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Role of deleted in colon carcinoma in osteoarthritis and in chondrocyte migration

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Objective. The concept of the chondrocyte as a stationary cell surrounded by an apparently impenetrable matrix has been challenged by *in vitro* observations in recent years. Chondrocyte migration may have a role in remodelling of the cartilage and pathological conditions. Candidate molecules are repellent factors for the regulation of chondrocyte migration, which are expressed in fetal and adult cartilage. We analysed the potential role of the receptor deleted in colon carcinoma (DCC) in chondrocytes, as this may exert attractive activities.

Methods. Gene expression was determined by quantitative RT–PCR and immunohistochemistry, and gene regulation by electro mobility shift assay and chromatin immunoprecipitation. Functional assays on migration and differentiation were done after cell treatment and transfection.

Results. DCC was shown to be specifically up-regulated in OA compared with normal chondrocytes *in vitro* and *in vivo*. Promoter analysis and transfection studies showed that the up-regulation of DCC in OA chondrocytes may be mediated by the transcription factors Sox9 and AP-2. Netrin-1, the ligand of DCC, was revealed to induce the migration of OA chondrocytes specifically. Expression of DCC in healthy chondrocytes by transient transfection significantly induced cell migration and chemotaxis to Netrin-1. DCC expression had no influence on cell differentiation; however, induction of MMP1 and -3 expression was observed.

Conclusion. Strong differential expression of DCC in OA compared with normal chondrocytes hints of a possible role of DCC in the pathophysiology of OA. The strong impact of the DCC receptor on cellular mobility of chondrocytes *in vitro* suggests a major relevance of migratory activities in physiological and pathological conditions of cartilage. However, definite proof of chondrocyte movements *in vivo* still has to be established.

Key words: Repellent factors, Deleted in colon carcinoma, Netrin, Migration, Proliferation, OA.

Introduction

The chondrocyte has, until recently, been viewed as a stationary cell encased in an apparently impenetrable matrix. This view has been challenged by *in vitro* observations in recent years. Several pioneering studies have shown that chondrocytes are able to migrate *in vitro* in response to chemoattractant factors such as cytokines, growth factors and components of the extracellular matrix [1]. Chondrocyte movements were observed in planar and 3D matrices [1]. Movements of chondrocytes *in vivo* in the growth plate [2] and during the development of the intervertebral disc [3, 4] are also possible. In a canine meniscus repair model, re-colonization of a cell-free autotransplant of meniscal tissue by α smooth muscle actin-expressing chondrocytes was observed, indicating the possibility of migration of chondrocytes into the transplant [5] and a possible relevance of chondrocyte migration to pathological conditions and repair.

The induction of chondrocyte movement by cytokines, growth factors and components of the extracellular matrix has already been demonstrated. A group of receptors and ligands known as 'repellent factors' is pertinent to guided migratory activities. Repellent factors regulate cell–cell interactions and cell–matrix interactions of migrating cells during embryonic development. The group consists of semaphorins and their receptors, Netrins and their receptors, deleted in colon carcinoma (DCC) and UNCs, slits and roundabout receptors (Robo) as well as ephrins and the eph receptors [6–8]. They direct the sprouting of nerve fibres as axons guiding cues by repelling or attracting the growth cone from or to certain structures in the developing CNS [6]. Apparently, repellent factors also contribute to the development of a variety of organs such as the lung, mammary gland, cardiovascular system and kidney by exerting repellent and attractant activities [6]. A significant fact that has not been noticed so far is that most of the members are expressed in the cartilage of developing limbs [9–15]. Interestingly, targeted disruption or over-expression of the repellent genes for semaphorin3A, neuropilin 1 and ephrin-A2 leads to reduplication, distortion or abnormal spatial arrangement of skeletal elements indicating a role in the definition of limb borders [9, 13, 14]. The formation of borders is probably associated with changes of the cytoskeleton and cell motility.

We concentrated, in this study, on changes in expression of the DCC molecule of the Netrin molecule family. This large family consists of Netrin-1, -3, -4, G1 and G2 and their DCC receptors, Neogenin and UNC5A–D. Netrin binding to DCC and Neogenin was shown to lead to cell attraction, whereas binding to the UNC receptor modulates repulsion [16]. Additionally, DCC and Neogenin belong to the family of dependence receptors. Certain cell types expressing DCC were shown to conduct apoptosis in the absence of ligand.

DCC functions in Netrin signalling as a component of a multi-protein receptor complex, interacting with other cell surface receptors, such as Robo, UNC5H or A2b [17–19]. On Netrin-1 binding, DCC activates the extracellular signal-regulated kinase-1/2 mitogen-activated protein kinase pathway [20, 21]. DCC was also recently shown to activate the small GTPases Cdc42 and Rac-1 when Netrin-1 is present [22, 23]. This activation seems to require the interaction of the intracellular domain of DCC with an adaptor molecule Nck-1 [24]. Small GTPases have been implicated in numerous cell processes, especially in actin organization and cell motility [25].

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In this study, we evaluate the expression and regulation of DCC in OA cartilage and its role in the regulation of migration of chondrocytes gained from OA joints.

Materials and methods

Primary human chondrocytes

Tissues were obtained from probands or patients after informed consent. The study was approved by the ethical committee of the University of Regensburg. For the study, we used four normal cartilage samples of two female and two male donors aged 49–74 years with no significant softening or surface fibrillation. The causes of death were pneumonia, right-sided heart failure, myocardial infarction and brain haemorrhage. The cartilage samples were obtained from autopsy within 48 h after death. OA cartilage was removed under sterile conditions from femoral condyles of knee joints of seven patients aged 40–65 years (three female and four male patients) undergoing total knee arthroplasty. Before RNA isolation, cartilage tissue was first classified macroscopically as either damaged or intact according to a pre-defined procedure comprising colour, surface integrity and tactile impression tested with a standard scalpel. Accordingly, diseased areas showed a colouration that was rather yellow than white. They showed erosions and discrete protuberances that rendered it impossible to smoothly move the scalpel over the tissue surface. In unaffected regions, there was a stronger resistance when cutting deeper into the tissue with the blade of the scalpel, which could not be done in the softened lesional areas [26]. Samples were taken from the better preserved cartilage regions to avoid the sampling of fibrous or fibrocartilaginous tissue and were evaluated using the Mankin score in hematoxylin and eosin- and Safranin O-stained tissue sections [27]. The average score in the normal cartilage group was 0.8 ranging from score values of 0–2. The average score in the OA group was 5.6 ranging from 5 to 7. Tissue fragments were cut into 1-mm slices and were washed four to five times with Krebs buffer containing 100 U/ml penicillin and 100 μg/ml streptomycin. After two further rinses with calcium-free DMEM (PAN Biotech, Aidenbach, Germany), the slices were further cut into small pieces and were incubated for 16–24 h with 1.5 mg/ml collagenase B (Roche Diagnostics, Mannheim, Germany) and 1 mM cysteine in DMEM. The cell suspension was filtered through nylon gauze (70 μm pore size) and was washed three times with calcium-free DMEM.

Cell lines and tissue culture

The chondrosarcoma cell line SW1353 was used in the experiments. Cells were grown at 37°C with 5% CO_2 in DMEM (PAN Biotech) supplemented with penicillin (100 U/ml), streptomycin (10 μg/ml) (both from Sigma, Deisenhofen, Germany), 10% fetal calf serum (FCS) (PAN Biotech) and split 1 : 2 at confluence. Cells were detached by incubation with 0.05% trypsin (Sigma) in phosphate buffered saline (PBS) for 5 min at 37°C.

Transient transfection

Primary chondrocytes derived from normal specimens were transfected using the Amaxa Nucleofector System (Amaxa, GmbH, Cologne, Germany) with the Amaxa Human Chondrocyte Nucleofector® Kit according to the manufacturer's instructions (program: U24). The transfection efficiency was ~20%, toxicity was 30%. Recombinant human pDCC-CMV-S was a kind gift of Patrick Mehlen (University of Lyon) [28].

Transfection of SW1353 cells was performed using Lipofectamin plus (Invitrogen, Carlsbad, CA, USA) with an efficiency of ~20–30% in this experiment. Briefly, the cells were cultured in six-well plates. For transient transfection with expression plasmids, each cationic lipid/plasmid DNA suspension of 0.5 μg plasmid within the transfection solution was prepared according to the manufacturer's instructions. The cells were harvested 24 h later, and RNA was isolated. The expression construct for Sox9 was a kind gift from Veronique Lefebvre, Cleveland [29]. An AP-2α expression plasmid was generated according to Imhof and colleagues [30, 31].

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed following the manufacturer's instructions (ChIP-IT^{TM} Express, Active Motif, Carlsbad, CA, USA). SW1353 cells were used for chromatin isolation. Samples were immunoprecipitated with a specific AP-2α or Sox9 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA; Millipore, Schwalbach, Germany). A polymerase II antibody was used as a positive control and an IgG antibody as a negative control following the protocol provided by the control kit (ChIP-IT^{TM} control Kit-human, Active Motif). DNA samples from the ChIP experiments were used for analysis by PCR. PCR was performed on four DNA templates: the input DNA, DNA isolated through RNA polymerase II ChIP (Pol II), DNA isolated through the negative control IgG ChIP (IgG) and DNA isolated through the AP-2α or Sox9 ChIP (AP-2α, Sox9). A control reaction with no DNA template was also performed (H_2O). Three sets of specific primer pairs were used: the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the negative control primer pairs provided by the kit, a primer pair spanning the AP-2 and a Sox9 binding site of the DCC promoter: promDCC_for: 5′-GCTGTGTGTTTCAGGATCTCG-3′ and promDCC_rev: 5′-CGTTTAAGGCGGAGGAGGTG-3′ (146-bp fragment). PCR fragments were analysed on a 2% agarose gel.

Proliferation assays

Proliferation was measured using the Cell Proliferation Kit II (Roche Diagnostics) according to the manufacturer's protocol [32].

Migration assay

Migration assays were done using Boyden Chambers containing polycarbonate filters with 8-μm pore size (NeuroProbe, Gaithersburg, MD, USA), essentially as described previously [32]. Filters were coated with gelatine from bovine skin (Sigma Aldrich, Taufkirchen, Germany) by treatment with 0.5% acetic acid for 20 min at 50°C followed by boiling in H_2O with 50 mg/l gelatine for 1 h. For the migration assay, the lower compartment of the Boyden Chamber was filled with fibroblast-conditioned medium (medium of primary dermal fibroblasts, cultured for 24 h with DMEM without FCS), used as a chemoattractant. Chondrocytes were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of 2×10^5 cells/ml and placed in the upper compartment of the chamber. After incubation at 37°C for 4 h, the filters were collected and the cells adhering to the lower surface were fixed, stained and counted. Recombinant Netrin-1 (100 ng/ml, Axxora, Lörrach, Germany) was added either to the upper or the lower chamber of the system together with the cells.

Isolation of RNA

Total cellular RNA was isolated using the RNeasy Mini Kit including an RNase-free DNase step following the manufacturer's manual (Qiagen, Hilden, Germany). RNA amounts were analysed by using a fluorescence microplate reader and following the instructions of the RiboGreen RNA Quantitation Reagent and Kit (MoBiTec, Göttingen, Germany).

Quantification of mRNA expression by real-time PCR

One-tenth of the RNA recovered from RNeasy spin columns (Qiagen) was used for RT–PCR as described previously [32]. cDNA fragments of DCC were amplified using the following primers (hDCC for: 5′-GACTCCAATCCCAGGTGACT-3′; hDCC rev: 5′-TGACTTCCTCGCCTCGTAAC-3′; β-actin for: 5′-CTACGTCGCCCTGGACTTCGAGC-3′; β-actin rev: 5′-GATGGAGCCGCCGATCCACACGG-3′; aggrecan for: 5′-CCTACCAAGTGGCATAGC-3′; aggrecan rev: 5′-TGTTGGAGCCTGGGTTAC-3′; colII for: 5′-AGGGCAATAGCAGGTTCACG-3′; colII rev: 5′-GGTCAGGTCAGCCATTCAGT-3′; Mia for: 5′-CATGCATGCGGTCCTATGCCCAAGCTG-3′; Mia rev: 5′-GATAAGCTTTCACTGGCAGTAGAAATC-3′; Sox9 for: 5′-CGAACGCACATCAAGACGGA-3′; Sox9 rev: 5′-AGGTGAAGGTGGAGTAGAGGC-3′; MMP1 for: 5′-TGGACCAAGGTCTCTGAGGGTCAA-3′; MMP1 rev: 5′-GGATGCCATCAATGTCATCCTGA-3′; MMP3 for: 5′-GGCACAATATGGGCACTTTAAAT-3′; MMP3 rev: 5′-GTCTACACAGATACAGTCACTG-3′). Reverse transcription was performed in 20 μl reaction mixture of 3 μl RNA, 4 μl 5× Superscript buffer (Invitrogen), 2 μl dithiothreitol (0.1 M), 1 μl antisense primer (1 μg/μl), 1 μl deoxyribonucleotide triphosphats (10 mM) and 1 μl reverse transcriptase (Superscript, Invitrogen). The mixture was incubated at 37°C for 60 min followed by 10 min at 70°C and RNaseA digestion at 37°C for 30 min. To precisely quantify gene expression, the real-time PCR LightCycler system (Roche Diagnostics) was used. For PCR, 1–3 μl cDNA preparation, 0.5 μM of forward and reverse primer and 10 μl of Takara Sybr-Green Mix (Lonza Walkersville, Cologne, Germany) in a total of 20 μl were applied. The following PCR program was performed: 30 s at 95°C (initial denaturation); 20°C/s temperature transition rate up to 95°C for 3 s, 10 s at 60°C, 8 s at 72°C, 10 s at 84°C acquisition mode single, repeated 40 times (amplification). Annealing temperature was optimized for each primer set. A standard curve method was used for quantification using β-actin for standardization. The PCR reaction was evaluated by melting curve analysis following the manufacturer's instructions and checking the PCR products on 1.8% agarose gels. Each quantitative PCR was performed at least in duplicate for two sets of RNA preparations.

Immunohistochemistry

For immunohistochemistry, standard 3-μm sections of formalin-fixed and paraffin-embedded tissue blocks were used. Immunohistochemical staining was performed using an indirect immunoperoxidase protocol according to the Vectastain ABC Universal kit (Vector Laboratories, Burlingame, CA, USA) [31]. Briefly, sections were deparaffinated by xylol. Then, endogenous peroxidase was inhibited by incubation in 3% H_2O_2 and unspecific binding was blocked by incubating the sections in 20% horse serum/PBS. Slides were incubated with the primary mouse polyclonal anti-DCC antibody (1 : 50; US Biological, Swampscott, MA, USA). The secondary antibody anti-mouse/-rabbit was biotinylated and used in a 1 : 50 dilution followed by treatment with streptavidin peroxidase. The red chromogen AEC (3-amino-9-ethylcarbazole) was used as substrate for peroxidase visualization in order to distinguish reliably endogenous pigment from immunosignals.

Western blotting

Protein extracts of cells were prepared as described previously [32]. For western blotting, a 40-μg aliquot of protein lysates was loaded and separated on 7.5% SDS–PAGE gels and subsequently blotted onto a poly(vinylidene fluoride) membrane. After blocking for 1 h with 5% milk powder/Tris-buffered saline Tween-20 (0.1% Tween), the membrane was incubated for 16 h at 4°C with the primary antibody (anti-human DCC antibody, 1 : 500, BD Biosciences, San Jose, CA, USA) and anti-β-actin (1 : 5000, Sigma). Subsequently, the membrane was washed three times in Tris-buffered saline, incubated for 1 h with alkaline phosphate-coupled secondary anti-mouse immunoglobulin antibody (1 : 4000, Millipore, Victoria, Australia) and then washed again. Finally, immunoreactions were visualized by nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt (Sigma) staining.

Statistical analysis

Results are expressed as mean ± S.D. (range) or per cent. Comparison between groups was made using the Student's unpaired *t*-test. Correlation between parameters was calculated with the Spearman test. A *P*-value of <0.05 was considered as statistically significant. All calculations were performed by using the SPSS 10 for Windows statistical computer package (SSPS, Chicago, IL, USA).

Results

Expression of DCC in normal and OA chondrocytes

The role of the repellent factor family of Netrins and their receptors has previously been mainly analysed in neuronal guidance. Due to the molecular function of these molecules, we developed an interest in characterizing a potential function of this family in healthy cartilage and OA.

First, we characterized the expression patterns of DCC in chondrocytes from healthy donors ($n=4$) and patients with OA ($n=7$) (Fig. 1A). Interestingly, DCC expression is newly significantly induced in OA chondrocytes and not detectable in chondrocytes from healthy donors. To confirm these results *in vivo*, we performed immunohistochemical staining for DCC in healthy and OA cartilage (Fig. 1B). Strong induction of DCC protein expression was observed in samples of OA cartilage (II–IV), whereas no staining was found in healthy tissue (I). DCC was expressed in almost all chondrocytes of the superficial and upper radial layer of OA samples. Expression appeared slightly more intense in the superficial layer and in clusters of chondrocytes. Within giant chondrones and regular chondrones of the radial layer, all chondrocytes of a clone showed a similar intensity of expression. The chondrocytes within multi-nuclear chondrones expressing DCC more intensely had a more abundant cytoplasm than chondrocytes in regular chondrones of the superficial and radial layer.

Regulation of DCC expression

We were interested to understand the reason for differential expression of DCC in OA cartilage and focused on transcription factors that could bind to the DCC promoter. Detailed analysis revealed a binding site for Sox9 (at −672 bp) and one binding site for AP-2 (at −643 bp, respectively, in relation to the A of the start codon), both transcription factors known to be differentially expressed in OA cartilage and to play a role in chondrogenesis [33, 34] (Fig. 2A). Consequently, we analysed DCC expression in the chondrocytic cell line SW1353 after transfection of an AP-2 or Sox9 expression plasmid and revealed significant induction of DCC expression by both factors in a synergistic manner (Fig. 2B). ChIP analysis confirmed binding of AP-2 and Sox9 to the DCC promoter (Fig. 2C and D).

Functional influence of DCC expression on chondrocytes

Encouraged by these highly specific changes in DCC expression, we asked whether treatment with Netrin-1, the ligand of this receptor, leads to changes in cellular function *in vitro*. The effect of Netrin-1 on the proliferation of OA chondrocytes in early passages and chondrocytes from healthy donors was determined

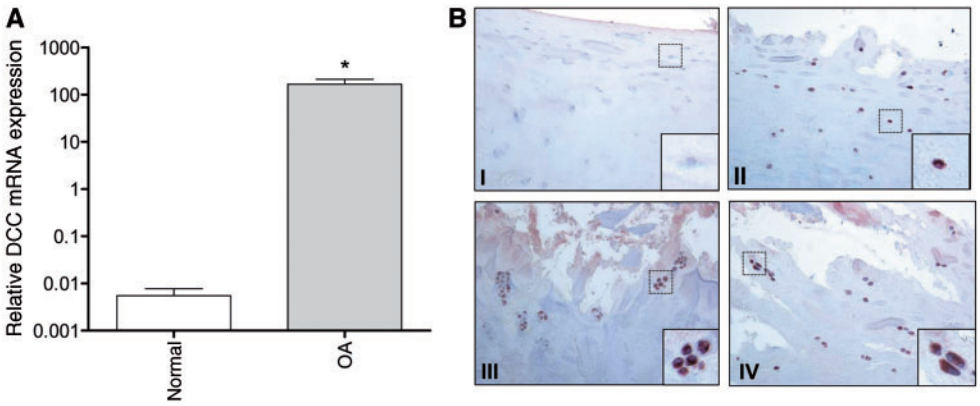

FIG. 1. Expression of DCC in cartilage. (**A**) mRNA expression of DCC was measured by quantitative RT-PCR (qRT–PCR) in healthy and OA chondrocytes. Analysis revealed significant induction of DCC expression in OA ($^*P < 0.05$, $n = 3$). (**B**) Immunohistochemical staining for DCC verified induction of DCC in OA cartilage. In healthy cartilage (I) no staining was found, whereas in OA samples (II–IV) strong staining was observed. Pictures are shown in a 400-fold magnification. Inserts in each picture show a large magnification of a section with single cells.

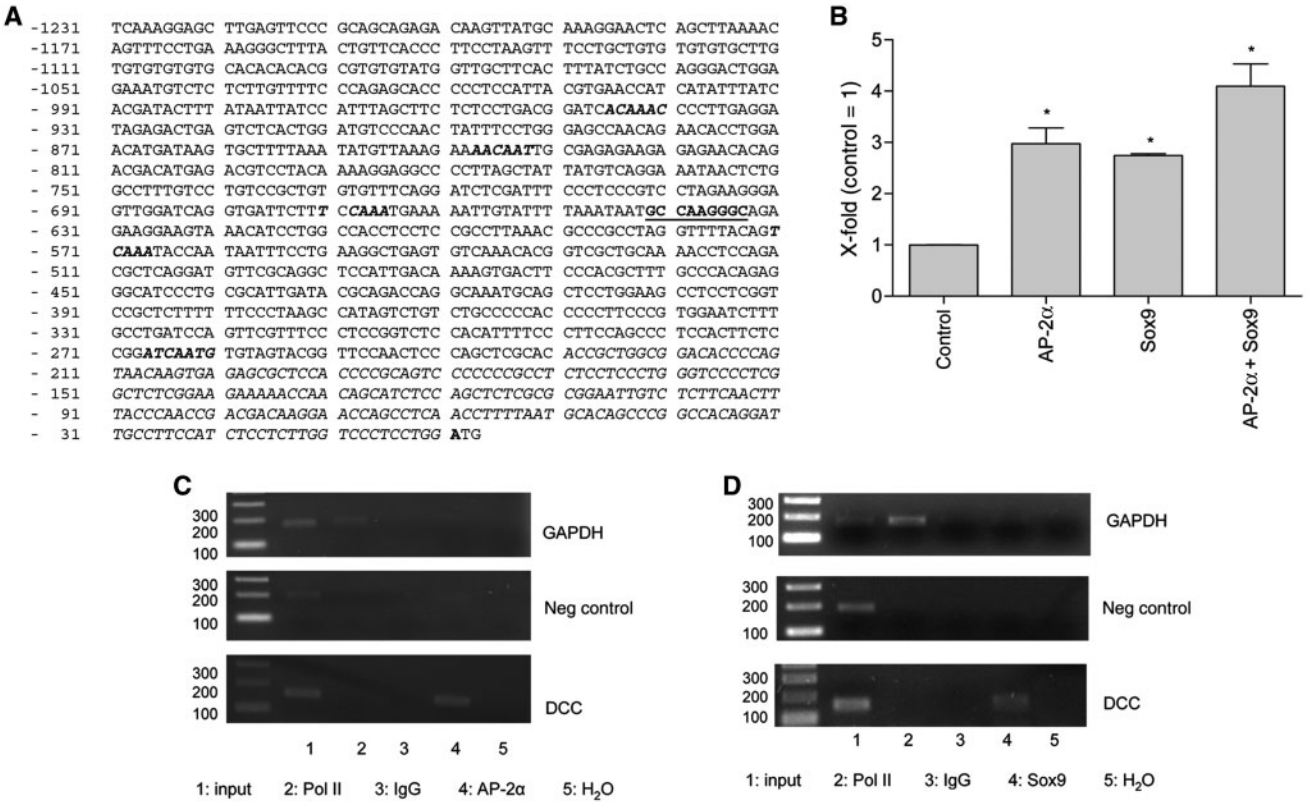

FIG. 2. Regulation of DCC in OA chondrocytes. (**A**) Nucleotide sequence analyses of the DCC promoter revealed five putative Sox9 binding sites (bold and italic) and one putative AP-2α binding site (bold and underlined) 5′ of the translation start (5′-untranscribed region: italic). (**B**) SW1353 chondrosarcoma cells were transfected with AP-2α or Sox9 expression plasmids, and mRNA expression of DCC was analysed by qRT–PCR ($^*P < 0.05$, $n = 3$). (**C** and **D**) Binding of AP-2α (C) and Sox9 (D) to the DCC promoter was confirmed by ChIP assays. Transcription factor-bound genomic DNA was isolated from OA chondrocytes and after co-immunoprecipitation, AP-2α or Sox9 binding to the respective binding site was confirmed by PCR [input: genomic DNA without ChIP; negative controls: water (H_2O) and IgG antibody (IgG); positive control: antibodies against Pol II binding to the GAPDH promoter].

by tetrazolium salt sodium 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assays (data not shown). Here, no changes in cell growth were observed. As DCC is known to have an attraction function, we analysed the effect of Netrin-1 on cell migration using a Boyden Chamber assay. No effect on migration of healthy chondrocytes was observed; however, Netrin-1 induced the migratory activity of OA chondrocytes (Fig. 3).

To analyse the functional effect of DCC expression in OA, we transfected normal chondrocytes with a DCC expression construct to achieve strong expression of DCC. Efficiency of transfection was confirmed by qRT–PCR (data not shown) and western blotting (Fig. 4A). Over-expression of DCC had no influence on cell proliferation. Interestingly, chondrocytes over-expressing DCC showed a higher migratory potential *in vitro*, which was inhibited by treating the cells directly with Netrin-1 (Fig. 4B). Using Netrin-1 as a chemoattractant stimulated the migration of DCC-transfected chondrocytes, but had no effect on DCC-negative cells (Fig. 4C). DCC expression did not induce random migration.

We also determined whether over-expression of DCC in chondrocytes influences the differentiation of the cells. Therefore, the expression of the differentiation markers, aggrecan, collagen type II and melanoma inhibitory activity/cartilage-derived retinoic acid-sensitive protein (MIA/CD-RAP), was analysed. Expression of aggrecan and collagen type II was not altered, but interestingly, there was a significant increase in MIA/CD-RAP expression after the over-expression of DCC (Fig. 5A). As MMPs are known to play a major role in cell migration in general [35] as well as in the pathophysiology of OA [36, 37], and as there is also a possible role for MMPs in chondrocyte migration [4], we analysed the expression of MMP1 and -3 after over-expression of DCC by qRT–PCR (Fig. 5B). Induction of both MMPs after DCC expression was observed.

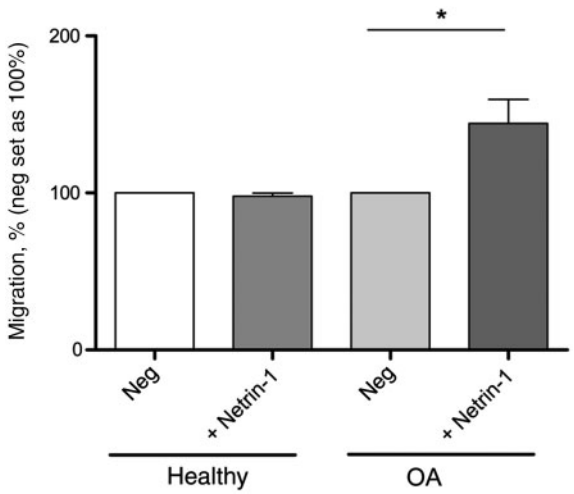

FIG. 3. Influence of Netrin-1 on migration. The effect of Netrin-1 on healthy chondrocytes and chondrocytes isolated from OA cartilage on migration was analysed in a Boyden Chamber model. Netrin-1 revealed no effect on healthy chondrocytes, but induced migration of OA chondrocytes (*$P < 0.05$, $n = 3$).

Discussion

Migration of chondrocytes starting from the articular margin, passing the superficial zone and ending in the transitional and radial zone was proposed as a key feature in the remodelling of cartilage during adulthood by Simkin [38]. Dysfunction of this hypothetical process, for instance, by misdirection of chondrocytes or disruption of migration, may be a possible cause of clustering of chondrocytes and changes in matrix composition in OA.

If there are migratory activities of chondrocytes during development, repair and pathological conditions, they have to be directed by chemoattractant and chemorepulsive factors. Otherwise, migrating chondrocytes could cross compartment borders of the joint possibly leading to alterations of the joint structure and disturbance of joint function. Crossing of joint compartment borders by synoviocytes is well documented in the case of RA. In this disease entity, the boundaries between the synovial and the cartilage compartment are crossed by synoviocytes that are capable of destroying the adjacent cartilage. In recent years, it has been assumed that a pathologically aggressive behaviour of synovial cells results in the transgression of compartment boundaries and, thus, to cartilage destruction [39, 40].

One mechanism involved in the migration of activated synovial fibroblasts (SFs) is attraction by chemotactic mediators such as chemokines [41]. However, the opposite mechanism of repulsion, so far only described in the nervous system, might also be relevant to alterations of joint compartments.

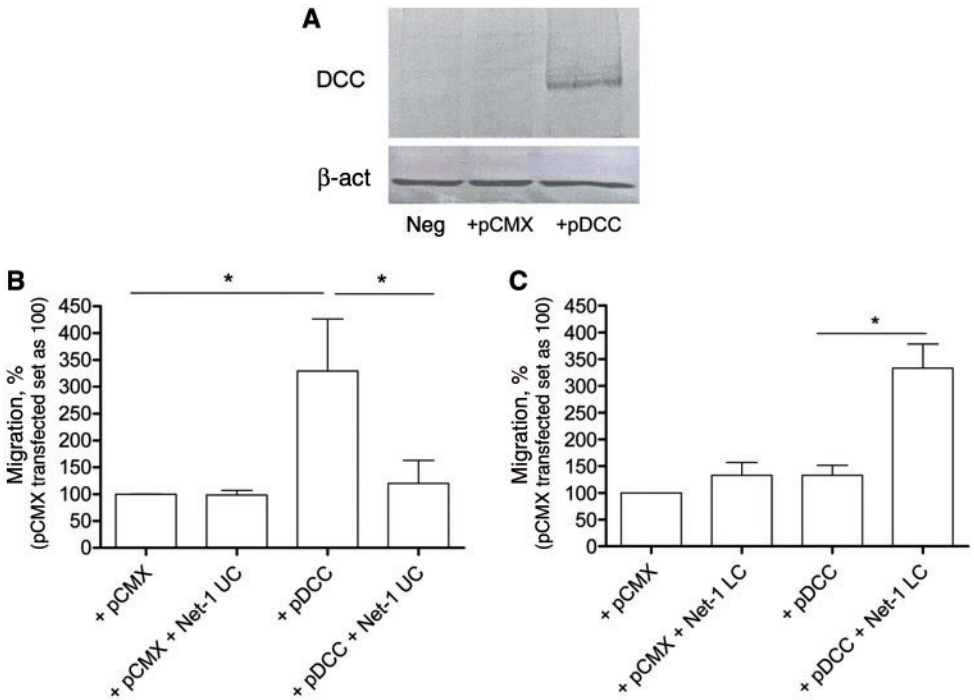

FIG. 4. Role of DCC in migration of chondrocytes. Chondrocytes were transfected with control plasmid (pCMX-pl1) or DCC expression plasmid (pDCC) and analysed in the Boyden Chamber model for migration. (**A**) Transfection efficiency was shown by western blotting. (**B**) Expression of DCC resulted in enhanced migration of chondrocytes towards a chemoattractive stimulus (fibroblasts conditioned medium). This induction was inhibited by adding Netrin-1 to the cells (+ Net-1 UC) (*$P < 0.05$, $n = 4$). (**C**) Netrin was used as a chemoattractant (+ Net-1 LC) and was shown to only attract DCC expressing chondrocytes. Random migration, as revealed in this assay, was not changed by DCC expression (*$P < 0.05$, $n = 3$).

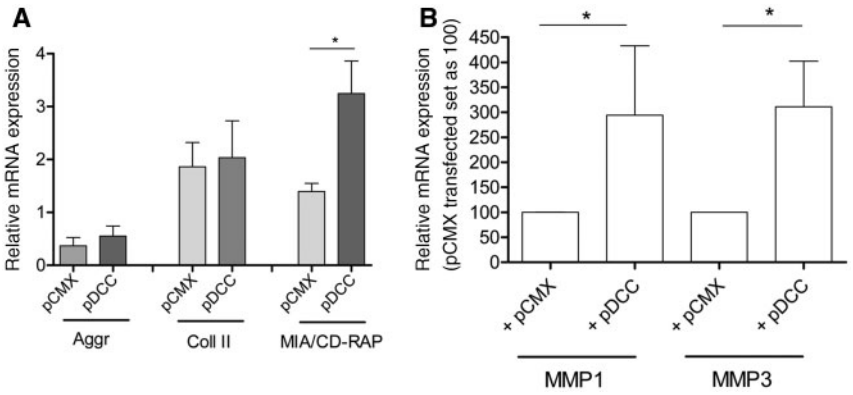

FIG. 5. MIA/CD-RAP and MMP expression is regulated by DCC. (**A**) Expression of the differentiation markers, aggrecan (Aggr), collagen type II (Coll II) and MIA/CD-RAP, was determined in chondrocytes transfected with a DCC expression plasmid by qRT–PCR. Expression of aggrecan and collagen type II was not altered but expression of MIA/CD-RAP was significantly increased after DCC transfection ($^*P < 0.05$, $n = 3$). (**B**) In the DCC transfected cells, MMP1 and MMP3 expression was also induced significantly ($^*P < 0.05$, $n = 3$).

In this study, we discovered strong induction of DCC in OA chondrocytes, indicating that altered regulation of DCC takes place in the course of OA. The presence of binding sites for the transcription factors Sox9 and AP-2 in the DCC promoter of chondrocytes, the demonstration of binding of Sox9 and AP-2 to the DCC promoter and the induction of DCC by both factors indicate that Sox9 and AP-2 have a role in up-regulating the DCC during OA.

Induction of Sox9 expression in OA was described by several groups [33]. In addition, AP-2 binding sites were also determined in several genes found to be up-regulated in OA [34]. Potentially, other factors are needed to control absence of expression of DCC in healthy cartilage as both transcription factors are known to be expressed, at lower levels, also in normal chondrocytes.

The differential expression of DCC is reflected in an increased migratory activity of OA chondrocytes *in vitro* compared with normal chondrocytes after exposure to recombinant Netrin-1, a ligand of the DCC receptor. Migration in response to Netrin-1 could also be induced *in vitro* in normal chondrocytes transfected with the DCC receptor. Netrins are involved in axon guidance. Growing axons will either move towards or away from a higher concentration of Netrin. Although the detailed mechanism of axon guidance is not fully understood, Netrin attraction is mediated through DCC or Neogenin cell surface receptors and repulsion is mediated through UNC receptors [42, 43]. Interestingly, Netrin-1, -2 and -3 and their receptors were shown to be expressed in activated SFs [44].

Our results suggest that migration of non-neuronal cells such as chondrocytes expressing DCC can also be induced by Netrin-1 *in vitro*. Furthermore, a possible significance of chondrocyte migration to the pathophysiology of OA is emphasized by the fact that DCC is up-regulated in OA chondrocytes and that DCC mediates a non-random migration *in vitro* directed to the chemoattractant Netrin-1. This observation can, in our opinion, be interpreted as a regulated process.

The induction of MMP1 and -3 expression in chondrocytes transfected with DCC may be potentially linked to the migratory effect. Additionally, or possibly, alternative expression of MMPs can be viewed as induction of a catabolic phenotype, reflecting the increased production of these matrix proteases in degrading cartilage of OA joints [45]. Furthermore, DCC over-expression in chondrocytes induces the differentiation marker MIA/CD-RAP, which was also found to be up-regulated in OA chondrocytes [46]. As aggrecan and collagen type II expression stayed unchanged after over-expression of DCC, we attributed the changes in Mia expression to the OA phenotype and not to differentiation.

To summarize, our findings show that DCC is up-regulated in OA chondrocytes and mediates directed chondrocyte migration *in vitro* in response to Netrin-1. In the light of new theories regarding a migratory capability of chondrocytes pertinent to cartilage remodelling, these findings may be a part of an altered migratory phenotype of chondrocytes or misdirected migration during OA. The induction of MMP1 and -3 in chondrocytes is an additional indication of a possible functional relevance of DCC in the pathophysiology of OA. However, definite proof of chondrocyte movements *in vivo* still has to be established.

Rheumatology key messages

- Induced expression of DCC mediates directed chondrocyte migration.
- DCC may be part of an altered migratory phenotype of chondrocytes during OA.
- DCC induces additional OA-associated genes.

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