Original Article

NOGGIN INHIBITS TGF-β1 OR TGF-β3 INDUCED CHONDROGENESIS OF MESENCHYMAL STROMAL CELLS

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Abstract

Noggin (NOG) is an antagonist of bone morphogenetic proteins (BMPs), which regulates development and homeostasis of bone and cartilage. NOG has also been discovered to be an antagonist of transforming growth factor-β1 (TGF-β1). However, the effect of NOG on chondrogenesis induced by TGF-β1 remains unknown. Interestingly, in previous work NOG did not appear to influence TGF-β3-driven chondrogenesis, implying isoform specificity. In our study, the impact of exogenous NOG on TGF-β-induced chondrogenesis of bone marrow derived mesenchymal stromal cells (MSCs) was further investigated. Both TGF-β1 and TGF-β3 supplementation increased NOG expression at day 7, 14, 21 and 28 in MSC pellet culture. Addition of NOG during chondrogenic differentiation *in vitro* reduced sGAG release into the medium and retention within the pellet induced by TGF-β1 or TGF-β3. This was further confirmed by Safranin O/Fast Green staining. Gene downregulation including *ACAN*, *COL2A1* and *SOX9*, was also observed downregulated by NOG at day 7. The same inhibitory role of NOG in TGF-β1 or TGF-β3-induced chondrogenesis suggests that the effect is not isoform-specific. We also observed differences mediated by NOG between the TGF-β1 and TGF-β3 groups. NOG suppresses cell proliferation during TGF-β1-induced chondrogenesis, whereas no significant alteration was observed in the TGF-β3 group. The effect of NOG on hypertrophy at day 7 was also investigated. In the TGF-β1 group, NOG resulted in alleviation of hypertrophy by downregulating *COL10A1* and *IHH* expression. In the TGF-β3 group, NOG reduced hypertrophy through downregulation of *COL10A1* and *RUNX2*.

Keywords: Noggin, TGF-β1, TGF-β3, chondrogenesis, hypertrophy.

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Introduction

Mesenchymal stromal cells (MSCs) are capable of self-renewal and multilineage differentiation (Ding *et al.*, 2011), which plays a significant role in tissue engineering and regenerative therapy, overcoming some limitations of autologous grafting (Vasanthan *et al.*, 2020). Bone marrow is currently the most common MSC source in clinical practice and has been widely used for cartilage repair either alone or in combination with scaffolds of various materials (Mohamed-Ahmed *et al.*, 2018). Understanding the molecular networks that drive or prevent chondrogenic differentiation is critical for controlling stable differentiation during stem cell-based therapy for cartilage repair (Augello and De Bari, 2010).

Transforming growth factor beta (TGF-β) 1 and TGF-β3 are isoforms of a multifunctional protein, that are widely used to promote *in vitro* chondrogenesis and cartilage extracellular matrix (ECM) synthesis (Mueller *et al.*, 2010). In a previous study (Wen *et al.*, 2021), Noggin (NOG), which is an antagonist of bone morphogenetic proteins (BMPs), was identified as a new inhibitor of TGF-β1. However, NOG did not impede the chondrogenic process of Wharton's jelly MSCs driven by TGF-β3 *in vitro* (Wen *et al.*, 2021). The role of NOG in chondrogenesis might therefore be determined by the TGF-β isoform. In addition, mice lacking noggin might lead to impaired joint formation (Reddi, 2001), which indicates essential role of noggin in joint development. In this study, we aim to explore the expression and effects of NOG during *in vitro* chondrogenesis triggered by different TGF-β isoforms of MSC to enhance our knowledge of the intricate chondrogenesis factor network.

To reveal the role of NOG in chondrogenic differentiation, the pellet culture system was used (Mackay *et*

al., 1998). sGAG retention in pellets and release into the medium from MSCs induced by TGF-β1 or TGF-β3 were quantified over four weeks. Furthermore, the impact of NOG on cellular proliferation was explored and chondrogenic marker gene expression including *ACAN*, *COL2A1*, and *SOX9* during chondrogenesis was assessed, thus uncover the role of NOG during human MSC chondrogenic differentiation. Avoiding hypertrophy in chondrogenesis progress is essential when related to cartilage regeneration (Armiento *et al.*, 2019), so we also explored the effect of NOG in hypertrophy through evaluating hypertrophic markers such as *COL10A1*, *IHH* and *RUNX2*.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) high glucose, α-modified essential medium (αMEM), penicillin-streptomycin (Pen/Strep), and non-essential amino acids were purchased from Gibco (Waltham, MA, USA). Recombinant human basic fibroblast growth factor (FGF-b) and recombinant human TGF-β1 protein were supplied by Fitzgerald (Ben Hill County, GA, USA). Recombinant human TGF-β3 protein was purchased from R&D Systems (MN, USA). Recombinant human Noggin was supplied by Peprotech (Cranbury, NJ, USA).

ITS supplement and fetal bovine serum (FBS) were supplied by Corning (Glendale, AZ, USA). V-bottom 96-well microplate and SuperScript Vilo RT Kit were purchased from Thermo Fisher (Waltham, MA, USA). TaqMan Gene Expression Master Mix was supplied by Applied Biosystems (Waltham, MA, USA). Quant-iT PicoGreen dsDNA Assay Kits were purchased from Invitrogen (Waltham, MA, USA). TRI reagent and PolyAcryl carrier were supplied by Molecular Research Center (Cincinnati, OH, USA). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). All other materials and reagents were purchased from Sigma-Aldrich (Waltham, MA, USA).

Isolation and Expansion of MSCs

Human bone marrow was obtained from 6 donors (male, 48, 49, 54, 55, 58, or 68 years old) with ethical approval from the local authorities (Ethik-Kommission der Albert-Ludwigs-Universität Freiburg, EK-326/08) and written consent of the patients undergoing total hip replacement. MSCs were isolated via density centrifugation separation and plastic adhesion and culture-expanded in αMEM supplemented with 10 % FBS, 1 % Pen/Strep and 5 ng/mL recombinant human basic fibroblast growth factor at 37 °C, 5 % CO_2, 95 % humidity (Armiento *et al.*, 2023). The medium was changed three times a week, and passage four was used for all experiments.

Pellet Culture of MSCs

MSCs were seeded at 0.2 million/well in a V-bottom 96-well microplate. The plate was centrifuged at 300 g for 5 minutes to pellet the cells, which were cultured in 250 µL chondrogenic differentiation medium (DMEM high glucose, 1 % Pen/Strep, 50 µg/mL ascorbic acid, 100 nM dexamethasone, 1 % ITS supplement, 1 % non-essential amino acids, 10 ng/mL TGF-β1 or TGF-β3). NOG was added at each medium change at concentrations of 0, 1, 10, or 100 ng/mL. The medium was collected and refreshed every three days. After 7, 14, 21 or 28 days of induction, the pellets were collected for further analysis.

sGAG and DNA Measurement

Each pellet was digested overnight at 56 °C in 250 µL proteinase K at 0.5 mg/mL. The total sulfated sGAG content in the pellet and in the medium were measured using the 1,9-dimethyl-methylene blue (DMMB) colorimetric method (Ladner *et al.*, 2023). Briefly, 20 µL of digested pellet samples or 50 µL of conditioned medium were added into each well followed by 200 µL DMMB reagent. Absorbance at 535 nm was immediately read using a Tecan microplate reader (Zürich, Switzerland). The proteinase K-digested samples were also used for measuring DNA content by the PicoGreen reagent, following the manufacturer's instructions. Briefly, 60 µL of each sample was added to 180 µL TE buffer, and the samples were pipetted in duplicates (100 µL) into a 96-well white plate. 100 µL PicoGreen working solution was added and the fluorescence of the samples was measured at an excitation wavelength of 485 nm and emission of 535 nm by using a Tecan reader after incubation for 3 minutes at room temperature.

Safranin O/Fast Green Staining

Pellets were fixed in 10 % buffered formalin overnight and dehydrated in an ascending ethanol series. Then, the samples were embedded in paraffin and sectioned at 5 µm thickness. To visualize the nuclei, slides were first stained with Weigert's iron Haematoxylin (Sigma-Aldrich, Burlington, MA, USA) for 10 minutes. After that, sections were stained with 0.02 % Fast Green in ddH_2O for 6 minutes to reveal collagen deposition, then stained with 0.1 % Safranin O for 12 minutes to show proteoglycan deposition, and finally rinsed in ddH_2O and sequentially differentiated in graded ethanol. Images were acquired on an Olympus BX63 microscope (Hachioji, Tokyo, Japan).

Gene Expression Analysis

RNA was extracted from a pool of three pellets with 1 mL TRI Reagent and 5 µL PolyAcryl carrier, then homogenized with a Retsch tissue lyser (Haan, Germany) at 30 Hz for 6 minutes. After centrifugation, the supernatant was collected. 0.1 mL 1-Bromo-3-chloropropane (BCP) was added to get the aqueous phase containing RNA, which was purified with RNeasy Mini Kit. Reverse transcription was con-

Table 1. Primers and probes used for PCR.

Human Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Probe (5′→3′)
RPLP0	TGG GCA AGA ACA CCA TGA TG	CGG ATA TGA GGC AGC AGT TTC	AGG GCA CCT GGA AAA CAA CCC AGC (5′FAM/3′TAMRA)
ACAN	AGT CCT CAA GCC TCC TGT ACT CA	CGG GAA GTG GCG GTA ACA	CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT (5′FAM/3′TAMRA)
COL10A1	ACG CTG AAC GAT ACC AAA TG	TGC TAT ACC TTT ACT CTT TAT GGT GTA	ACT ACC CAA CAC CAA GAC ACA GTT CTT CAT TCC
RUNX2	AGC AAG GTT CAA CGA TCT GAG AT	TTT GTG AAG ACG GTT ATG GTC AA	TGA AAC TCT TGC CTC GTC CAC TCC G
COL2A1	Hs00264051_m1		
SOX9	Hs00165814_m1		
NOG	Hs00271352_s1		
IHH	Hs01081800_m1		

Primers and probes presented with sequences were custom designed; primers and probes presented with catalogue numbers were from Applied Biosystems.

ducted with Vilo RT Kit. qRT-PCR was performed with TaqMan gene expression master mix on a QuantStudio™ 6 Pro Real-Time PCR system (Thermo Fisher, Waltham, MA, USA). Primer and probes sequences or assay on demand numbers are presented in Table 1. Relative quantification of mRNA was performed according to the $2^{-\Delta\Delta Ct}$ method with RPLP0 as the endogenous control. Gene expression values were normalized to the average values of MSCs before inducing chondrogenesis (day 0).

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) of six independent experiments. Shapiro–Wilk normality test was used to define whether data were normally distributed in GraphPad Prism 8.1.0 software (La Jolla, CA, USA). Two-way analysis of variance (ANOVA) was conducted to determine differences with normal distribution. Multiple comparison was corrected by Sidak test. Friedman test was conducted to evaluate the differences for non-normally distributed data. p value < 0.05 was considered significant.

Results

NOG Expression was Upregulated by TGF-β1 or TGF-β3

The expression patterns of *NOG* during chondrogenesis were determined at different time points (Fig. 1). Compared to non-induced cells, all donors showed higher expression levels of *NOG* on day 7, 14, 21 and 28 upon TGF-β1 stimulation. In TGF-β3-treated cells, donors all showed the same trend of *NOG* expression although not statistically significant after day 7.

Effects of Exogenous NOG on MSC Pellet Chondrogenesis

To assess whether NOG regulates sGAG retention, sGAG content in pellets were quantified. The retention of sGAG in pellets stimulated by TGF-β1 or TGF-β3 was reduced by 100 ng/mL NOG at day 7, 14, 21 and 28 (Fig. 2**A**). We also explored the effect of NOG on cellular proliferation by measuring DNA content. In all donors, proliferation was inhibited by 100 ng/mL NOG at day 7, 14 and 21 when treated with TGF-β1 but not with TGF-β3 (Fig. 2**B**). sGAG in pellet was then normalized with DNA content to account for ECM production relative to cell number. In the TGF-β1 group, sGAG normalized to DNA remained unaltered until day 21 when exposed to concentrations of 10 ng/mL or 100 ng/mL of NOG, as depicted in Fig. 2**C**. In contrast, in the TGF-β3 group, sGAG relative to cell number exhibited a decline commencing from day 7 and con-

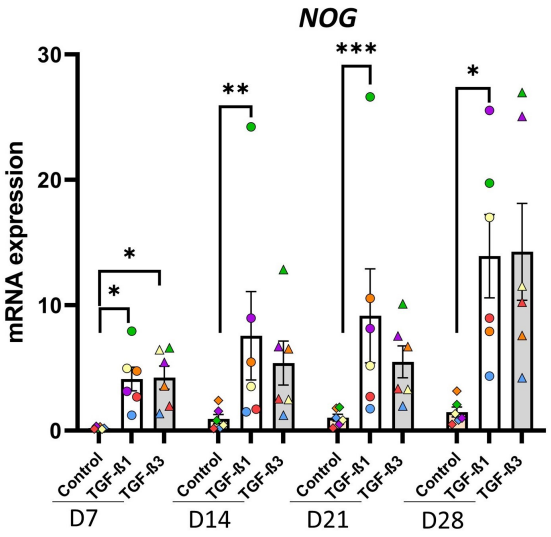

Fig. 1. *NOG* mRNA expression in control and chondrogenic MSC was measured over four weeks of culture. Dots or triangles of different colors represent individual donors (n = 6). Relative quantification of mRNA was performed according to the $2^{-\Delta\Delta Ct}$ method with RPLP0 as the endogenous control. Gene expression values were normalized to the average values of MSCs before inducing chondrogenesis (day 0). NOG, noggin; MSCs, mesenchymal stromal cells. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$.

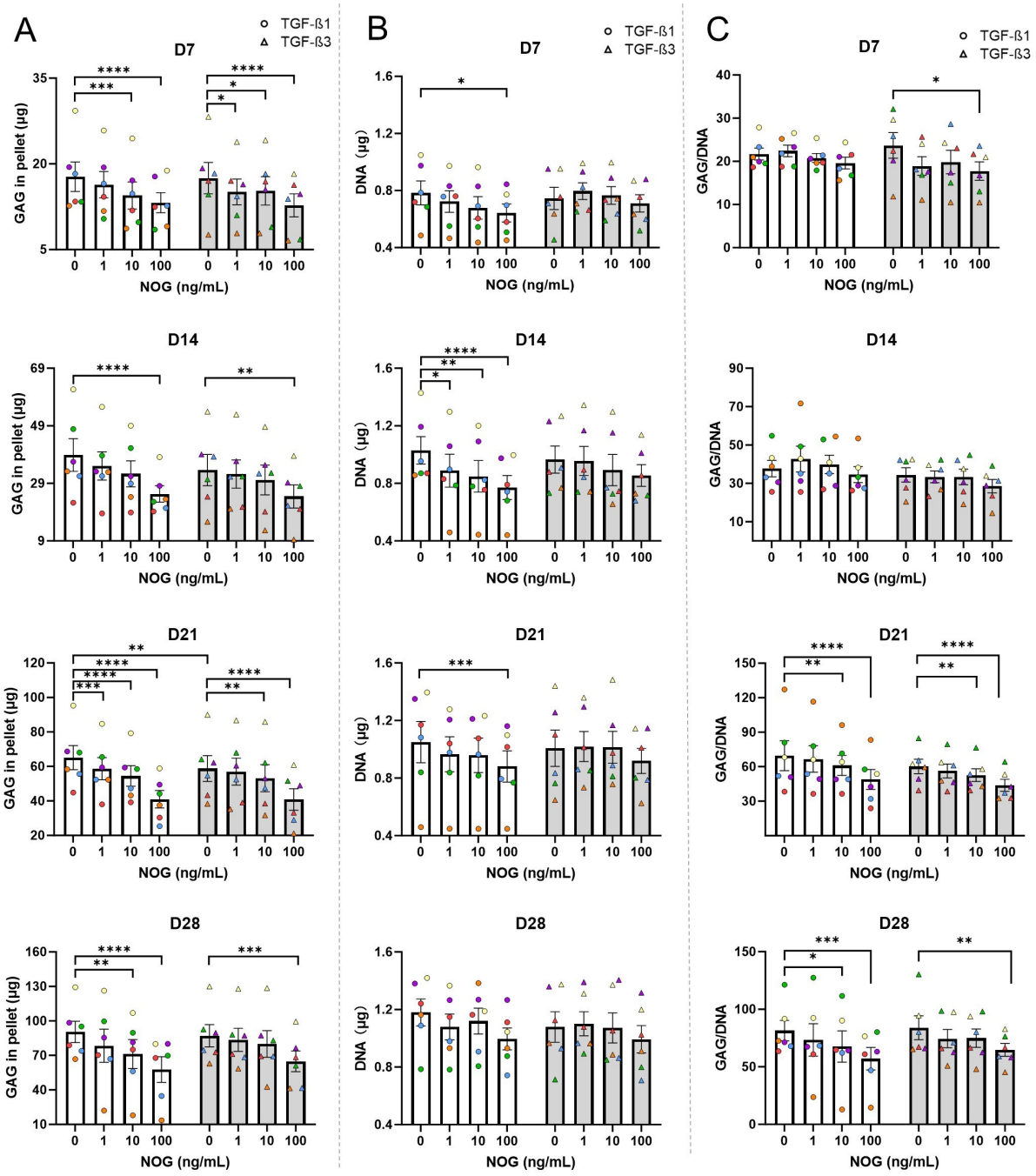

Fig. 2. sGAG retention and DNA content during chondrogenic differentiation were measured. (**A**) The impact of NOG on sGAG retention in pellets at different time points. (**B**) Quantitative analysis of DNA content after adding NOG. (**C**) Normalization of sGAG retained in pellet to DNA content. *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Dots or triangles of different color represent individual donors.

tinuing through day 28 when 100 ng/mL NOG were added, although no significant differences were observed on day 14.

NOG Inhibited sGAG Generation during Chondrogenic Differentiation

sGAG retention is dependent on incorporation within the proteoglycans-collagen network, while release commonly signifies production rather than degradation. Release of the matrix can also be an indication of an imma-

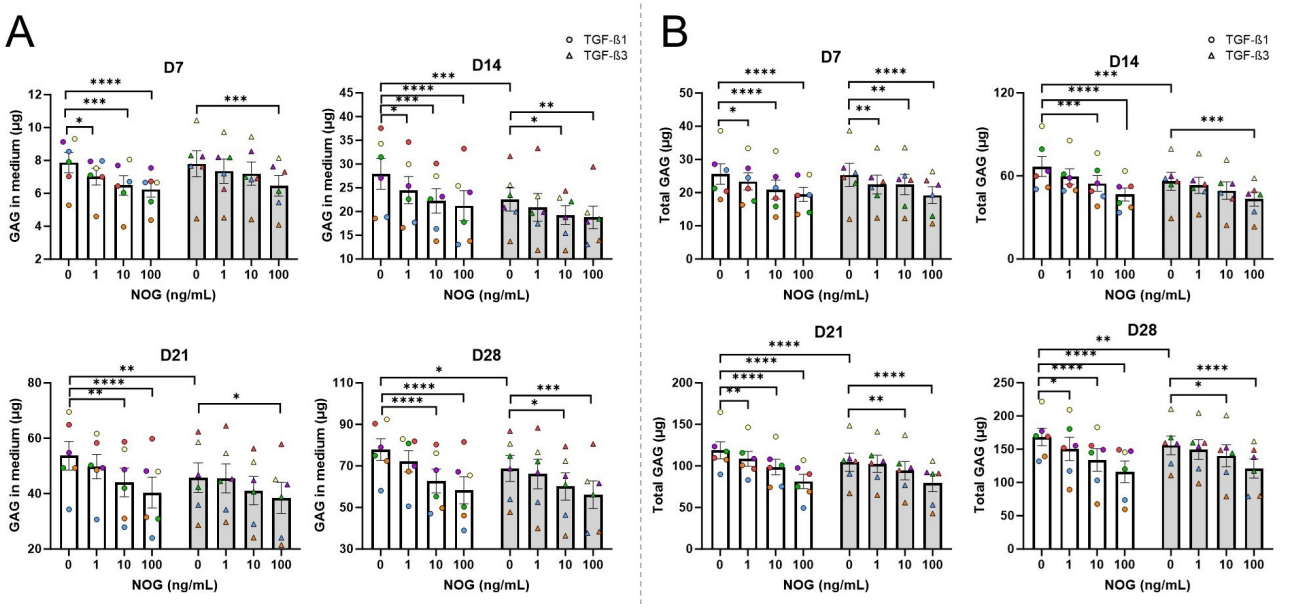

Fig. 3. Cumulative release of sGAG into the medium and total GAG generation during chondrogenic differentiation was measured after treatment with different NOG concentrations (0, 1, 10, 100 ng/mL). (**A**) sGAG release into the medium. (**B**) Total sGAG generation (GAG Retention in Pellet and Release into the Medium). Dots or triangles of different color represent individual donors (n = 6). *$p < 0.05$. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

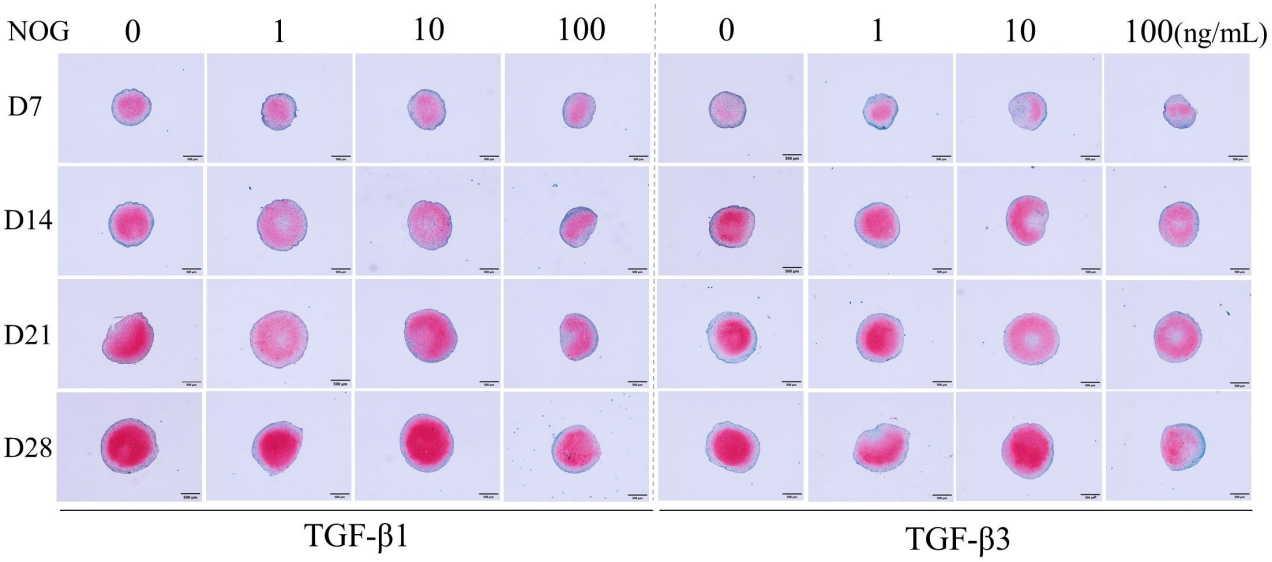

Fig. 4. Light microscopy images of cross-sectioned pellets. Safranin O/Fast Green staining was performed during TGF-β1 or TGF-β3 induced chondrogenesis at day 7, 14, 21 and 28. 100 ng/mL NOG inhibits proteoglycan generation of MSCs during chondrogenesis (Scale bar: 500 µm). TGF-β1, transforming growth factor-β1.

ture percellular matrix, which is unable to retain the newly synthesized matrix. Release of sGAG into the medium of MSCs treated with TGF-β1 was inhibited by 10 or 100 ng/mL NOG for 28 days compared to groups without NOG addition (Fig. 3**A**). sGAG release stimulated by TGF-β3 was also reduced by 100 ng/mL NOG for 28 days. TGF-β1 also exhibited a notably higher release of sGAG into the medium during the period from day 14 to day 28 compared to TGF-β3, indicating that TGF-β1 is a more effective stimulant of sGAG release than TGF-β3. Certainly, the total sGAG content, encompassing both release into the medium and retention in the pellet (Fig. 3**B**), demonstrates analogous outcomes.

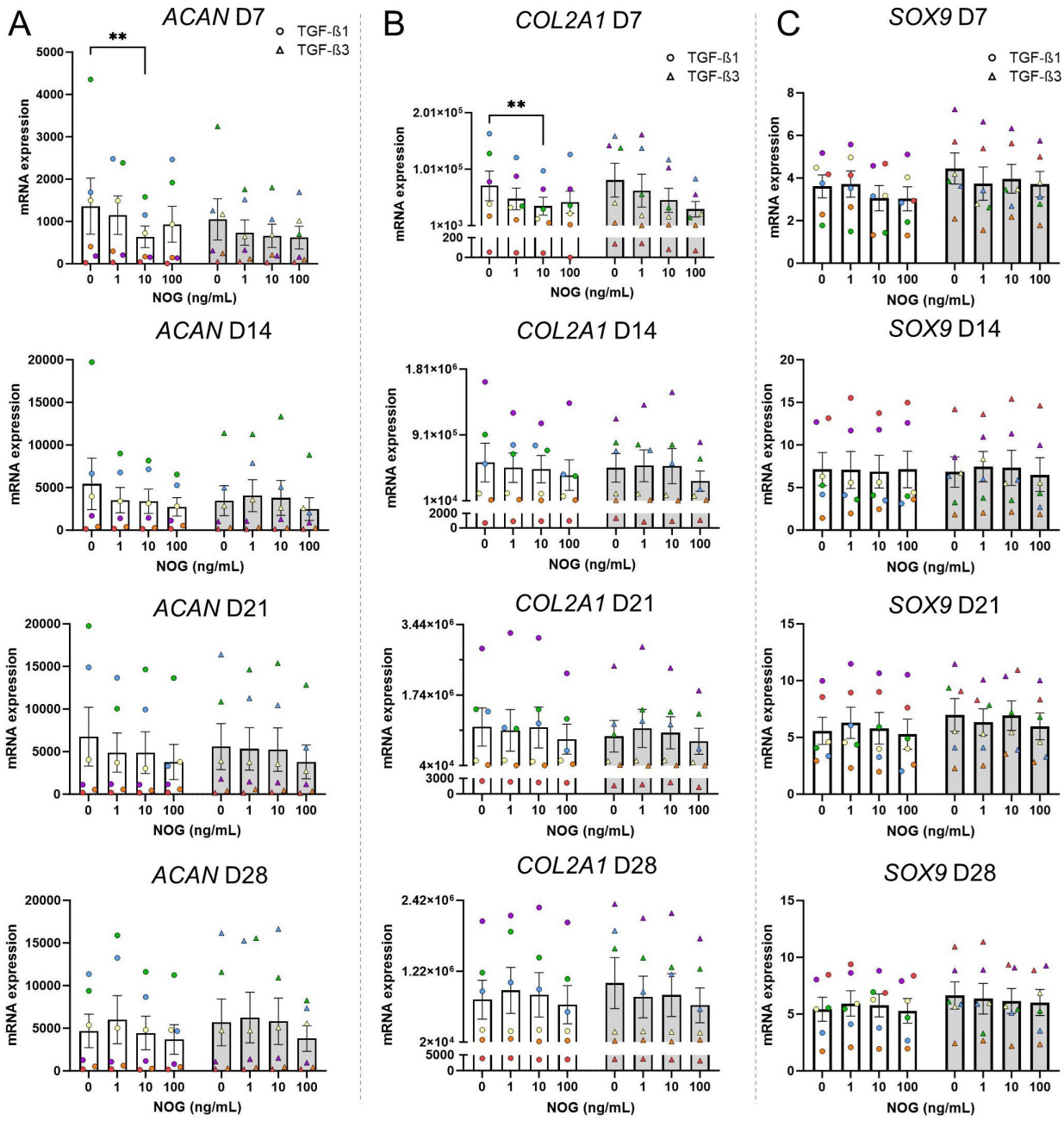

Fig. 5. mRNA expressions of (A) *ACAN*, (B) *COL2A1*, and (C) *SOX9* in MSCs treated with NOG at day 7, 14, 21 and 28 were evaluated. Relative quantification of target mRNA was performed according to the $2^{-\Delta\Delta Ct}$ method with RPLP0 as the endogenous control. Gene expression values were normalized to the average values of MSCs before inducing chondrogenesis (day 0). Dots or triangles of different color represent individual donors (n = 6). $^{**}p < 0.01$.

Generation and Distribution of Proteoglycans were Affected by NOG

Safranin O/Fast Green staining, where red staining indicated synthesis of proteoglycans and green staining showed generation of collagens (Rosenberg, 1971) was used to visualize chondrogenesis. Pellets supplemented with TGF-β1 or TGF-β3 all showed positive Safranin O staining (Fig. 4). Compared to pellets cultured in the absence of NOG, pellets treated with 10 ng/mL NOG showed a lighter red staining or smaller red area on day 14 and day 21 in both TGF-β1 or TGF-β3 pellets. Additionally, the center of the sections was less intensely stained. MSCs supplemented with 100 ng/mL NOG all showed reduced Safranin-O staining on day 14, 21 and 28 both in TGF-β1 and TGF-β3 groups, indicating less pronounced chondrogenic differentiation.

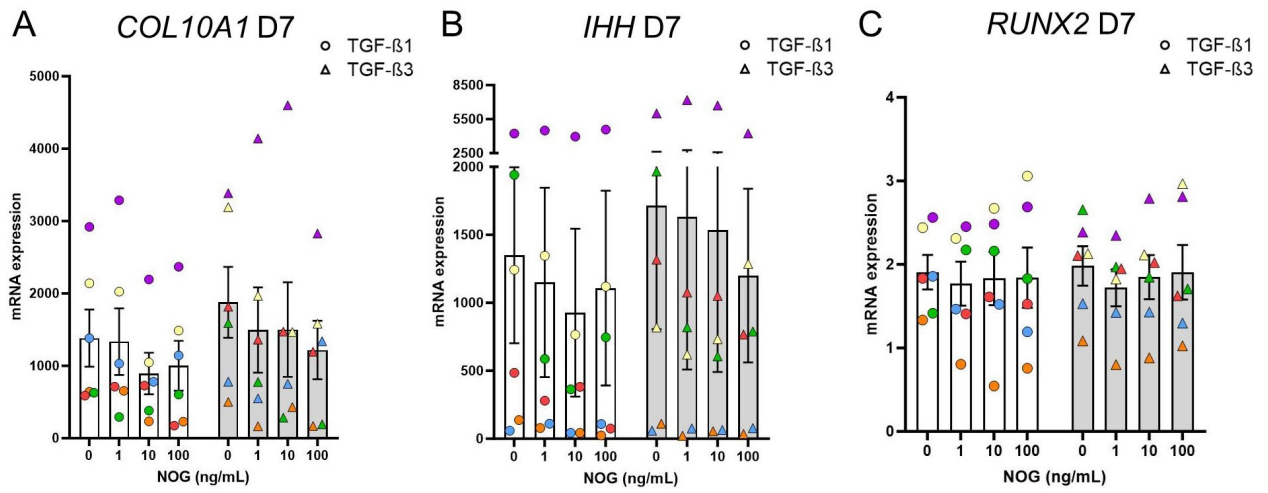

Fig. 6. Gene expressions of (A) *COL10A1*, (B) *IHH*, and (C) *RUNX2* in MSCs treated with NOG at day 7 were assessed. Relative quantification of target mRNA was performed according to the $2^{-\Delta\Delta Ct}$ method with RPLP0 as the endogenous control. Gene expression values were normalized to the average values of MSCs before inducing chondrogenesis (day 0). Dots or triangles of different color represent individual donors (n = 6).

10 ng/mL or 100 ng/mL NOG Downregulated Chondrogenic Gene Expressions at the Early Stage of Chondrogenesis

To assess the role of NOG more comprehensively, we examined gene expression levels of chondrogenic markers including *ACAN*, *COL2A1*, *SOX9* by qRT-PCR for four weeks (Fig. 5). *ACAN* and *COL2A1* expression induced by TGF-β1 was downregulated at day 7 when adding 10 ng/mL NOG. In the TGF-β1 group at day 7, supplementation with 100 ng/mL NOG resulted in decreased expression of *ACAN* in at least five donors, excluding those represented by the blue circle. Furthermore, all donors exhibited reduced expression of *COL2A1* upon addition of 100 ng/mL NOG at day 7 in TGF-β1 group, although without statistical significance. Five donors also consistently demonstrated decreased *SOX9* expression following supplementation with either 10 ng/mL or 100 ng/mL NOG. However, the expression of chondrogenic markers remains unchanged after day 7.

In the TGF-β3 group on day 7, all six donors exhibited decreased expression of *ACAN* upon addition of 10 ng/mL NOG. Additionally, five donors, excluding the blue triangle, showed reduced *ACAN* expression after supplementation with 100 ng/mL NOG. Moreover, all donors displayed decreased expression of *COL2A1* following the addition of both 10 ng/mL and 100 ng/mL NOG, although statistical significance was not observed. Five donors consistently demonstrated decreased *SOX9* expression following supplementation with 10 ng/mL NOG, while six donors exhibited lower expression of *SOX9* after the addition of 100 ng/mL NOG. However, there was no observed alteration in the expression of these genes after day 7.

Exogenous Noggin Alleviated the Extent of Hypertrophy in the Early Stage of Chondrogenesis

We also assessed the gene level of hypertrophic markers including *COL10A1*, *IHH* and *RUNX2* at day 7 (Fig. 6). In TGF-β1 induced chondrogenesis group, five donors, excluding one indicated by a red dot, exhibited a tendency for downregulation of *COL10A1* following treatment with 10 ng/mL NOG. At a concentration of 100 ng/mL NOG, *COL10A1* expression was downregulated in six donors within the TGF-β1 group. Furthermore, all donors demonstrated a reduction in *IHH* expression after adding 10 ng/mL NOG in TGF-β1 group. In the TGF-β3 group, 10 ng/mL NOG led to a downregulation of *COL10A1* in five donors, excluding one marked by a purple triangle. Additionally, 100 ng/mL NOG reduced *COL10A1* expression in five donors, except for one marked by a blue triangle. 10 ng/mL NOG resulted in downregulation of *RUNX2* in five donors, excluding one identified by a purple triangle in TGF-β3 group. Consistent alterations were not observed at day 14, day 21, and day 28 (**Supplementary Fig. 1**).

Discussion

The TGF-β superfamily, composed of the TGF-β subfamily and bone morphogenetic protein (BMP) subfamily, is indispensable for development and homeostasis of articular cartilage (Yang and Yang, 2008). TGF-β1 and TGF-β3, which belong to TGF-β subfamily, have been considered as major driving force in chondrogenesis by enhancing production and accumulation of extracellular matrix proteins (Johnstone *et al.*, 1998; Tang *et al.*, 2009). BMP antagonist NOG (Krause *et al.*, 2011) was identified as a new endogenous antagonist of TGF-β1, and NOG did not affect MSC chondrogenesis induced by TGF-β3 (Wen *et al.*,

2021). However, the effect of NOG on MSC chondrogenesis driven by TGF-β1 still remained unclear.

Firstly, we observed upregulation of *NOG* in MSC chondrogenesis by TGF-β1 or TGF-β3 stimulation for four weeks. In our study, we also provide sGAG level and histological evidence for the role of NOG in human MSC chondrogenesis for four weeks.

It was demonstrated that 100 ng/mL NOG reduced both the sGAG content in pellets and release into the medium induced by TGF-β1 or TGF-β3. In the TGF-β1 group, 100 ng/mL NOG inhibited cellular proliferation from day 0 to day 21 and reduced sGAG production relative to cell number at day 21 and 28. However, 100 ng/mL NOG only affected suppressed sGAG production normalized to DNA from day 0 to day 28 in TGF-β3 group, with no differences in DNA content. Also in an embryonal carcinoma-derived mesodermal cell line C1 overexpression of Noggin inhibited chondrogenesis by downregulating *COL2A1* and *SOX9* expression at day 15 (Nifuji *et al.*, 2004). However, our results partially conflict with another study, where addition of NOG during MSC chondrogenesis induced by TGF-β1 showed no effect on proteoglycan production and *COL2A1* expression at day 28 (Karl *et al.*, 2014).

Safranin O/Fast Green staining also revealed that sGAG deposition in pellets was inhibited by addition of 100 ng/mL NOG to both TGF-β isoforms. However, proteoglycan staining after adding 10 ng/mL NOG both in TGF-β1 group and TGF-β3 group at day 28 was not consistent with the quantitative detection of glycosaminoglycans in total pellets. The inconsistency might arise because extracellular matrix generation in the central part was not representative of the total pellet. In addition, we observed downregulation of chondrogenic markers only by 10 ng/mL or 100 ng/mL NOG at the early stage of chondrogenesis rather than four weeks in the TGF-β1 group. The possible reason of non-consistent and non-dose dependent ACAN mRNA expression might be that mRNA expression is a snapshot, but sGAG production and histology are a result of long-term accumulation. There may also be post translational protein modifications that are differentially affected. In this study, the statistically significant effects of Noggin administration were predominantly observed solely at the high dose of NOG. Exogenous growth factors, due to their physical distance from cells, often require supraphysiological doses for application. *In vivo*, similar responses are expected at significantly lower doses.

In vitro chondrogenesis, the process of differentiating stem cells into chondrocytes, is indeed often accompanied with hypertrophy (Armiento *et al.*, 2019), which is characterized by expression of hypertrophic markers such as *COL10A1*, *IHH*, and *RUNX2*. At concentrations of 10 ng/mL and 100 ng/mL, NOG consistently downregulated the expression of *COL10A1* at least five donors both TGF-β1 and TGF-β3 isoforms at day 7. Additionally, at 10 ng/mL, NOG specifically downregulated *IHH* in the TGF-β1 group and *RUNX2* in the TGF-β3 group. These findings highlight NOG's role in modulating hypertrophy in early chondrogenesis process. In a previous study, under chondrogenic conditions, NOG treatment did not influence on hypertrophic extent (Karl *et al.*, 2014). However, under hypertrophic conditions induced by triiodothyronine, NOG treatment was found to inhibit hypertrophy (Karl *et al.*, 2014). The reason might be the timepoint of NOG addition, as they added NOG at day 14, not at day 0.

sGAG production and matrix staining at four weeks showed evidence that NOG impeded the progress of TGF-β1 or TGF-β3 induced chondrogenesis in MSCs. NOG seems plays a dual role in the regulation of chondrogenesis and hypertrophy. Although it inhibits hypertrophy at day 7, it also prevented chondrogenesis. In a previous study (Wen *et al.*, 2021), NOG did not show an inhibitory effect on TGF-β3-induced chondrogenesis. The reason for the differing results might be the source of MSCs, which previously were from Wharton's jelly of human umbilical cords, while bone marrow derived MSCs were used here. Also, the previous study used 2 % oxygen while the current study was performed at atmospheric oxygen. Chondrogenic differentiation of MSCs was reported to be enhanced by hypoxia (Lee *et al.*, 2013), so NOG might not show any inhibitory effects at low oxygen, which is a much more favorable condition for cartilage formation. This observation underscores that *in vitro* culture results need to be interpreted with caution as elements of the culture conditions might contribute to artifactual results, emphasizing the importance of carefully considering experimental parameters.

The mechanism of inhibitory role of NOG in TGFβ-1 induced chondrogenesis might be the physical interaction between NOG and TGFβ-1 (Wen *et al.*, 2021). Indirect effects of secreted BMP's (Deng *et al.*, 2018) that are blocked by external NOG might also lead to sGAG production decrease, although we did not observe any significant alterations in the mRNA levels of BMPs, including *BMP2*, *BMP4*, *BMP6*, *BMP7*, *GDF5*, and *GDF6* (Data not shown) after adding exogenous Noggin. However, the mechanism underlying the inhibitory role of NOG in TGFβ-3-induced chondrogenesis remains unknown.

In conclusion, endogenous NOG expression increased during TGF-β induced chondrogenesis of human bone marrow derived MSCs, and NOG inhibited chondrogenic differentiation induced by TGF-β1 or TGF-β3. Our study provides sGAG level, gene expression and histological evidence for the inhibitory role of NOG *in vitro* chondrogenic differentiation of adult mammalian progenitor cells derived from bone marrow. Notably, in TGFβ-1 induced chondrogenesis, NOG also inhibited cellular proliferation. The complex factor network affecting chondrogenic differentiation and the observed differences in gene or protein expression remain to be fully elucidated in the future.

List of Abbreviations

NOG, Noggin; BMPs, bone morphogenetic proteins; TGF-β1, transforming growth factor-β1; MSCs, mesenchymal stromal cells; ECM, extracellular matrix; DMEM, Dulbecco's Modified Eagle's Medium; αMEM, α-modified essential medium; Pen/Strep, penicillin-streptomycin; FGF-b, basic fibroblast growth factor; FBS, fetal bovine serum; DMMB, 1,9-dimethyl-methylene blue; BCP, 1-Bromo-3-chloropropane; SEM, standard error of the mean; ANOVA, analysis of variance.

Availability of Data and Materials

Data available upon justified request.

Author Contributions

LW performed the experiments, analyzed the data, and wrote the manuscript. ARA and MJS designed the experiments. AGG, ARA and LC reviewed and edited the manuscript. AGG and LC analyzed the data. SG and MJS worked on funding acquisition and supervised the whole work. All authors reviewed the results and approved the final version of the manuscript.All authors contributed to editorial changes in the manuscript, read and approved the final manuscript, and have participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors have agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Human bone marrow was obtained from 6 donors (male, 48, 49, 54, 55, 58, or 68 years old) with ethical approval from the local authorities (Ethik-Kommission der Albert-Ludwigs-Universität Freiburg, EK-326/08) and written consent of the patients undergoing total hip replacement.

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Conflict of Interest

All authors declare no conflict of interest. MJS and SG are serving as the Editorial Board members of this journal. We declare that MJS and SG had no involvement in the peer review of this article and have no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to CH. ARA is an employee of UCB Pharma and has no conflict of interest with this study.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.22203/eCM.v047a10.

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