Alternative splicing of type II procollagen pre-mRNA in chondrocytes is oppositely regulated by BMP-2 and TGF-β1

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Abstract Type II collagen is the major protein of cartilage and is synthesized as a procollagen in two forms (IIA and IIB), generated by differential splicing of the gene primary transcript. Previous studies have indicated that only type IIB is expressed in differentiated chondrocytes. Here, we examined the effects of bone morphogenetic protein (BMP)-2 and transforming growth factor (TGF)-β1 on the expression of IIA and IIB forms expressed in de-differentiated chondrocytes grown in monolayer. Our results demonstrate that BMP-2 favors expression of type IIB whereas TGF-β1 potentiates expression of type IIA induced by subculture. These observations reveal the specific capability of BMP-2 to reverse the de-differentiation state of chondrocytes.

Key words: Bone morphogenetic protein; Transforming growth factor-β; Type II collagen; Chondrocyte

1. Introduction

Cartilage development is an early morphogenetic step in skeletogenesis. This process involves the commitment, condensation and differentiation of mesenchymal cells into chondrocytes which ultimately leads to the synthesis and secretion of proteins characteristic of the cartilage matrix, such as type II, IX and XI collagens, and the large proteoglycan aggrecan [1]. Type II collagen is the major component of the cartilage collagen fibrils and is classically considered a reference marker for the differentiated chondrocyte phenotype. This protein is a homotrimer of three α_1(II) chains, which are products of one gene (*Col2a1*). Two forms of type II collagen are generated by alternative RNA splicing [2]. The type IIA form includes and the type IIB form excludes a 69 amino acid cysteine-rich (CR) domain encoded by exon 2 in the NH2-propeptide. CR domains are present in a variety of other extracellular proteins including fibrillar type I, III and V procollagens, chordin in *Xenopus* [3], and the chordin homologue sog in *Drosophila* [4]. Recent studies indicate that CR domains could provide binding modules for diverse transforming growth factor (TGF)-β superfamily members, such as bone morphogenetic proteins (BMPs). Zhu et al. [5] showed that the NH2-propeptide containing the CR domain of type IIA procollagen binds to BMP-2 and TGF-β1, two regulators of various developmental processes as well as chondrogenesis. This finding led to the hypothesis that type IIA procollagen could play a broader role than a purely structural one, by controlling in the extracellular space the amount of specific morphogenetic agents [5]. In support of this hypothesis, procollagen IIA mRNA, like chordin mRNA, showed dorsalizing activity in *Xenopus* embryo microinjection assays and the CR domain was required for this anti-BMP activity [6].

In supplement of the developmental standpoint mentioned above, it is of particular interest to note that type IIA procollagen is expressed in pre-chondrocytes, mesenchymal and epithelial cells, and in a number of embryonic, non-chondrogenic tissues such as somites and intervertebral disks, whereas type IIB procollagen is expressed in differentiating and adult cartilage and in adult tissues such as sclera [7–14]. Thus, in regard of cartilage biology, the shift from type IIA to type IIB is a sign of chondrocyte differentiation. On the other hand, re-expression of type IIA procollagen has been reported in human osteoarthritic cartilage and this could represent a potential reversion towards a chondroprogenitor cellular phenotype [15].

Therefore, it is important to examine the spliced forms of type II procollagen mRNA expressed by the chondrocyte for an accurate determination of its phenotype in an in vivo or in vitro situation. In this latter case, several studies that monitored the stability of the chondrocyte phenotype have shown that culture of these cells in monolayers on plastic substrata for a long time or upon repeated passages leads to the loss of their spherical shape and to the acquisition of a fibroblastic morphology. These shape changes are linked to biochemical changes, including the progressive reduction of type II collagen synthesis and other cartilage-characteristic proteins [16–25], a process designated 'de-differentiation'.

Only a few studies have been performed to understand the molecular mechanisms underlying chondrocyte de-differentiation. The transcription factors SP1 and SP3 and their ratio control the expression of the *Col2a1* promoter during chondrocyte de-differentiation [26,27]. Since de-differentiation of chondrocytes represents a limiting factor in mass cell production required for the treatment of articular cartilage defects [28], further analysis is needed to better define how cartilage-specific genes are modulated in monolayer culture, to ulti-

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mately prevent de-differentiation or promote re-differentiation of chondrocytes. In this context, growth factors are good candidates to influence the transcriptional machinery of the chondrocytes. In particular, the BMPs promote chondrogenic and osteogenic phenotypes in vitro [29–31]. Recently, we showed that BMP-2 stimulates type II collagen expression and synthesis in mouse chondrocytes and TGF-β signaling antagonizes this effect [32,33]. This observation prompted us to investigate the role of BMP-2 and TGF-β1 in the regulation of de- and re-differentiation of mouse chondrocytes in monolayer culture, with particular attention given to the expression of spliced forms of type II procollagen mRNA.

2. Materials and methods

2.1. Cell culture

Embryonic mouse chondrocytes were isolated from the ventral parts of the rib cages of 17.5 dpc mice, as described [34]. These chondrocytes were seeded on 90 mm Corning dishes with 1×10^6 cells/dish and cells at this stage were designated P0. Chondrocytes were cultured with 1:1 high-glucose Dulbecco's modified Eagle's medium/Ham F12 containing 10% fetal bovine serum (FBS) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all products from Invitrogen), at 37°C in 5% CO_2. Confluent cells were detached using 0.05% trypsin–EDTA and seeded onto 90 mm Corning dishes for amplification. The progressive loss of the differentiated phenotype was achieved by serial subcultures, up to six passages (cells were then designated P1–P6). P1 and P6 chondrocytes were seeded in 6 well culture dishes with 1.5×10^5 cells/well and grown for 3 or 6 days in medium containing 1% FBS supplemented with BMP-2 (0–100 ng/ml) or TGF-β1 (0–5 ng/ml). Recombinant human BMP-2 was produced and purified by Wyeth Research (Cambridge, MA, USA). Recombinant human TGF-β1 was purchased from Sigma. The culture medium was replaced every day. In experiments dedicated to the Western blotting analysis of type II procollagen synthesized by P1 chondrocytes, 20 μg/ml ascorbic acid (Sigma) was added daily to the culture medium.

2.2. Immunofluorescence

P0 and P1 chondrocytes were seeded on glass coverslips in 6 well culture dishes with 4×10^5 cells/well (P0) or 1.5×10^5 cells/well (P1). P0 chondrocytes were grown for 24 h and P1 chondrocytes were grown for 3 and 6 days, in the presence of 1% FBS supplemented or not with 100 ng/ml BMP-2 or 5 ng/ml TGF-β1. The cell cultures were then treated as previously described [33], followed by an incubation for 1 h with the 2B1 monoclonal antibody recognizing an epitope located in the triple helix of type II collagen [35], or with polyclonal rabbit antibodies raised against mouse type I collagen (Novotec ref. 20151) [36]. Cells were then washed with phosphate-buffered saline (PBS) and incubated for 1 h with Cy3- or Cy2-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson) in PBS containing 0.25 μg/ml Hoechst 33258 (Sigma) to visualize nuclei. Chondrocytes were again washed with PBS and coverslips were mounted in glycerol/PBS (1:1) for their observation by epifluorescence with a Zeiss Axioplan 2 microscope equipped with a CoolSNAP Fx camera (Roper Scientific). Image acquisition and overlay were achieved with Metaview software.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were extracted and reverse-transcribed as previously described [33]. RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was performed to ascertain that an equivalent amount of cDNA was synthesized from the different samples. The conditions for the PCR amplifications and the sequences of the primers for GAPDH and type IIA/IIB procollagen have been previously reported [32,33]. The total amount of type II procollagen (IIA+IIB) transcripts was determined by designing a sense oligonucleotide (5′-GGTTTGGAGAGACCATGAAC-3′) in exon 50 and an antisense primer (5′-TGGGTTCGCAATGGATTGTG-3′) in exon 52 of the mouse *Col2a1* gene, from a sequence available in GenBank (M65161). Samples were amplified for 20 cycles, with denaturation at 95°C (30 s), annealing at 55°C (30 s), and extension at 72°C (30 s), giving a product size of 463 bp. The RT-PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. Amplicons of the expected size were obtained, cloned in a pCR2.1-TOPO vector (Invitrogen, San Diego, CA, USA) and sequenced (Genome Express, France) to confirm the identity of the sequences. Photographs of gels of RT-PCR experiments using cells obtained from three independent dissections were analyzed with Image Quant software (Amersham Biosciences).

2.4. Western blot analysis

Cells were washed three times with PBS and scraped into 100 μl of Laemmli sample buffer. After boiling for 5 min in the presence of 2-mercaptoethanol, the proteins were separated on 6% polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). Non-specific binding sites were blocked by incubating the membrane overnight at 4°C in 0.2% (w/v) non-fat dry milk blocker (Bio-Rad) dissolved in TBS (20 mM Tris–HCl, 0.5 M NaCl, pH 7.5). After rinsing with TBS-T (TBS containing 0.1% Tween 20), the membrane was incubated for 2 h with polyclonal rabbit antibodies raised against mouse type II collagen (Novotec, ref. 20251) [36], at 2.5 μg/ml in TBS-T, 0.2% blocker. The membrane was washed with TBS-T and incubated for 1 h with goat alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad). After multiple washes in TBS-T, bound antibodies were detected on X-ray films using a Bio-Rad Immun-star chemiluminescent substrate. Either Ponceau S staining of immunoblots or parallel Coomassie brilliant blue-stained gels confirmed equivalent protein loading.

3. Results and discussion

When chondrocytes were isolated from mouse embryos and seeded for 24 h at subconfluence, they presented round and polygonal shapes and were all positively stained by immunofluorescence for type II collagen, attesting the purity of the chondrogenic population (Fig. 1A). The gene expression of

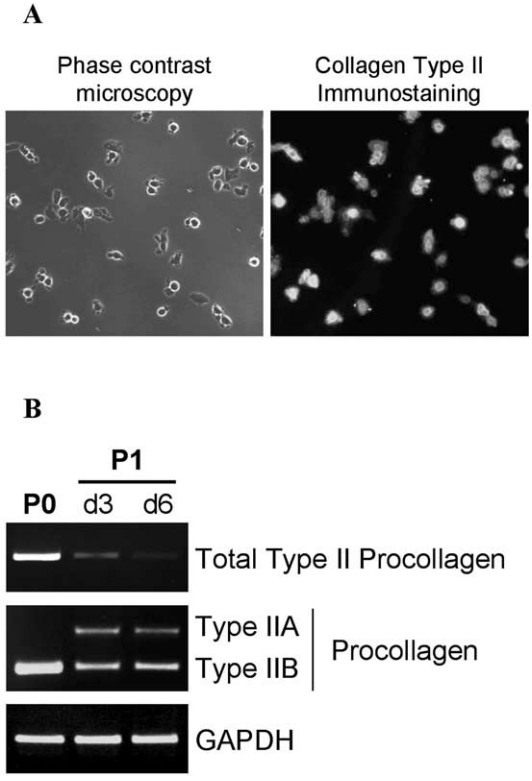

Fig. 1. A: Morphology and staining of P0 chondrocytes seeded for 24 h. B: RT-PCR analysis of expression of type II procollagen in P0 chondrocytes seeded for 24 h, and in P1 chondrocytes grown for 3 and 6 days after their first passage.

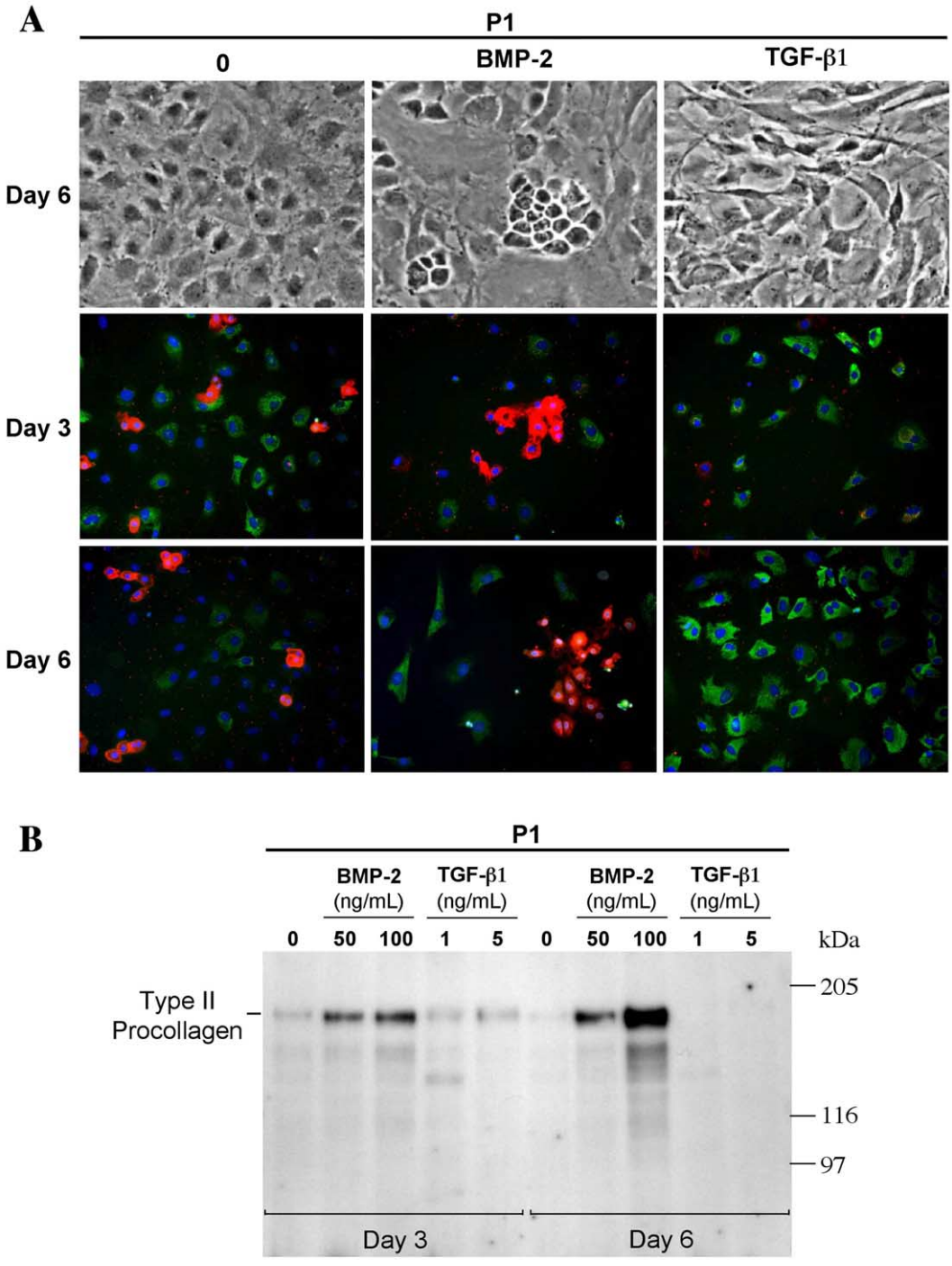

Fig. 2. A: Phase contrast microscopy and staining of P1 chondrocytes grown for 3 and 6 days in the presence of 1% FBS alone (0) or supplemented with 100 ng/ml BMP-2 or 5 ng/ml TGF-β1, as indicated. At the top, control cells (0) show spread or fibroblastic morphology whereas BMP-2-treated cells are organized in refractile clusters. TGF-β1-treated cells show flattened or fibroblastic morphology. In the middle and at the bottom, cells are double-stained for type II collagen with Cy3-conjugated secondary antibodies (red) and for type I collagen with Cy2-conjugated secondary antibodies (green). Nuclei are stained with Hoechst dye (blue). B: Western blotting analysis of type II procollagen accumulation in P1 chondrocyte monolayers. Cells were grown for 3 and 6 days in the presence of 1% FBS (0) or in the presence of 1% FBS supplemented with BMP-2 or TGF-β1, at the indicated concentrations. The positions of globular molecular size markers are shown on the right.

total type II procollagen decreased gradually with time from P0 to P1, illustrating the de-differentiation process classically observed for chondrocytes maintained in monolayer culture on plastic (Fig. 1B). More precisely, the type IIB form of type II procollagen mRNA was the only form expressed by P0 chondrocytes, as expected from differentiated chondrocytes, whereas the type IIA form was expressed only after subculture, in P1 chondrocytes (Fig. 1B). Recently, Kim et al. [37] showed that alternative splicing of type II procollagen pre-mRNA occurs during de-differentiation of rat chondrocytes in monolayer, but both type IIA and type IIB transcripts were already expressed in chondrocytes on the day of their isolation. This discrepancy between their results and ours could be explained by species differences, since we previously showed

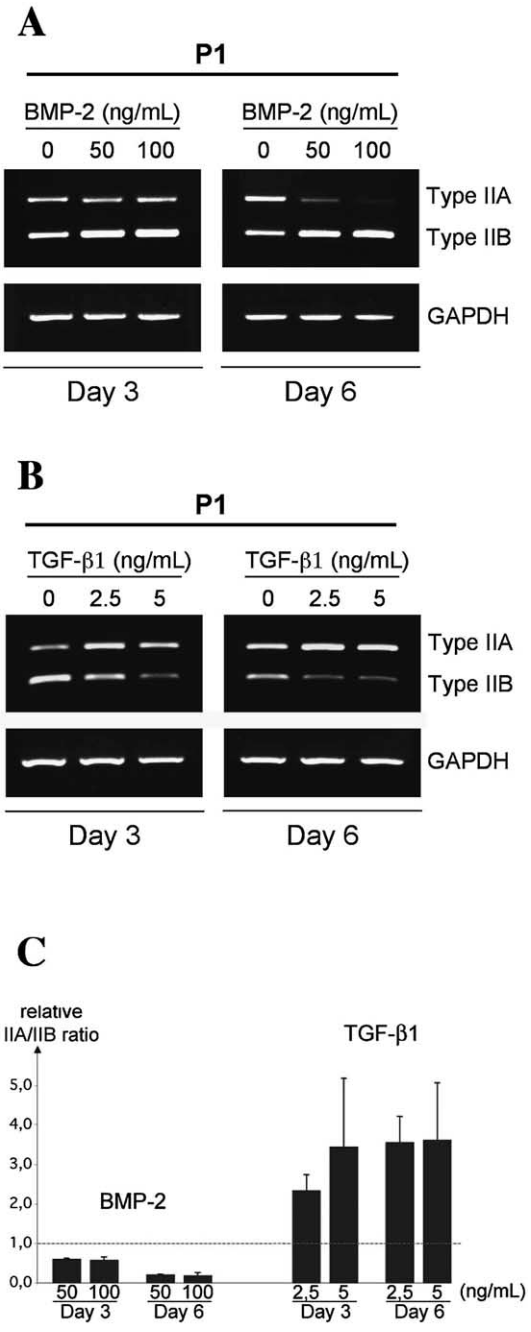

Fig. 3. A,B: RT-PCR analysis of type IIA and type IIB procollagen spliced forms in P1 chondrocytes grown for 3 and 6 days in the presence of 1% FBS alone (0) or supplemented with BMP-2 or TGF-β1, at the indicated concentrations. C: Densitometric quantitation of the type IIA/IIB ratio, shown relative to the level of control cells grown in the presence of 1% FBS alone (1.00), at the indicated days. Values given are the arithmetic means, and the error bars represent standard deviations of three independent experiments.

that type IIB mRNA is the only spliced variant expressed in mouse rib cage [33].

We further investigated the responsiveness of de-differentiated P1 chondrocytes to BMP-2 and TGF-β1, during a 3 and 6 day culture period. Cells treated with BMP-2 showed polygonal and round shapes and were organized in refractile clusters, a trait of differentiated chondrocytes (Fig. 2A). In contrast, control chondrocytes grown in the presence of 1% serum showed a spread or fibroblastic morphology and TGF-β1 treatment accentuated this effect. The addition of BMP-2 increased the number of cells positively stained for type II collagen on days 3 and 6, as compared to untreated cells (Fig. 2A). It should be noted that the positive staining for type II collagen observed after BMP-2 treatment was mainly localized in cells organized in clusters. Inversely, TGF-β1 suppressed type II collagen-positive staining (Fig. 2A). Concomitantly, some cells were positively stained for type I collagen in the control and BMP-2-treated cultures while most cells were positive for type I collagen in the presence of TGF-β1, whatever the time in culture (Fig. 2A). Type I collagen has been previously described as a marker of de-differentiated chondrocytes [16]. Thus, our data together indicate that (i) embryonic mouse chondrocytes undergo de-differentiation after subculture and (ii) TGF-β1 potentiates this de-differentiation process whereas BMP-2 reverses it. We further evaluated this differential responsiveness by a Western blotting analysis of the amount of type II procollagen present in cell monolayers. Our results showed that BMP-2, but not TGF-β1, favors type II procollagen accumulation in P1 chondrocyte monolayers in a time- and dose-dependent manner (Fig. 2B).

Subsequently, we investigated whether alternative splicing of type II procollagen pre-mRNA in de-differentiated chondrocytes was modulated by BMP-2 or TGF-β1 treatment. Indeed, RT-PCR analyses of P1 chondrocytes indicated that BMP-2 favors the expression of type IIB transcript whereas TGF-β1 favors the expression of type IIA transcript (Fig. 3A,B). The effect of each growth factor on the ratio of expression of the two transcripts was significant, as shown after densitometric analysis (Fig. 3C). Furthermore, when the effects of BMP-2 were evaluated on late de-differentiated chondrocytes (P6), we found again that BMP-2 was able to stimulate *Col2a1* expression, particularly that of type IIB transcript (Fig. 4).

Thus, this study shows for the first time that BMP-2 and TGF-β1 have specific, opposite effects on the alternative splicing of type II procollagen pre-mRNA. In regard of this splicing, BMP-2 reverses the program of chondrocyte de-differentiation, as attested by re-expression of the cartilage-characteristic type IIB procollagen. This important feature needs to be considered during the cell amplification procedure for the currently used autologous chondrocyte transplantation, even though other skeletal markers need to be evaluated, as well as the ability of human articular chondrocytes to re-

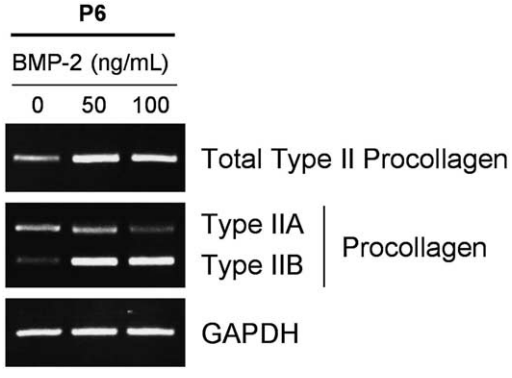

Fig. 4. RT-PCR analysis of type II procollagen in P6 chondrocytes grown for 3 days in the presence of 1% FBS alone (0) or supplemented with 50 or 100 ng/ml BMP-2, as indicated.

spond to BMP-2. In addition, we have established a novel cellular model to study the role of the *Col2a1* exon 2-encoded peptide and to decipher the molecular mechanisms underlying alternative splicing of type II procollagen pre-mRNA. Such studies should contribute to gain insight into the regulation of the chondrocyte phenotype and represent a first step to further control this phenotype in vitro.

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