Osteoarthritis and Cartilage 18 (2010) 1518–1527

Osteoarthritis and Cartilage

Endoglin differentially regulates TGF-β-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes

K.W. Finnson †, W.L. Parker †, Y. Chi †, C.D. Hoemann ‡, M.B. Goldring §, J. Antoniou ||, A. Philip †*

† *Division of Plastic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada*
‡ *Department of Chemical Engineering, University of Montreal, Montreal, Quebec, Canada*
§ *Research Division, Hospital for Special Surgery, Weill Cornell Medical College, New York, NY 10021, USA*
|| *Division of Orthopaedic Surgery, Department of Surgery, McGill University, Montreal, Quebec, Canada*

ARTICLE INFO

Article history:
Received 12 April 2010
Accepted 3 September 2010

Keywords:
Endoglin
Chondrocyte
TGF-beta
Smad
Dedifferentiation
Osteoarthritis

SUMMARY

Objective: Transforming growth factor-β (TGF-β) plays a critical role in cartilage homeostasis and deregulation of its signalling is implicated in osteoarthritis (OA). TGF-β isoforms signal through a pair of transmembrane serine/threonine kinases known as the type I and type II TGF-β receptors. Endoglin is a TGF-β co-receptor that binds TGF-β with high affinity in the presence of the type II TGF-β receptor. We have previously shown that endoglin is expressed in human chondrocytes and that it forms a complex with the TGF-β signalling receptors. However, the functional significance of endoglin expression in chondrocytes is unknown. Our objective was to determine whether endoglin regulates TGF-β/Smad signalling and extracellular matrix (ECM) production in human chondrocytes and whether its expression varies with chondrocyte differentiation state.
Method: Endoglin function was determined by overexpression or antisense morpholino/siRNA knockdown of endoglin in human chondrocytes and measuring TGF-β-induced Smad phosphorylation, transcriptional activity and ECM production. Alterations in endoglin expression levels were determined during subculture-induced dedifferentiation of human chondrocytes and in normal vs OA cartilage samples.
Results: Endoglin enhances TGF-β1-induced Smad1/5 phosphorylation and inhibits TGF-β1-induced Smad2 phosphorylation, Smad3-driven transcriptional activity and ECM production in human chondrocytes. In addition, the enhancing effect of endoglin siRNA knockdown on TGF-β1-induced Smad3-driven transcription is reversed by ALK1 overexpression. Furthermore, endoglin levels are increased in chondrocytes following subculture-induced dedifferentiation and in OA cartilage as compared to normal cartilage.
Conclusion: Together, our results suggest that endoglin regulates the balance between TGF-β/ALK1/Smad1/5 and ALK5/Smad2/3 signalling and ECM production in human chondrocytes and that endoglin may represent a marker for chondrocyte phenotype.

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional regulator of cartilage repair and homeostasis with critical roles in chondrocyte proliferation, differentiation and extracellular matrix (ECM) production[1–3]. Studies in mice have shown that disruption of TGF-β signalling leads to the development of degenerative joint disease resembling human osteoarthritis (OA)[4,5]. Furthermore, TGF-β receptor expression and TGF-β signalling are markedly reduced in murine models of OA[6]. Conversely, TGF-β treatment has been reported to result in OA-like lesions in rat knee joints suggesting that excessive TGF-β action may also play a role in OA pathogenesis[7,8]. Thus, factors that increase or decrease TGF-β activity may be important in maintaining chondrocyte phenotype and preventing cartilage diseases such as OA.

TGF-β isoforms (β1, β2, β3) signal through a pair of transmembrane serine/threonine kinases known as the type I (TβRI or activin receptor-like kinase-5; ALK5) and the type II (TβRII)

* Address correspondence and reprint requests to: Anie Philip, Division of Plastic Surgery, Montreal General Hospital, 1650 Cedar Avenue, Room C9-158, Montreal, QC, Canada H3G 1A4. Tel: 1-514-934-1934x44533; Fax: 1-514-934-8289.
E-mail address: anie.philip@mcgill.ca (A. Philip).

1063-4584/$ – see front matter © 2010 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.joca.2010.09.002

receptors[9]. TGF-β binds TβRII which, in turn, recruits ALK5 resulting in the formation of a heteromeric receptor complex[10,11]. TβRII is a constitutively active kinase and phosphorylates ALK5 within the TGF-β receptor complex leading to activation of ALK5 kinase activity. The activated ALK5 propagates the TGF-β signal by phosphorylating intracellular Smad2 and Smad3. Besides signalling through the ALK5/Smad2/3 pathway, TGF-β has also been shown to signal through another type I TGF-β receptor named ALK1 leading to phosphorylation of Smad1 and Smad5[12,13]. Phosphorylated Smad2/3 and phosphorylated Smad1/5 associate with Smad4 and translocate to the nucleus where they interact with various transcriptional coactivators, corepressors and transcription factors to modulate target gene expression[14].

Endoglin is a TGF-β co-receptor that has been shown to modulate TGF-β signalling in endothelial cells[15,16]. Endoglin binds TGF-β1 and -β3 with high affinity in the presence of TβRII but does not bind TGF-β2[15,16]. Although endoglin is not a signalling receptor, it has been shown to associate with the TGF-β signalling receptors (TβRII, ALK1 and ALK5) and to modulate their phosphorylation status as well as downstream Smad-dependent and Smad-independent signalling in endothelial cells[17]. We have previously shown that endoglin is expressed in human chondrocytes and that it forms a heteromeric complex with TβRII and TβRI (ALK1 and ALK5)[18]. However, the functional significance of endoglin expression in chondrocytes is unknown. Here we show that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation but inhibits TGF-β1-induced Smad2 phosphorylation, Smad3-driven transcriptional activity and ECM protein (type II collagen and PAI-1) expression in human chondrocytes. We also demonstrate that the enhanced TGF-β-induced Smad3-driven transcription by endoglin siRNA knockdown is reversed by ALK1 overexpression. Furthermore, we show that endoglin expression is increased in human chondrocytes following subculture-induced dedifferentiation *in vitro* and in OA cartilage as compared to normal cartilage *in vivo*. Taken together, our results establish endoglin as a regulator of TGF-β/Smad signalling and ECM production in human chondrocytes and suggest that endoglin may represent a potential marker for chondrocyte dedifferentiation and/or phenotype.

Method

Cell culture

The SV40-immortalized human chondrocyte cell lines, C28/I2 and tsT/AC62, have been described previously[19]. The C28/I2 cell line was developed from chondrocytes isolated from juvenile human costal cartilage and immortalized by retroviral infection of the SV40 large T antigen[20]. The tsT/AC62 cells were developed from chondrocytes isolated from adult human articular cartilage and immortalized by retroviral infection of a temperature-sensitive mutant of SV40 large T antigen that is functional at 32°C but not at 37°C[19]. Primary chondrocytes were isolated from human osteoarthritic articular cartilage obtained intraoperatively from adult arthroplasty specimens or from normal adult cartilage obtained from traumatic open joint injuries with no evidence of degenerate joint disease (normal cartilage, N). All procedures were approved by the Research Ethics Board of the McGill University Health Center and all cartilage samples were obtained with informed consent. The cartilage was rinsed with cold phosphate buffered saline (PBS), minced and then incubated for 1 h at 37°C with 1 mg/ml Pronase followed by overnight digestion at 37°C with 1 mg/ml collagenase. The suspension was passed through a 70 μm cell sieve and chondrocytes were collected by 5 min centrifugation, washed with PBS and plated at a density of 2.5×10^5 cells/cm^2. Alternatively, chondrocyte cultures were established by outgrowth of explants as described previously[21]. All cells were cultured in DMEM/F12 containing 10% fetal bovine serum and antibiotics and maintained at 37°C in a humidified incubator under 5% CO_2/air (Supplementary information for details).

The 3-dimensional (3-D) alginate culture system for chondrocyte redifferentiation has been described previously[19,20]. Briefly, chondrocytes (4×10^6 cells/ml) were suspended in a 1.2% alginate solution and passed drop-wise through a 25-gauge needle into a 102 mM $CaCl_2$ solution resulting in the formation of alginate beads. The beads were washed with 0.15 M NaCl and transferred to culture flasks with serum-containing media. The alginate beads were cultured for 21 days and the media changed every third day. Alginate beads were depolymerised with 55 mM sodium citrate/0.15 M NaCl and the recovered chondrocytes washed in PBS, resuspended in serum-containing media, plated (2.5×10^5 cells/cm^2) and grown to confluence. Subculture-induced dedifferentiation was performed by serially passaging confluent chondrocytes at a 1:2 split ratio for up to 12 passages. Cells at passage 1–2 are designated as 'early passage' and cells at passage 10–12 are designated as 'late passage'. Chondrocyte morphology was visualized by phase contrast microscopy and images were captured with a digital camera.

Affinity labelling

Affinity labelling was performed as described previously[13]. Briefly, chondrocytes were washed with cold binding buffer (Supplementary information for details) and incubated for 3 h at 4°C with 100 pM of [^{125}I]TGF-β1. [^{125}I]TGF-β1-labelled complexes were covalently cross-linked with 1 mM bis-sulfosuccinimidyl suberate. Membrane extracts were prepared in solubilisation buffer (Supplementary information for details). Protein concentrations were determined using a Protein Assay Kit. Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the gels stained with Coomassie blue and radioactive bands were visualized by autoradiography.

Transfections

Endoglin overexpression: Chondrocytes were transiently transfected with endoglin or its empty vector (EV) using Lipofectamine 2000 according to the manufacturers' instructions. *Endoglin knockdown*: Chondrocytes were transfected with endoglin-specific antisense morpholinos or control morpholinos using i-PEI transfection reagent according to the manufacturers' instructions. Alternatively, chondrocytes were transfected with endoglin-specific siRNA, ALK1-specific siRNA or control siRNA as described previously[22].

Western blot analysis

Chondrocytes transfected with (1) endoglin or EV, (2) endoglin-specific or control morpholinos or (3) endoglin-specific siRNA, ALK1-specific siRNA or control siRNA were grown overnight in serum-containing media. Chondrocytes were washed with PBS, serum-starved for 3 h and then treated for 45 min or 24 h with 0–100 pM TGF-β1. Cell lysates were prepared in RIPA buffer (Supplementary information for details). Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with Tris–buffered saline–Tween 20 (Supplementary information for details) and incubated overnight at 4°C with the indicated primary antibodies (Supplementary Table 1 for antibody information). Membranes were washed with TBST and incubated with a horseradish peroxidase (HRP)-conjugated

secondary antibody. Detection was performed using the enhanced chemiluminescence (ECL) system.

Luciferase assays

C28/I2 cells were transfected with (1) Eg or EV plasmids or (2) endoglin-specific siRNA or control siRNA. In some experiments, endoglin-specific siRNA and control siRNA transfected cells were co-transfected with wild-type (WT) ALK1, constitutively active ALK1 (QD) or EV. Cells were co-transfected with Smad3-responsive $CAGA_{12}$-lux[23] or Smad2/3-responsive 3TP-lux[24] luciferase reporter constructs. All cells were co-transfected with pCMV-β-galactosidase. Cells were then treated for 24 h with 0–100 pM of TGF-β1 or TGF-β2 under serum-free conditions. Cell lysates were prepared and analyzed for luciferase and β-galactosidase activity as described previously[13]. The data are presented as a fold-change in luciferase activity from control (absence of ligand addition and/or transfection with control siRNA/EV alone).

Statistical analysis

Means calculated from replicates of three or more independent experiments were used for statistical analysis. Differences between the means were compared using Analysis of Variance (ANOVA) followed by *post hoc* pair-wise comparision using Bonferroni *t*-test (Systat 11.0; Systat, Chicago, IL).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from chondrocytes and reverse transcribed using (MMLV)-RT and oligo-dT primer. PCR was performed using human endoglin[25] and human GAPDH primers[26] and Taq polymerase (Supplementary information for details). PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining[13].

Cartilage extraction

Total protein was extracted from human articular cartilage as described previously[27]. Briefly, cartilage tissue was pulverized under liquid nitrogen using a biopulverizer (Biospec Products Inc., Bartlesville, OK) and then homogenized in GuCl extraction buffer [4 M GuCl/50 mM Tris, pH 7.5 and 1 mM ethylenediaminetetraacetic acid (EDTA)]. Samples were vortexed at 4°C for 30 min, centrifuged at 15,000×*g* for 10 min at 4°C and the supernatants were collected and precipitated with 5 vol 100% ethanol at −20°C. Pellets were washed with 75% ethanol, dried, and resuspended in 8 M urea. Samples containing equivalent amounts of protein were analyzed by Western blot using an anti-endoglin antibody. Membranes were stained with Ponceau to confirm equal protein loading.

Immunohistochemistry

Cartilage tissue was fixed in a periodate-lysine-paraformaldehyde solution[28] and embedded in a solution containing two parts 20% sucrose phosphate buffer to one part O.C.T. embedding medium (Tissu-Tek, Miles, USA). Cryostat sections (8 μm) were obtained, fixed in 4% paraformaldehyde, washed in PBS and treated with chondroitinase ABC for 1 h at 37°C. The sections were washed in PBS, blocked for 1 h at room temperature with 5% normal goat serum and then incubated overnight at 4°C with anti-endoglin

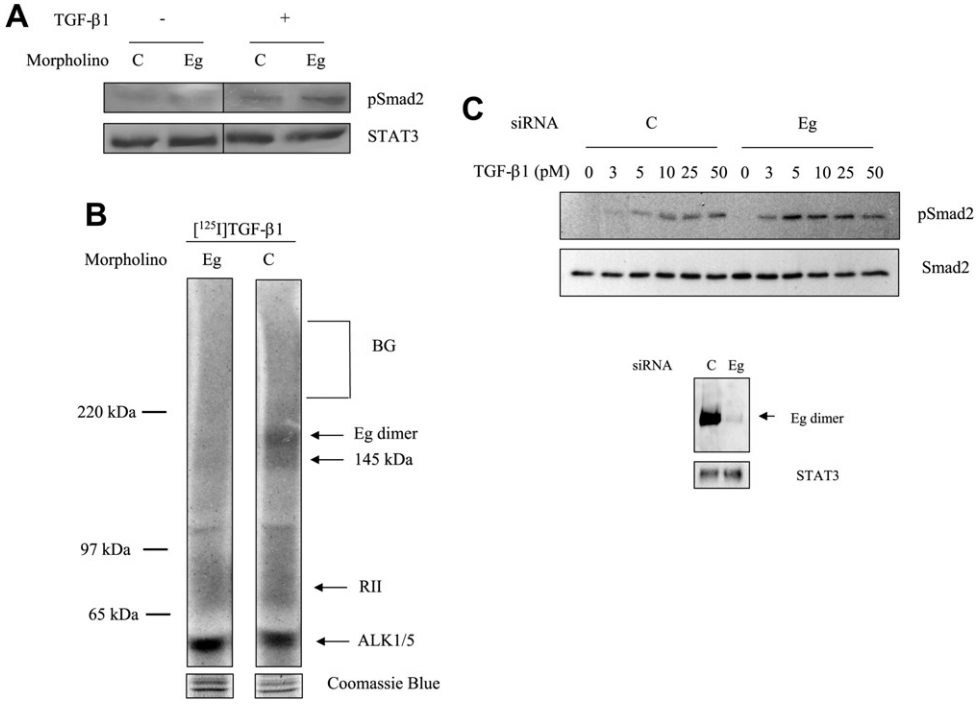

Fig. 1. Endoglin inhibits TGF-β1-induced Smad2 phosphorylation in human chondrocytes. (A) Primary human articular chondrocytes treated with endoglin-specific (Eg) antisense morpholino oligonucleotides or control (C) morpholino oligonucleotides were incubated for 15 min with or without 100 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by Western blot using anti-phosphoSmad2 or anti-STAT3 antibodies as indicated. (B) Primary human articular chondrocytes treated with endoglin-specific (Eg) or control (C) morpholinos were affinity labelled with [^{125}I]TGF-β1. Cell lysates were prepared and analyzed under non-reducing conditions by SDS-PAGE/autoradiography. Coomassie blue staining confirms that equal amounts of protein were loaded in each lane. (C) Primary human articular chondrocytes transfected with endoglin-specific (Eg) siRNA or control (C) siRNA were treated for 30 min with 0–50 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by Western blot using anti-phospho-Smad2, anti-Smad2, anti-endoglin or anti-STAT3 antibodies as indicated. For A and B, the lanes were selected from non-adjacent regions of the same gel. Results shown are representative of three independent experiments. Eg = endoglin; BG = betaglycan.

(Dako, SN6h, 1:100) antibody. Sections were washed in PBS and incubated at room temperature for 30 min with a HRP-conjugated anti-mouse secondary antibody. Immune complexes were detected using the Vectastain ABC kit according to the manufacturers' instructions (Vector Laboratories, Burlington, ON).

Results

Endoglin inhibits TGF-β1-induced Smad2 phosphorylation and enhances TGF-β1-induced Smad1/5 phosphorylation in human chondrocytes

The ability of endoglin to regulate TGF-β/Smad signalling in human chondrocytes was examined by blocking endoglin expression in primary human articular chondrocytes using antisense morpholinos and measuring TGF-β1-induced Smad2 phosphorylation. Fig. 1(A) demonstrates that blocking endoglin expression is associated with enhanced TGF-β1-induced Smad2 phosphorylation [Fig. 1(A) top panel]. Reprobing the membrane with an anti-STAT3 antibody confirms that equivalent amounts of protein were loaded in each lane [Fig 1(A), bottom panel]. Fig. 1(B) demonstrates that cells transfected with Eg-specific antisense morpholinos display markedly reduced levels of [^{125}I]TGF-β1-bound (cell surface) endoglin as compared to cells transfected with control morpholinos. Western blot analysis also demonstrated that chondrocytes transfected with endoglin-specific morpholinos display a marked reduction in endoglin protein levels as compared to control morpholino transfected cells (data not shown).

The effect of blocking endoglin expression on TGF-β1-induced Smad2 phosphorylation was also examined using a siRNA-based approach. Fig. 1(C) (top panel) shows that primary articular chondrocytes transfected with endoglin-specific siRNA display enhanced TGF-β1-induced Smad2 phosphorylation as compared to control morpholino transfected cells, particularly at lower doses (i.e., $\leq$10 pM) of TGF-β1, without altering total Smad2 protein levels [Fig. 1(C), second panel]. Fig. 1(C) (third panel) shows that endoglin siRNA treated cells display markedly reduced levels of endoglin as compared to control siRNA transfected cells. Reprobing the membrane with an anti-STAT3 antibody confirms that equal amounts of protein were loaded in each lane [Fig. 1(C), bottom panel].

We next examined whether endoglin regulates TGF-β/ALK1-mediated phosphorylation of Smad1/5 in primary human articular chondrocytes. Fig. 2(A) (top panel) shows that ALK1-specific siRNA transfected primary chondrocytes display a marked reduction in TGF-β1-induced Smad1/5 phosphorylation, without altering total Smad1 levels [Fig. 2(A), bottom panel], confirming the importance of ALK1 in mediating TGF-β-induced Smad1/5 phosphorylation in human chondrocytes[13]. Importantly, Fig. 2(B) demonstrates that endoglin siRNA transfected chondrocytes display a marked reduction in TGF-β1-induced Smad1/5 phosphorylation as compared to control cells [Fig. 2(B), top panel] without altering total Smad1 levels [Fig. 2(B), second panel]. The relative levels of endoglin and STAT3 (loading control) in endoglin-specific siRNA and control siRNA transfected cells are indicated [Fig. 2(B), panels 3 and 4, respectively].

Endoglin inhibits TGF-β1-induced Smad3-driven transcriptional activity in human chondrocytes

The effect of endoglin on Smad3-driven signalling was examined using the Smad3-responsive CAGA$_{12}$-lux and Smad2/3-responsive 3TP-lux luciferase reporter constructs in C28/I2 cells. Results shown in Table I demonstrate that endoglin siRNA transfection leads to a significant increase ($P = 0.006$) in

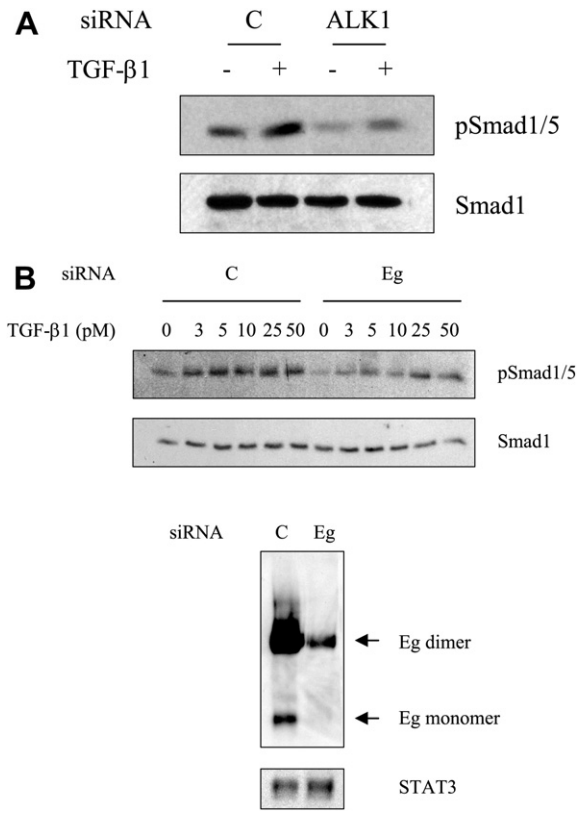

Fig. 2. Endoglin enhances TGF-β1/ALK1-induced Smad1/5 phosphorylation in human chondrocytes. (A) Primary human articular chondrocytes transfected with ALK1-specific (ALK1) siRNA or control (C) siRNA were treated for 30 min without or with 50 pM TGF-β1 under serum-free conditions. (B) Primary human articular chondrocytes transfected with endoglin-specific (Eg) siRNA or control (C) siRNA were treated for 30 min with 0–50 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by Western blot using anti-phosphoSmad1/5, anti-Smad1, anti-endoglin or anti-STAT3 antibodies. Results shown are representative of three independently performed experiments.

TGF-β1-induced CAGA$_{12}$-lux activity as compared to control siRNA transfection. Similarly, endoglin siRNA treatment results in increased TGF-β1-induced 3TP-lux activity (Supplementary Fig. 1). Interestingly, endoglin siRNA transfection also leads to enhanced TGF-β2-induced CAGA$_{12}$-lux activity in these cells (Supplementary Fig. 2), suggesting that endoglin regulates TGF-β signalling in a TGF-β isoform-independent manner in human chondrocytes. We also found that overexpression of endoglin inhibited TGF-β1-induced CAGA$_{12}$-lux activity (Supplementary Fig. 3), supporting the results obtained with siRNA.

We next examined whether the stimulatory effect of endoglin siRNA knockdown on TGF-β1-induced Smad3-driven transcriptional activity could be reversed by ALK1 overexpression.

Table I
Endoglin siRNA knockdown enhances TGF-β1-induced Smad3-driven (CAGA$_{12}$-lux*) transcriptional activity in human chondrocytes (C28/I2 cells)

siRNA	TGF-β1	Fold-change	95% CI
Control	–	1.0 ± 0	0
Control	+	100 ± 15.8†	39.2
Endoglin	–	1.7 ± 0.21	1.1
Endoglin	+	276.2 ± 28.6†	70.9

* CAGA$_{12}$-lux is a TGF-β responsive luciferase reporter gene.
† Significantly different from each other; $P = 0.006$.

Fig. 3(A) shows that Eg siRNA transfected C28/I2 cells display enhanced TGF-β1-induced $CAGA_{12}$-lux activity, as expected. Importantly, co-transfection of WT or constitutively active (QD) ALK1 leads to a significant reduction in the stimulatory effect of endoglin siRNA knockdown on TGF-β1-induced $CAGA_{12}$-lux activity [Fig 3(A), $P = 0.006$ and 0.002, respectively]. Expression levels of endoglin and ALK1 proteins are depicted in Fig. 3(B) and (C), respectively.

Endoglin inhibits TGF-β1-induced ECM production in human chondrocytes

Our previous results have shown that TGF-β1 stimulates type II collagen and PAI-1 protein production in human chondrocytes[13]. The role of endoglin in regulating ECM production was first examined in primary human articular chondrocytes. Fig. 4(A) demonstrates that endoglin siRNA transfected chondrocytes display an increase in TGF-β1-induced type II collagen (top panel) and an increase in (non-glycosylated) PAI-1 protein expression as compared to control cells [Fig. 4(A), second panel]. PAI-1 is detected as a double band and may represent non-glycosylated (lower band) and glycosylated (upper band) forms of PAI-1 which have been reported previously[29]. Equal protein loading is verified by reprobing the membrane with an anti-actin antibody [Fig. 4(A), third panel]. The role of endoglin in regulating ECM production was also examined in C28/I2 cells. Fig. 4(B) illustrates that endoglin siRNA knockdown increases TGF-β1-induced type II collagen protein production as compared to control siRNA transfected cells [Fig. 4 (B), top panel]. Endoglin siRNA transfected C28/I2 cells also display a marked increase in TGF-β1-induced PAI-1 protein (at a dose of 100 pM TGF-β1) as compared to the control siRNA transfected cells [Fig. 4(B), second panel].

Endoglin expression is increased following subculture-induced dedifferentiation

The effects of subculture-induced dedifferentiation on endoglin expression were examined in early and late passage primary human articular chondrocytes. Fig. 5(A) (top panel) shows that endoglin protein levels are higher in late passage as compared to early passage chondrocytes. Type II collagen protein levels are lower in late passage as compared to early passage chondrocytes [Fig. 5(A), middle panel] consistent with the process of chondrocyte dedifferentiation[30,31]. Reprobing the membrane with an anti-STAT3 antibody confirms that equal amounts of protein are loaded in each lane [Fig. 5(A), bottom panel].

Endoglin expression in early and late passage primary articular chondrocytes was also examined at the mRNA level. RT-PCR analysis performed on total RNA extracted from early and late passage chondrocytes indicates that endoglin mRNA levels are higher in late passage as compared to early passage chondrocytes [Fig. 5(B), top panel]. RT-PCR for GAPDH was performed as a control [Fig. 5(B), second panel]. Western blot analysis of cell lysates prepared from the same cells confirms that endoglin protein levels are higher in late passage as compared to early passage chondrocytes [Fig. 5(B), third panel]. Reprobing the membrane with an anti-STAT3 antibody confirms that equal amounts of protein are loaded in each lane [Fig. 5 (B), bottom panel]. Subculture-induced changes in morphology of the human articular chondrocytes are shown [Fig 5(C)].

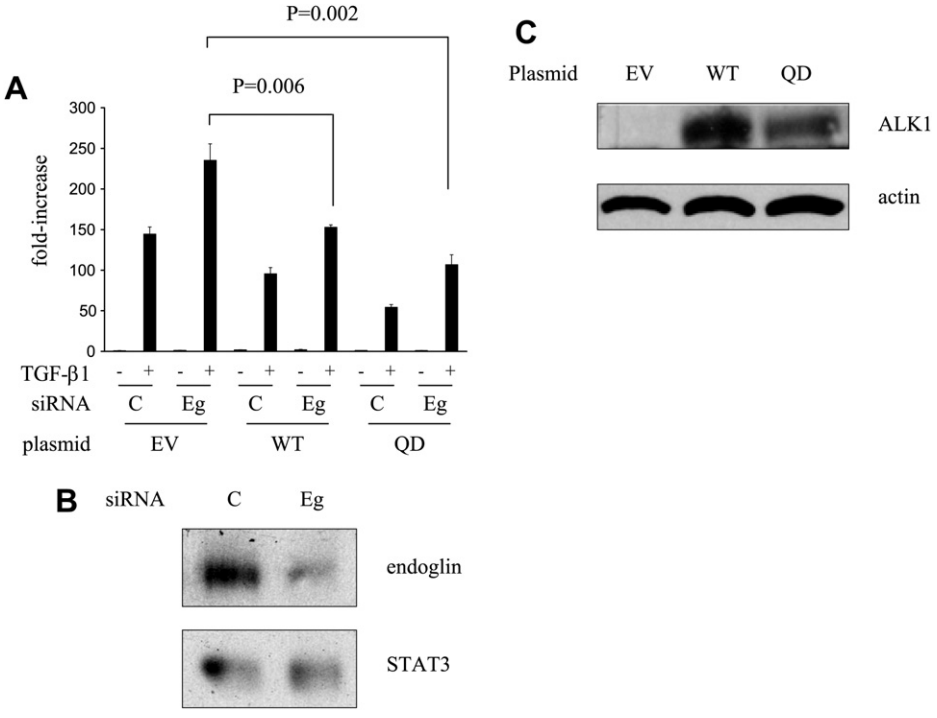

Fig. 3. ALK1 overexpression reverses the effect of endoglin siRNA knockdown on TGF-β1-induced Smad3-driven transcriptional activity in human chondrocytes. (A) C28/I2 cells were transfected with endoglin-specific (Eg) siRNA or control (C) siRNA and WT or constitutively active (QD) ALK1 or corresponding EV with $CAGA_{12}$-lux (luciferase reporter gene) and CMV-β-galactosidase. Cells were treated for 24 h without or with 100 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and assayed for luciferase and β-galactosidase activities. Luciferase activity was normalized to β-galactosidase activity and data are presented as fold-increase [mean ± 95% confidence interval (CI), $N = 3$] in luciferase activity relative to the control (EV and control siRNA transfected cells not treated with TGF-β1; *P*-values are indicated). (B) Cell lysates from C28/I2 cells transfected with endoglin-specific siRNA or control siRNA were analyzed by Western blot using an anti-endoglin. The membrane was reprobed with anti-STAT3 antibody to verify equal protein loading. (C) Cell lysates from C28/I2 cells transfected with ALK1-WT, ALK1-QD or EV were analyzed by Western blot using an anti-ALK1 antibody. The membrane was reprobed with an anti-actin antibody to confirm equal protein loading. The results shown are representative of three independent experiments.

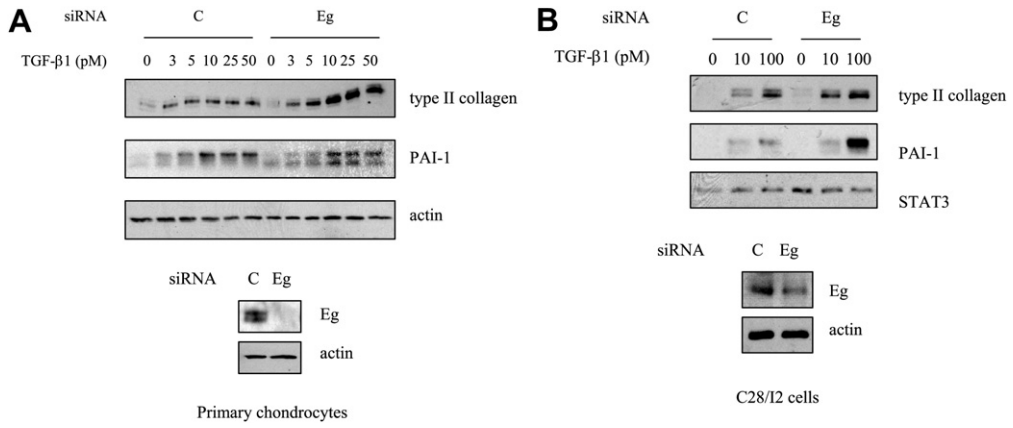

Fig. 4. Endoglin inhibits TGF-β1-induced type II collagen and PAI-1 protein expression in human chondrocytes. (A) primary human articular chondrocytes or (B) C28/I2 cells were transfected with endoglin-specific (Eg) or control (C) siRNA. The cells were treated for 24 h with 0–100 pM TGF-β1 under serum-free conditions. Cell lysates were prepared and analyzed by Western blot using anti-type II collagen, anti-PAI-1, anti-endoglin and anti-actin antibodies. Results shown in A and B are each representative of two independent experiments. Results shown are representative of four (A) and two (B) independent experiments, respectively.

Endoglin expression was also examined in early and late passage tsT/AC62 cells. Western blot analysis indicates that total endoglin protein levels are higher in late passage as compared to early passage tsT/AC62 cells [Fig. 6(A), top panel]. The type II collagen protein levels are lower and the type I collagen (third panel) are higher in late passage as compared to early passage tsT/AC62 cells [Fig. 6(A), second and third panel, respectively], consistent with the process of chondrocyte dedifferentiation[30,31]. Reprobing the membrane with an anti-actin antibody confirms that equal amounts of protein are loaded in each lane. Cell surface endoglin levels were also examined in early (E) and late (L) passage tsT/AC62 by affinity labelling with [^{125}I]TGF-β1. Fig. 6(B) shows that late passage tsT/AC62 cells display higher levels of [^{125}I]TGF-β1-associated endoglin as compared to early passage cells. The morphologies of early and late passage tsT/AC62 cells are shown in Fig. 6(C). Immediately (24 h) following transfer from alginate to monolayer cultures, polygonal-shaped cells are observed [Fig 6(C), early passage]. However, the cells progressively acquire a more elongated 'fibroblastic' morphology following several passages in monolayer [Fig 6(C), late passage].

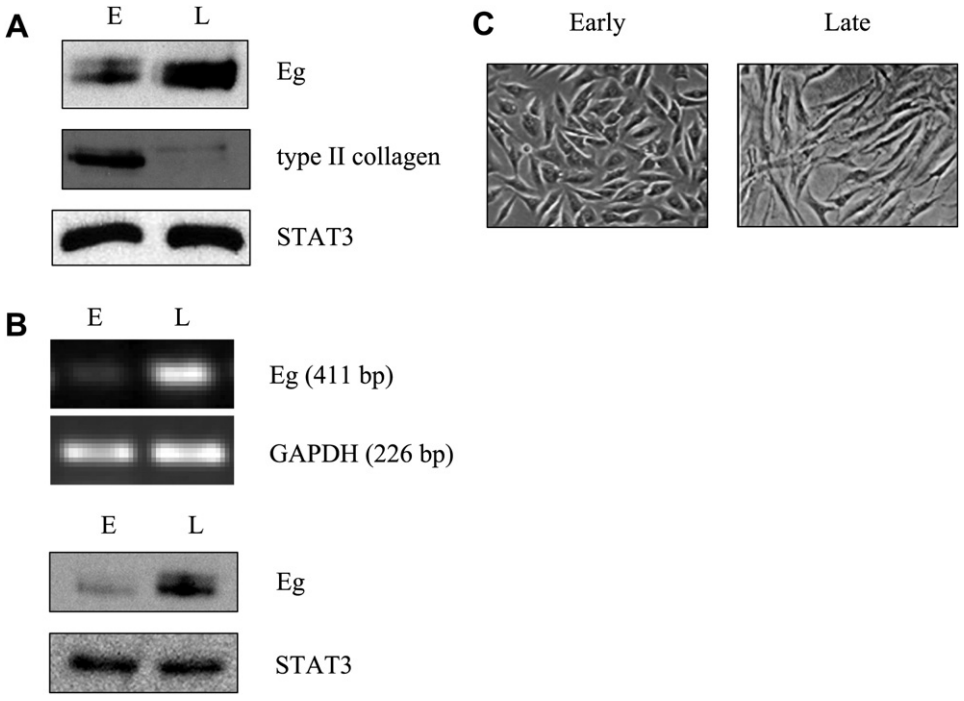

Fig. 5. Endoglin expression is increased in primary human articular chondrocytes following subculture-induced dedifferentiation. (A) Cell lysates from early and late passage primary human articular chondrocytes were analyzed by Western blot using anti-endoglin (top panel) or anti-type II collagen (middle panel) antibodies. Membranes were reprobed with an anti-STAT3 antibody (bottom panel) to confirm that equal amounts of protein were loaded in each lane. (B) Total RNA and cell lysates were prepared from early and late passage primary human articular chondrocytes. Total RNA was analyzed by RT-PCR using endoglin- (top panel) and GAPDH-specific (second panel) oligonucleotide primers. PCR product sizes for endoglin (e.g., 411 bp) and GAPDH (226 bp) are indicated. Cell lysates were analyzed by Western blot using anti-endoglin (third panel) and anti-STAT3 (bottom panel) antibodies. (C) *Cell morphology*: Phase contrast microscopy of early and late passage primary human articular chondrocytes in monolayer culture. Results shown are representative of three independent experiments.

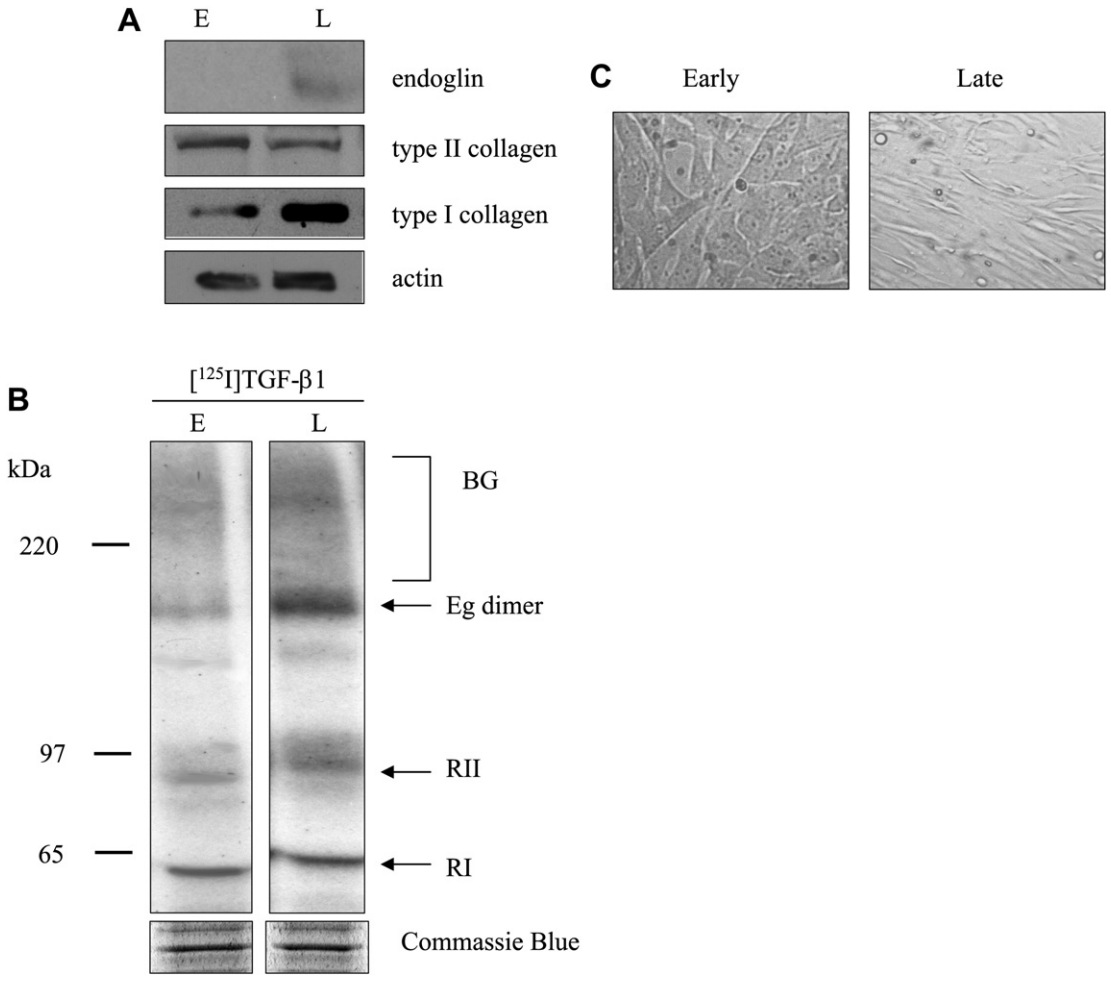

Fig. 6. Endoglin expression is increased in tsT/AC62 cells following subculture-induced dedifferentiation. (A) Cell lysates from early and late passage tsT/AC62 cells were analyzed by Western blot using anti-endoglin (top panel), anti-type II collagen (second panel) or anti-type I collagen (third panel) antibodies. Membranes were reprobed with an anti-actin antibody (bottom panel) to confirm that equal amounts of protein were loaded in each lane. (B) Early and late passage tsT/AC62 cells were affinity labelled with 100 pM of [^{125}I]TGF-β1. Membrane extracts were prepared, electrophoretically separated by 3–11% gradient SDS-PAGE under non-reducing conditions and levels of [^{125}I]TGF-β1-associated endoglin visualized by autoradiography. The lanes were selected from non-adjacent regions of the same gel. Coomassie blue staining confirms that equal amounts of protein were loaded in each lane. (C) *Cell morphology*: Phase contrast microscopy of early and late passage tsT/AC62 cells in monolayer culture. Results shown are representative of three independent experiments.

Endoglin protein expression in OA and normal cartilage

We next examined endoglin protein expression *in vivo* in cartilage samples obtained from the knee of OA patients and normal controls. Cartilage protein was extracted as described in the Method section and analyzed by Western blot using an anti-endoglin antibody. Results shown in Fig. 7(A) demonstrate that endoglin protein levels are higher in OA as compared to normal cartilage. Ponceau staining of the membrane confirms that equal amounts of protein were loaded in each lane. Similar results were obtained when comparing endoglin protein levels in three different pairs of OA and normal cartilage samples (data not shown). Endoglin expression was also examined in human OA and normal cartilage by immunohistochemistry using an anti-endoglin (SN6h, Dako) antibody. Fig. 7(B) demonstrates that OA cartilage displays enhanced staining for endoglin as compared to normal cartilage. No staining was detected when the primary antibody was omitted in parallel experiments (data not shown).

Discussion

TGF-β is a pleiotropic cytokine that plays a critical role in maintaining cartilage homeostasis[6,32]. Although deregulation of TGF-β signalling has been implicated in cartilage diseases such as OA[6,32], the factors that regulate TGF-β signalling in normal or diseased human chondrocytes are unclear. We have previously shown that human chondrocytes express several different TGF-β receptors including two isoforms of TβRII (RII and RIIB), two different TβRIs (ALK5 and ALK1) and the TGF-β co-receptors endoglin and betaglycan[13,18,33]. We have shown that endoglin forms a heteromeric complex with TβRII and TβRI (ALK5 and ALK1) in human chondrocytes[13,18] as has been demonstrated for endoglin in endothelial cells[11,34–37]. However, the functional significance of endoglin expression in human chondrocytes has not been examined. Here we show that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation but inhibits TGF-β1-induced Smad2 phosphorylation, Smad3-driven ($CAGA_{12}$-lux and 3TP-lux) transcriptional activity and type II collagen and PAI-1 protein expression in human chondrocytes. Moreover, we present evidence that endoglin expression is increased in human chondrocytes following subculture-induced dedifferentiation *in vitro* and that endoglin expression levels are increased in human OA cartilage as compared to normal cartilage *in vivo*. Collectively, our results establish endoglin as a regulator of TGF-β signalling and ECM protein production with opposing effects on the ALK1/Smad1/5 and ALK5/Smad2/3 pathways in human chondrocytes and suggest that endoglin

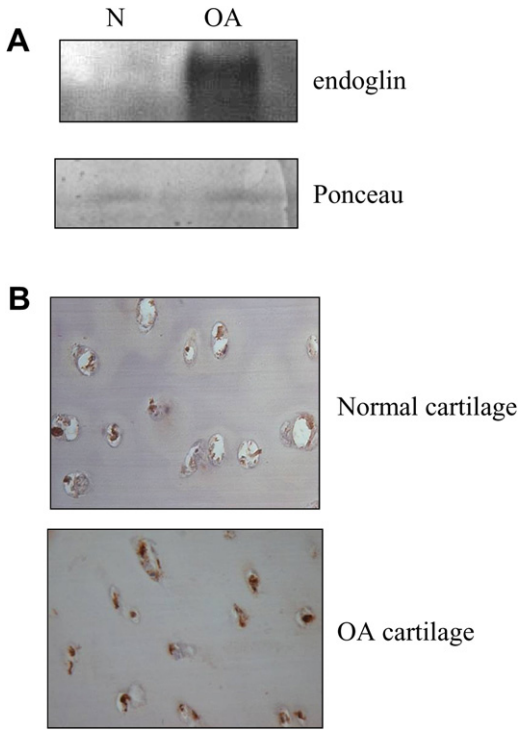

Fig. 7. Endoglin expression is increased in OA cartilage as compared to normal cartilage. (A) *Western blot*: Protein was extracted from OA and normal cartilage as described in Methods section. Samples were analyzed by Western blot using an anti-endoglin antibody (top panel). The membrane was stained with Ponceau S to confirm that equal amounts of protein were loaded in each lane (bottom panel). The lanes were selected from non-adjacent regions of the same gel. Results shown are representative of three independent experiments performed on cartilage of three different OA and three different normal donors. (B) *Immunohistochemistry*: Cryostat sections of OA and normal cartilage were analyzed by immunohistochemistry using an anti-endoglin (Dako, SN6h, 1:100 dilution) antibody. Detection was performed using Vectostain ABC immunostaining kit. Results shown are representative of two independently performed experiments.

may represent a potential marker for chondrocyte dedifferentiation or the loss of chondrogenic phenotype.

Endoglin is a TGF-β co-receptor that has been shown to regulate TGF-β signalling in endothelial cells[12,17,38]. Our present results show that endoglin enhances TGF-β1-induced Smad1/5 phosphorylation but decreases TGF-β1-mediated Smad2 phosphorylation in human chondrocytes. In comparison, other studies report conflicting data on the role of endoglin in regulating TGF-β-mediated Smad1/5 and Smad2 signalling in different cell types. For example, ectopic expression of endoglin in rat myofibroblasts was reported to increase phosphorylation (and total levels) of Smad2[39] whereas another study using ectopic expression of endoglin in rat myofibroblasts reported no significant effect on TGF-β-induced phosphorylation of Smad1 or Smad2[40]. Conversely, studies using endoglin-null mouse embryonic endothelial cells reported that endoglin promotes TGF-β-mediated Smad1/5 signalling[36,41] whereas another study using endoglin siRNA knockdown in human bone marrow stromal cells reported that endoglin acts as a positive regulator of both ALK1-induced Smad1 activation and ALK5-induced Smad2 activation[42]. Taken together, these studies suggest that endoglin differentially regulates TGF-β signalling *via* ALK1/Smad1/5 and ALK5/Smad2 depending on cell-type and cellular context. Our results suggest that endoglin differentially regulates TGF-β/Smad signalling in human chondrocytes by promoting TGF-β signalling through the ALK1/Smad1/5 pathway and decreasing signalling through the ALK5/Smad2/3 pathway.

Our previous work has demonstrated that ALK1 inhibits TGF-β/ALK5-dependent Smad3-driven transcriptional activity and ECM production in human chondrocytes[13]. Our present results indicate that endoglin also inhibits TGF-β1/ALK5-induced Smad3-driven transcriptional activity and ECM production in human chondrocytes. Because we have shown that endoglin interacts with ALK1 in human chondrocytes[13] and that others have shown endoglin to interact with, and promote signalling through, ALK1 in endothelial cells to inhibit ALK5 signalling[36], it is plausible that endoglin regulates TGF-β/ALK5 signalling through a similar mechanism in human chondrocytes. Our results showing that endoglin promotes TGF-β1/ALK1 signalling (i.e., Smad1/5 phosphorylation) and that overexpression of ALK1 can reverse the stimulatory effect of endoglin siRNA knockdown on TGF-β1-induced $CAGA_{12}$-lux activity are consistent with this notion. Whether endoglin inhibits TGF-β/ALK5/Smad2/3 signalling by promoting the TGF-β/ALK1/Smad1/5 pathway or by regulating other pathways remains to be determined. Importantly, our finding that endoglin inhibits TGF-β-induced Smad3-driven transcription provides further evidence for endoglin's role in inhibiting TGF-β-mediated ALK5/Smad2/3 signalling in human chondrocytes.

Primary human articular chondrocytes maintain a rounded, polygonal morphology in monolayer culture but undergo a progressive change to an elongated fibroblast-like morphology over time, particularly after subculture[43]. Accompanying these morphological changes is the loss of the chondrogenic (differentiated) phenotype or 'chondrocyte dedifferentiation' which is characterized by a decrease in type II collagen expression[43]. Our results demonstrate that primary human articular chondrocytes and tsT/AC62 cells display such changes in morphology and reduction in type II collagen expression following serial passage in monolayer suggesting that these cells undergo subculture-induced dedifferentiation. Human costal chondrocytes (C28/I2) also show a decrease in type II collagen expression after serial passage in monolayer although morphological changes were not observed in these cells (data not shown). Importantly, our results showing that endoglin protein levels are higher in late passage as compared to early passage chondrocytes suggest that endoglin expression is increased following subculture-induced dedifferentiation. The increased endoglin protein expression in late passage chondrocytes is mirrored by an increase in endoglin mRNA expression indicating that the mechanism of its up-regulation involves an increase in endoglin gene expression and/or an increase in endoglin mRNA stability. Our results are consistent with a previous report identifying endoglin as one of many cell surface molecules upregulated in primary articular chondrocytes during cell expansion in monolayer[44]. Thus, our results suggest that endoglin may represent a novel marker for chondrocyte dedifferentiation and/or the loss of normal 'differentiated' chondrocyte phenotype.

Factors that regulate TGF-β signalling in normal and 'phenotypically altered' chondrocytes are ill-defined. A previous study has shown that ALK1 mRNA levels are increased during *in vitro* expansion of primary human articular chondrocytes[45] and a more recent study has reported an increase in the ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in OA in humans and mice[46]. Our results showing that endoglin levels are increased in human chondrocytes following subculture-induced dedifferentiation *in vitro* and in human OA cartilage as compared to normal cartilage *in vivo* extend these findings and raise the possibility that increased expression of endoglin and ALK1 together leads to a shift in TGF-β signalling away from the ALK5/Smad2/3 pathway in favour of ALK1/Smad1/5 pathway. Further studies using increased numbers of age-, sex- and gender-matched OA and normal human cartilage samples will be needed to confirm these findings.

In conclusion, our results establish endoglin as a regulator of TGF-β signalling and ECM protein production with opposing effects on the ALK1/Smad1/5 and ALK5/Smad2/3 pathways in human chondrocytes and suggest that endoglin may represent a potential marker for chondrocyte dedifferentiation or loss of chondrogenic phenotype. Delineation of the precise mechanism by which endoglin may regulate the balance of ALK1 and ALK5 signalling in human chondrocytes will be a fruitful avenue for future studies.

Role of the funding source

This study was supported by a CIHR Grants MOP 69060 and MOP 13732 (to AP) and the McGill Surgeon Scientist Training Award (to WLP).

Contributions

Kenneth Finnson contributed to the conception and design, collection and assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

Wendy Parker contributed to the conception and design, collection and assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

Yoonyoung Chi contributed to the collection and assembly of the data, critical revision of the article for important intellectual content and final approval of the article.

Caroline Hoemann contributed to the collection of the data, critical revision of the article for important intellectual content and final approval of the article.

Mary Goldring contributed to analysis and interpretation of data, critical revision of the article for important intellectual content and final approval of the article.

John Antoniou contributed to the collection of the data, critical revision of the article for important intellectual content and final approval of the article.

Anie Philip contributed to the conception and design of the study, obtaining funding, the assembly of the data, analysis and interpretation of data, drafting of the article, critical revision of the article for important intellectual content and final approval of the article.

Conflict of interest

The authors state that they have no conflicts of interest.

Acknowledgements

We thank Genzyme Inc. (Framingham, MA) for the gift of TGF-β1 and TGF-β2, Calvin Vary (Maine Medical Center Research Institute, Scarborough, ME) for the endoglin cDNA cloned into pCDNA3.1/myc-HIS, Kohei Miyazono (The University of Tokyo, Tokyo, Japan) for ALK1-WT and ALK1-QD plasmids, Lucia Lesec (Jewish General Hospital, Montreal, QC) for collection of human articular cartilage, Mohammed Zafarullah (University of Montreal and Research Centre Notre-Dame Hospital, Montreal, QC) for assistance with the calcium phosphate transfection method. Dr S. Huet (Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) for (CAGA)12-lux.

Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.joca.2010.09.002.

References

1. Blom AB, van der Kraan PM, van den Berg WB. Cytokine targeting in osteoarthritis. Curr Drug Targets 2007;8:283–92.
2. Goldring MB. Update on the biology of the chondrocyte and new approaches to treating cartilage diseases. Best Pract Res Clin Rheumatol 2006;20:1003–25.
3. Grimaud E, Heymann D, Redini F. Recent advances in TGF-β effects on chondrocyte metabolism. Potential therapeutic roles of TGF-β in cartilage disorders. Cytokine Growth Factor Rev 2002;13:241–57.
4. Serra R, Johnson M, Filvaroff EH, LaBorde J, Sheehan DM, Derynck R, *et al*. Expression of a truncated, kinase-defective TGF-β type II receptor in mouse skeletal tissue promotes terminal chondrocyte differentiation and osteoarthritis. J Cell Biol 1997;139:541–52.
5. Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, *et al*. Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β. EMBO J 1999;18:1280–91.
6. Blaney Davidson E, van der Kraan P, van den Berg W. TGF-β and osteoarthritis. Osteoarthritis Cartilage 2007;15:597–604.
7. Itayem R, Mengarelli-Widholm S, Hulth A, Reinholt FP. Ultrastructural studies on the effect of transforming growth factor-β1 on rat articular cartilage. Apmis 1997;105:221–8.
8. Itayem R, Mengarelli-Widholm S, Reinholt FP. The long-term effect of a short course of transforming growth factor-β1 on rat articular cartilage. APMIS 1999;107:183–92.
9. Massague J, Gomis RR. The logic of TGF-β signaling. FEBS Lett 2006;580:2811–20.
10. Yamashita H, ten Dijke P, Franzen P, Miyazono K, Heldin CH. Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-β. J Biol Chem 1994;269:20172–8.
11. Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-β receptor function in the endothelium. Cardiovasc Res 2005;65:599–608.
12. Pardali E, ten Dijke P. Transforming growth factor-β signaling and tumor angiogenesis. Front Biosci 2009;14:4848–61.
13. Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. J Bone Miner Res 2008;23:896–906.
14. ten Dijke P, Hill C. New insights into TGF-β-Smad signalling. Trends Biochem Sci 2004;29:265–73.
15. Bernabeu C, Lopez-Novoa JM, Quintanilla M. The emerging role of TGF-β superfamily co-receptors in cancer. Biochim Biophys Acta 2009;1792:954–73.
16. Mokrosinski J, Krajewska WM. TGF-β signalling accessory receptors. Postepy Biochem 2008;54:264–73.
17. ten Dijke P, Goumans MJ, Pardali E. Endoglin in angiogenesis and vascular diseases. Angiogenesis 2008;11:79–89.
18. Parker WL, Goldring MB, Philip A. Endoglin is expressed on human chondrocytes and forms a heteromeric complex with betaglycan in a ligand and type II TGFβ receptor independent manner. J Bone Miner Res 2003;18:289–302.
19. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, *et al*. Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1β. Arthritis Rheum 2000;43:2189–201.
20. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, *et al*. Interleukin-1 β-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94:2307–16.
21. Qiu W, Murray MM, Shortkroff S, Lee CR, Martin SD, Spector M. Outgrowth of chondrocytes from human articular cartilage

explants and expression of alpha-smooth muscle actin. Wound Repair Regen 2000;8:383–91.
22. Qureshi HY, Ahmad R, Zafarullah M. High-efficiency transfection of nucleic acids by the modified calcium phosphate precipitation method in chondrocytes. Anal Biochem 2008;382:138–40.
23. Dennler S, Huet S, Gauthier J-M. A short amino-acid sequence in MH1 domain is responsible for functional differences between Smad2 and Smad3. Oncogene 1999;18:1643–8.
24. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, Laiho M, *et al*. TGF-β signals through a heteromeric protein kinase receptor complex. Cell 1992;71:1003–14.
25. Bellon T, Corbi A, Lastres P, Cales C, Cebrian M, Vera S, *et al*. Identification and expression of two forms of the human transforming growth factor-β-binding protein endoglin with distinct cytoplasmic regions. Eur J Immunol 1993;23:2340–5.
26. Yoshimura K, Uchida G, Okazaki M, Kitano Y, Harii K. Differential expression of heparin-binding EGF-like growth factor (HB-EGF) mRNA in normal human keratinocytes induced by a variety of natural and synthetic retinoids. Exp Dermatol 2003;12:28–34.
27. Hoemann CD, Sun J, Chrzanowski V, Buschmann MD. A multivalent assay to detect glycosaminoglycan, protein, collagen, RNA, and DNA content in milligram samples of cartilage or hydrogel-based repair cartilage. Anal Biochem 2002;300:1–10.
28. McLean IW, Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. J Histochem Cytochem 1974;22:1077–83.
29. Gils A, Pedersen KE, Skottrup P, Christensen A, Naessens D, Deinum J, *et al*. Biochemical importance of glycosylation of plasminogen activator inhibitor-1. Thromb Haemost 2003; 90:206–17.
30. Duval E, Leclercq S, Elissalde JM, Demoor M, Galera P, Boumediene K. Hypoxia-inducible factor 1α inhibits the fibroblast-like markers type I and type III collagen during hypoxia-induced chondrocyte redifferentiation: hypoxia not only induces type II collagen and aggrecan, but it also inhibits type I and type III collagen in the hypoxia-inducible factor 1alpha-dependent redifferentiation of chondrocytes. Arthritis Rheum 2009;60:3038–48.
31. Domm C, Schunke M, Christesen K, Kurz B. Redifferentiation of dedifferentiated bovine articular chondrocytes in alginate culture under low oxygen tension. Osteoarthritis Cartilage 2002;10:13–22.
32. van der Kraan PM, Blaney Davidson EN, Blom A, van den Berg WB. TGF-β signaling in chondrocyte terminal differentiation and osteoarthritis modulation and integration of signaling pathways through receptor-Smads. Osteoarthritis Cartilage 2009;17:1539–45.
33. Parker WL, Finnson KW, Soe-Lin H, Knaus P, Philip A. Expression and function of TβRII-B, a variant of the type II TGF-β receptor, in human chondrocytes. Osteoarthritis Cartilage 2007;15:442–53.
34. Blanco FJ, Santibanez JF, Guerrero-Esteo M, Langa C, Vary CP, Bernabeu C. Interaction and functional interplay between endoglin and ALK-1, two components of the endothelial transforming growth factor-β receptor complex. J Cell Physiol 2005;204:574–84.
35. Koleva RI, Conley BA, Romero D, Riley KS, Marto JA, Lux A, *et al*. Endoglin structure and function: determinants of endoglin phosphorylation by transforming growth factor-β receptors. J Biol Chem 2006;281:25110–23.
36. Lebrin F, Goumans MJ, Jonker L, Carvalho RL, Valdimarsdottir G, Thorikay M, *et al*. Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. Embo J 2004;23:4018–28.
37. Lee NY, Blobe GC. The interaction of endoglin with β-arrestin2 regulates transforming growth factor-b-mediated ERK activation and migration in endothelial cells. J Biol Chem 2007;282:21507–17.
38. Garcia-Pozo L, Miquilena-Colina ME, Lozano-Rodriguez T, Garcia-Monzon C. Endoglin: structure, biological functions, and role in fibrogenesis. Rev Esp Enferm Dig 2008; 100:355–60.
39. Santibanez J, Letamendia A, Perez-Barriocanal F, Silvestri C, Saura M, Vary C, *et al*. Endoglin increases eNOS expression by modulating Smad2 protein levels and Smad2-dependent TGF-β signaling. J Cell Physiol 2007;210:456–68.
40. Velasco S, Alvarez-Munoz P, Pericacho M, Ten Dijke P, Bernabeu C, Lopez-Novoa JM, *et al*. L- and S-endoglin differentially modulate TGF-β1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. J Cell Sci 2008;121:913–9.
41. Lee NY, Ray BN, How T, Blobe GC. Endoglin promotes transforming growth factor-β-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC. J Biol Chem 2008;283:32527–33.
42. O'Connor JC, Farach-Carson MC, Schneider CJ, Carson DD. Coculture with prostate cancer cells alters endoglin expression and attenuates transforming growth factor-β signaling in reactive bone marrow stromal cells. Mol Cancer Res 2007;5:585–603.
43. Goldring MB. Human chondrocyte cultures as models of cartilage-specific gene regulation. Methods Mol Med 2005;107:69–95.
44. Diaz-Romero J, Gaillard JP, Grogan SP, Nesic D, Trub T, Mainil-Varlet P. Immunophenotypic analysis of human articular chondrocytes: changes in surface markers associated with cell expansion in monolayer culture. J Cell Physiol 2005; 202:731–42.
45. Dell'Accio F, De Bari C, Luyten FP. Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. Arthritis Rheum 2001; 44:1608–19.
46. Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, *et al*. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol 2009;182:7937–45.