Elsevier Editorial System(tm) for Biochemical and Biophysical Research Communications
Manuscript Draft

Manuscript Number:

Title: Immunohistochemical localization of mesenchymal stem cells in ossified human spinal ligaments

Article Type: Regular Article

Keywords: Spinal ligament ossification; Mesenchymal stem cells; Immunohistochemistry; Localization; Neovascularization; Chondrocytes.

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Dr. Ernesto Carafoli
Editor-in-Chief
Biochemical and Biophysical Research Communications

4 Jun., 2013

Dear Dr. Carafoli,

Please find enclosed our manuscript entitled “Immunohistochemical localization of mesenchymal stem cells in ossified human spinal ligaments”, which we would like to submit for publication as an Original Article in *Biochemical and Biophysical Research Communications*.

Spinal ligament ossification is a frequently observed phenomenon stemming from progressive calcification of ligaments surrounding the spinal column. While the causes and severity vary amongst patients, it can lead to spinal canal stenosis, resulting in symptoms reminiscent of spinal cord injury or myelopathy. Consequently, drug treatment or corrective surgery is usually indicated in these patients. To date, the pathogenesis of spinal ligament ossification is still unclear. We previously showed the existence of mesenchymal stem cells (MSCs) in these ossified ligaments [1]. Building on these findings, here we sought to identify the source of MSCs in ossified (OLF) and non-ossified ligament flavum (non-OLF) from thoracic vertebrae.

We found that MSCs were localized to blood vessel perivascular regions and within the collagenous matrix of spinal ligament sections, with a higher prevalence of MSCs and neovascularization found in OLF tissues than in non-OLF tissues. In addition, MSCs colocalized with markers of pericytes, not endothelial cells. In OLF tissues, chondrocytes at the ossification front also expressed MSC-specific markers. Together, these results suggest that MSCs may be involved in chondrocyte differentiation and/or endochondral ossification during the pathogenesis of OLF.

Understanding stem cell involvement in the progression of various diseases is becoming an important consideration in tissue repair and regeneration. The link between stem cells and the progression of spinal ligament ossification will provide important insight into the treatment of spinal diseases. As such, we believe that our manuscript will be of significance to the broad readership of *Biochemical and Biophysical Research Communications*, particularly those in the stem cell field.

We confirm that this manuscript has not been published elsewhere and is not under consideration by another journal. All authors have approved the manuscript and agree with submission to *Biochemical and Biophysical Research Communications*. The authors have no conflicts of interest to declare.

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We look forward to hearing from you at your earliest convenience.

Yours sincerely,

Ken-Ichi Furukawa

[1] T. Asari, K.-I. Furukawa, S. Tanaka, et al., Mesenchymal stem cell isolation and characterization from human spinal ligaments, Biochem. Biophys. Res. Commun. 417 (2012) 1193–1199.

***Highlights (for review)**

>Mesenchymal stem cells (MSCs) are localized in pericytes and collagenous matrix. >High prevalence of MSCs in regions of ossification of the ligamentum flavum (OLF).>Chondrocytes near the ossification front were positive for MSCs markers.>MSCs may participate in chondrocyte differentiation in ectopic ossification.>Analysis of MSC-directed chondrogenesis in OLF may elucidate ectopic ossification.

Immunohistochemical localization of mesenchymal stem cells in ossified human spinal ligaments

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Abstract:

Mesenchymal stem cells (MSCs) have been isolated from various tissues and used for elucidating the pathogenesis of numerous diseases. In our previous *in vitro* study, we showed the existence of MSCs in human spinal ligaments and hypothesized that these MSCs contributed to the pathogenesis of ossification of spinal ligaments. The purpose of this study was to use immunohistochemical techniques to analyze the localization of MSCs in ossified human spinal ligaments *in situ*. Ossified (OLF) or non-ossified ligamentum flavum (non-OLF) samples from the thoracic vertebra were obtained from patients who had undergone posterior spinal surgery. Serial sections were prepared from paraffin-embedded samples, and double immunofluorescence staining was performed using antibodies against markers for MSCs (CD73, CD90 and CD105), endothelial cells (CD31), pericytes (α-smooth muscle actin), and chondrocytes (S100). Immunolocalization of MSCs was observed in the perivascular area and collagenous matrix in spinal ligaments. Markers for MSCs and pericytes were co-expressed in the perivascular area. Compared with non-OLF, OLF had a large amount of neovascularization in the fragmented ligament matrix, and a high accumulation of MSCs around blood vessels. The prevalence of MSCs in OLF within collagenous matrix was significantly higher than that in non-OLF. Chondrocytes near the ossification front in OLF also presented expression of MSC markers. MSCs may contribute to the ectopic ossification process of OLF through endochondral ossification.

Keywords:

Spinal ligament ossification; Mesenchymal stem cells; Immunohistochemistry; Localization; Neovascularization; Chondrocytes.

1. Introduction

Human spinal ligaments adjacent to the spine contribute to its flexibility and stabilization by guiding segmental motion and limiting excessive motion [1,2]. Ossification of spinal ligaments, such as ossification of the posterior longitudinal ligament (OPLL) and the ligamentum flavum (OLF), can lead to narrowing of the spinal canal and eventually cause serious damage to the spinal cord, with patients suffering from various symptoms. These patients require pharmacotherapy and in severe cases surgery may be required to remove the ossified components and release the compression on the spinal cord [3]. The etiology of ectopic ossification of spinal ligaments has been analyzed extensively and linked to various epidemiological, genetic, metabolic, and mechanical factors [4-7]; however, the pathogenesis of the disease is still unknown.

Mesenchymal stem cells (MSCs) have been isolated from various human tissues including muscle, synovium, meniscus, intra-articular ligament, bone marrow, and adipose tissue, among others [8-12]. MSCs with multilineage potential have been used in regenerative therapy [13] and to elucidate the pathogenesis of numerous diseases in animal experimental models [14-16]. Furthermore, a number of studies have separated and identified MSCs in spinal ligaments and focused on the role of the MSCs in the pathogenesis of hypertrophy of spinal ligaments [17].

Ectopic bone formation in spinal ligamentous tissues has been shown to occur through endochondral ossification [18,19]. However, until recently, the source of these cells remained to be clarified. We recently identified the presence of MSCs in human spinal ligaments *in vitro* and showed their capacity to differentiate into the chondrocytic and osteocytic lineages. We hypothesized that these cells may contribute to the pathogenesis of ectopic ossification [20]. Using this previous work as a basis, it is now

important to determine the localization of MSCs in ossified spinal ligaments as compared with non-ossified spinal ligaments, with the goal to determine how these cells commit to the ossification site. One possibility is that MSCs undergo chondrocytic differentiation, resulting in spinal ligament ossification. These findings would provide valuable insight into the treatment of ectopic ossification in spinal ligaments.

Therefore, the purpose of this study was to use immunohistochemical techniques to analyze the localization of MSCs in ossified and non-ossified human spinal ligaments *in situ* and investigate a possible role of MSCs and/or chondrocytes in spinal ligament ossification.

2. Materials and Methods

2.1. Clinical diagnosis and spinal ligament samples

Clinical diagnosis was confirmed by X-ray, computed tomography and magnetic resonance imaging of the spine. Samples of the thoracic vertebra ligamentum flavum plaque were obtained *en bloc* from 12 patients. The plaques of ossified ligament tissues and ligamentum flavum (LF) were taken from six patients (four males, two females; mean age at surgery, 69.2 years; range 56-77 years) who underwent posterior decompression surgery for thoracic OLF. As a control, non-ossified LF plaques were obtained from six patients (five males, one female; mean age, 49.8 years, range, 22-81 years) who underwent posterior surgery for spinal tumor, syringomyelia or burst fracture at the thoracic vertebral level. None of the patients had evidence of congenital bone or joint disorders or was positive for rheumatoid factor. The Human Ethics Review Committee of the Hirosaki University Hospital approved the study protocol, and a signed informed consent form was obtained from each patient for all procedures.

2.2. Tissue Preparation

Samples were immediately fixed with 10% formaldehyde at 4℃ for 7 days. Samples with ossified tissue or bony tissue were further decalcified with KC-X solution (Falma, Tokyo, Japan) for 4–7 days at room temperature. Then, samples were bisected sagittally in the median plane, and embedded in paraffin. Serial, 4-μm-thick sections were prepared and subjected to hematoxylin and eosin (H&E) staining and immunohistochemical staining, using antibodies against markers for MSCs (CD73, CD90 and CD105), endothelial cells (CD31), pericytes (α-smooth muscle actin (SMA)), and chondrocytes (S100).

2.3. Immunohistochemical staining

Immunohistochemical analysis was performed with fluorescence antibody double staining. Sections were deparaffinized with xylene and treated with ethanol. After washing in phosphate buffered saline (PBS) at room temperature for 5 min, antigen retrieval was performed by heating samples in a PASCAL pressure chamber (Dako Cytomation, Produktionsvej, Glostrup, Denmark) to 125℃ for 3 min in Tris/EDTA buffer (Tris 10 mM, EDTA 1 mM, pH 9.0). After washing with PBS containing 0.01% Tween 20 (PBS-T), the sections were treated with 1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS-T at room temperature for 30 min to block non-specific protein binding. Next, the samples were incubated overnight at 4℃ with a mixture of two primary antibodies diluted with 1% BSA from the following list of antibodies. Monoclonal anti-CD73 antibody (Abcam, Cambridge, MA, USA.; mouse); polyclonal anti-CD73 antibody (Abcam; rabbit);

monoclonal anti-CD90 antibody (Abcam; rabbit); and monoclonal anti-CD105 antibody (Dako; mouse) were used to detect the expression of MSC markers. Polyclonal anti-CD31 antibody (Abcam; rabbit) and monoclonal anti-CD31 antibody (Dako; mouse) were used to identify vascular endothelial cells. Polyclonal anti-alpha smooth muscle actin (α-SMA) antibody (Abcam; rabbit) and monoclonal anti-α-SMA antibody (Dako; mouse) were used to identify pericytes. Monoclonal anti-S100 antibody (ab14849; Abcam; mouse) and polyclonal anti-S100 antibody (ab76729; Abcam; rabbit) were used to identify chondrocytes. Sections were then washed with PBS and incubated at room temperature for 2 h with a mixture of two secondary antibodies: Alexa Fluor® 594 donkey anti-mouse IgG (H+L) conjugate and Alexa Fluor® 488 goat anti-rabbit IgG (H+L) conjugate (Life Technologies, Carlsbad, CA). Finally, the sections were lightly counterstained with 4', 6-diamidino-2-phenylindole (DAPI).

2.4. Observation and quantification

Following double immunofluorescence staining, the sections were examined by confocal laser scanning microscope (Digital Eclipse C1si/C1 plus; Nikon Instruments, Japan) equipped with a charge-coupled device (CCD) camera and EZ-C1 3.90 Free Viewer software (Nikon Instruments, Japan). Images were collected sequentially as raw TIFF files and analyzed without further thresholding or filtering (e.g., no background subtraction). MSCs are phenotypically characterized by the expression of CD73, CD90 and CD105 [21]. Thus, in this study, double staining of the pairs of anti-CD73/CD90 antibodies, anti-CD73/CD105 antibodies, and anti-CD90/CD105 antibodies were performed to identify MSCs. For MSC marker expression, we focused on regions surrounding blood vessels, within collagenous matrix, and near the ossification front in

ossified ligamentous plaques. Furthermore, double staining of the pairs of anti-CD31 antibody with anti-CD73, anti-CD90, or anti-CD105 antibodies, and the pairs of anti-α-SMA antibody with anti-CD73, anti-CD90, or anti-CD105 antibodies were performed to confirm the association between MSCs with endothelial cells and pericytes in the blood vessel regions.

Next, we calculated the prevalence of MSC marker-positive cells in the collagenous matrix area of all samples and compared the prevalence of the OLF group with the non-OLF control group in the MSC marker pairs of anti-CD73 and anti-CD90 antibodies, anti-CD73 and anti-CD105 antibodies, and anti-CD90 and anti-CD105 antibodies. Prevalence was defined as the ratio of MSC marker-double positive cells to nucleated cells. For each MSC marker pair, three serial sections per sample were prepared and subjected to double immunofluorescence staining. On each section, multiple sites within the collagenous matrix were examined and MSC marker-double positive cells and nucleated cells were counted. Values were expressed as the percentage of MSC marker-double positive cells compared with the total number of DAPI-counterstained cells within each section, counting at least 1,000 cells per section.

2.5. Statistical analysis

The prevalence of each MSC marker pair was compared between OLF and non-OLF groups using the Mann-Whitney U-test. Statistical analysis was performed with SPSS ver. 12.0J (SPSS Inc., Chicago, IL, USA), and the level of significance was set at a P value of less than 0.05.

3. Results

3.1. Localization of MSCs in blood vessel region

Double immunofluorescence staining for MSC markers (CD73, CD90 or CD105) showed the existence of MSC marker-double positive cells around blood vessels within and surrounding the spinal ligaments for both OLF and non-OLF samples (Fig. 1). In the control non-OLF tissues, few MSC marker-double positive cells were detected sparsely distributed around the blood vessels in intact ligament tissues (Fig. 1A, C-E). On the other hand, in OLF tissues, there was a large amount of neovascularization in the fragmented ligament matrix (Fig. 1B), and a higher number of MSC marker-double positive cells around blood vessels (Fig. 1F-H). These differences between the OLF group and non-OLF group suggested that there is a close relationship between neovascularization and the accumulation of MSCs during damage ligament repair.

Since these MSC marker-positive cells were located close to blood vessels, we investigated the relationship between MSC marker-positive cells and the presence of vascular endothelial cells and pericytes in these regions with non-OLF samples. Double immunofluorescence staining showed no co-localization between MSC marker-positive cells and CD31-positive endothelial cells (Fig. 2A-C, G). In contrast, double immunofluorescence staining for MSC markers and the pericyte marker, α-SMA, showed co-expression of these two cell types in the perivascular area (Fig. 2D-G). Thus, this staining revealed that MSCs are distinct from endothelial cells, but exist at the perivascular area, possibly in close relationship with pericytes.

3.2. Localization and prevalence of MSCs in collagenous matrix

Next, we investigated the expression of MSC markers within the collagenous

matrix, and observed fibroblast-like cells with double positive expression of MSC markers (Fig. 3). In control non-OLF tissues, the collagenous fibers showed regular arrangement (Fig. 3A), but only few fibroblast-like cells were observed that were double positive for the expression of MSC markers (Fig. 3D-F). On the other hand, in OLF tissues, numerous fibroblast-like cells were double positive for MSC markers, situated amongst the irregular arrangement and fragmented collagenous fibers (Fig. 3B, G-I). The statistical analysis showed a significant increase in the prevalence of MSC marker expression coincident with ossified ligament plaques than in non-ossified ligament plaques ($p < 0.05$, each) (Fig. 3C). These results suggest that MSCs migrated from certain locations (such as the perivascular area) to accumulate at micro-injured ligament tissue sites to restore damaged ligamentous tissues.

3.3. Localization of MSCs near the ossification front

In OLF tissue samples, we identified chondrocytes using a characteristic marker of morphology (S100), and identified a large number of chondrocytes around the ossification front (Fig. 4A-E; B shows a higher magnification of A). In addition, we also observed that chondrocytes around the ossification front showed double positive expression of MSC markers (Fig. 4F-H). Together, these observations may suggest a role for MSCs in chondrocyte differentiation or endochondral ossification during the pathogenesis of OLF.

4. Discussion

Human MSCs have been identified in multiple organs *in vivo*. Using various methods of immunodetection, these MSCs have been shown to reside both in alignment

with the collagenous matrix and adjacent to small blood vessels [11,22,23]. However, until our recent study [20], there was no report to describe the detailed localization of MSCs from human spinal ligaments *in situ*. Here, we build on our previous findings, and show, for the first time, the existence of MSCs in both non-ossified and ossified human spinal ligaments *in vivo*. In spinal ligaments, a distinct pattern of MSC localization was observed, with positive MSC marker expression found in regions of vascularization and within the collagenous matrix. Furthermore, chondrocytes around the ossification front in ossified spinal ligaments showed positive expression of MSC markers.

In the current study, the existence of MSCs and blood vessels in collagenous matrix was minimal in non-OLF tissues. On the other hand, a high number of MSCs and a large amount of vascularization were observed in OLF tissues. Recently, some researchers have demonstrated that blood vessels are the source or niche of MSCs, providing convincing evidence that angiogenesis is associated with ectopic calcification in human tissues, such as in blood vessel walls, heart valves, and skeletal muscle. It has been hypothesized that angiogenesis may regulate ectopic calcification via various angiogenic factors, cytokines, oxygen and nutrients [24] and that new blood vessels can serve as a conduit for osteoprogenitor cells, which may be derived from the circulation or from pericytes present in the neovessels themselves and have the functions of vessel stabilization, synthesis of matrix proteins, and providing immunological properties. [25,26]. From the results of our current study and in line with previous reports, we consider that vascularization occurs as part of the repair process brought about by mechanical stress that leads to collagen tears and other microdamage in the ligament. Active vascularization derives a large number of MSCs from the circulation or pericytes

from capillary walls, and consequently changes the microenvironment of the extracellular matrix by secreting various factors or cytokines. These various growth factors and cytokines create an environment that leads to ectopic ossification within the ligament. However, the precise suite of factors responsible for this process is still unknown. In the future, a better understanding of the underlying mechanisms that link angiogenesis, pericytes, and MSCs should provide a basis for understanding the pathogenesis of ectopic ossification in spinal ligaments.

In spinal ligaments, we showed MSCs localized around blood vessels, coincident with the expression of the pericyte marker (α-SMA) in the perivascular area. However, the MSCs were distinct from the endothelial cells, as indicated by CD31 staining in the endothelial cells layer. In recent years, pericytes that surround blood vessels have been identified in multiple human organs including skeletal muscle, pancreas, adipose tissue, and placenta. Moreover, irrespective of their tissue of origin, long-term cultured pericytes are able to give rise to adherent, multilineage progenitor cells that exhibit the features of MSCs. Some studies have hypothesized that MSCs are pericytes, or could be derived from pericytes [11,24,27]. Overall, the results of the co-expression of the pericyte marker and MSC markers in our study are consistent with the previous studies and support the current hypothesis. Furthermore, we believe that an ancestor of the MSC is firmly associated with human perivascular cells, pericytes in particular.

Near the ossification front, the immunohistochemical analysis revealed the presence of numerous chondrocytes that were also positive for MSC markers. Several studies concluded that the process of ectopic ossification of the spinal ligament occurs through endochondral ossification and clustering of abnormal fibrocartilage or

cartilaginous cells [19]. Our previous studies have shown that various cytokines are involved in the presence/development of ectopic ossification in human spinal ligaments, and chondrocytes around the ossification front were stained with the antibody against CTGF/Hcs24, which plays an important role in endochondral ossification and osteogenesis in spinal ligament cells. [18; 28-32]. Uchida and colleagues demonstrated that chondrocytes around the ossification front had strong immunoreactivity using antibodies against several transcription factors, including Sox9, Runx2, and Osterix, among others, and demonstrated that chondrocyte differentiation around the ossification front is influenced by these transcription factors [33]. With this in mind, and given the positive expression of MSC markers in chondrocytes, our study supports the involvement of MSCs in the process of ectopic ossification in human spinal ligaments. Future experiments will hope to elucidate the role of MSCs in chondrocyte differentiation and the relationship between the cytokines that induce chondrometaplasia.

There were several limitations in this study. First, we employed double staining instead of triple staining (CD73/CD105, CD90/CD105 and CD90/CD73). Since MSCs have no unique specific marker, identification of the expression of CD73, CD90 and CD105 surface markers is required to verify the cell type. In future studies, triple immunohistochemical staining analysis will provide a more accurate representation of MSC populations for the identification of the MSCs. Second, we only used S100 as a marker for the presence of chondrocytes. As S100 also stains other cells of neural crest origin, additional staining using chondrocyte-specific markers, such as Type II collagen, osteonectin, aggrecan, chondroitin-S or other markers should be considered in future experiments. Third, the current study included a relatively limited number of subjects (n

= 6) and was not adequately powered to perform all statistical analyses. It would be necessary to conduct a further study with a larger sample size in the future.

In conclusion, our study showed the localization of MSCs in human spinal ligaments in the perivascular area and within the collagenous matrix. In addition, the co-expression of MSC and pericyte markers was observed in the perivascular area. Chondrocytes near the ossification front in OLF were also positive for MSC marker expression. The prevalence of MSCs in OLF was significantly higher than that of non-OLF in collagenous matrix. We suspect that MSCs play a key role in the ectopic ossification process of OLF.

Acknowledgments

This work was supported by a Grant-in-Aid from the Investigation Committee on Ossification of the Spinal Ligaments, the Public Health Bureau of the Japanese Ministry of Labour, Health and Welfare and by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grants-in-Aid 24590310). We thank Drs. Kazumasa Ueyama, Naoki Echigoya, Akio Sannohe, Gentaro Kumagai, Toru Yokoyama, Kazunari Takeuchi, Takuya Numasawa, Shuichi Aburakawa, Yoshihito Yamasaki, Taisuke Nitobe, and Kenji Kowatari for their contributions to sample collection, and thank Medical Corporation Seijin Society Ono Hospital for financial support. We also thank Drs. Kenzo Uchida and Takafumi Yayama of the Fukui University Department of Orthopaedics and Rehabilitation Medicine for their valuable suggestions.

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Figure/Table Legends

Fig. 1. Mesenchymal Stem Cell (MSC) marker-double positive cells in blood vessel regions. (A, B) H&E staining of (A) control non-OLF shows the sparse distribution of microvasculature (arrow: blood vessel), whereas a rich neovascularization is observed in the (B) OLF ligament matrix. Representative images of double immunofluorescence staining for MSC markers (CD73, CD90, and CD105) were shown in non-OLF (C, D, E) and OLF (F, G, H). The immunoexpression was detected around the blood vessel region. Merged images for CD90 (green) and CD73 (red) (C, F), for CD90 (green) and CD105 (red) (D, G), and for CD73 (green) and CD105 (red) (E, H) are shown. MSC marker-double positive cells are shown in yellow or orange. OLF: ossification of the ligamentum flavum; scale bar = 50 μm.

Fig. 2. Relationship between mesenchymal stem cell (MSC) marker (CD73, CD90, and CD105)-positive cells with vascular endothelial cells and pericytes in non-OLF samples. Merged images of double immunofluorescence staining for MSC markers and endothelial cell marker, CD31: (A) CD90 (green) and CD31 (red); (B) CD31 (green) and CD73 (red); (C) CD31 (green) and CD105 (red). Merged images of double immunofluorescence staining for MSC markers and pericyte marker, α-smooth muscle actin (SMA): (D) CD90 (green) and α-SMA (red); (E) α-SMA (green) and CD73 (red); (F) α-SMA (green) and CD105 (red). Enlarged images (G) show an absence of co-localization with CD90-positive cells (green) and CD31-positive cells (red), but co-expression of CD90-positive (green) and α-SMA positive (red) staining (merged, yellow and orange) for cells in the perivascular area. α-SMA: α-smooth muscle actin; scale bar = 10 μm.

Fig. 3. Mesenchymal Stem Cell (MSC) marker-double positive cells in collagenous matrix. In the H&E staining, (A) a section of the control non-OLF shows regular arrangement of fiber bundles, whereas (B) OLF shows marked irregular and fragmented fibers. Representative images of double immunofluorescence staining for MSC markers (CD73, CD90, and CD105) are shown in (D, E, F) non-OLF and (G, H, I) OLF. The fibroblast-like cells in the collagenous matrix show immunoexpression of MSC markers. Merged images for (D, G) CD90 (green) and CD73 (red); (E, H) CD90 (green) and CD105 (red); and (F, I) CD73 (green) and CD105 (red). (C) The prevalence of MSC marker-double positive cells in OLF (red) was compared with the prevalence in non-OLF (blue) in all of three MSC marker pairs. Values are the mean ± SEM (standard error of the mean) from six samples per group. *$p < 0.05$, compared with the control. OLF: ossification of the ligamentum flavum; scale bar = 50 μm.

Fig. 4. Mesenchymal Stem Cell (MSC) marker-double positive cells near the ossification front in OLF. (A) Histological findings using H&E staining of the ossification front show irregular calcification and many chondrocytes. (B) Higher magnification of the boxed area in (A). The chondrocyte-like cells near the ossification front showed immunoexpression of the chondrocyte marker (S100) and MSCs markers (CD73, CD90, and CD105). Merged images of double immunofluorescence staining for S100 and MSCs markers in the calcified cartilage area (CCA) area: (C) CD90 (green) and S100 (red); (D) S100 (green) and CD73 (red); (E) S100 (green) and CD105 (red); (F) CD90 (green) and CD73 (red); (G) CD90 (green) and CD105 (red); and (H) CD73 (green) and CD105 (red). FCA: fibrocartilage area; OA: ossified area; OLF: ossification

447 of the ligamentum flavum; Scale bar = 50μm.

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Figure 1

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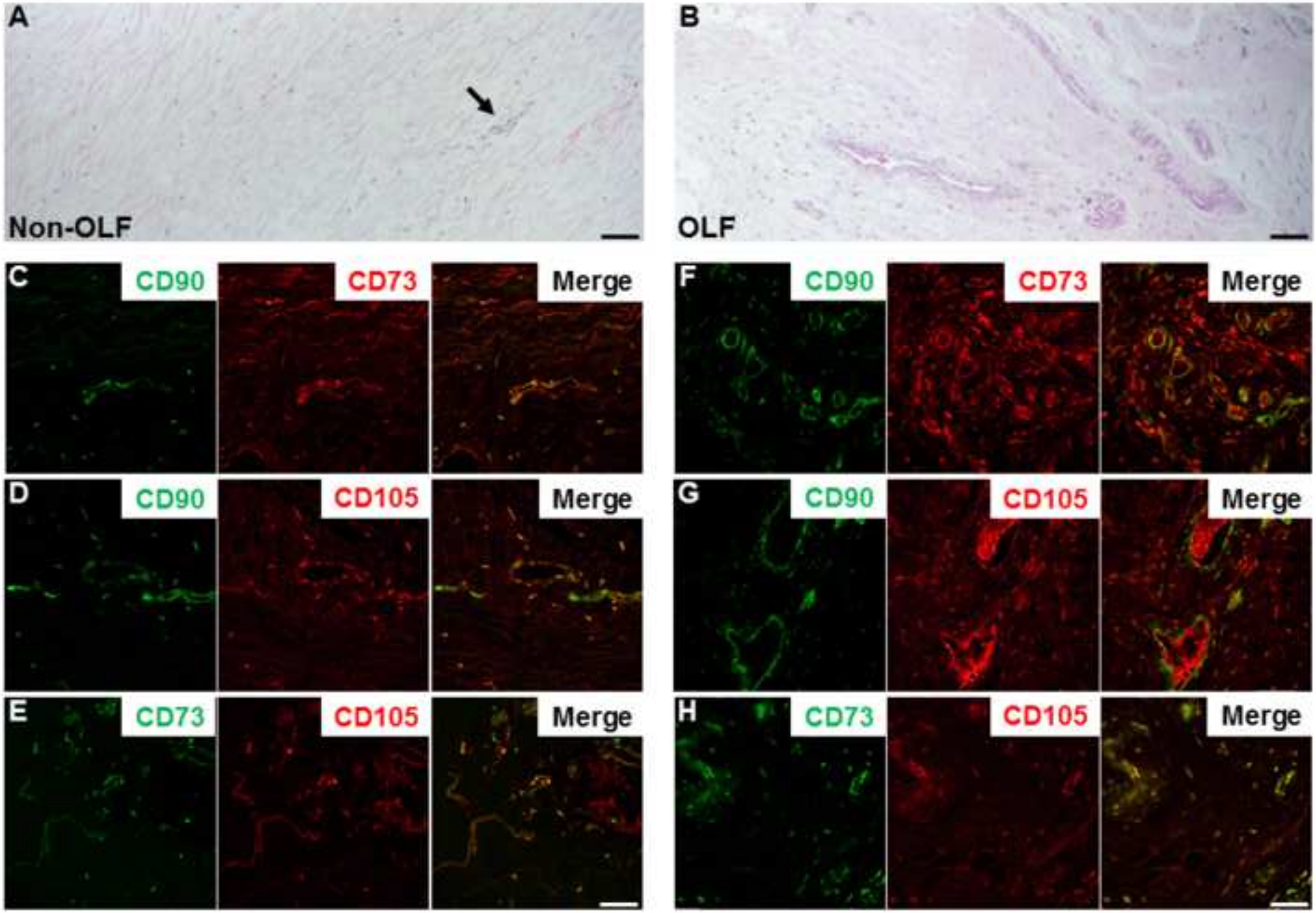

Figure 2

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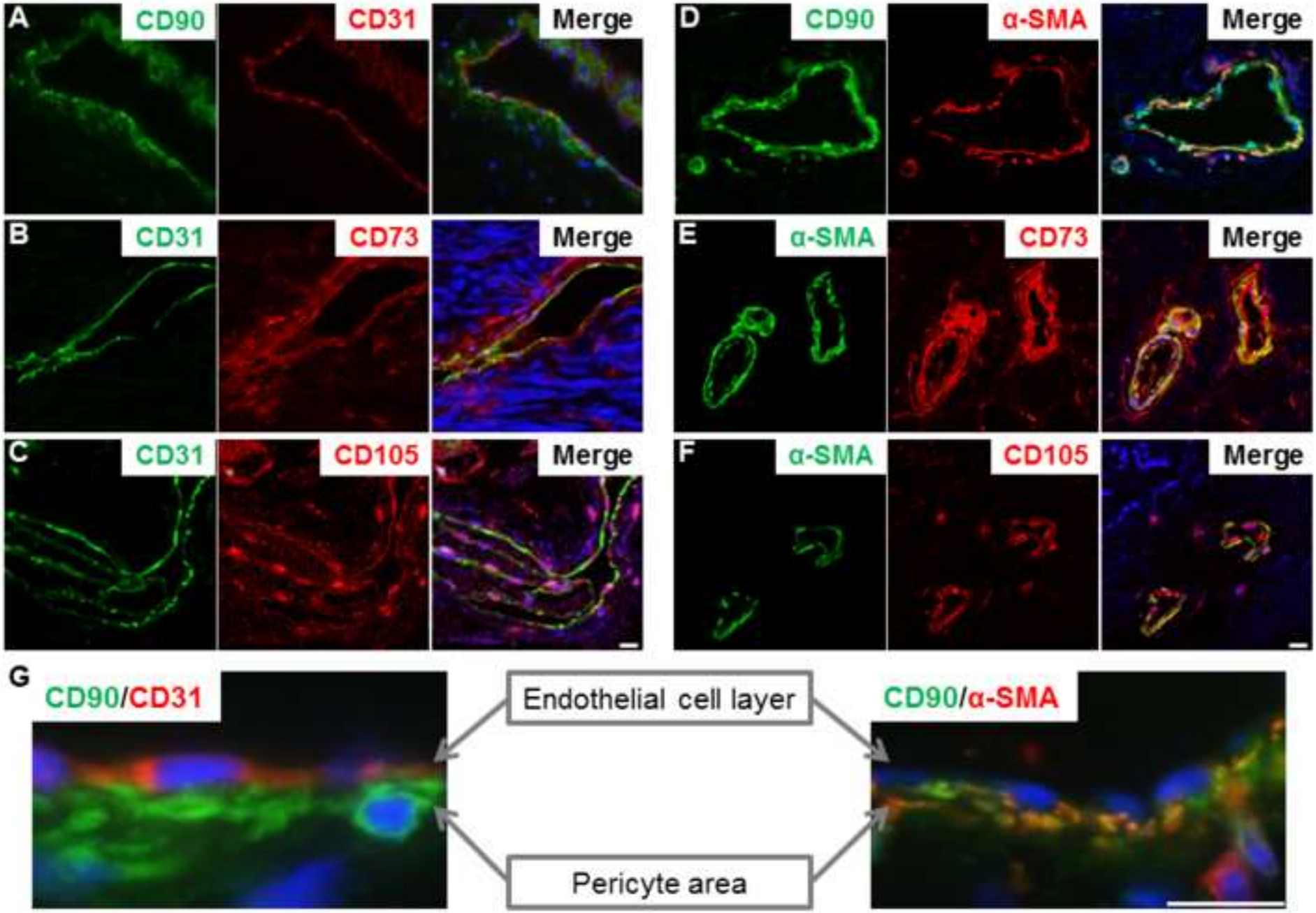

Figure 3

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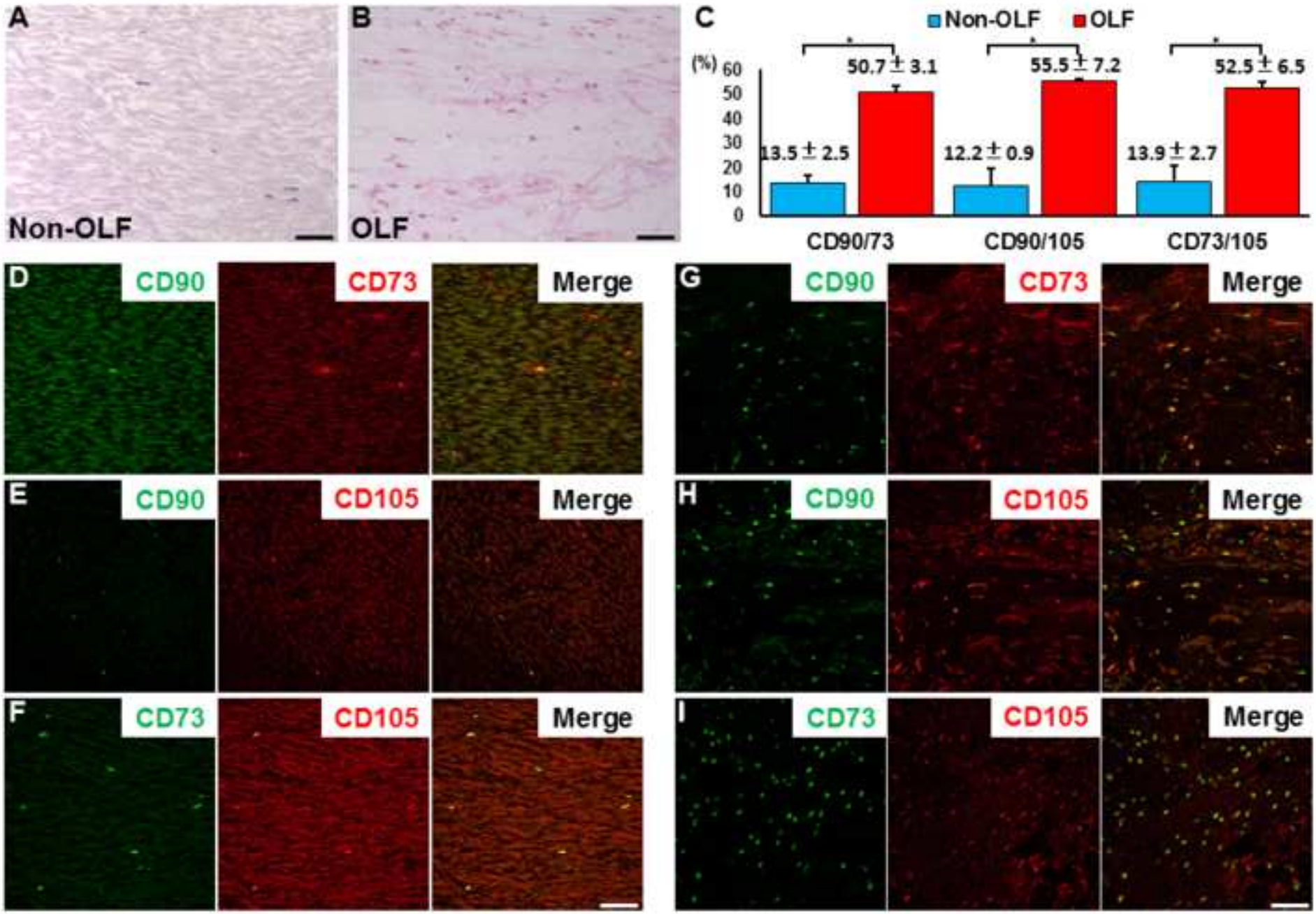

Figure 4

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