively affects cell proliferation and lens differentiation, as shown by morphological as well as molecular criteria. Notably, we found that B4 is specific to oocyte and early embryo before the onset of zygotic gene expression (7–12).

In addition to showing expression of B4 in PECs, we have also demonstrated that B4 has a function during lens transdifferentiation. Knocking down B4 induces apoptosis and negatively affects cell proliferation and lens differentiation, as shown by morphological as well as molecular criteria. Notably, we found that B4 is specific to oocyte and early embryo before the onset of zygotic gene expression (7–12).
replacement. According to this, B4 would replace H1 nonselectively. This replacement would cause genome-wide chromatin decondensation similar to the reprogramming mediated by SCNT (16, 17). Such chromatin decondensation might allow transcriptional factors to interact with the promoter region of lens differentiation genes. The other model is selective replacement. In this case, B4 would affect expression of specific factors that need to interact with H1 for regulation. An example of such regulation has been shown in the case where Msx1 cooperates with H1b for repression of MyoD to inhibit muscle differentiation (24). To address those hypotheses, we need genome-wide ChIP-on-chip analysis. Such an experiment is not possible at present because the new genome has not been sequenced.

Collectively, our expression and functional experiments identified a novel role of the linker histone B4. This is the first time that this oocyte-specific linker histone, which has been associated with reprogramming, was found to be expressed in adult somatic cells and, moreover, controls the process of transdifferentiation and lens regeneration. This finding suggests that reprogramming in germ cells and regenerating newt cells share similar strategies, thus providing a novel paradigm about cellular plasticity. In this case our results open new avenues for experimentation in regenerative biology.

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