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FBXW2 localizes with osteocalcin in bovine periosteum on culture dishes as visualized by double immunostaining

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Abstract

Osteocalcin (OC) is a well-known protein related to bone, however, the role of F-box and WD-40 domain-containing protein 2 (FBXW2) in bone remains unclear. In 2016, the presence of FBXW2 in bovine periosteum was reported. In this study, double immunostaining was used to investigate the relationship between OC and FBXW2. FBXW2 showed tubular structures, and OC showed a similar localization pattern as FBXW2. Double immunostaining findings suggested that FBXW2 tubes were coated with OC. To the author's knowledge, this is the first study to reveal the interaction between OC and FBXW2.

Keywords: Biochemistry, Cell biology, Structural biology

1. Introduction

Osteocalcin (OC) is involved in bone regeneration. However, the role of F-box and WD-40 domain-containing protein 2 (FBXW2) is unknown. Paraffin sections of bovine periosteum were prepared after 5 weeks of culture. Immunostaining

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confirmed that OC and FBXW2 are expressed in the bovine periosteum. However, the relationship between these proteins is unclear. Double immunostaining showed that FBXW2 has a tubular structure and that OC is present on the perimeter of FBXW2-containing tubes. This study suggests that FBXW2 localizes with OC on culture dishes.

F-box proteins promote the ubiquitin-proteasome system (Halbach et al., 2015; Murdoch et al., 2016; Sandoval et al., 2015; Teixeira et al., 2013; Trausch-Azar et al., 2015; Yalla et al., 2018; Zheng et al., 2016) and interact with cell cycle progression (Randle and Laman, 2016; Zhang et al., 2017). One F-box protein, FBXW2, has been identified as a housekeeping gene (Almeida et al., 2014), but the functions of FBXW2 are largely unknown. FBXW2 is expressed in placenta cells (Chiang et al., 2008; Wang et al., 2013) and lung cancer cell lines and tissue (Xu et al., 2017). A combination of mass spectrometry and immunohistochemistry has been used to analyze proteins expressed in periosteum tissue and cultured periosteum-derived cells, and FBXW2 expression has been confirmed along with other proteins (Akiyama, 2014a, 2014b, 2016). A previous study by Akiyama showed that FBXW2 is expressed in periosteum tissue (Akiyama, 2016). Periosteum tissue and cultured periosteum-derived cells are known to possess osteogenic ability (Li et al., 2016; Sung et al., 2015), and they have been studied in bone regeneration (Akiyama et al., 2006; Akiyama and Nakamura, 2009). The histology of the periosteum has been widely studied (Duchamp de Lageneste et al., 2018; Fan et al., 2010; Ferretti and Mattioli-Belmonte, 2014; Kim et al., 2016; Roberts et al., 2015) as either the periosteum in living bodies or immediately fixed tissue after death. Additionally, Akiyama (2016) studied periosteum tissue containing periosteum-derived cells after 5 weeks of culture on dishes.

Immunohistochemistry was previously performed using single staining (Akiyama, 2014a, 2014b, 2016), but immunostaining using more than one antibody in the same tissue has been used to characterize protein interactions (Isidro et al., 2015; Rozenvald et al., 2017). In this study, double immunohistochemistry was performed in order to determine the potential interaction between OC and FBXW2. The findings showed that both FBWX2 and OC were expressed in periosteum tissue. Furthermore, OC appeared to coat FBWX2 tubes.

2. Materials and methods

2.1. Periosteum extraction and section preparation

Periosteum tissue and sections were obtained as described previously (Akiyama, 2014b). The periosteum was removed from bovine legs (Kobe Chuo Chikusan, Kobe, Japan) at 24 h after death. All protocols were approved by Osaka Dental University animal experiments committee (approval number 17-02015) and

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complied with fundamental guidelines for proper conduct of animal experiment and related activities in academic research institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology (the Ministry of Education, Culture, Sports, Science and Technology directive 2006, Notice No. 71). To obtain periosteum-derived cells, the periosteum was used for explant culture on dishes with 10 ml Medium 199 (Gibco by Life Technologies, Grand Island, NY, USA) supplemented with ascorbic acid for 5 weeks. Both the periosteum and periosteum-derived cells were fixed in 4% paraformaldehyde, and paraffin blocks were prepared.

2.2. Double immunostaining

Three primary antibodies were used: anti-FBXW2 antibody (1:100, ab5309; Abcam, Cambridge, UK), anti-exosome complex component RRP45 (EXOSC9) (1:200, H-300 for 300 human amino acids; sc-135118; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and anti-osteocalcin (1:500, code no. M042, clone no. OCG2; Takara Bio Inc., Otsu, Japan). Anti-human procollagen type 1 c-peptide (PIP) (1:500, code no. M011, clone no. PC5-5; Takara Bio Inc.) and anti-Rho GTPase activating protein 36 (ARHGAP36) (1:100, D-14; sc-138596; Santa Cruz Biotechnology, Inc.) were used as negative controls. Anti-EXOSC9 antibody was reported to stain cell nuclei of bovine periosteum-derived cells (Akiyama, 2014b). Three secondary antibodies were used: mouse anti-goat IgG-AP (1:200, sc-2355; Santa Cruz Biotechnology, Inc.) with anti-FBXW2 antibody; N-Histofine Simple Stain AP (R) (#414251, Nichirei Biosciences Inc., Tokyo, Japan) with anti-EXOSC9, and N-Histofine Simple Stain AP (M) (#414241, Nichirei Biosciences Inc.) with anti-osteocalcin. PermaRed/AP (Diagnostic BioSystems, Pleasanton, CA, USA) and PermaBlue/AP (Diagnostic BioSystems) were used for visualization. Antigens in sections were activated with proteinase K (Dako Cytomation, Glostrup, Denmark).

Four double immunostaining procedures were carried out:

A. OC (blue) + EXOSC9 (red)

B. PIP (negative control; blue) + EXOSC9 (red)

C. OC (red) + FBXW2 (blue)

D. OC (red) + no FBXW2 (antibody diluent; blue)

Single immunostaining with FBXW2 was performed as previously described (Akiyama, 2016) and stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB; brown color). ARHGAP36 was set as a negative control of FBXW2 immunostaining. Finally, hematoxylin counterstaining was performed.

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3. Results

Akiyama reported that FBXW2 is expressed in the bovine periosteum (Akiyama, 2016) and recently found that FBXW2 forms tubular structures (Fig. 1a). The diameter of the tubular structures (arrows) was 1−2 microns. While FBXW2 expression was distinct in the periosteum after 5 weeks of culture (Fig. 1a), FBXW2 expression in bone and periosteum on day 0 was faint (Fig. 1b, c). Tubular structures were restricted to between the bone and periosteum, and parts of the tubular structure entered into the bone from the periosteum (Fig. 1b, c). FBXW2 peptide blocked reaction (Fig. 1d, e). Figure 2a, b shows a comparison of OC and FBXW2 staining. In Fig. 2a, double immunostaining of OC (blue) and EXOSC9 (red) coincidentally revealed areas of dense OC expression (arrows) and widespread OC expression in the periosteum. As both OC and FBXW2 were expressed in the periosteum, the relationship between OC and FBXW2 was subsequently investigated. In Fig. 2b, double immunostaining of OC (red) and FBXW2 (blue) revealed similar localization patterns for OC and FBXW2. Fig. 3a (condition A) shows outlined tubular structures of OC (blue). Fig. 3b (condition B) shows PIP (blue) did not form tubular structures. In

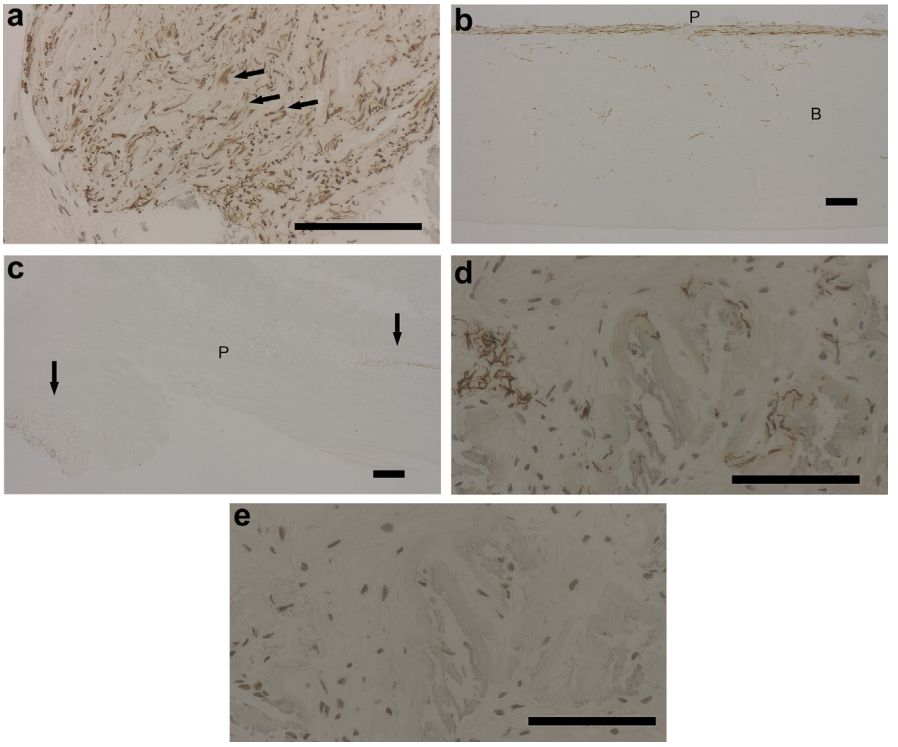

Fig. 1. (a) FBXW2 in bovine periosteum after 5 weeks of culture with periosteum-derived cells. FBXW2 immunostaining was previously reported in 2016 (Akiyama, 2016), but Fig. 1a is a unique image. (b) FBXW2 in bovine bone (day 0), (c) FBXW2 in bovine periosteum (day 0), (d) with FBXW2 antibody, (e) with FBXW2 peptide. Arrows: tubular structure of FBXW2, B: bone, P: periosteum. Scale bar: 100 μm.

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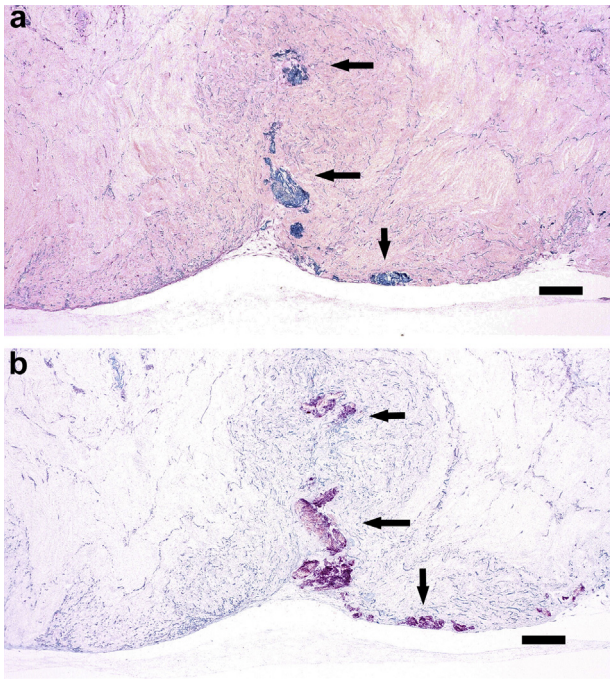

Fig. 2. Comparison of OC and FBXW2 localization. Both OC and FBXW2 showed similar staining patterns. Arrows: OC.

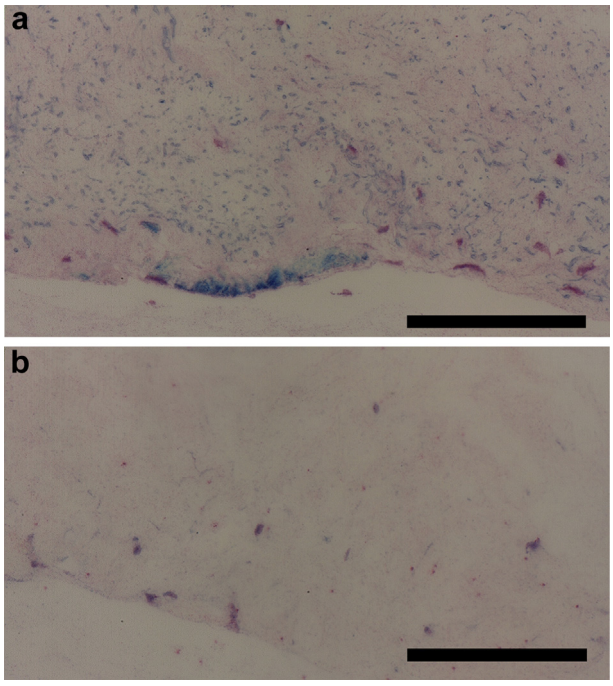

Fig. 3. (a, b) Double immunostaining under conditions A and B. (a) OC: blue + EXOSC9: red. (b). PIP (negative control): blue + EXOSC9: red. Scale bar: 100 μm.

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Figure 3a, b EXOSC9 (red) expression was observed in periosteum cell nuclei. Figure 4a–d shows the comparison of OC and FBXW2 localization. Figure 4a, c (condition C) showed that OC (red) is present on the perimeter of tubular structures of FBXW2 (blue). Figure 4b, d (condition D) showed that, because OC was thinly deposited, OC expression in condition C was indistinct. ARHGAP36 did not form tubular-shaped structures (Fig. 4e).

4. Discussion

In this study, the potential interaction between OC and FBXW2 were investigated using double immunostaining. The host species of antibodies against OC, EXOSC9, and FBXW2 are mouse, rabbit, and goat, respectively, which prevented potential unexpected interactions (Isidro et al., 2015). OC and PIP antibodies are mouse monoclonal IgG1 and were diluted 1:500. As in condition B, PIP did not express tubular structures and was thus used as a negative control. FBXW2 and ARHGAP36 antibodies are polyclonal goat IgG and were diluted 1:100. ARHGAP36 was also used as a negative control. For double immunostaining using EXOSC9, OC and EXOSC9 were stained in different ways to show the difference between OC and EXOSC9 staining (Fig. 3a). In conditions A, C, D, OC staining was carried out first as the

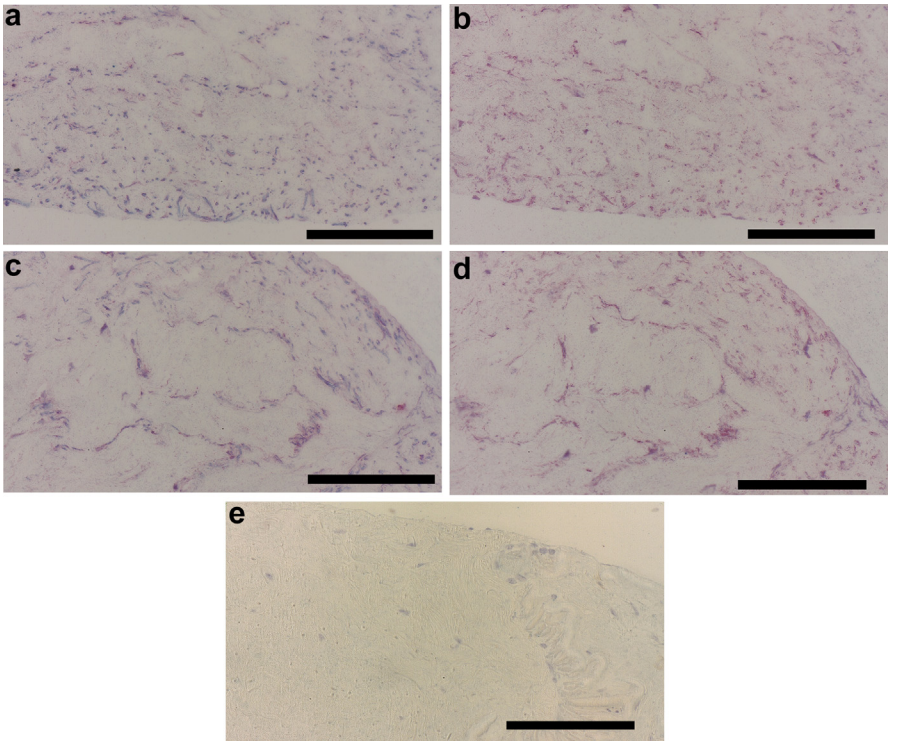

Fig. 4. (a–e) Double immunostaining under conditions C and D. (a, c) OC: red + FBXW2: blue. (b, d) OC: red + no FBXW2: blue. (e) ARHGAP36 (negative control). Scale bar: 100 μm.

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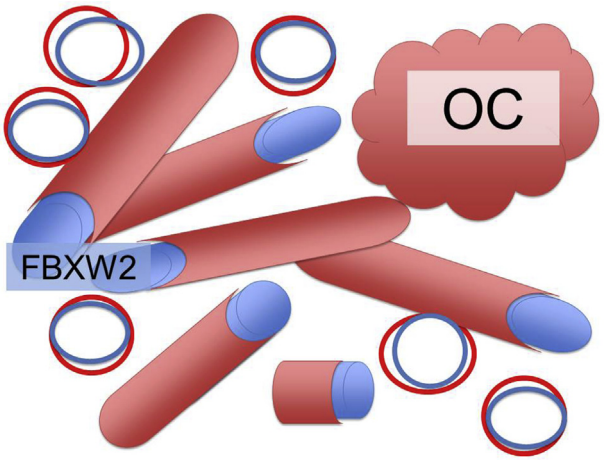

Fig. 5. Hypothesis for OC and FBXW2 co-localization.

OC outline was very thin. Although previous reports on single immunostaining of OC, EXOSC9, and FBXW2 utilized horseradish peroxidase (HRP) labeling and DAB staining (Akiyama, 2014b, 2016), we utilized alkaline phosphatase (AP) labeling to improve sensitivity in paraffin sections (Rozenvald et al., 2017). FBXW2 expression was weak and restricted to a specific area immediately after bovine death. In addition, FBXW2 localized between the bone and periosteum with some tubes entering the bone (Fig. 1b, c). FBXW2 staining subsequently increased after 5 weeks of culture.

Figs. 1a, 3a, and 4a–d show that FBXW2 and OC have similar tubular shapes. Based on the findings of this study, FBXW2 tubes appear to be coated with OC around OC-positive areas (Fig. 5). The interaction between OC and other proteins, particularly during glucose metabolism, has been studied (Garbossa and Folli, 2017; Kanazawa, 2015, 2017; Levinger et al., 2017; Liu et al., 2015; Takeno et al., 2018; Thomas et al., 2017), whereas the interaction between OC and FBXW2 has not been previously studied. To the author's knowledge, this is the first study to reveal an interaction between OC and FBXW2. Future clarification of the roles of OC and FBXW2 in bone regeneration is considered necessary.

5. Conclusions

1. FBXW2 formed tubular structures in the periosteum.
2. OC also formed tubular structures and OC and FBXW2 exhibited the same expression pattern.
3. FBXW2 tubes were coated with OC.

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Declarations

Author contribution statement

Mari Akiyama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The author declares no conflict of interest.

Additional information

No additional information is available for this paper.

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