

# Osteoarthritis and Cartilage



Brief Report

## Slug transcription factor and nuclear Lamin B1 are upregulated in osteoarthritic chondrocytes



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### SUMMARY

**Objective:** To contribute to clarify molecular mechanisms supporting senescence and de-differentiation of chondrocytes in chondrocyte pathologies such as osteoarthritis (OA). Specifically, we investigated the relationship between the nuclear lamina protein Lamin B1 and the negative regulator of chondrogenesis Slug transcription factor in osteoarthritic chondrocytes.

**Methods:** Lamin B1 and Slug proteins were analyzed in cartilage explants from normal subjects and OA patients by immunohistochemical technique. Their expression was confirmed on isolated chondrocytes both at passage 0 and passage 2 (de-differentiated chondrocytes) by immunofluorescence and western blot. Subsequently, we explored the “*in vivo*” binding of Slug on *LMNB1* promoter by chromatin immunoprecipitation assay (ChIP).

**Results:** In this study we demonstrated that nuclear lamina protein Lamin B1 and anti-chondrogenic Slug transcription factor are upregulated in cartilage and OA chondrocytes. Furthermore, we found that Slug is “*in vivo*” recruited by *LMNB1* gene promoter mostly when chondrocytes undergo de-differentiation or OA degeneration.

**Conclusions:** We described for the first time a potential regulatory role of Slug on the *LMNB1* gene expression in OA chondrocytes. These findings may have important implications for the study of premature senescence, and degeneration of cartilage, and may contribute to develop effective therapeutic strategies against signals supporting cartilage damage in different subsets of patients.

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### Introduction

Lamins (A, C, B1 and B2) are nuclear intermediate filament proteins with multifunctional characteristics<sup>1</sup>. They support the structural integrity of the nucleus, and play a key role in a wide range of nuclear functions, including transcription, DNA replication and repair, control of cell cycle and stem cell niche function, cell proliferation and differentiation of specific lineages during development and adult life<sup>1,2</sup>. It has been demonstrated that tissue homeostasis can be disrupted by different mutations or abundance

variation in lamins causing a variety of diseases, collectively termed laminopathies, including the premature aging syndrome Hutchinson–Gilford progeria<sup>3</sup>. In recent years, the study on the lamins contributed to a better understanding of the phenomenon of cellular aging, tissue degeneration and pathogenesis of age-related diseases. Interestingly, changes of A:B lamin stoichiometry is correlated with stiffness, and tissues such as cartilage and bone have a high A:B ratio<sup>4</sup>. Over-expression of lamin A or the presence of its mutant form progerin leads to defects in chondrogenic differentiation potential of mesenchymal stem cells (MSCs)<sup>5</sup>. High levels of lamin A were found in osteoarthritic chondrocytes<sup>6</sup>, whilst lamin B's contribution remains unexplored. Osteoarthritis (OA) is a common degenerative joint disease characterized by cellular senescence, loss of chondrocyte activity and degradation of articular cartilage<sup>7</sup>. Due to the complexity of these phenomena, there is a limited understanding in OA pathogenesis. The hypothesis to be

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explored is the possibility that chondrocyte response to various OA inducing conditions converges on de-differentiation and acquisition of stem cell-like properties. This might occur before reentry into the cell cycle or acquisition of the senescent phenotype. Clarifying this issue may be important to devise new approaches for OA prevention and treatment.

We recently found that Slug, an antichondrogenic transcription factor, is a marker of chondrocyte de-differentiation<sup>8</sup>. In order to find new Slug target molecules and to explore the participation of lamin B1 in the cartilage damage, we investigated their expression and molecular relationship in OA cartilage explants and *in vitro* de-differentiated chondrocytes.

## Material and methods

### Cartilage explant analysis

Knee cartilage explants from normal subjects (at autopsy,  $n = 3$ , age =  $55 \pm 3$  years, sex = male, Mankin score = 0–3) and OA patients (from joint replacement,  $n = 6$ , age =  $58 \pm 7$  years, sex = male, Mankin score = 8–10) were fixed in a freshly prepared 9:1 mixture of B5 solution (mercuric-chloride containing fixative)/40% formaldehyde at room temperature for 2 h, dehydrated and embedded in paraffin as previously described<sup>9</sup>. The slides were then incubated using the following antibodies:  $\beta$ -galactosidase (goat anti-human 1:200) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Slug (mouse anti-human, 1:300) from Origene (Rockville, MD, USA), Lamin A (rabbit anti-human 1:200) and Lamin B1 (rabbit anti-human 1:200) from Abcam (Cambridge, UK) overnight at 4°C, washed and incubated with anti-goat- or goat-anti-mouse/rabbit-biotinylated and alkaline phosphatase-conjugated streptavidin (Kit BioGenex, San Ramon, CA, USA) at room temperature for 30 min. Slides were developed using new fast red as substrate and evaluated using a brightfield microscope. Safranin O staining was also performed. The study was approved by local ethical committee at Istituto Ortopedico Rizzoli.

### Cell culture, immunofluorescence and gene expression analysis

Normal ( $n = 3$ ) and osteoarthritic chondrocytes ( $n = 6$ ) were isolated from articular cartilage by sequential enzymatic digestion as previously described<sup>10</sup>. Cells from passage 0 (p0) to passage 2 (p2) were grown on glass coverslips were fixed in methanol at  $-20^\circ\text{C}$  for 7 min, blocked in 4% BSA-PBS and incubated with rabbit anti-human-Slug (clone H140, 1:10, Santa Cruz Biotechnology) and mouse anti-human Lamin B1 (clone 8D1, 1:100, Santa Cruz Biotechnology) overnight at 4°C. Coverslips were next washed and incubated with fluorescence-labeled secondary antibodies (Sigma–Aldrich Chemical Co., St. Louis, MO) for 1 h at room temperature. Nuclei were counterstained with DAPI (4,6-diamino-2-phenylindole). Slides were mounted with antifade reagent in glycerol and observed with a Nikon E600 epifluorescence microscope (100 $\times$  magnification, 1.3 NA – numerical aperture) equipped with a digital camera. Images were processed using Adobe Photoshop (Adobe Systems).

Total RNA was extracted from cells at p0 and p2, reverse transcribed and PCR performed for collagen type I, collagen type II and Sox9 as previously described<sup>10</sup>.

### Western blotting

20  $\mu\text{g}$  of whole cell lysates were resolved by SDS-PAGE, transferred to PVDF (Millipore, Billerica, MA, USA) and blocked with PBS-0.05% Tween 20/5% NFD, then probed overnight (4°C) with the following antibodies: Slug (mouse anti-human, clone A-7, 1:1000),

Actin (mouse anti-human, clone C-2, 1:5000) from Santa Cruz Biotechnology, and Lamin B1 (rabbit anti-human 1:1500) from Abcam. After washing, the membranes were incubated with anti-mouse or anti-rabbit HRP conjugated antibodies (DAKO, Glostrup, Denmark) 1:2000, and signals were detected by Supersignal West Femto Substrate (Pierce, Rockford, IL, USA). Actin was used to confirm equal protein loading.

### Chromatin immunoprecipitation assay (ChIP)

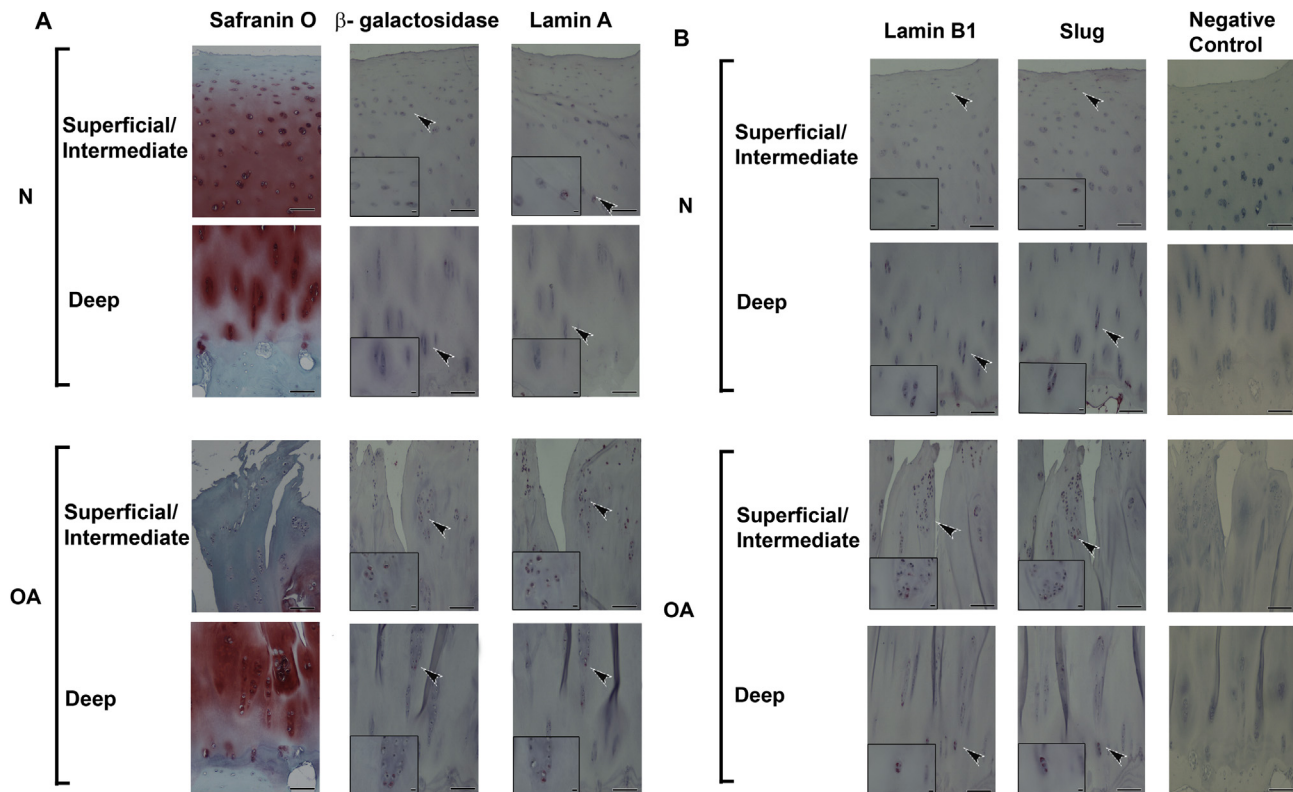
ChIP was performed using a CHIP assay kit (Upstate Biotechnology, Waltham, MA, USA) according to the manufacturer's instructions as previously described<sup>8</sup>. Chromatin samples from normal or OA chondrocytes were immunoprecipitated with a specific primary Slug antibody. IgG antibody was used as a negative control (all antibodies were purchased from Santa Cruz Biotechnology).

PCR was carried out with three primer sets (Set 1: upper: 5'-GTCACCTCGTCTTGCATTT-3', lower: 5'-GCGTTTAGAGGAACGGAGAA-3'; Set 2: upper: 5'-GTAGACACGGGGTTTACCA-3', lower: 5'-TGGATTACCATCCACACAA-3'; and Set 3: upper: 5'-ACGGGGTTTCACTATGTTGG-3' lower: 5'-TTGGTTGTGAGACCCTTTCC-3'), spanning Slug binding sites in the hLMNB1 gene promoter.

## Results

Loss of chondrocyte-specific properties was detected in degenerated cartilage tissue from OA patients. As shown in Fig. 1(A), OA cartilage showed a low level of Safranin O staining for sulfated glycosaminoglycans, and increased senescence-associated  $\beta$ -galactosidase and Lamin A immunostaining. This confirmed that a combination of phenomena including deterioration of tissue function and senescence occurs in OA cartilage. In search for new mediators of cartilage damage, we next analyzed tissue sections for the presence of another key player in cellular senescence, Lamin B1, and an anti-chondrogenic molecule, Slug transcription factor. Interestingly, the expression of Lamin B1 was increased in OA cartilage compared to normal samples and Slug colocalized with Lamin B1 [Fig. 1(B)]. Noteworthy, in normal cartilage few Slug-positive cells were present, both in the superficial/intermediate and deep area of the tissue. Conversely, a higher number of Slug-positive cells, located in all cartilage layers, were detected in OA cartilage. As senescence of chondrocytes is inherent to the OA process<sup>7</sup>, we state that Slug, in addition to being a marker of cell de-differentiation<sup>8</sup>, can also be associated to senescence.

We confirmed this hypothesis in isolated normal and OA chondrocytes that were de-differentiated through prolonged passages in monolayer culture. In accordance with literature data<sup>10,11</sup>, we confirmed the loss of the cartilaginous phenotype associated with de-differentiation process by demonstrating a decrease in the expression of typical chondrogenic markers, including collagen type II and Sox9, and an increase of collagen type I [Fig. 2(A)]. As previously demonstrated<sup>8</sup>, Slug protein gradually increased to very high levels during de-differentiation process of normal chondrocytes. This phenomenon was observed here, for the first time, also in OA chondrocytes, as shown by the immunoblot analysis reported in Fig. 2(A). The same analysis revealed the presence of Lamin B1 in p0 chondrocytes and de-differentiated p2 chondrocytes from healthy subjects and OA patients. Higher Slug levels were determined in OA samples compared with controls. This was confirmed by immunofluorescence analysis showing that Lamin B1 localized at the nuclear periphery, while Slug was mostly recruited in the nuclear interior [Fig. 2(B)].



**Fig. 1.** (A) Safranin O staining of the superficial/intermediate and deep zone of representative normal (N) and OA cartilage showing the composition of the cartilages analyzed. Immunohistochemical analysis of  $\beta$ -galactosidase, Lamin A on serial sections of cartilage from normal and OA cartilage. Bar = 100  $\mu$ m, Inset bar = 10  $\mu$ m. (B) Immunohistochemical analysis of Lamin B1 and Slug on serial sections of cartilage from normal and OA cartilage. Bar = 100  $\mu$ m, Inset bar = 10  $\mu$ m. Negative Control, Bar = 100  $\mu$ m.

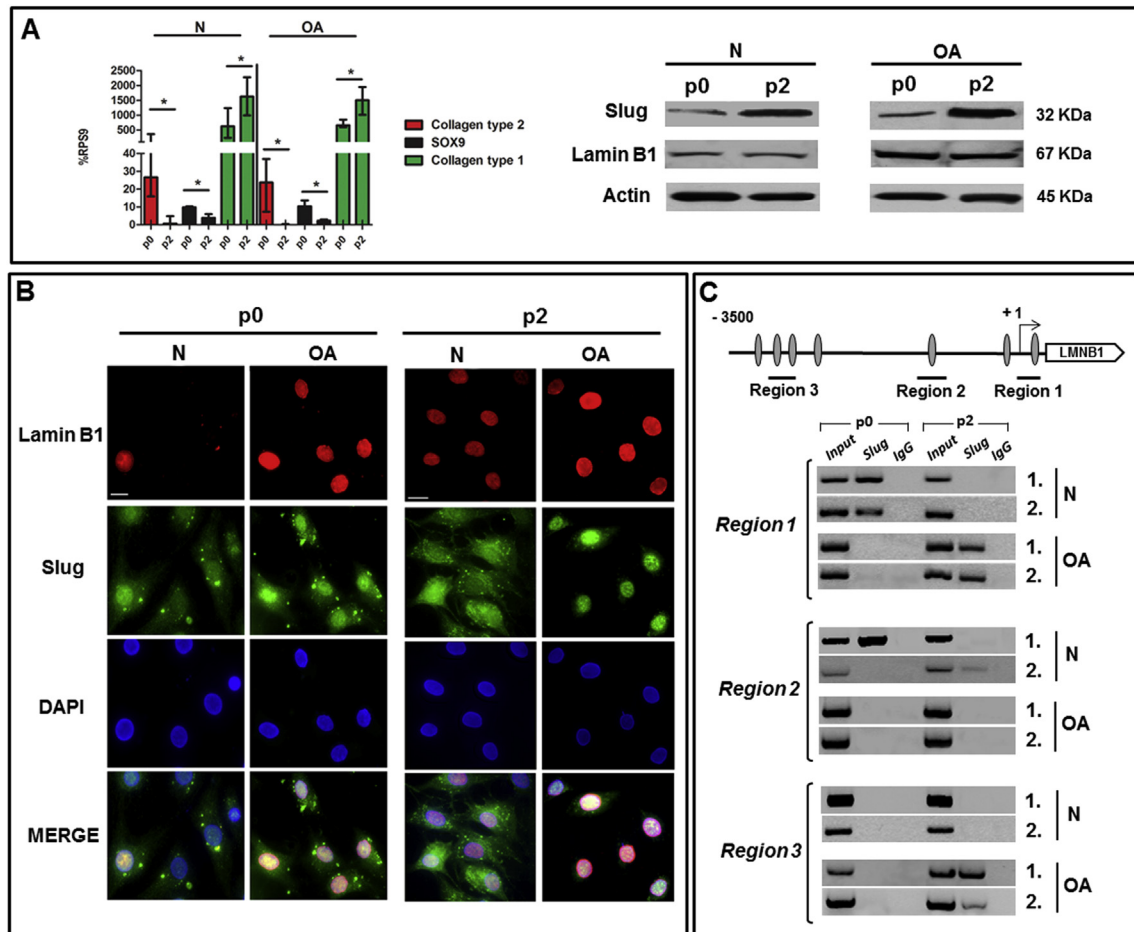
These findings prompted us to establish the functional link between Slug and Lamin B1, investigating a possible role of Slug as regulator of Lamin B1 expression during chondrocyte de-differentiation process. The possibility that Lamin B1 is a direct transcriptional target of Slug was examined by ChIP assay. By using the Transcription Element Search Software (TESS) for transcription factor search and MatInspector 7.4, we identified seven potential Slug binding sites (E-boxes, 5'-CANNTG/CANNTG-3') in the *LMNB1* gene 5' regulatory region, six upstream and one downstream of the transcription start site. We focused on three chromatin subregions of *LMNB1* promoter for "in vivo" E-box occupancy analysis. We observed Slug binding to Regions 1 and 3 in p2 OA chondrocytes, whereas Slug was mostly recruited at Region 1 and 2 in p0 normal chondrocytes [Fig. 2(C)]. Concomitant binding of acetylated histone H3 (Ac-H3) confirmed the active transcription of the locus (data not shown).

## Discussion

An effective strategy to prevent cartilage loss or treat cartilage diseases is still lacking. Therefore, the search for potential molecular targets that are involved in the mechanisms of pathogenesis represents an important challenge. In this study, we described, for the first time, the upregulation of Slug and lamin B1 both in OA cartilage explants and *in vitro* de-differentiated chondrocytes, suggesting the implications of these molecules in two critical phenomena involved in onset of OA, the loss of cell differentiation and cellular senescence. We previously demonstrated that the inhibition of Slug, a transcriptional repressor of cartilage formation<sup>12</sup>, is effective in inducing chondrogenesis of mesenchymal stem cells<sup>8</sup>. Our data evidence that Slug is involved in chondrocyte senescence,

possibly due to its *in vivo* recruitment on the promoter of *LMNB1* gene. However, further studies will be needed to dissect the way Slug can accomplish its task. Our results suggest that Slug might drive the loss of chondrocytic phenotype through regulation of Lamin B1 expression and lead us to speculate that Slug inhibition in culture-expanded chondrocytes could prevent the de-differentiation process. Contrasting roles of Lamin B1 in cellular senescence have been described<sup>13</sup>. Reduced levels of Lamin B1 have been observed in HGPS patient-derived fibroblasts and senescent normal fibroblasts *in vitro*<sup>13</sup>. In one report, Lamin B1 silencing led to apoptosis in HeLa cells<sup>14</sup>, whereas recent work suggested that inhibition of Lamin B1 causes senescence in WI-38 cells<sup>15</sup>. Conversely, Lamin B1 overexpression appeared to enhance proliferation and delay the onset of senescence, whereas a second study showed that elevated levels of Lamin B1 triggered senescence<sup>13</sup>. Our data demonstrate a marked increase of Lamin B1 in OA chondrocytes, in favor of the hypothesis of its association with tissue degeneration and senescence.

Tissue degeneration and senescence involve a plethora of mediators so it is difficult to establish a hierarchy of events. Therefore, the key question is whether Slug and Lamin B1 are upstream or downstream the OA pathogenetic process. However, the proof of the *in vivo* molecular relationship between these two proteins could help to identify potential mechanisms by which catabolic changes in the articular cartilage lead to the onset of OA and to devise new approaches for the prevention and treatment of OA. Moreover, the evidences here described may help to improve cell-based therapies that use chondrocytes, helping to solve the problems of cellular senescence and de-differentiation that occur during *in vitro* expansion that represent one of the major practical barriers to clinical application.



**Fig. 2.** (A) Collagen type II, SOX9 and Collagen type I expression levels were determined by qRT-PCR in p0 chondrocytes and p2 de-differentiated chondrocytes from healthy subjects (N) and OA patients. Real time PCR was run in a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the SYBR Premix Ex Taq (TaKaRa Biomedicals, Tokyo, Japan). For each target gene, mRNA levels were calculated, normalized to RPS9 according to the formula  $2^{-\Delta Ct}$  and expressed as a percentage of the reference gene. Statistical analysis was performed comparing p0 to p2 chondrocytes, using non parametric Wilcoxon matched pair test. Significant differences \* $P < 0.05$ . Slug and Lamin B1 expression levels were determined by Western Blotting in p0 chondrocytes and de-differentiated p2 chondrocytes from healthy subjects (N) and OA patients (OA). 20 μg of each samples were assayed on SDS-PAGE and proteins were visualized by ECL method (PIERCE). Actin was used as loading control. (B) Representative immunofluorescence analysis performed on p0 and de-differentiated p2 chondrocytes from healthy subjects (N) and OA patients (OA) using anti-Lamin B1 (red) and Slug (green) antibodies. Nuclei were counterstained with DAPI. Merge contains the combined image of Slug and Lamin B1 immunostaining and DAPI staining. Determinations were done in triplicate in three independent cell cultures. Bar = 10 μm. (C) Schematic representation of the human LMNB1 promoter region; putative Slug binding sites are indicated with grey ovals. ChIP assay of “in vivo” Slug binding to the Lamin B1 proximal promoter was performed. DNA templates were obtained from p0 chondrocytes and de-differentiated p2 chondrocytes from two healthy subjects (N) and two OA patients (OA). Immunoprecipitation was performed using an anti-Slug antibody or preimmune rabbit IgG. DNA samples of ChIP reactions (Slug, IgG) and Input DNA, collected before precipitation, were used in PCRs with different primer pairs and amplicons are reported: region 1 = 174 bp, region 2 = 189 bp, region 3 = 178 bp. Each PCR reaction was performed with 5 μl of the bound DNA fraction or 2 μl of the Input. PCR fragments were analyzed on 1.5% agarose gel.

### Author's contributions

Prof Piva, Dr Lattanzi and Dr Lisignoli were responsible for conception and design of the study.

All authors contributed to acquisition and interpretation of data.

The article was drafted by Prof Piva and Dr Lisignoli, and was revised by Dr Lattanzi.

All authors have approved the final version for submission.

### Conflict of interests

The authors have none to declare.

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