学位論文

Histomorphometry of ectopic mineralization using undecalcified frozen bone sections

香川大学大学院医学系研究科

機能構築医学専攻　組織化学部門

藤原龍史

1 **Title**

2   Histomorphometry of ectopic mineralization using undecalcified frozen bone sections

3

4 **Running title**

5   Frozen section evaluating mineralization

6

7 **Authors**

8 Ryuji Fujihara, MD,[1,2]

9 Yoichi Chiba, MD, PhD,[1]

10 Toshitaka Nakagawa, PhD,[3]

11 Ryuta Murakami, MD,[1]

12 Koichi Matsumoto, MD, PhD,[1]

13 Machi Kawauchi,[1]

14 Takayuki Fujii, MD,[4]

15 Ryuichi Shimono, MD, PhD,[4]

16 Tetsuji Yamamoto, MD, PhD,[2]

17 Masaki Ueno, MD, PhD,[1]

18 1: Department of Pathology and Host Defense, Faculty of Medicine, Kagawa University,

19 Kagawa, Japan

20 2: Department of Orthopaedic Surgery, Faculty of Medicine, Kagawa University, Kagawa,

21 Japan

22 3: Life Science Research Center, Faculty of Medicine, Kagawa University, Kagawa, Japan

23 4: Department of Pediatric Surgery, Faculty of Medicine, Kagawa University, Kagawa,

24 Japan

25

26 **Correspondence author**

27 Masaki Ueno, MD, PhD

28 Department of Pathology and Host Defense, Faculty of Medicine, Kagawa University,

29 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan

30 Tel.: +81-87-891-2115, FAX: +81-87-891-2116

31 Email: masaueno@med.kagawa-u.ac.jp

32

Abstract

Objective: To investigate the correlation between mineral formation and enhanced

expressions of some proteins using undecalcified frozen bone sections.

Summary of Background Data: Histological studies have revealed that some proteins,

such as BMP2, BMPR1A, and Connexin 43, are expressed in and around sites of ectopic

ossification. However, the relationship between the expressed proteins considered to be

associated with the ossification and mineral formation in vivo is not clear.

Methods: Ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1)-mutant spinal

hyperostotic TWY mice and ICR mice as controls were euthanized after calcein labeling,

and undecalcified frozen sections were obtained from the middle thoracic spine.

Intervertebral disc areas were examined histologically and by measuring calcein-labeled

areas and areas showing immunoreactivity for BMP2, BMPR1A, and Connexin 43.

Results: Calcein-labeled areas, indicating mineralization in the ectopic mineralization sites,

were significantly larger in the mutant mice than in controls. The expression of Connexin

43 was elevated in the annulus fibrosus. Increases in the calcein-labeled areas was not

1   correlated with increases in the areas showing immunoreactivity for Connexin 43 in the

2   annulus fibrosus.

3   **Conclusions:** There was no statistical correlation between enhanced immunohistochemical

4   expression and elevated calcein-labeled areas. By applying the morphometrical analysis

5   method using undecalcified frozen sections to ENPP1-mutant mice, quantitative evaluation

6   of the mineralization and proteins expressed in the surrounding area in the same animal

7   became possible.

8

9

10   **Key words:** BMP2, BMPR1A, Connexin 43, ENPP1, histomorphometry, mineralization,

11   ossification of the posterior longitudinal ligament, undecalcified frozen section

12

1   **Research highlights**

2   By applying the morphometrical method using undecalcified frozen sections, evaluation

3   of the mineralization and proteins in the same animal became possible. The correlation

4   between them was analyzed in vivo.

5

6

7

8

9

1 Introduction

2

3   Since the ossification of the posterior longitudinal ligament (OPLL) in an autopsy case

4 was initially reported in 1960, many histological studies have been conducted to clarify the

5 pathogenesis (Tsuzuki, 2006). Some studies using decalcified paraffin-embedded sections

6 from human OPLL showed the specific localization of collagen subtypes and

7 immunoreactivity of bone morphogenetic protein (BMP)-2, BMP receptor (BMPR), and

8 Connexin 43 (Chen et al., 2016; Kawaguchi et al., 1992; Sato et al., 2007; Yasui, Ono,

9 Yamaura, Konomi, & Nagai, 1983; Yonemori et al., 1997). These proteins have been

10 considered to be factors involved in ectopic mineralization, but few papers have reported

11 the quantitative evaluation of immunohistochemical findings.

12   The use of animal models has facilitated analysis of the mineralization front in ectopic

13 ossification because labeling by fluorescent calcein injected in a few days before

14 euthanization makes it possible to evaluate new bone formation. A TWY mouse, with a

15 nonsense mutation in the ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1)

16 gene, was used to analyze ectopic mineralization (Okawa et al., 1998), because of

17 association between ENPP1 gene polymorphism and human OPLL (Koshizuka et al.,

1   2002). The mutant mouse was discovered during brother-sister mating of ICR mouse

2   (Hosoda, Yoshimura, & Higaki, 1981) and showed elevated bone formation compared with

3   the control ICR mouse in resin-embedded sections (Okawa, Goto, & Moriya, 1999; Tanno,

4   1992). However, useful immunohistochemical findings could not be obtained from the

5   resin-embedded sections.

6   From the above, it remains to be clarified whether some index values of mineral

7   formation in the mineralization front are related to immunohistochemically expressed

8   proteins. Only by conducting experiments using undecalcified frozen bone sections

9   (Kawamoto, 2003) obtained from the ENPP1-mutant spinal hyperostotic mice,

10   quantification of mineral formation at the ectopic mineralization and immunohistochemical

11   expression of some involved proteins in the same animal became possible. The present

12   study aimed: (1) to verify the findings obtained from undecalcified frozen bone sections by

13   comparing with previous reports, (2) to quantify both parameters of immunoreactivity for

14   some proteins and mineral formation, and (3) to analyze the correlation between

15   immunohistochemical findings and mineral formation. By applying the method using

16   undecalcified frozen sections, the role of expressed proteins could be analyzed in relation to

17   mineral formation in vivo.

6

1

2

Materials and Methods

4

Experimental animals

Eight-week-old male ICR (Jcl:ICR) mice and TWY (TWY/Jic-ttw) mice were

purchased from CLEA Japan, Inc. (Tokyo, Japan). They were kept under controlled

conditions with a 12-h light/dark cycle at 22–26°C, and maintained on commercial food

(MF, Oriental Yeast Corp., Ltd., Japan) and tap water ad libitum. All experimental

procedures complied with the guidelines for the care and use of animals established by

Kagawa University and the National Institute of Health Guide for the Care and Use of

Laboratory Animals. The experimental protocols were approved by the Kagawa University

Animal Care and Use Committee.

14

Experimental protocol

At 15 weeks of age, five ICR mice and five TWY mice were euthanized, with calcein

(20 mg/kg, IP; Dojindo Laboratory, Kumamoto, Japan) intraperitoneally administered 8 and

1   1 day before death. Mice were anesthetized intraperitoneally with an overdose of sodium

2   pentobarbital, and perfused transcardially with phosphate-buffered saline (PBS) and then

3   with a fixative containing 10% buffered formalin. After formalin fixation for one day at

4   4°C, the thoracic spine was removed from mice and embedded in super cryoembedding

5   medium (SECTION-LAB Co., Ltd., Hiroshima, Japan) with hexane on dry ice. Frozen

6   sections were cut at -25°C on a cryostat (LEICA CM1950, Leica Microsystems, Tokyo,

7   Japan) with a tungsten-carbide blade. Supported with adhesive films (Cryofilm type 2C (9),

8   SECTION-LAB Co., Ltd., Hiroshima, Japan), 8-μm-thick sections were obtained. Sagittal

9   sections around the median plane including the nucleus pulposus and gray matter of the

10   spinal cord were obtained. Freeze-dried sections that had been placed in a cryochamber

11   maintained at -25°C for 1 h were used to analyze mineralization. For histological

12   examination, frozen sections were thawed at room temperature for 30 sec and put in 99%

13   ethanol for 30 sec before staining.

14

15   **Histological procedure**

16   Hematoxylin & eosin (HE) staining was performed according to the previous report

17   (Kawamoto & Kawamoto, 2014). In brief, the sections were stained with Hematoxylin for 1

1   minute. After washing the sections in running water, the sections were stained with eosin

2   for approximately 10 sec and rinsed with 99% ethanol. Then we dropped mounting medium

3   (SCMM-R2, SECTION-LAB Co., Ltd., Hiroshima, Japan) on the sections, and fixed them

4   under ultraviolet light.

5   Modified von Kossa staining was performed using a calcium stain kit according to the

6   attached instructions (ScyTek, Utah, USA). In brief, we incubated the sections in 5% silver

7   nitrate solution for 10 min under ultraviolet light. After rinsing the sections in 3 changes of

8   distilled water, the sections were incubated in 5% sodium thiosulfate solution for 2 min.

9   The nucleus was stained with nuclear fast red solution for 5 min. After rinsing the sections

10   in distilled water, the sections were preserved by mounting medium as described above.

11   For azan staining, we treated the sections with mordanting solution (10% potassium

12   dichromate, 10% trichloroacetic acid) for 10 min. We incubated the sections in azocarmine

13   G solution (0.15 g azocarmine G, 150 ml distilled water, 1.5 ml acetate) for 30 min. After

14   differentiation in anilin alcohol (0.15 ml anilin blue, 150 ml 95% ethanol) for several

15   seconds followed by rinse in acetic alcohol, the sections were incubated in 5%

16   phosphotungstic acid for 30 min. Following the incubation with anilin blue/orange G

1   solution (0.5 g anilin blue, 2 g orange G, 200 ml distilled water, 8 ml acetate) for 30 min,

2   the sections were rinsed in 99% ethanol for 5 min.

3   For alcian blue staining, we treated sections with 3% acetate for 3 min. The sections

4   were incubated in alcian blue solution (1 g alcian blue 8GX, 100 ml 3% acetate) for 10 min,

5   followed by rinse in 3% acetate for 3 min. After rinsing the sections in distilled water, the

6   nucleus was stained with nuclear fast red solution for 3 min.

7

8   **Immunohistochemical procedure**

9   For immunohistochemical examination, the sections were treated with 0.3% H_2O_2 for

10   10 min, blocked with normal horse serum for 30 min, and incubated in the presence of the

11   primary antibody (Bridgewater et al., 2013; Fujishiro, Kaneko, Kawashima, Ishida, &

12   Kawano, 2014; Gu et al., 2014; Hwang et al., 2016; Tu et al., 2016; Xia et al., 2017; Yeom

13   et al., 2013) (Table 1) at 4°C over night. To reduce the non-specific reactions, we diluted

14   secondary antibody twice times with normal horse serum (ImmPRESS HRP REAGENT

15   KIT, Vector Laboratories, Burlingame, USA), and the sections were incubated in the diluted

16   solution for 30 min. The color was developed with 3,3'-diaminobenzidine

1 tetrahydrochloride and hydrogen peroxide (Nichirei) at RT for 10 min. The sections were

2 counterstained with hematoxylin for several seconds.

3

4 **Qualitative analysis of extracellular matrix**

5 Each vertebra of the middle thoracic spine was imaged using a microscope (Olympus

6 BX51, Tokyo, Japan) with a 4x objective lens. The histological architecture of the spine

7 was evaluated by HE staining. Mineral apposition was evaluated by modified von Kossa

8 staining. Collagen fibers were evaluated by azan staining and immunostaining of collagen

9 subtypes, while the deposition of acid mucopolysaccharides was evaluated with alcian blue

10 staining.

11

12 **Quantitative analysis of immunohistochemistry**

13 Each vertebra of the middle thoracic spine was imaged using a microscope (Olympus

14 BX51, Tokyo, Japan) with a 20x objective lens. The stained areas obtained with

15 immunohistochemical techniques were measured semi-automatically with WinROOF2015

16 (Mitani Corporation, Tokyo, Japan). We measured annulus fibrosus area as follows: the

17 region of interest (ROI) of the annulus fibrosus area was determined at intervertebral disc

1   area except for ectopic mineralization and nucleus pulposus. The proportion of the stained

2   area (%) was obtained by dividing the stained area by the area of ROI. The mean value of

3   each vertebra was used for statistical analysis.

4

5   **Evaluation of mineralization**

6   Each vertebra in the middle thoracic spine was imaged using a fluorescence phase-

7   contrast microscope (BZ-X710, Keyence, Osaka, Japan) with a 4x objective lens. A

8   fluorescence image using 1/200-second exposure with a GFP filter (Keyence) and a phase-

9   contrast image using 1/7,500-second exposure were obtained. The ROI for the quantitative

10   evaluation of the fluorescence and phase-contrast images was determined in the ectopic

11   mineralization area. The fluorescence area was measured semi-automatically with graphic

12   software (WinROOF2015, Mitani Corporation) (Andreassen & Oxlund, 2003). The

13   proportion of the calcein-labeled area (%) was obtained by dividing the fluorescence area

14   by the area of ROI.

15   The dynamic parameters were measured manually using WinROOF2015 (Mitani

16   Corporation). The single-labeled surface (sLS), double-labeled surface (dLS), and label

17   thickness were measured at the periosteal surface. The mineralizing surface (Ps.MS/BS)

1   was calculated by dividing the total extent of dLS plus half the extent of sLS by the

2   periosteal perimeter. The mineral apposition rate (Ps.MAR) was calculated by dividing the

3   label thickness by the labelling interval. The bone formation rate (Ps.BFR/BS) was

4   calculated as the product of the mineralizing surface and mineral apposition rate (Mashiba

5   et al., 2000). The nomenclature and symbols were used according to the recommendations

6   of the Histomorphometry Nomenclature Committee of the American Society for Bone and

7   Mineral Research (Dempster et al., 2013). The mean value of each vertebra was used for

8   statistical analysis.

9

10   **Statistical analysis**

11   Values are shown as the mean ± SD. Differences between the groups were analyzed

12   using the Mann-Whitney test. Spearman's rank correlation analysis was performed to

13   assess the relationship between mineralization and immunohistochemical parameters. SPSS

14   statistics 22 (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Differences

15   were considered significant at $p < 0.05$.

16

17

Results

The body weight before euthanasia was 43.2 g ± 2.9 for ICR mice and 32.1 g ± 3.9 g

for TWY mice. The difference between them was significant (p=0.009 Mann-Whitney test).

Qualitative analysis of extracellular matrix

HE staining and modified von Kossa staining showed mineral apposition at the annulus

fibrosus in TWY mice but not in ICR mice (Fig. 1 A, B, E, F). Alcian blue staining revealed

deposition of acid mucopolysaccharides in the intervertebral disc area, and distribution of

mucopolysaccharides exhibited an unclear boundary between the ectopic mineralization

and annulus fibrosus (Fig. 1 C, G). Through azan staining, the annulus fibrosus was stained

blue, while ectopic mineralization showed focal staining with a purple color (Fig. 1 D, H).

In contrast with the negative control for immunohistochemistry (Fig. 2 A, B, I, J), collagen

type I was immunohistochemically expressed in the outer layer of the annulus fibrosus

(Fig. 2 C, K), while collagen type II was immunohistochemically expressed in endplates

(Fig. 2 D, L). Immunoreactivity of collagen type II was observed in ectopic mineralization

of TWY mice, while that of collagen type I and negative control was scarce (Fig. 2 J-L).

14

1

Quantitative analysis of immunohistochemistry

Immunoreactivity of BMP2, BMPR1A, and Connexin 43 was observed in ectopic

mineralization of TWY mice (Fig. 2 M-O), however that of normal rabbit IgG was also

observed in ectopic mineralization (Fig. 2 J). By contrast, immunoreactivity of Connexin

43 was observed at annulus fibrosus of ICR and TWY mice (Fig. 2 G, O) without that of

normal rabbit IgG at annulus fibrosus. Connexin 43 immunohistochemical expression was

significantly enhanced in the annulus fibrosus of TWY mice, compared with that in the area

of ICR mice (p=0.009 Mann-Whitney test) (Table 2). There was no immunoreactivity of

BMP2 and BMPR1A at annulus fibrosus.

Evaluation of mineralization

The calcein-labeled area in the ectopic mineralization was significantly larger in TWY

mice than in ICR mice, and dynamic parameters at the periosteal surface were higher in

TWY mice than in ICR mice (Table 2).

Correlation between mineralization and immunohistochemistry

1   There were no associations between any parameters of mineralization and

2   immunohistochemically stained area with Connexin 43.

3

4

5   **Discussion**

6

7   First, we verify the validity of the findings obtained from undecalcified frozen bone

8   sections by comparing them with published observations (Hong et al., 2012). In the present

9   study, the rate of periosteal mineralization was significantly higher in the TWY mouse,

10   consistent with the previous findings obtained from resin-embedded sections (Okawa et al.,

11   1999). Next, we investigated the immunohistochemical distribution of collagen subtypes, to

12   verify the immunohistochemical findings. Collagen type I was mainly expressed in the

13   outer layer of the annulus fibrosus, and collagen type II was expressed in the endplate and

14   annulus fibrosus (Fig. 1). The areas expressing these proteins were similar to the published

15   findings obtained from decalcified paraffin sections in ICR and TWY mice (Furusawa,

16   Baba, Imura, & Fukuda, 1996; Hirakawa et al., 2004). These findings indicate that it is

16

1 effective to evaluate the mineralization and immunohistochemical expression of some

2 proteins using undecalcified frozen bone sections.

3 The relationships between mineralization and the extracellular matrix have been

4 reported in human OPLL and in the TWY mouse. In human OPLL, the ossified area shows

5 collagen type I expression, while the calcified cartilaginous layer shows collagen type II

6 expression and toluidine blue staining (Sato et al., 2007). In the TWY mouse, ectopic

7 ossification expressed collagen type I, while protruding cartilaginous tissue expressed

8 collagen type II (Furusawa et al., 1996). In this study, several staining patterns revealed

9 ectopic mineralization accompanied by the deposition of mucopolysaccharides, collagen

10 type I, and collagen type II. At present, it remains to be fully clarified whether the

11 pathogenesis of the ENPP1 mutant mouse actually reflects that of human OPLL, because

12 the mutant mouse exhibited ectopic mineralization at many sites beyond the posterior

13 longitudinal ligament (Hosoda et al., 1981). However, the findings suggest that ectopic

14 ossification begins in the calcified cartilaginous tissues.

15 It was reported that BMP2, BMPR1A, and Connexin 43 were expressed in and around

16 sites of ectopic ossification in decalcified paraffin sections (Chen et al., 2016; Kawaguchi

17 et al., 1992; Sato et al., 2007; Tu et al., 2016; Yonemori et al., 1997). An in vitro experiment

reported that expressions of BMP2, BMPR, and Connexin 43 were induced by mechanical

stress on OPLL-derived cells (Tanno, Furukawa, Ueyama, Harata, & Motomura, 2003;

Yang et al., 2011). However, it remains unclear where these factors are expressed in TWY

mice or how they are correlated with mineralization. In this study, using undecalcified

frozen sections, the immunoreactivity of BMP2, BMPR1A, and Connexin 43 were

observed ectopic mineralized areas, while that of Connexin 43 expression was enhanced in

the annulus fibrosus of TWY mice. However, there were no correlations between any

parameters of mineralization and immunohistochemically stained area. These findings

suggest that immunoreactivity of these substances may be involved in the pathogenesis of

ectopic mineralization, however, there was discrepancy between enhanced

immunoreactivity of Connexin 43 and elevated mineralization. There are several

explanations for this discrepancy. First, ectopic mineralization may require multiple

pathological steps such as hyperplasia, metaplasia, neovascularization (Uchida et al., 2012),

before mineralization. Expression of Connexin 43 in annulus fibrosus may not be directly

involved in mineralization. Second, immunohistochemical findings in ectopic

mineralization site was not evaluated in relation to mineralization in this study because the

ectopic mineralization was stained with normal rabbit IgG. Although undecalcified frozen

1   sections may preserve the antigenicity, non-specific reactions for mineralized tissue were

2   reported (Carter, Sloan, & Aaron, 1994). If the complete negative control is achieved by

3   histological procedures, such as pretreatment of the section by EDTA (Carter et al., 1994),

4   direct comparison between parameters of the immunohistochemistry and mineralization in

5   ectopic mineralization site become possible. Finally, quantification of

6   immunohistochemical findings has no guarantee of accurate amount of expressed proteins.

7   This is the first paper to report semi-quantitative results of ectopic mineralization and

8   analysis of whether the association between mineralization and immunohistochemical

9   findings is significant. However, there are some limitations. First, immunohistochemical

10   findings were not confirmed using other methods, such as in situ hybridization.

11   Immunoreactivity of several proteins at ectopic mineralization site was hard to conclude

12   that these proteins were expressed there. Second, histological site-specific quantification

13   was semi-quantitatively performed in only one sagittal section near the median plane, and

14   not multiple planes. Third, the evaluation of the calcein-labeled area was performed in the

15   ectopic mineralization sites. However, the boundary of the ectopic mineralization was not

16   clear in freeze-dried sections. The application of an improved mineral staining method,

17   which can minimize the elution of water-soluble calcein, would generate better images of

19

1   the ectopic mineralization front. Further investigation is required.

2   In summary, a morphometrical method for analysis of the mineralization front and

3   immunohistochemically expressed proteins in the same animal was established using

4   undecalcified frozen bone sections. Enhanced immunoreactivity for Connexin 43 was not

5   correlated with elevated mineralization in hyperostotic mice, indicating the multiple

6   pathological steps before the development of ectopic mineral formation. Application of the

7   method using undecalcified frozen bone sections would make it possible to analyze the

8   pathophysiology of ectopic mineralization in more detail.

9

10

11   **Acknowledgements**

12

17

1   **References**

2

3   Andreassen, T. T., & Oxlund, H. (2003). Local anabolic effects of growth hormone on

4   intact bone and healing fractures in rats. *Calcified Tissue International*, *73*(3), 258–

5   264. https://doi.org/10.1007/s00223-002-2074-6

6   Bridgewater, L. C., Mayo, J. L., Evanson, B. G., Whitt, M. E., Dean, S. A., Yates, J. D., …

7   Hancock, C. R. (2013). A novel bone morphogenetic protein 2 mutant mouse,

8   nBmp2NLS(tm), displays impaired intracellular Ca2+ handling in skeletal muscle.

9   *Biomed Res Int*, *2013*, 125492. https://doi.org/10.1155/2013/125492

10 Carter, D. H., Sloan, P., & Aaron, J. E. (1994). The cryomicrotomy of rat dental tissues: a

11   technique for histological and immunohistochemical analysis. *The Histochemical*

12   *Journal*, *26*(2), 103–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7512083

13 Chen, D., Liu, Y., Yang, H., Chen, D., Zhang, X., Fermandes, J. C., & Chen, Y. (2016).

14   Connexin 43 promotes ossification of the posterior longitudinal ligament through

15   activation of the ERK1/2 and p38 MAPK pathways. *Cell and Tissue Research*, *363*(3),

16   765–773. https://doi.org/10.1007/s00441-015-2277-6

17 Dempster, D. W., Compston, J. E., Drezner, M. K., Glorieux, F. H., Kanis, J. A., Malluche,

21

1   H., … Parfitt, A. M. (2013). Standardized nomenclature, symbols, and units for bone

2   histomorphometry: A 2012 update of the report of the ASBMR Histomorphometry

3   Nomenclature Committee. *Journal of Bone and Mineral Research*, *28*(1), 2–17.

4   https://doi.org/10.1002/jbmr.1805

5   Fujishiro, A., Kaneko, H., Kawashima, T., Ishida, M., & Kawano, T. (2014). In vivo

6   neuronal action potential recordings via three-dimensional microscale needle-electrode

7   arrays. *Scientific Reports*, *4*, 1–9. https://doi.org/10.1038/srep04868

8   Furusawa, N., Baba, H., Imura, S., & Fukuda, M. (1996). Characteristics and mechanism of

9   the ossification of posterior longitudinal ligament in the tip-toe walking Yoshimura

10   (twy) mouse. *European Journal of Histochemistry*, *40*(3), 199–210.

11   Gu, S., Wu, W., Liu, C., Yang, L., Sun, C., Ye, W., … Chen, Y. (2014). BMPRIA

12   mediated signaling is essential for temporomandibular joint development in mice.

13   *PLoS ONE*, *9*(8), e101000. https://doi.org/10.1371/journal.pone.0101000

14   Hirakawa, H., Kusumi, T., Nitobe, T., Ueyama, K., Tanaka, M., Kudo, H., … Harata, S.

15   (2004). An immunohistochemical evaluation of extracellular matrix components in the

16   spinal posterior longitudinal ligament and intervertebral disc of the tiptoe walking

17   mouse. *Journal of Orthopaedic Science*, *9*(6), 591–597.

https://doi.org/10.1007/s00776-004-0823-2

Hong, S.-H., Jiang, X., Chen, L., Josh, P., Shin, D.-G., & Rowe, D. (2012). Computer-automated static, dynamic and cellular bone histomorphometry. *Journal of Tissue Science & Engineering*, *Suppl 1*(1), 4. https://doi.org/10.4172/2157-7552.S1-004

Hosoda, Y., Yoshimura, Y., & Higaki, S. (1981). A new breed of mouse showing multiple osteochondral lesions--twy mouse. *Ryumachi. [Rheumatism]*, *21 Suppl*, 157–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7344126

Hwang, P. Y., Jing, L., Chen, J., Lim, F.-L., Tang, R., Choi, H., … Setton, L. A. (2016). N-cadherin is key to expression of the nucleus pulposus cell phenotype under selective substrate culture conditions. *Scientific Reports*, *6*(1), 28038. https://doi.org/10.1038/srep28038

Kawaguchi, H., Kurokawa, T., Hoshino, Y., Kawahara, H., Ogata, E., & Matsumoto, T. (1992). Immunohistochemical demonstration of bone morphogenetic protein-2 and transforming growth factor-beta in the ossification of the posterior longitudinal ligament of the cervical spine. *Spine*, *17*(3 Suppl), S33-6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1566182

Kawamoto, T. (2003). Use of a new adhesive film for the preparation of multi-purpose

1   fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Archives of*

2   *Histology and Cytology*. https://doi.org/10.1679/aohc.66.123

3   Kawamoto, T., & Kawamoto, K. (2014). Preparation of thin frozen sections from nonfixed

4   and undecalcified hard tissues using Kawamot's film method (2012). *Methods in*

5   *Molecular Biology (Clifton, N.J.)*, *1130*, 149–164. https://doi.org/10.1007/978-1-

6   62703-989-5_11

7   Koshizuka, Y., Kawaguchi, H., Ogata, N., Ikeda, T., Mabuchi, A., Seichi, A., … Ikegawa,

8   S. (2002). Nucleotide pyrophosphatase gene polymorphism associated with

9   ossification of the posterior longitudinal ligament of the spine. *Journal of Bone and*

10   *Mineral Research*, *17*(1), 138–44. https://doi.org/10.1359/jbmr.2002.17.1.138

11   Mashiba, T., Hirano, T., Turner, C. H., Forwood, M. R., Johnston, C. C., & Burr, D. B.

12   (2000). Suppressed bone turnover by bisphosphonates increases microdamage

13   accumulation and reduces some biomechanical properties in dog rib. *Journal of Bone*

14   *and Mineral Research*, *15*(4), 613–620. https://doi.org/10.1359/jbmr.2000.15.4.613

15   Okawa, A., Goto, S., & Moriya, H. (1999). Calcitonin simultaneously regulates both

16   periosteal hyperostosis and trabecular osteopenia in the spinal hyperostotic mouse

17   (twy/twy) in vivo. *Calcified Tissue International*, *64*(3), 239–247.

1   https://doi.org/10.1007/s002239900610

2   Okawa, A., Nakamura, I., Goto, S., Moriya, H., Nakamura, Y., & Ikegawa, S. (1998).

3   Mutation in Npps in a mouse model of ossification of the posterior longitudinal

4   ligament of the spine. *Nature Genetics*, *19*(3), 271–273. https://doi.org/10.1038/956

5   Sato, R., Uchida, K., Kobayashi, S., Yayama, T., Kokubo, Y., Nakajima, H., … Baba, H.

6   (2007). Ossification of the posterior longitudinal ligament of the cervical spine:

7   histopathological findings around the calcification and ossification front. *Journal of*

8   *Neurosurgery. Spine*, *7*, 174–183. https://doi.org/10.3171/SPI-07/08/174

9   Tanno, M., Furukawa, K. I., Ueyama, K., Harata, S., & Motomura, S. (2003). Uniaxial

10   cyclic stretch induces osteogenic differentiation and synthesis of bone morphogenetic

11   proteins of spinal ligament cells derived from patients with ossification of the

12   posterior longitudinal ligaments. *Bone*, *33*(4), 475–484.

13   https://doi.org/10.1016/S8756-3282(03)00204-7

14   Tanno, T. (1992). [Experimental study on the spinal lesions in hyperostotic mouse

15   (twy/twy): special reference to the pathogenesis of the ossification of the spinal

16   ligaments and to the action of ethane-1-hydroxy-1, 1-diphosphonate (EHDP)]. *Nihon*

17   *Seikeigeka Gakkai Zasshi*, *66*(10), 1073–83. Retrieved from

1   http://www.ncbi.nlm.nih.gov/pubmed/1460378

2   Tsuzuki, N. (2006). Review of histopathological studies on OPLL of the cervical spine,

3   with insights into the mechanism. In K. Yonenobu, K. Nakamura, & Y. Toyama

4   (Eds.), *Ossification of the posterior longitudinal ligament* (2nd edi, pp. 41–47).

5   Tokyo: Springer.

6   Tu, B., Liu, S., Liu, G., Li, Z., Sun, Y., & Fan, C. (2016). Inhibition of connexin 43

7   prevents trauma-induced heterotopic ossification. *Scientific Reports*, *6*(1), 37184.

8   https://doi.org/10.1038/srep37184

9   Uchida, K., Yayama, T., Sugita, D., Nakajima, H., Guerrero, A. R., Watanabe, S., … Baba,

10   H. (2012). Initiation and progression of ossification of the posterior longitudinal

11   ligament of the cervical spine in the hereditary spinal hyperostotic mouse (twy/twy).

12   *European Spine Journal*, *21*(1), 149–155. https://doi.org/10.1007/s00586-011-1971-7

13   Xia, C., Chen, P., Mei, S., Ning, L., Lei, C., Wang, J., … Fan, S. (2017). Photo-crosslinked

14   HAMA hydrogel with cordycepin encapsulated chitosan microspheres for

15   osteoarthritis treatment. *Oncotarget*, *8*(2), 2835–2849.

16   https://doi.org/10.18632/oncotarget.13748

17   Yang, H., Lu, X., Chen, D., Yuan, W., Yang, L., Chen, Y., & He, H. (2011). Mechanical

strain induces Cx43 expression in spinal ligament fibroblasts derived from patients

presenting ossification of the posterior longitudinal ligament. *European Spine Journal*,

20(9), 1459–1465. https://doi.org/10.1007/s00586-011-1767-9

Yasui, N., Ono, K., Yamaura, I., Konomi, H., & Nagai, Y. (1983). Immunohistochemical

localization of types I, II, and III collagens in the ossified posterior longitudinal

ligament of the human cervical spine. *Calcified Tissue International*, *35*(2), 159–163.

Yeom, M., Kim, S.-H., Lee, B., Zhang, X., Lee, H., Hahm, D.-H., … Lee, H. (2013).

Effects of laser acupuncture on longitudinal bone growth in adolescent rats. *Evidence-*

Based Complementary and Alternative Medicine, *2013*, 424587.

https://doi.org/10.1155/2013/424587

Yonemori, K., Imamura, T., Ishidou, Y., Okano, T., Matsunaga, S., Yoshida, H., … Sakou,

T. (1997). Bone morphogenetic protein receptors and activin receptors are highly

expressed in ossified ligament tissues of patients with ossification of the posterior

longitudinal ligament. *The American Journal of Pathology*, *150*(4), 1335–47.

Retrieved from

http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1858169&tool=pmcentrez

&rendertype=abstract

1

2

1   **Tables**

2

Table 1. Summary of primary antibodies used in this study

Antibody	Source	Host	Concentration	Specificity
BMP2	Novus Biologicals (NBP1-19751SS)	Rabbit	10 μg/ml	Growth plate[a] Skeletal muscle[b]
BMPR1A	Abcam (ab38560)	Rabbit	20 μg/ml	Articular cartilage[c]
Connexin 43	Abcam (ab11370)	Rabbit	5 μg/ml	Heterotopic ossification[d]
Collagen I	Abcam (ab34710)	Rabbit	20 μg/ml	Annulus fibrosus[e]
Collagen II	Abcam (ab116242)	Rabbit	5 μg/ml	Articular cartilage[f]
Normal IgG	R&D Systems (AB-105-C)	Rabbit	20 μg/ml	Negative control[g]

Please refer to papers written by [a]Yeom et al. (2013), [b]Bridgewater et al. (2013), [c]Gu et al. (2014), [d]Tu et al. (2016), [e]Hwang et al. (2016), [f]Xia et al. (2017), [g]Fujishiro et al. (2014).

3

1

Table 2. Quantitative analysis of immunohistochemistry and mineralization

	ICR（n=5）	TWY（n=5）
Immunohistochemistry		
Connexin 43		
annulus fibrosus (%)	1.5 ± 0.6	6.7 ± 5.2*
Mineralization		
calcein-labeled area (%)	0.0 ± 0.0	8.7 ± 4.9*
Ps.MAR (µm/day)	0.1 ± 0.2	0.6 ± 0.5*
Ps.MS/BS (%)	0.03 ± 0.03	0.3 ± 0.09*
Ps.BFR/BS ($\mu m^3/\mu m^2$/day)	0.01 ± 0.02	0.2 ± 0.2*

Values are expressed as mean ± SD.

*: $P < 0.05$ vs. ICR.   Mann-Whitney test

2

3

4

1   Figure legends

2

3   Fig. 1

4   Representative histochemical images of the extracellular matrix obtained from serial

5   sections. Hematoxylin and eosin (HE) staining (A,E), modified von Kossa staining (B,F),

6   alcian blue staining (C,G), and azan staining (D,H) in ICR (A-D) and TWY mice (E-H).

7   There is no ectopic mineralization in ICR mice (A-D), while TWY mice exhibit ectopic

8   mineralization in the intervertebral disc area (E-H: arrowhead). The ectopic mineralization

9   is stained clearly by HE staining (E: arrowhead) and modified von Kossa staining (F:

10   arrowhead). Alcian blue staining shows an unclear boundary between the ectopic

11   mineralization and annulus fibrosus (G: long arrow). Collagen fibers are focally stained in

12   the ectopic mineralization (H: short arrow). Scale bars show 500 μm.

13

14   Fig. 2

15   Representative immunohistochemical images and a fluorescence image of the

16   mineralization front obtained from serial sections. Immunohistochemistry of normal rabbit

17   IgG incubated for 30 min (A,I), for over-night (B,J), Collagen type I (C,K), Collagen type

31

II (D,L), BMP2 (E,M), BMPR1A (F,N), Connexin 43 (G,O), and a fluorescence image of

the mineralization front (H,P) of ICR (A~H) and TWY (I~P) mice. Although the ectopic

mineralization and the nucleus pulposus are not stained with normal rabbit IgG incubated

for 30 min (A,I), they are positively stained if incubated for over-night (B,J: long arrow).

Immunoreactivity of collagen type I is observed at outer layer of the annulus fibrosus,

cortical or cancellous bone (C,K), while immunoreactivity of collagen type II is observed at

endplates in addition to above (D,L). Although immunoreactivity of collagen type I,

collagen type II, BMP2, BMPR1A, and Connexin 43 is observed at the ectopic

mineralization site (K-O: black arrowhead), its intensity is the same as that of normal rabbit

IgG (J: long arrow). Immunoreactivity of BMP2 is observed at spinal cord (E,M: short

arrow). Immunoreactivity of Connexin 43 is observed at annulus fibrosus (G,O: thick

arrow). Diffuse fluorescence due to calcein labeling between growth plates (P: white

arrowhead) is considered to reflect ectopic mineralization in the TWY mice. Scale bars

show 500 μm.