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Validation in mesenchymal progenitor cells of a mutation-independent *ex vivo* approach to gene therapy for osteogenesis imperfecta

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Over 100 dominant-negative mutations within the *COL1A1* gene have been identified in osteogenesis imperfecta (OI). In terms of human therapeutics, targeting each of these mutations independently is unlikely to be feasible. Here we show that the hammerhead ribozyme Rzpol1a1, targeting a common polymorphism within transcripts from the *COL1A1* gene, downregulates *COL1A1* transcript in human mesenchymal progenitor cells at a ribozyme to transcript ratio of only 1 : 1. Downregulation was confirmed at the protein level. Transducing stem cells with Rzpol1A1 *ex vivo* followed by autologous transplantation could provide a gene therapy for a large proportion of OI patients with gain-of-function mutations using a single therapeutic.

INTRODUCTION

Osteogenesis imperfecta (OI) is a debilitating disorder resulting in the formation of brittle bones. Types II, III and IV generally arise from gain-of-function mutations in either of the two type I collagen genes, *COL1A1* and *COL1A2*, and represent serious, sometimes lethal, disorders. Type I OI, however, usually results from null mutations in the *COL1A1* gene and is phenotypically milder (1–3). In this study we investigated a gene therapy for *COL1A1*-associated dominant-negative forms of OI in human mesenchymal progenitor cells (MPCs) using a hammerhead ribozyme. Notably, ribozymes have been shown to down-regulate *COL1A1* transcripts specifically at mutation sites both *in vitro* (4) and *in cellulo* (5,6). In addition, mutation-independent ribozymes, targeting the *COL1A1* and *COL1A2* transcript *in vitro*, have been described (7,8).

The design of gene therapies for dominant hereditary diseases is greatly complicated by genetic heterogeneity. Gene therapies for dominant-negative diseases, such as many forms of OI, will probably require suppression of a mutant allele while still maintaining normal expression of a wild-type allele. Since over 100 dominant-negative mutations in the *COL1A1* gene alone have been identified, the development of mutation-independent methods of suppressing mutant alleles (9) is imperative (for known mutations see www.le.ac.uk/ge/collagen/COL1A1.html). In this study, we examine the ability of a hammerhead ribozyme, Rzpol1a1, targeting a common C/T polymorphism at position 3210 (GenBank accession no. K01228) in the 3′UTR of the *COL1A1* transcript (7,8,10), to downregulate *COL1A1* in human MPCs. Rzpol1a1 can cleave the T allele of the polymorphism, but not the C allele *in vitro* (7,8). Since over 40% of Caucasian populations in the USA and Europe are heterozygous for the polymorphism (3,8), 20% of *COL1A1*-associated OI patients will harbour a mutation on the cleavable T allele and may therefore benefit from the effects of Rzpol1a1. Interestingly, the homozygous T/T population, representing an additional 40% of OI patients, may also be treated with Rzpol1a1. Since Rzpol1a1 would target both *COL1A1* alleles, the therapy would need to include a replacement *COL1A1* gene, altered at the ribozyme target site, such that it escapes ribozyme suppression. We cloned Rzpol1a1 into two different positions of a Moloney murine leukaemia viral vector (Mo-MLV), RNL-Pol (Fig. 1A), which we used to transfect human MPCs, homozygous for the T allele of *COL1A1*. Since RNL-Pol contains three different promoters, the 3′-LTR, Rous sarcoma virus (RSV) and cytomegalovirus (CMV) promoters, the virus should express three different RNA populations (Fig. 1B), enhancing ribozyme numbers and maximizing the chance of ribozyme activity.

Human MPCs were deemed to constitute a suitable system for testing Rzpol1a1 activity, since these cells can differentiate *in vivo* into osteoblasts, the cells involved in OI (11–13). It is notable that donor human MPCs have been administered to five children with severe OI. Engraftment was shown in three children, increasing their bone mineral content by between 44% and

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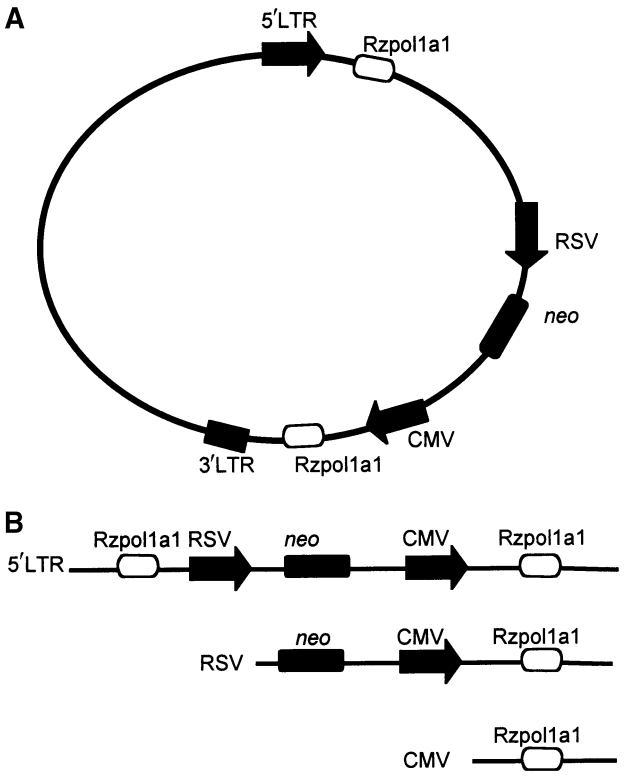

Figure 1. MLV retroviral vector RNL-Pol. The 5′-LTR, RSV and CMV promoters (arrows), Rzpol1a1 (open symbols), cloned at two positions in RNL-Pol, and the *neomycin* gene (closed symbols) are shown. (**A**) Schematic of plasmid used for generating RNL-Pol. (**B**) Schematic of the three RNA populations which RNL-Pol will generate using the 3′-LTR, RSV and CMV promoters.

77% of baseline values 3 months after treatment (14,15). Engrafted MPCs have been shown to constitute between 1% and 5% of bone marrow cells (14–19). While this demonstrates the administration of MPCs to patients and the cells' survival, it is, however, necessary to administer immunosuppressant drugs to patients treated in this manner, since acute and chronic graft versus host disease (GVHD) is a risk associated with bone marrow transplantation (15). In an alternative approach, utilizing autologous MPCs from OI patients as gene delivery vehicles in order to avoid the risk of GVHD, we have tested the mutation-independent hammerhead ribozyme, Rzpol1a1, in human MPCs. It is perhaps notable that Rzpol1a1 may also be delivered by other means. Regardless of the method of gene delivery, the functionality of any therapeutic drug in its target cell type must be investigated first. Thus, this study describes the functionality of a novel mutation-independent ribozyme, Rzpol1a1, targeting an intragenic polymorphism in *COL1A1*, in human MPCs, the target stem cell type for OI therapies.

RESULTS

COL1A1 transcript downregulation in MPCs expressing Rzpol1a1

Having identified an MPC sample homozygous for the T allele of the *COL1A1* gene, we stably transduced these cells with retroviral vector RNL-Pol (Fig. 1). Two MPC samples, MPC-Pol1 and MPC-Pol2, were thus generated. RNL-Pol carries the mutation-independent hammerhead ribozyme Rzpol1a1, which targets the T allele of *COL1A1* specifically. *COL1A1* transcript levels were assessed in MPC-Pol1, MPC-Pol2 and untransduced MPCs using real-time RT–PCR standardized with *COL1A2* (the natural associate of *COL1A1*) and the housekeeping genes *GAPDH* and *β-actin.* Downregulation of the *COL1A1* transcript was confirmed 36 times in both MPC-Pol1 (Table 1) and MPC-Pol2 (Table 2). Mean levels of downregulation standardized with *COL1A2*, *GAPDH* and *β-actin* of 40.31% (34.97%, 44.41% and 41.55%) and 47.08% (36.44%, 54.67% and 50.13%) were achieved in MPC-Pol1 and MPC-Pol2 respectively. Notably, differences in levels of *COL1A1* suppression observed when RT–PCR reactions were standardized with *COL1A2*, *GAPDH* or *β-actin* as internal controls were not significant in either MPC-Pol1 or MPC-Pol2 ($P = 0.72$ and $P = 0.090$ respectively).

Retroviral gene expression levels in MPC-Pol1 and MPC-Pol2

We compared levels of retroviral gene expression to *COL1A1* expression levels in MPC-Pol1 and MPC-Pol2 in order to establish the level of Rzpol1a1 contributing to the observed downregulation of *COL1A1.* Expression levels were compared in terms of absolute copy number using real-time RT–PCR. Since RNL-Pol expresses three populations of RNA (Fig. 1B), two RT–PCR reactions were carried out. The first RT–PCR reaction amplified all three RNA populations arising from RNL-Pol. The second RT–PCR reaction amplified only the two larger RNA molecules from RNL-Pol. Results (Table 3) indicate that in both MPC-Pol1 and MPC-Pol2, a ratio of retroviral RNA to *COL1A1* RNA of less than 1 : 1 is sufficient to achieve a downregulation of *COL1A1* transcript of over 40% in human MPCs. In addition, these data indicate that in human MPCs the CMV promoter is estimated to be over five times stronger than the 5′-LTR and RSV promoters together.

Type I collagen protein downregulation in MPC-Pol1 and MPC-Pol2

To determine whether suppression of *COL1A1* transcript is reflected at the protein level, we performed immunocytochemistry on untransduced MPCs, and MPC-Pol1 and MPC-Pol2 cells using a fluorescence isothiocyanate (FITC)-labelled anti-human type I collagen antibody. We used fibronectin as a control. Qualitative assessment after 4 and 7 days of cell growth showed reproducibly a marked downregulation of *de novo* type I collagen in MPC-Pol1 and MPC-Pol2 in terms of intensity of fluorescence, while fibronectin levels appeared to be unchanged. A representation of these data is presented in Figure 2.

Using ELISA as an alternative, quantifiable approach to monitoring protein downregulation, we assayed the amount of *de novo* type I collagen formed and excreted into the culture medium over a 24 h time period by untransduced MPCs, MPC-Pol1 and MPC-Pol2 cells. The relative amounts of *de novo* type I collagen found in media harvested from untransduced MPCs, MPC-Pol1 and MPC-Pol2 cells were

Table 1. Mean levels of downregulation of the *COL1A1* transcript in MPC-Pol1

	COL1A1 levels standardized with *COL1A2* (%)	*COL1A1* levels standardized with *GAPDH* (%)	*COL1A1* levels standardized with *β-actin* (%)
MPC	100.00 ± 6.04	100.00 ± 10.15	100.00 ± 2.78
MPC-Pol1	65.03 ± 10.89	55.59 ± 9.76	58.45 ± 1.16
Suppression	34.97 ($n = 12$, $P = 0.0008$)	44.41 ($n = 12$, $P = 0.0044$)	41.55 ($n = 12$, $P < 0.0001$)

Table 2. Mean levels of downregulation of the *COL1A1* transcript in MPC-Pol2

	COL1A1 levels standardized with *COL1A2* (%)	*COL1A1* levels standardized with *GAPDH* (%)	*COL1A1* levels standardized with *β-actin* (%)
MPC	100.00 ± 5.24	100.00 ± 4.37	100.00 ± 2.05
MPC-Pol2	63.56 ± 13.58	45.33 ± 3.36	49.87 ± 0.70
Suppression	36.44 ($n = 12$, $P = 0.0043$)	54.67 ($n = 12$, $P = 0.0001$)	50.13 ($n = 12$, $P < 0.0001$)

58.56 ± 6.46 ng/ml, 36.67 ± 4.68 ng/ml and 26.62 ± 2.26 ng/ml respectively, indicating that the reduction in type I collagen levels found in MPC-Pol1 and MPC-Pol2 was 37.4% and 54.5%.

DISCUSSION

Rzpol1a1 is, to our knowledge, the first mutation-independent ribozyme to be active in cells and the first gene suppression agent that uses an single nucleotide polymorphism (SNP) to direct a therapy to a mutant allele in a mutation-independent manner. Polymorphisms have been invaluable in the generation of high-resolution genome maps and may have multiple future uses in the design of novel therapeutics. The current study provides a prototype for such a use.

We do not know what levels of mutant *COL1A1* downregulation will be required to be of therapeutic benefit to OI patients. However, the quantification of relative levels of ribozyme to target transcript, suggests that even 1 : 1 ratios of Rzpol1a1 to *COL1A1* transcript result in 40–50% suppression. This is encouraging, since the ratio of ribozyme to target may be augmented, resulting in an increase in levels of suppression. Notably, the suppression levels seen at the RNA level are almost exactly mirrored at the protein level as seen by ELISA and confirmed by immunocytochemistry. While measured levels of *COL1A1* suppression varied between experiments, downregulation of RNA was significant and observed in all 72 experiments (Tables 1 and 2). Because expression levels of housekeeping genes are sometimes influenced by external factors (20), *COL1A1* levels were compared to three very different genes, *COL1A2*, *GAPDH* and *β-actin*. While measured levels of downregulation did vary depending on the internal control gene used, this variance was not significant. The variance is therefore likely to be a due to the resolution of real-time RT–PCR. Thus, we believe that suppression of *COL1A1* is indeed due to Rzpol1a1 and not for example the retrovirus. Notably, real-time RT–PCR is the most accurate method of RNA quantification available at this time (http://dorakmt.tripod.com/genetics/realtime.html). The efficient downregulation of *COL1A1* in MPCs and the use of a recombinant retrovirus to introduce multiple copies of the suppression agent, Rzpol1a1, represent additional novel aspects of the study. This is of particular interest given that the same stem cell type has previously been used in human clinical trials in OI patients. Results showing that the *COL1A1* and CMV promoters are similar in strength in human MPCs highlight the necessity of using an extremely strong promoter to drive any gene therapeutic targeting *COL1A1*.

Hence, since Rzpol1a1 has been shown to promote significant suppression of *COL1A1* at both the RNA and protein levels, we suggest that Rzpol1a1 may benefit over 20% (50% of the C/T 3210 *COL1A1* heterozygous OI population) of OI patients with gain-of-function mutations in the *COL1A1* gene. Interestingly, however, it may also be possible to treat homozygous *COL1A1* 3210 T-allele OI patients with Rzpol1a1 using a combined suppression and replacement strategy—representing an additional 40% of OI patients with *COL1A1* mutations. In this situation, a replacement *COL1A1* gene must be introduced concurrently with RNL-Pol and should be of the uncleavable 3210 C-allele type. In addition, the *COL1A1* replacement gene may be altered at various sites surrounding the 3210 C polymorphism in the 3′-UTR, thus protecting replacement *COL1A1* transcripts from ribozyme cleavage and suppression by the antisense arms of the ribozyme, without altering the collagen protein produced. Consequently, a single ribozyme, Rzpol1a1, may potentially be of therapeutic value to both *COL1A1* 3210C/T and 3210T/T OI patients, representing over 60% of *COL1A1*-linked OI cases, clearly demonstrating the power of polymorphism to overcome mutational heterogeneity in diseases such as OI. Despite significant downregulation at the RNA and protein levels of the target *COL1A1* in human MPCs, it is worth noting that the degree of downregulation of mutant protein that would be required to provide therapeutic benefit to OI patients remains to be established.

In summary, this study amalgamates a novel aspect of the human genome (polymorphism) with suppression technologies (ribozymes), stem cell technologies (MPCs) and *ex vivo* delivery using retroviral vectors to overcome mutational heterogeneity, one of the most significant hurdles in the development of therapies for OI. It is of note that the genetic heterogeneity inherent in OI is mirrored in many other

Table 3. Mean ($n = 6$) amounts of *COL1A1* and retroviral transcript

	COL1A1 RNA (%)	All three retroviral RNAs (%)	Larger retroviral RNAs (%)
MPC-Pol1	100.00 ± 4.74	76.76 ± 5.70	14.97 ± 1.67
MPC-Pol2	100.00 ± 4.66	62.57 ± 4.33	12.34 ± 0.60

dominant disorders where hundreds of different mutations can give rise to clinically similar phenotypes. The immense number of SNPs now characterized in the human genome represents a powerful tool with which to attempt to overcome this heterogeneity when developing therapies, as has been clearly demonstrated in the current study.

MATERIALS AND METHODS

MPC isolation and culture

Heparinized human bone marrow cells, obtained from leftover materials of bone marrow transplantations, were used with informed consent. MPCs were isolated and cultured (21,22).

Retroviral construct and viral transfection of MPCs

Rzpol1a1 (8) was cloned into the *Sal*I and *Bam*HI sites of retroviral plasmid pLRNL. In addition, Rzpol1a1 driven by a CMV promoter was cloned into the *Cla*I site of pLRNL (23). An MLV virus, RNL-Pol, was generated with this construct (Fig. 1A) by the UCSD Program in Human Gene Therapy. MPCs were seeded at 2000 cells/cm^2 and transfected at a multiplicity of infection (MOI) = 5 in 83 µl/cm^2 of α-MEM, 20% fetal bovine serum (GibcoBRL, Paisley, UK; Cat. no. 10270–106) and 20 µg/ml of polybrene. Selection with 400 µg/ml G418 was started after 48 h. RNA was isolated using TriZol (GibcoBRL; Cat. no. 15596–018) and standard procedures 14 days later.

Cloning and *in vitro* transcription

Part of the *COL1A1* coding sequence and 3′-UTR, spanning nucleotides 2977–3347 (GenBank accession no. K01228), was cloned into the *Hind*III and *Xba*I sites of pcDNA3 (Invitrogen, Groningen, The Netherlands; no longer available). In addition, a part of the *neomycin* gene, present in RNL-Pol (Fig. 1A), was cloned into the same sites of pcDNA3 using PCR and primers NEO F and NEO R (Table 4). Also, a section of the MLV *envelope* gene, 24 bp upsteam of the 3′-LTR in RNL-Pol, was cloned into the *Hind*III and *Xba*I sites of pcDNA3 using PCR and primers pLRNL F and pLRNL R (Table 4). The *COL1A1, neo* and *env* clones were maxiprepped using the HiSpeed Plasmid Maxi Kit (Qiagen, Crawley, UK; Cat. no. 12663) and linearized with *Bbs*I. Digested clones were cleaned extensively with phenol, phenol–chloroform, chloroform and ethanol and used to express *COL1A1*, *neo* and *env* RNA using the T7 promoter present in pcDNA3, the Ribomax kit (Promega, Madison, Wisconsin, USA; Cat. no. P1300) and standard procedures. RNAs were treated with DNase for 20 min at 37°C.

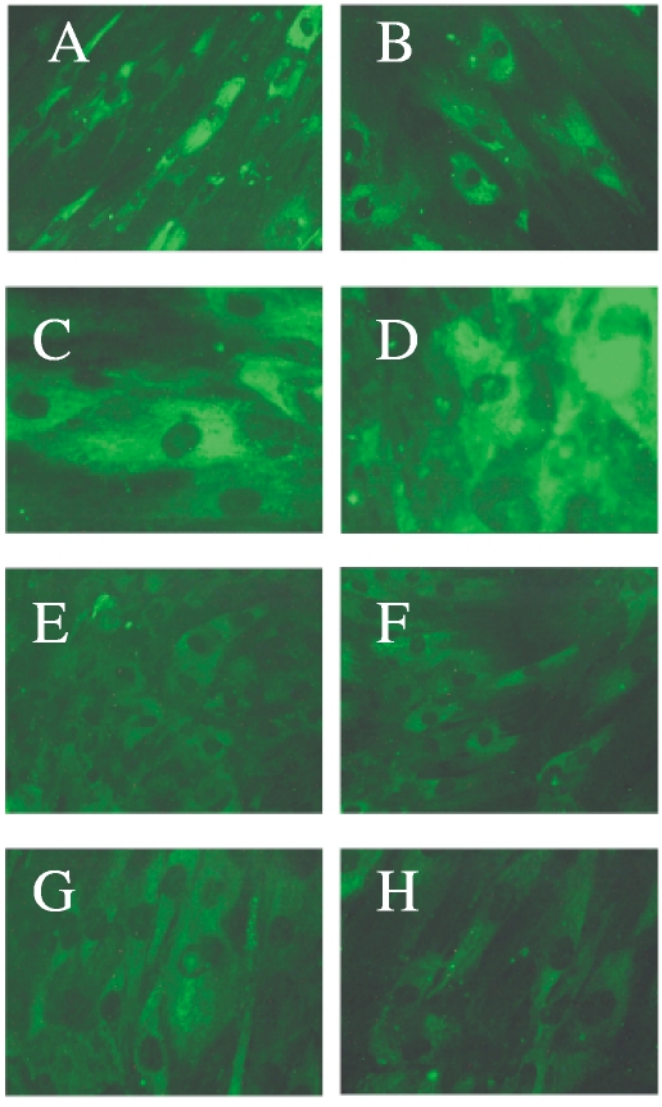

Figure 2. Immunocytochemical analysis of type I collagen in cells grown for 7 days. **A**–**D** represent untransduced MPCs and **E**–**H** MPC-Pol2. Cells were analysed at either 100× magnification (A, B, E and F) or 200× magnification (C, D, G and H). Untransduced MPCs have visibly larger amounts of type I collagen than MPC-Pol2 cells.

DNase was heat-inactivated for 5 min at 80°C. Copy numbers of RNA products were calculated from OD_{260} readings, performed in triplicate.

Real-time RT–PCR

COL1A1 downregulation experiments were performed four times in triplicate using real-time RT–PCR and a LightCycler (Roche, Lewes, UK). In addition, *COL1A1* transcript levels were standardized using *COL1A2*, *GAPDH* and *β-actin* as internal controls. Thus, in both MPC-Pol1 and MPC-Pol2, *COL1A1* levels were quantified 36 times. RT–PCR reactions were carried out using the QuantiTect SYBR Green 1-step RT–PCR kit (Qiagen, UK; Cat. no. 204243), 57°C annealing

Table 4. Primer sequences

Primer	Primer sequence	Product size (bp)	Amplified gene
GAPDH F	5′-CAGCCTCAAGATCATCAGCA-3′	100	*GAPDH*
GAPDH R	5′-CATGAGTCCTTCCACGATAC-3′	100	*GAPDH*
COL1A2 F	5′-CAAGGATGCACTATGGATGC-3′	111	*COL1A2*
COL1A2 R	5′-GGAGCTCCTATACCAGTTCT-3′	111	*COL1A2*
COL1A1 F	5′-CAGGAATTCGGCTTCGA-3′	125	*COL1A1*
COL1A1 R	5′-GGTTCAGTTTGGGTTGCTTG-3′	125	*COL1A1*
ACTIN F	5′-TCACCCACACTGTGCCCATCTACGA-3′	295	*β-actin*
ACTIN R	5′-CAGCGGAACCGCTCATTGCCAATGG-3′	295	*β-actin*
pLRNL F[a]	5′-AAGACAGGATATCAGTGGTC-3′	80	*env*
pLRNL R[a]	5′-CTATGGCTCGTACTCTATAG-3′	80	*env*
NEO F[a]	5′-GATGCCTGCTTGCCGAATAT-3′	127	*neo*
NEO R[a]	5′-CAACGCTATGTCCTGATAGC-3′	127	*neo*

[a]Primers were also generated with *Hind*III and *Xba*I sites 5′ and 3′ respectively and used in cloning experiments.

temperatures, 5 s elongation times for *COL1A1*, *COL1A2* and *GAPDH*, 10 s elongation times for *β-actin* and the manufacturer's protocols. PCRs were carried out for between 28 and 35 cycles. RNA products expressed *in vitro* (see above) were used to generate standard curves of absolute copy numbers on a LightCycler, making the measurement of *COL1A1* and retroviral transcript copy number in MPC-Pol1 and MPC-Pol2 possible. Thus the ratios of retroviral RNA to *COL1A1* RNA were determined six times each in MPC-Pol1 and MPC-Pol2. All primers (Table 4) were HPLC-purified.

Immunocytochemistry

MPC-Pol1 and MPC-Pol2 cells were seeded at 2000 cells/cm^2, grown for 4 or 7 days, fixed with 50% acetone and 50% methanol, and stained for 30 min at room temperature. Antibodies were anti-human type I collagen (BioDesign, Saco, Maine, USA; Cat. no. MIA340M) and monoclonal anti-human fibronectin (Sigma, Dublin, Ireland; Cat. no. F0916). The secondary antibody was anti-mouse IgG FITC conjugate (Sigma; Cat. no. F2012). Ninety-three fields of these cells were then photographed and assessed by 10 individuals in a blind test (data not shown).

ELISA assays

Two samples of untransduced MPCs, and MPC-Pol1 and MPC-Pol2 cells, were seeded at 2000 cells/cm^2 and grown. Medium was removed and cells were washed three times with phosphate buffered saline (PBS). One millilitre of fresh medium, which did not contain fetal calf serum or antibiotics, was placed on cells, which were then grown for an additional 24 h. Medium was then removed and immediately stored at −80°C for use in ELISA assays. The numbers of MPC, MPC-Pol1 and MPC-Pol2 cells were then counted four times using a hemacytometer to determine the quantity of cells which had contributed to generating the *de novo* type I collagen excreted into the medium over 24 h. ELISAs on the six medium samples were performed in duplicate using the Metra CICP EIA kit (Metra Biosystems, San Diego, CA, USA; Cat. no. 8003) and the manufacturer's protocol. ELISA assays were analysed using MetraFIT, version 1.1.

Statistical analysis

Paired *t*-tests were performed using Data Desk 6.0 (Data Descriptions Inc., New York, New York, USA) to determine levels of *COL1A1* downregulation. ANOVA tests were carried out to analyse whether results standardized with *COL1A2, GAPDH* and *β-actin* were the same. Differences were considered significant at $P < 0.05$. Standard deviations of ELISA results were calculated using Microsoft Excel 2002.

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