Signaling cross-talk between IGF-binding protein-3 and transforming growth factor- β in mesenchymal chondroprogenitor cell growth

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Abstract

Cartilage formation is driven by mesenchymal chondroprogenitor cells (MCCs) that proliferate and differentiate into chondrocytes. The molecular mechanisms by which growth factors regulate MCC fate are not well defined. Insulin-like growth factor binding protein-3 (IGFBP-3) has intrinsic bioactivity that is independent of IGF binding. We previously reported that IGFBP-3 has IGF-independent antiproliferative and apoptotic effects in MCCs, and requires STAT-1 activation to mediate its apoptotic effect. Transforming growth factor- β (TGF- β) is a key chondroinductive growth factor. The objective of the study is to define the interactions between IGFBP-3 and TGF- β in MCC growth and their intracellular signaling pathways. We used the RCJ3-1C5-18 mesenchymal chondrogenic cells that without biochemical or oncogenic transformation progress in culture from MCCs to differentiated chondrocytes. Cell proliferation was assessed in MCCs treated with IGFBP-3 or transfected with IGFBP-3, in the presence or absence of TGF- β . To demonstrate that IGFBP-3 effects were IGF-independent an IGFBP-3 analog that lacks IGF binding was used (GGG-IGFBP-3). To determine the functional roles of the TGF- β -mediated signaling and the STAT-1 pathway, cells were either stably transfected with a dominant negative TGF- β type II receptor (MCC-DNT β RII) or treated with a STAT-1 morpholino antisense oligonucleotide. We found that in MCCs, TGF- β antagonized the antiproliferative effect of IGFBP-3. IGFBP-3 increased the cyclin-dependent kinase inhibitor p21 expression and this effect was abolished by TGF- β . Furthermore, TGF- β inhibited STAT-1 phosphorylation induced by IGFBP-3. Similarly to TGF- β , STAT-1 antisense oligonucleotide inhibited the IGFBP-3 antiproliferative action. Although TGF- β in MCC-DNT β RII lacked Smad-mediated signaling, it persistently antagonized the IGFBP-3 antiproliferative action. However, TGF- β even in MCC-DNT β RII cells induced ERK1/2 phosphorylation, and treatment with MEK inhibitor, UO126, inhibited the antagonistic effects of TGF- β on IGFBP-3. Furthermore, UO126 blocked the TGF- β inhibition of STAT-1 phosphorylation induced by IGFBP-3. Collectively, these results demonstrate cross-talk between the IGFBP-3-dependent STAT-1 signaling and the TGF- β -dependent ERK pathway that regulates MCC proliferation.

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Introduction

Cartilage is essential for the skeletal growth and the functional integrity of the skeleton. Cartilage formation is driven by mesenchymal chondroprogenitor cells (MCCs) that condensate, proliferate and differentiate into chondrocytes. Cartilage is not able to repair itself and cartilage degeneration, such as in osteoarthritis, leads to serious disabilities. Several studies have demonstrated that MCCs are potentially useful for cellular and gene therapy to repair damaged cartilage (Johnstone *et al.* 1998, Pittenger *et al.* 1999, Sekiya *et al.* 2002). However, much of the basic biology of MCCs

and in particular the molecular signals by which growth factors regulate MCC fate are basically unknown.

Insulin-like growth factor-binding protein-3 (IGFBP-3) is the predominant insulin-like growth factor (IGF) circulating carrier in postnatal life (Spagnoli & Rosenfeld 1996). Well characterized as an IGF carrier, IGFBP-3 has been extensively reported to exert pleiotropic effects on diverse cell types and to have a broad range of functions that are independent from its binding to IGF. Through this IGF-independent action, IGFBP-3 has been shown to control cell proliferation and to induce or enhance apoptosis (Cohen *et al.* 1993, Oh *et al.* 1995b, Valentini *et al.* 1995, Lalou *et al.* 1996, Spagnoli

et al. 2001, 2002, Firth & Baxter 2002, Longobardi *et al.* 2003). Using RCJ3·1C5·18 chondrogenic cells as an established model to study MCC fate during the chondrogenesis process *in vitro*, we have previously reported that: IGFBP-3 has IGF-independent antiproliferative and apoptotic effects in MCCs; the IGFBP-3 apoptotic effect in MCCs requires STAT-1 expression and activation; and IGFBP-3 modulates the chondrocytic differentiation rate by regulating MCC growth (Spagnoli *et al.* 2001, 2002, Longobardi *et al.* 2003). RCJ3·1C5·18 cells are derived from rat calvaria mesenchymal cells and over 2 weeks of culture, without requiring biochemical and oncogenic transformation, they sequentially differentiate from MCCs to terminally differentiated chondrocytes in a highly predictable and reproducible manner (Grigoriadis *et al.* 1996, Lunstrum *et al.* 1999, Spagnoli *et al.* 2001). Furthermore, RCJ3·1C5·18 cells do not express IGFs or IGFBP-3, so the action of these peptides can be studied without interference from endogenous proteins (Spagnoli *et al.* 2001).

Transforming growth factor- β (TGF- β) plays a central role in the chondrogenesis process (Sanford *et al.* 1997, Serra *et al.* 1997, Dunker & Kriegstein 2000, Dunker *et al.* 2002, Grimaud *et al.* 2002). TGF- β s and TGF- β receptors are expressed in developing and adult cartilage (Sandberg *et al.* 1988, Pelton *et al.* 1990, 1991, Horner *et al.* 1998, Matsunaga *et al.* 1999). Mice carrying null mutations of the gene encoding TGF- β 2 have skeletal abnormalities (Sanford *et al.* 1997). Mice lacking TGF- β 3 exhibit cleft palate, a defect in the epithelial–mesenchymal interaction (Proetzel *et al.* 1995). Mice carrying a dominant negative mutation for the TGF- β type II receptor (DNT β RII) that abolishes Smad-mediated signaling develop a degenerative joint disease that resembles human osteoarthritis (Serra *et al.* 1997). A similar phenotype is observed in *Smad3* $-/-$ mice (Yang *et al.* 2001). TGF- β upregulates a number of molecules associated with prechondrogenic mesenchyme condensation, a critical step in the chondrogenesis process (Chimal-Monroy & Diaz de Leon 1999). Furthermore, TGF- β determines the exclusive commitment of mesenchymal stem cells derived from bone marrow to MCCs (Cassiede *et al.* 1996, Mackay *et al.* 1998, Yoo *et al.* 1998, Pittenger *et al.* 1999, 2000, Sekiya *et al.* 2002). TGF- β elicits its biological effects by binding to a heteromeric complex of type I and type II TGF- β receptors (T β RI, T β RII), each containing serine/threonine kinase domains that interact leading to T β RI phosphorylation and activation (Wrana *et al.* 1992, Yamashita *et al.* 1994). A central paradigm to explain TGF- β signaling has been established, in which the activated T β RI phosphorylates receptor-associated Smads (Smad-2 and Smad-3), which then bind Smad-4 and translocate to the nucleus where they regulate transcription of target genes (Nakao *et al.* 1997, Zhang

et al. 1998). However, T β Rs transduce signals through Smad-independent pathways. Indeed, data are now rapidly accumulating to implicate a variety of alternative pathways, including the extracellular signal-regulated kinase (ERK) signaling pathway (Bakin *et al.* 2000, 2002, Bhowmick *et al.* 2001a,b, 2003, Shin *et al.* 2001, Derynck & Zhang 2003, Dumont *et al.* 2003). It is likely that TGF- β pathways vary according to conditions and cell types.

In several cell lines, TGF- β effects on cell growth and apoptosis correlate with the induction of IGFBP-3 at both the transcriptional and translational levels (Oh *et al.* 1995a, Rajah *et al.* 1997, Cohen *et al.* 2000). Furthermore, IGFBP-3 has been shown to enhance the TGF- β -induced phosphorylation of Smad-2 and Smad-3 (Fanayan *et al.* 2000). More recently, Fanayan *et al.* (2002), have reported that IGFBP-3 stimulates phosphorylation of T β RI and activates the promoter for plasminogen activator inhibitor-1 (PAI-1), a gene characterized as being TGF- β responsive (Fanayan *et al.* 2002). The functional interaction between specific IGFBP-3 and TGF- β signaling pathways has not been identified.

The present study was aimed at defining the molecular mechanisms that determine the interactions between IGFBP-3 and TGF- β signaling in MCCs and in turn in the chondrogenesis process. In particular, our study was designed to identify the intracellular signaling pathways involved in the cross-talk between IGFBP-3 and TGF- β signaling pathways in MCC growth.

Materials and methods

Chemical reagents

Recombinant non-glycosylated human (h) IGFBP-3 and the ND-IGFBP-3 variant expressed in *E. coli* were generously supplied by Celtrix Pharmaceuticals, Inc. (Santa Clara, CA, USA). Purified porcine TGF- β 1 was supplied by R&D Systems (Minneapolis, MN, USA). des-(1–3)-IGF-I (des-IGF-I) was purchased from Diagnostic System Laboratories, Inc. (Webster, TX, USA). des-IGF-I exhibits 30- to 100-fold reduced affinity for IGFBP-3, but unaltered affinity for the type I IGF receptor compared with IGFs (Francis *et al.* 1992, Oh *et al.* 1993). Recombinant human bone morphogenic protein-6 (BMP-6) was obtained from R&D Systems. Fetal bovine serum was obtained from Hyclone (Logan, UT, USA). MEM- α medium and sodium pyruvate were purchased from Gibco-BRL (Gaithersburg, MD, USA). Dexamethasone was obtained from Sigma Chemical (St Louis, MO, USA). UO126, a specific MEK inhibitor, was purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phosphorylated STAT-1 (Tyr701), anti-STAT-1, anti-phosphorylated Smad-1, anti-phosphorylated ERK1/2, anti-ERK1/2 and anti-p38

polyclonal antibodies were obtained from Cell Signaling. Anti-phosphorylated Smad-2 was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-p21 monoclonal antibody was obtained from EMD Biosciences (La Jolla, CA, USA). Anti-p21 polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin polyclonal antibody was obtained from Sigma. Alexa-conjugated Fluor 488 anti-rabbit secondary antibody and Hoechst 33256 were obtained from Molecular Probes (Eugene, OR, USA). Trypan Blue was from CellGro (Herndon, VA, USA).

Plasmid constructs and STAT-1 morpholino antisense oligonucleotide

The GGG-IGFBP-3 mutant cDNA generated by site-directed mutagenesis at residues I56, L80 and L81 to G56 G80 G81 was generously donated by Dr Ron Rosenfeld (Stanford University, CA, USA). Binding studies (including BIAcore analysis) showed that the GGG-IGFBP-3 mutant protein, generated in *E. coli* and baculovirus expression systems, had abolished affinity for IGFs (Buckway *et al.* 2001). For transfection, hIGFBP-3 and GGG-IGFBP-3 mutant cDNAs were subcloned into the pCMV6 vector as previously described (Spagnoli *et al.* 2002). One day after seeding, cells were transfected with expression vector plasmids using Mirus Transit LT-1, as described by the manufacturer (PanVera; Madison, WI, USA).

A truncated T β RII cDNA was used. This construct encodes a T β RII lacking the cytoplasmic region of the receptor that contains the serine/threonine kinase domain (Chen *et al.* 1993). The truncated receptor is still able to bind TGF- β and recruit T β RI, but is unable to signal via the Smad signaling pathway (generously donated by Dr R Derynck; University of California at San Francisco). The expressed construct acts as a dominant negative receptor (DNT β RII) (Chen *et al.* 1993, Serra *et al.* 1997).

The Smad-dependent p(CAGA)12-Lux construct was provided by Dr J M Gauthier, (Laboratoire Glaxo Wellcome, Les Ulis Cedex, France). The p(CAGA)12-Lux construct contains 12 CAGA repeats that bind TGF- β -dependent Smad-2, -3 and -4 complexes (Dennler *et al.* 1998).

To inhibit STAT-1 transcription in cells a STAT-1 morpholino antisense oligonucleotide (GeneTools LLC, Philomath, OR, USA) was designed based upon the published rat STAT-1 cDNA sequence (GenBankTM accession number AF205604: 5'-GCTGAAGCTCGAA CCACTGTGACAT-3') and which corresponded to the first 25 nucleotides of the STAT-1 open reading frame (Spagnoli *et al.* 2002). Antisense oligonucleotide was delivered by using the Special Delivery Morpholino System as described by the manufacturer (GeneTools LLC) and previously reported (Spagnoli *et al.* 2002).

Cell culture

RCJ3-1C5-18 cells generously donated by Dr Jane E Aubin (University of Toronto), were grown in MEM- α medium supplemented with 15% heat-inactivated fetal bovine serum, 10^{-7} M dexamethasone and 2 mM sodium pyruvate. Cells plated at a density of 6×10^4 cells/well in six-well dishes, without requiring biochemical or oncogenic transformation undergo, a reproducible, time-dependent progression from MCCs, during the first 4 days of culture, to early (7–10 days of culture) and then terminally differentiated chondrocytes (14 days of culture) when fresh medium with 50 μ g/ml ascorbic acid and 10 mM β -glycerophosphate is added (Grigoriadis *et al.* 1988, 1989, Lunstrum *et al.* 1999, Spagnoli *et al.* 2001). As previously reported, the morphology, the histochemical markers and the temporal sequential acquisition of the chondrocytic phenotype in the RCJ3-1C5-18 cell system mimics the chondrogenesis process that occurs *in vivo* (Lunstrum *et al.* 1999, Spagnoli *et al.* 2001).

Generation of MCCs stably expressing DNT β RII (MCC-DNT β RII)

DNT β RII cDNA, subcloned into the pBabe retroviral vector, was transfected into Phoenix amphotropic packaging cells (provided by G Nolan, Stanford University) using Lipofectamine according to manufacturer recommendations (Invitrogen Life Technologies, Carlsbad, CA, USA). Culture supernatants containing virus were collected 3 days after transfection, filtered through a 0.45 μ m filter (Pall Gelman Sciences; Ann Arbor, MI, USA). RCJ3-1C5-18 cells were seeded at a density of 6×10^4 cells in a 75 cm² flask and, 24–48 h later, filtered medium containing virus and 8 μ g/ml polybrene (Sigma) was added. After 72 h, the medium was replaced with fresh complete medium for MCCs containing 2.5 μ g/ml puromycin (Sigma) for 3 weeks. Cells were then expanded in MCC medium containing 2.5 μ g/ml puromycin (Sigma). At least three clones of MCCs expressing either the empty vector pBabe or the DNT β RII were generated.

Measurement of cell proliferation and cell number

Cells were seeded in six-well plates and after 4 days of culture were changed to serum-free medium for 4 h. Cells were then incubated with specified peptides and [³H]thymidine (1 μ Ci/ml) in serum-free medium for 18 h. Incubations were terminated by washing with ice-cold PBS. Incorporation of [³H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material, as previously described (Spagnoli *et al.* 2001). The MEK inhibitor UO126 (10 μ M) was added 2 h before peptides were added. Cell

counting experiments were performed in cells treated with exogenous IGFBP-3 as well as in cells transfected with IGFBP-3 or GGG-IGFBP-3. For cell counting done with exogenous IGFBP-3, cells at day 4 of culture were changed to serum-free medium with or without IGFBP-3 (0.5, 2, 5, 15, 30 nM) for 24 h; cells were also treated with IGFBP-3 (2 nM) with or without TGF- β (5 ng/ml) in the presence or absence of des-IGF-I (15 nM). For cell counting done in cells transfected with IGFBP-3 or GGG-IGFBP-3, cells were transfected the day after seeding with either IGFBP-3 cDNAs or empty vector and treated, 17 h later, with or without TGF- β (5 ng/ml) for 24 h. For experiments in which UO126 was employed, cells were pre-incubated with UO126 (10 μ M) for 2 h in serum-free medium and then treated with IGFBP-3 (2 nM) with or without TGF- β (5 ng/ml) for 24 h or were transfected with IGFBP-3 cDNAs or empty vector and treated, 17 h later, with or without UO126 (10 μ M) for 2 h and then with TGF- β (5 ng/ml) for 24 h. Viable floating and attached cells (after trypsinization) were counted on a hemocytometer after Trypan Blue exclusion.

In the experiments where STAT-1 antisense oligonucleotide was used, cells (24 h after seeding) were treated with or without the STAT-1 morpholino antisense oligonucleotide. Thirty-six hours after antisense treatment, cells were changed to serum-free medium for 4 h and then incubated with specific peptides and [3 H]thymidine, as described above.

Cell extracts and WIB analysis

Cell extracts were obtained at different time points after transfection from cells transfected with expression vector plasmid (IGFBP-3 or GGG-IGFBP-3 or empty vector), or from untransfected cells. Two-day-old transfected or untransfected cells were treated with TGF- β (5 ng/ml) for different time periods. To block activation of the ERK1/2 pathway, cells were treated with UO126 for 2 h prior to TGF- β treatment. To inhibit STAT-1 expression, 1-day-old cells were incubated with STAT-1 morpholino antisense oligonucleotide. Twenty-four hours after antisense treatment, cells were transfected with 4 μ g expression vector plasmid (IGFBP-3 or GGG-IGFBP-3 or empty vector), using Mirus Transit LT-1 as described above. Twenty-four hours after transfection, cell extracts were obtained. BMP-6 (300 ng/ml) was used in cells that were seeded for 48 h and incubated with or without BMP-6 for 1 h. Cell extracts were obtained using two different protocols. The first one involved lysis of cells for 30 min at 4 °C in lysis buffer containing 1% NP-40, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 10% glycerol, and a cocktail of protease inhibitors (Boehringer-Mannheim, Germany) including 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and

20 mM sodium fluoride. The suspension was sonicated for 5 s twice and cleared by centrifugation and resulting supernatants were collected and frozen at -80 °C or used immediately. The second protocol involved lysis with boiling modified Laemmli buffer (60 mM Tris, pH 6.8, 10% glycerol, and 2% SDS, without β -mercaptoethanol and bromophenol blue). Cell extracts were passed through a 27-gauge needle, followed by centrifugation and supernatants were collected and frozen at -80 °C or used immediately. Protein concentrations were determined in the supernatants (Bio-Rad Laboratories, Hercules, CA, USA) and 20–30 μ g of proteins were subjected to WIB with specific primary antibodies, using ECL (Pierce; Rockford, IL, USA) or alkaline phosphatase reagents (Sigma), as previously described (Spagnoli *et al.* 1995). Membranes were probed either with an anti-actin antibody or p38 antibody as internal controls for the protein amount loaded. Densitometric analysis was done with a GS700 Imaging Densitometer (Bio-Rad Laboratories).

Transcriptional assay

Cells were seeded in six-well dishes and transfected the following day with 0.5 μ g/ml p(CAGA)12-Lux in conjunction with 0.052 μ g/ml cytomegalovirus-driven renilla luciferase plasmid (pCMV-R1) (Promega, Madison, WI, USA) using Mirus Transit LT-1 transfection reagents, as described by the manufacturer (PanVera). The day after, cells were treated overnight with TGF- β (5 ng/ml). Firefly luciferase (Luc) and *Renilla reniformis* luciferase (RLuc) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 3010 luminometer (BD Biosciences, San Diego, CA, USA). Luc activity was normalized to R1 Luc activity.

Immunofluorescence

Cells were plated at a density of 1×10^4 cells/chamber and 24 h after plating were transfected with 1 μ g IGFBP-3 or GGG-IGFBP-3 mutant expression vector plasmids or empty vector. Twelve hours after transfection, cells were incubated for 3 h with TGF- β (5 ng/ml) and then fixed in 4% paraformaldehyde for 10 min at 4 °C, washed with Tri-HCl 50 mM pH 7.4, NaCl 150 mM, 0.1% Triton X-100 and permeabilized in 100% methanol for 10 min at 4 °C. After washing in TBS, samples were incubated for 1 h at room temperature (RT) with blocking buffer (5% normal goat serum and 1% BSA in TBS), washed with TBS, incubated overnight at 4 °C with anti-p21 antibody (1:1000 dilution in TBS with 1% BSA), washed, incubated for 30 min at RT with Alexa-conjugated Fluor 488 anti-rabbit secondary antibody (1:5000) and

with Hoechst (1:1000) in TBS with 1% BSA, washed with TBS and water, mounted with coverslips, and visualized under fluorescence microscope.

Measurement of IGFBP-3 and GGG mutant

Conditioned media were obtained 24 and 48 h after transfection with IGFBP-3, GGG-IGFBP-3, or with empty vector or left untransfected. Media were concentrated 4- to 7-fold using Centricon 3 columns (Amicon, Boston, MA, USA), and IGFBP-3 and GGG concentrations were determined using a commercial IRMA kit for hIGFBP-3 (Diagnostic Systems). The minimum detection limit of the assay is 0.5 ng/ml; the intra-assay coefficient of variation ranges from 1.8 to 3.9% and the inter-assay coefficient of variation ranges from 0.5 to 1.9%. It has been previously reported that the GGG mutation does not interfere with the ability of the peptide to be recognized by the anti-IGFBP-3 antibodies used in the assay (Buckway *et al.* 2001).

Measurement of apoptosis

A cell death detection ELISA kit was used to measure cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) generated in the early phase of apoptosis (Roche Molecular Biochemicals, Indianapolis, IN, USA). The assay is based on a quantitative sandwich enzyme immunoassay, using antibodies directed against DNA and histones. This allows the specific determination of mono- and oligo-nucleosomes, which are released into the cytoplasm of apoptotic cells. Cells were seeded in 24-well dishes and at day 4 of culture were treated with IGFBP-3 (2 nM) and/or TGF- β (5 ng/ml) in serum-free medium. Four and 24 h later, cell lysates were prepared and subjected in duplicate to the cell death detection assay as previously described (Spagnoli *et al.* 2002).

Statistics

Data are presented as means $\pm$ S.D. Statistical differences between means were assessed by an unpaired Student's *t*-test or one-way ANOVA followed by the Student–Newman–Keuls test for all pairwise multiple comparisons, or, when necessary, by one-way ANOVA on ranks (Kruskal–Wallis) followed by Dunn's test for all pairwise multiple comparisons by using Sigmapstat Software from Jandel Scientific, San Rafael, CA, USA. Sigmoidal dose–response curves with relative EC₅₀ and logEC₅₀ values as well as comparison of best-fit values were obtained by using GraphPad Prism, Inc. software (San Diego, CA, USA). Statistical significance was set at $P < 0.05$.

Results

TGF- β antagonizes the IGFBP-3 antiproliferative effect and inhibits the activation of STAT-1 and p21 expression induced by IGFBP-3

TGF- β inhibits the antiproliferative action of IGFBP-3 in MCCs

We found that in MCCs, TGF- β had an antagonistic effect on the growth inhibitory action of IGFBP-3. As shown in Fig. 1A, IGFBP-3 had a dose-dependent antiproliferative effect in MCCs and TGF- β treatment induced a 2-fold increase in the EC₅₀ of the IGFBP-3 growth inhibitory response (EC₅₀ IGFBP-3: 2.0 nM vs EC₅₀ IGFBP-3+TGF- β : 4.0 nM, $P < 0.05$; logEC₅₀ IGFBP-3: 0.3 vs logEC₅₀ IGFBP-3+TGF- β : 0.6, $P < 0.05$; $n = 6$). IGFBP-3 had a significant antiproliferative effect that started at 1 nM and gradually increased to 15 nM and had a plateau at 30 nM. Furthermore, as shown in Fig. 1B, TGF- β antagonized the IGFBP-3 antiproliferative effect in a dose-dependent manner. TGF- β in the presence or absence of serum and, with or without des-IGF-I had no effect on MCC growth. We also evaluated the antagonistic effect of TGF- β on the IGFBP-3 effect on the number of viable cells, as determined by cell counting after Trypan Blue exclusion of dead cells. As shown in Fig. 2A, IGFBP-3 inhibited the des-IGF-I action $\sim 25\%$ and TGF- β antagonized the inhibitory effect of IGFBP-3. TGF- β antagonized also the action of IGFBP-3 and GGG-IGFBP-3 on cell number when these cDNAs were transfected in MCCs (Fig. 2B). MCC transfection with empty vector had no effect on cell counting as compared with control untransfected cells, indicating that the empty vector and the transfection reagents had no effect on cell survival. A direct inhibitory effect of IGFBP-3 on cell count was found; in cells treated with IGFBP-3 (in the absence of des-IGF-I) a dose-dependent decrease of cell number was detected with maximal effect at 30 nM; IGFBP-3, 0.5 nM: $95.1 \pm 4.5\%$ of control untreated cells; IGFBP-3, 2 nM: $74.5 \pm 0.9\%$ of control; IGFBP-3, 5 nM: $70.1 \pm 1.8\%$ of control; IGFBP-3, 15 nM: $64.3 \pm 3.7\%$ of control; IGFBP-3, 30 nM: $61.4 \pm 1.2\%$ of control; $P < 0.05$ $n = 6$. As shown in Fig. 2C, TGF- β antagonized also the direct effect of IGFBP-3. Taken together these results demonstrate that IGFBP-3 has growth inhibitory effects in MCCs, in exogenously treated cells as well as in transfected cells, the IGFBP-3 effect is clearly IGF-independent and TGF- β is an antagonist. In all the following experiments exogenous IGFBP-3 was used at the dose of 2 nM that corresponds to the EC₅₀ of its antiproliferative effect. Since IGFBP-3 induces apoptosis in MCCs, and cell number results from cell proliferation, apoptosis and death, we evaluated whether the antagonistic effect of TGF- β was mediated by inhibition of IGFBP-3 apoptotic action. We found that TGF- β had no effect on cell apoptosis

Figure 1 TGF- β antagonizes the IGFBP-3 antiproliferative action in MCCs as determined by [3 H]thymidine incorporation. (A) MCCs cultured for 4 days were changed to serum-free medium for 4 h and then incubated for an additional 18 h with [3 H]thymidine with increasing doses of IGFBP-3 (0.01 nM; 0.1 nM; 1 nM; 7.5 nM; 15 nM and 30 nM) and des-IGF-I (15 nM), with or without TGF- β (5 ng/ml). Incorporation of [3 H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. Results are expressed as percent of maximal growth inhibitory response; sigmoidal dose-response curves with relative EC_{50} and $logEC_{50}$ values were obtained using GraphPad Prism Software. (B) MCCs cultured for 4 days were changed to serum-free medium for 4 h and then incubated for an additional 18 h with [3 H]thymidine with IGFBP-3 (2 nM) and increasing doses of TGF- β (0.01 ng/ml; 0.1 ng/ml; 1 ng/ml; 2.5 ng/ml; 5 ng/ml; 10 ng/ml) in the presence of des-IGF-I (15 nM). Incorporation of [3 H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. Results are expressed as percent of maximal TGF- β effect on IGFBP-3 inhibitor response.

induced by exogenous IGFBP-3 treatment for 4 h, as determined by quantifying cytoplasmic histone-associated DNA fragments (control untreated: 0.9 ± 0.3 absorbance/well; TGF- β : 1.1 ± 0.2 ; IGFBP-3: 1.7 ± 0.3 ; IGFBP-3+TGF- β : 1.7 ± 0.3 ; TGF- β : 1.1 ± 0.2 ; $n=8$). Furthermore, we found that in cells treated for

Figure 2 TGF- β antagonizes the antiproliferative action of exogenous IGFBP-3 (A, C) in the presence (A) or in the absence of des-IGF-I (C) as well as IGFBP-3 and GGG-IGFBP-3 transfected into MCCs (B) as determined by cell counting. (A) MCCs at day 4 of culture were changed to serum-free medium with IGFBP-3 (2 nM) with or without TGF- β (5 ng/ml) and des-IGF-I (15 nM) for 24 h. Results are expressed as percentage of control untreated cells that had a total cell number of $120 \times 10^4 \pm 6$ /well. ^a $P < 0.05$ vs des-IGF-I; ^b $P < 0.05$ vs des-IGF-I+IGFBP-3; $n=6$. (B) MCCs were transfected, the day after seeding, with either IGFBP-3 cDNAs or empty vector and treated, 17 h later, with or without TGF- β (5 ng/ml) for 24 h. Results are expressed as control untransfected cells that had a total cell number of $195 \times 10^4 \pm 5$ /well. ^c $P < 0.05$ vs IGFBP-3; ^d $P < 0.05$ vs GGG-IGFBP-3; $n=6$. (C) Cells at day 4 of culture were changed to serum-free medium with IGFBP-3 (2 nM) with or without TGF- β (5 ng/ml) for 24 h. ^c $P < 0.05$ vs IGFBP-3; $n=6$. Results are expressed as percentage of control untreated cells that had a total cell number of $105 \times 10^4 \pm 7$ /well. Viable floating and attached cells (after trypsinization) were counted on a hemocytometer after Trypan Blue exclusion.

24 h with exogenous IGFBP-3 in serum-free medium, the IGFBP-3 apoptotic effect was blunted. We conclude that the IGFBP-3 effect on cell count in 24 h exogenously treated cells, in serum free, is mostly due to its antiproliferative action and TGF- β antagonizes this effect.

Figure 3 STAT-1 is required for the CDK inhibitor p21 expression induced by IGFBP-3 and GGG-IGFBP-3. MCCs were treated with or without STAT-1 antisense oligonucleotide and, 24 h later were transfected with IGFBP-3 or GGG-IGFBP-3 (GGG). Forty-eight hours after treatment with or without STAT-1 antisense, cell lysates were obtained and subjected to WB analysis for p21 (A), total STAT-1 (B) and phosphorylated STAT-1 (C). Membranes were probed with an anti-p38 antibody to demonstrate equal protein loading. This is a representative gel that has been repeated at least three times.

STAT-1 mediates the cyclin-dependent kinase (CDK) inhibitor p21 expression induced by IGFBP-3 and its IGFBP-3 antiproliferative action in MCCs

We next examined whether the growth inhibitory action of IGFBP-3 was associated with an increase in expression of the CDK inhibitor p21. We found that in MCCs, IGFBP-3 transfection resulted in an increase in p21 expression, compared with untransfected cells or cells transfected with empty vector (Fig. 3A). To determine whether STAT-1 was a mediator of the p21 expression induced by IGFBP-3 and GGG-IGFBP-3 we used a STAT-1 antisense oligonucleotide to inhibit the endogenous expression of STAT-1. As shown in Fig. 3, in cells pre-treated with STAT-1 morpholino antisense oligonucleotide, p21 expression induced by either IGFBP-3 or GGG-IGFBP-3 was significantly reduced to the level observed in control cells treated with the empty vector or left untransfected. The STAT-1 antisense was able to knock down STAT-1 expression (Fig. 3B) and activation (Fig. 3C) induced by IGFBP-3 or GGG-IGFBP-3 by at least 80% and had no effect on control cells. In cells transfected with the empty vector no effect on STAT-1 activation and expression and p21 were

Figure 4 STAT-1 has a functional role in the growth inhibitory action of IGFBP-3. MCCs were treated with (B) or without (A) a STAT-1 antisense oligonucleotide; 36 h later they were changed to serum-free medium for 4 h and then incubated for an additional 18 h with [3 H]thymidine and des-IGF-I (15 nM) with or without IGFBP-3 (2 nM) and with or without TGF- β (5 ng/ml). Incorporation of [3 H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. Results are expressed as percentage of the control (cells not treated with peptides), which was given an arbitrary value of 100%; [3 H]thymidine incorporation in controls was respectively 550 $\pm$ 90 c.p.m. (A) and 681 $\pm$ 105 c.p.m. (B). ^a P <0.05 vs des-IGF-I; n =6.

found, indicating that the vector per se and the transfection reagents did not interfere with these cell signaling components. Furthermore, we found that STAT-1 had a functional role in the antiproliferative action of IGFBP-3 in MCCs. As shown in Fig. 4, in cells pre-treated with STAT-1 antisense oligonucleotide the antiproliferative response to IGFBP-3 was blunted. The STAT-1 antisense oligonucleotide was able to hamper the growth inhibitory effect of IGFBP-3 to the same extent as TGF- β (Fig. 4A). Since we noted that treatment with STAT-1 morpholino antisense oligonucleotide increased des-IGF-I mitogenic response, in order to determine whether IGFBP-3 response was blunted because the IGFBP-3 used was insufficient to inhibit the increased des-IGF-I response we increased IGFBP-3 to 30 nM. Despite the maximal dose used, IGFBP-3 inhibitory action was blocked in MCCs pre-treated with STAT-1 antisense oligonucleotide. Taken together, these data indicate that STAT-1 signaling is required for the antiproliferative response and the p21 expression induced by IGFBP-3.

In the media of cells transfected with IGFBP-3 or GGG-IGFBP-3, 24 h after transfection, 56 $\pm$ 15 ng/ml IGFBP-3 and 42 $\pm$ 8 ng/ml GGG mutant were measured (n =21). Furthermore, IGFBP-3 and GGG-IGFBP-3 levels expressed in the media of transfected cells were stable during the period we performed all our

Figure 5 TGF- β inhibits STAT-1 phosphorylation induced by GGG-IGFBP-3. Cell lysates were obtained from MCCs transfected with GGG-IGFBP-3 (GGG) or with empty vector for 24 h and treated with or without TGF- β (5 ng/ml) for 16 h. Cell extracts were made using either a non-ionic detergent lysis buffer followed by sonication (A) or boiling SDS containing lysis buffer (B). The 91 kDa (STAT-1 α) and 84 kDa (STAT-1 β) bands, representing alternatively spliced products of STAT-1 gene, are visualized. Membranes were probed with an anti-actin antibody to demonstrate equal protein loading. This is a representative gel that has been repeated at least three times.

experiments (between 24 and less than 48 h). In fact 48 h after transfection IGFBP-3 and GGG-IGFBP-3 levels were not significantly different from the levels found at 24 h (respectively 49 ± 5 ng/ml IGFBP-3 and 56 ± 9 ng/ml GGG-IGFBP-3). Levels of IGFBP-3 and GGG-IGFBP-3 expressed in the media of transfected cells are in the same order of magnitude of the exogenous IGFBP-3 that we have used in our experiments (2 nM equals 58 ng/ml). In the media of untransfected cells or cells transfected with empty vector, IGFBP-3 was undetected even after TGF- β treatment, confirming that cells do not express IGFBP-3 (Spagnoli *et al.* 2001).

TGF- β inhibits STAT-1 activation and p21 expression induced by IGFBP-3

Next we addressed whether the antagonistic effects of TGF- β on IGFBP-3 antiproliferative action correlated with an inhibition of STAT-1 signaling and p21 expression. As shown in Fig. 5, TGF- β treatment resulted in a decrease of STAT-1 phosphorylation induced by GGG-IGFBP-3 to the level observed in the control cells (untransfected or transfected with empty vector). TGF- β had similar effects on cells transfected with IGFBP-3 (data not shown). We also examined the

Figure 6 TGF- β inhibits p21 expression induced by IGFBP-3 and GGG-IGFBP-3. (A) Cell lysates were obtained from MCCs transfected with either IGFBP-3 or GGG-IGFBP-3 (GGG) or empty vector or left untransfected (Untran) for 18 h followed by incubation with or without TGF- β for 6 h. Cell lysates were subjected to WIB analysis for p21. Membranes were probed with an anti-p38 antibody to demonstrate equal protein loading. This is a representative gel that has been repeated at least three times. (B) MCCs were transfected with GGG-IGFBP-3 (GGG) or empty vector, treated with TGF- β (5 ng/ml) and subjected to immunofluorescence for p21.

effects of TGF- β on STAT-1 phosphorylation induced by GGG-IGFBP-3 using a harsher condition to obtain cell extracts (SDS lysis) (Fig. 5B) and found that results were similar to a gentler method to obtain cell extracts (non-ionic detergent followed by sonication) (Fig. 5A). Furthermore, TGF- β decreased p21 expression upregulated by the GGG mutant and IGFBP-3 to the level of the control (Fig. 6A). The apparent decreases of p21 expression in cells treated with TGF- β alone was not confirmed in repeated experiments and WIB analyses for p21. The effect of TGF- β on IGFBP-3-mediated p21 expression was confirmed by immunofluorescence. As shown in Fig. 6B, in cells transfected with GGG-IGFBP-3 an increase of cells positive for the p21 nuclear signal was noted compared with control cells (transfected with empty vector) ($32 \pm 6\%$ positive cells in GGG-IGFBP-3 vs $5 \pm 2\%$ in control; $P < 0.01$), whereas, very few cells with a positive p21 signal were seen in the nuclei of cells transfected with GGG-IGFBP-3 and treated with TGF- β (Fig. 6B) ($4 \pm 1\%$; $P < 0.01$ vs GGG-IGFBP-3-only transfected cells).

Negative cross-talk between TGF- β and IGFBP-3 signaling in MCC proliferation

Smad-mediated signaling is not required for the inhibitory action of TGF- β on the IGFBP-3 antiproliferative effect

To characterize the TGF- β pathway(s) that modulate its effect on the IGFBP-3 growth response and signaling, we stably expressed in MCCs a truncated T β RII that lacks most of the cytoplasmic domain including the kinase domain (Chen *et al.* 1993). This truncated T β RII is still able to bind TGF- β , it interacts with type I receptor, and it acts as a dominant negative mutation in cell culture and in transgenic mice, but lacks the Smad-signaling pathway (Chen *et al.* 1993, Serra *et al.* 1997). Expression of the DNT β RII in MCCs resulted in a functional loss of Smad signaling as indicated by the fact that the ability of TGF- β to induce Smad-2 phosphorylation was impaired (Fig. 7A). Next we examined the effect of DNT β RII expression in the TGF- β -dependent Smad-mediated transcriptional activity using the p(CAGA)12 Lux reporter construct in a dual-luciferase assay. The p(CAGA)12 Lux is a reporter construct containing 12 Smad-2–3 complex DNA-binding domains repeated in tandem (Dennler *et al.* 1998). As shown in Fig. 7B, TGF- β could induce transcription of p(CAGA)12 Lux reporter construct in MCCs expressing pBabe (control) but its ability to do so was impaired in MCC-DNT β RII. To assess stability of MCC-DNT β RII expression, p(CAGA)12 Lux luciferase reporter assays were performed every 3–4 weeks. As shown in Fig. 7B, the insensitivity of MCC-DNT β RII to TGF- β Smad-mediated transcriptional activity was persistent in cells expanded for up to 4 months. All the following experiments were performed using cells, transduced with MCC-DNT β RII or with the empty vector pBabe, that were not expanded for more than 2 months. In order to determine whether expression of DNT β RII perturbed BMP signaling, we examined its effect on BMP6-induced phosphorylation of Smad-1. As shown in Fig. 7C, in MCC-DNT β RII the Smad-1 phosphorylation induced by BMP6 was intact, indicating that DNT β RII does not interact with BMP type I receptor and in MCC-DNT β RII the BMP signaling is preserved.

We next examined the repercussions of DNT β RII expression in the TGF- β effect on IGFBP-3 antiproliferative action. We found that in MCC-DNT β RII, although it did not induce the Smad-mediated signaling, TGF- β persistently antagonized the IGFBP-3 growth inhibitory action (Fig. 8). These results indicate that Smad signaling is not required for the antagonistic effect of TGF- β on IGFBP-3-mediated MCC growth inhibition.

Role of the ERK pathway in the antagonistic effect of TGF- β on IGFBP-3 signaling and growth inhibition

To determine whether signaling pathways other than the Smad pathway were involved in the antagonistic effect

Figure 7 Expression of DNT β RII in MCCs impairs Smad-2 phosphorylation and Smad-mediated transcriptional activity induced by TGF- β but the BMP–Smad-1 signaling is preserved. (A) cell lysates were obtained from three clones of MCCs expressing DNT β RII and from control MCCs expressing pBabe alone. MCC-DNT β RII and control MCCs were treated with or without TGF- β (5 ng/ml) for 24 h. Cell lysates were subjected to WB analysis for phosphorylated Smad-2. Membranes were rehybridized with an anti-actin antibody to demonstrate equal protein loading. (B) MCC-DNT β RII (expanded for 1, 3 and 4 months) and control MCCs expressing pBabe 24 h after transfection with p(CAGA)12 Lux and pCMV-R1, were incubated with or without TGF- β (5 ng/ml) for 24 h. Firefly luciferase (Luc) and *renilla reniformis* (R1 Luc) activities in cell lysates were determined using a dual luciferase reporter assay system. Luc activity was normalized to R1 Luc. Results are expressed as percentage of the control (cells not treated with TGF- β), which was given an arbitrary value of 100%.

^a $P < 0.05$ vs control; $n = 3$. (C) cell lysates were obtained from MCC-DNT β RII and from control parental MCCs treated with or without BMP-6 (300 ng/ml) for 1 h and, were subjected to WB analysis for phosphorylated Smad-1. To verify equal loading blots were probed with an antibody directed against actin. This is a representative gel that has been repeated at least three times.

of TGF- β on IGFBP-3 signaling and growth inhibition we examined the ERK pathway, which has been reported to be induced by TGF- β in other cell systems including chondrocytes (Frey & Mulder 1997, Miyazaki *et al.* 2000, Watanabe *et al.* 2001). As shown in Fig. 9A, we found that TGF- β induced ERK1/2 phosphorylation in MCCs with maximal effect after 20 min. Furthermore, we found that TGF- β was still capable of inducing activation of ERK1/2 in MCC-DNT β RII

Figure 8 TGF-β persistently antagonized the growth inhibitory effect of IGFBP-3 in DNTβRII. MCC-DNTβRII and MCCs transduced with empty pBabe were cultured for 4 days, changed to serum-free medium for 4 h and then incubated for an additional 18 h with [³H]thymidine, IGFBP-3 (2 nM), des-IGF-I (15 nM) with or without TGF-β (5 ng/ml). Incorporation of [³H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. Results are expressed as percentage of control untreated cells which was given an arbitrary value of 100%; [³H]thymidine incorporation in controls was respectively 690±80 c.p.m. (pBabe) and 780±115 c.p.m. (DNTβRII). ^a*P*<0.05 vs des-IGF-I; ^b*P*<0.05 vs des-IGF-I+IGFBP-3; *n*=12.

(Fig. 9B). Pre-incubation of cells with the MEK inhibitor, UO126, inhibited ERK1/2 phosphorylation induced by TGF-β in the MCC-DNTβRII expressing cells, as well as in the pBabe-transduced and parental cells (Fig. 9B).

We next examined the functional role of the ERK pathway in the antagonistic effect of TGF-β on IGFBP-3 antiproliferative action and STAT-1 signaling. Pre-incubation of MCCs with UO126 blocked the TGF-β effect on IGFBP-3 growth inhibitory action in the presence (Fig. 10A) as well as in the absence of des-IGF-I (Fig. 10B) as determined by [³H]thymidine incorporation (Fig. 10A) and cell counting (Fig. 10B). Although UO126 blocked in part the mitogenic action of des-IGF-I, IGFBP-3 was still capable of inhibiting the des-IGF-I effect and UO126 completely blocked the antagonistic effect of TGF-β on IGFBP-3 (Fig. 10A). Similar effects were found when MCCs were transfected with GGG-IGFBP-3. In MCCs transfected with GGG-IGFBP-3, pre-incubation with UO126 was capable of blocking the antagonistic effect of TGF-β on GGG-IGFBP-3 growth inhibitory action (GGG-IGFBP-3+TGF-β: 105±6% of control transfected with empty vector; GGG-IGFBP-3: 66±7% vs GGG-IGFBP-3+UO126+TGF-β: 68±8%, *P*=NS; *n*=6) as determined by cell counting. UO126 alone had no effect on cell proliferation. Furthermore, pre-incubation of the MCCs with UO126 blocked the ability of TGF-β to inhibit STAT-1 phosphorylation induced by IGFBP-3 (Fig. 11). Taken together, these data indicate that the ERK signal-

Figure 9 TGF-β is capable of inducing ERK1/2 phosphorylation in parental MCCs (A) as well as in MCC-DNTβRII (B); the MEK inhibitor UO126 inhibits TGF-β-mediated ERK1/2 phosphorylation. (A) Cell lysates were obtained from MCCs cultured for 2 days, changed to serum-free medium for 12 h, washed and treated with or without TGF-β (5 ng/ml) respectively for 10 min, 20 min and 3 h. (B) Cell lysates were obtained from MCC-DNTβRII, parental MCCs and MCCs transduced with empty pBabe cultured for 2 days, changed to serum-free medium for 12 h, washed, treated with or without UO126 (10 μM) for 2 h, and treated with or without TGF-β (5 ng/ml) for 20 min. Cell lysates were subjected to WB analysis using an antibody directed against phosphorylated ERK1/2 and antibody directed against the un-phosphorylated form (total) of ERK1/2. This is a representative gel that has been repeated at least three times.

ing mediates the TGF-β inhibitory effects of IGFBP-3 on MCC growth and the STAT-1 pathway.

Discussion

In the present study, we have identified a cross-talk between IGFBP-3 and TGF-β signaling pathways that regulates MCC proliferation. It was determined that TGF-β, through activation of the ERK pathway, inhibits STAT-1 signaling and in turn p21 expression and growth arrest induced by IGFBP-3. Our data provide critical information into the molecular mechanisms by which these two growth factors control MCC biology and in turn the cartilage formation process.

We found that TGF-β antagonized the antiproliferative effect of IGFBP-3 in MCCs expressing IGFBP-3

Figure 10 In MCCs, ERK signaling mediates the antagonistic effects of TGF- β on the IGFBP-3 growth inhibitory action. (A) MCCs were cultured for 4 days, changed to serum-free medium for 4 h, treated with or without UO126 (10 μ M) for 2 h and then incubated for an additional 18 h with [3 H]thymidine, IGFBP-3 (2 nM) or des-IGF-I (15 nM) with or without TGF- β (5 ng/ml). Incorporation of [3 H]thymidine into DNA was determined as uptake of radioactivity in trichloroacetic acid-precipitable material. Results are expressed as percentage of the control (cells not treated with peptides), which was given an arbitrary value of 100%; [3 H]thymidine incorporation in the control was 650 ± 90 c.p.m. ^a $P < 0.05$ vs des-IGF-I; ^b $P < 0.05$ vs des-IGF-I+IGFBP-3; ^c $P < 0.05$ vs des-IGF-I+UO126; $n=6$. (B) Cells at day 4 of culture were changed to serum-free medium with or without UO126 (10 μ M) for 2 h and then incubated with or without IGFBP-3 (2 nM) in the presence or absence of TGF- β (5 ng/ml). Results are expressed as percentage of control untreated cells that had a total cell number of $145 \times 10^4 \pm 4$ /well. Viable floating and attached cells (after trypsinization) were counted on a hemocytometer after Trypan Blue exclusion. ^d $P < 0.05$ vs IGFBP-3; ^e $P < 0.05$ vs IGFBP-3+TGF- β ; $n=6$.

and GGG-IGFBP-3 mutant as well as in MCCs treated with exogenous IGFBP-3. TGF- β can act as both a growth suppressor and a growth promoter. In most epithelial cells TGF- β elicits growth inhibitory effects while in mesenchymal cell types it stimulates cell proliferation (Derynck & Zhang 2003). The canonical model of TGF- β -induced signaling is a linear pathway from the T β RII and T β RI heterodimer activation to the formation of activated Smad complexes that results in gene transcription (Derynck & Zhang 2003). Indeed, data are now rapidly accumulating to implicate a variety of TGF- β -induced Smad-independent pathways

Figure 11 In MCCs, ERK signaling mediates the TGF- β inhibition of STAT-1 phosphorylation induced by IGFBP-3. Cell lysates were obtained from MCCs transfected with IGFBP-3 or empty vector for 24 h, incubated with or without UO126 (10 μ M) for 2 h and treated with or without TGF- β (5 ng/ml) for 16 h. Cell lysates were subjected to WB analysis for phosphorylated STAT-1 and total STAT-1. Membranes were probed with an anti-actin antibody to demonstrate equal protein loading. Optical density measurements of phosphorylated STAT-1 bands are normalized for those of actin. ^a $P < 0.05$ IGFBP-3+TGF- β vs IGFBP-3 alone; ^b $P < 0.05$ UO126+IGFBP-3+TGF- β vs IGFBP-3+TGF- β ; $n=3$.

(Derynck & Zhang 2003). It is likely that TGF- β activation of different signaling pathways is cell type specific and may be important for the paradoxically opposite TGF- β responses on growth. In our study we have found that TGF- β antagonizes the growth inhibitory action of IGFBP-3 in MCCs treated or expressing IGFBP-3. Furthermore we found that TGF- β antagonized STAT-1 activation induced by IGFBP-3 and GGG-IGFBP-3. Our studies have clearly demonstrated that these TGF- β responses are Smad-independent as indicated by the fact that they were not inhibited in MCC-DNT β RII, although in these cells, TGF- β lacked the activation of the Smad signaling and transcriptional activity. On the other hand, we have found that TGF- β antagonizes IGFBP-3 effects and STAT-1 phosphorylation activating the ERK pathway. The direct links of the ERK pathway activation by TGF- β to the receptors are still unknown. The rapid activation that we have observed, and the fact that it was still activated in the MCC-DNT β RII, clearly indicate independence from a Smad-dependent transcription response that has been reported in other studies

(Massague 2000). Dumont *et al.* (2003) have hypothesized that different signaling pathways require different thresholds of T β R activation and the level of expression of dominant negative T β RII may result in a differential blockage of specific signaling pathways. Alternatively, other not well characterized T β Rs can be hypothesized to induce Smad-independent pathways. IGFBP-3 has been shown to bind T β RV/LRP-1 and to compete with TGF- β for this receptor binding (Leal *et al.* 1997, Huang *et al.* 2003). It may be possible that in MCCs, TGF- β antagonizes IGFBP-3 antiproliferative action competing for the T β RV/LRP-1. However, we have previously reported that in MCCs, IGFBP-3 binds to a cell membrane protein of ~ 21 kDa while the T β RV/LRP-1 is ~ 400 kDa (Leal *et al.* 1997). Few studies have reported the characterization of TGF- β signaling in chondrogenic cell systems. In ATDC5 chondrogenic cells, Watanabe *et al.* (2001) have reported that the TGF- β activation of Smad-dependent and -independent pathways (including ERK) is related to the expression of aggrecan, a marker of chondrogenic differentiation. In our study, we have identified in chondroprogenitors a TGF- β -activated Smad-independent signaling pathway that mediates a regulatory cell growth response through negative cross-talk with the STAT-1 pathway. We have previously reported that IGFBP-3 growth inhibitory action on MCCs causes a decrease in the chondrocyte differentiation rate (Longobardi *et al.* 2003). In the light of the present results, we hypothesize that in MCCs, activation of the ERK pathway may represent a mechanism by which TGF- β suppresses the growth inhibitory IGFBP-3 and preserves the number of progenitors in favor of the differentiation process. It has been largely reported that TGF- β induces commitment of bone marrow derived mesenchymal stem cells to MCCs and stimulates chondrocyte differentiation (Pittenger *et al.* 1999, Yoo *et al.* 2000). We hypothesize that the maintenance of the MCC pool from growth inhibitory factors may represent an important Smad-independent mechanism through which TGF- β exerts its chondroinductive action. Our hypothesis fits with the observation that TGF- β induces the commitment of hematopoietic precursors to the osteoclastic lineage inhibiting the JAK/STAT signaling (Fox *et al.* 2003). In MCCs, des-IGF-I as well as TGF- β induced MAPK/ERK activation as indicated by the fact that the MEK inhibitor blocked in part the mitogenic response of des-IGF-I. However, TGF- β had no mitogenic effects on MCCs while it was capable of antagonizing the growth inhibitory response of IGFBP-3 in the presence or absence of des-IGF-I. We hypothesize that the activation of the MAP/ERK pathway requires additional distinct mediators downstream to ERK1/2 that determine the mitogenic response of IGF-I or the antagonistic action of TGF- β on the STAT-1-mediated antiproliferative response.

In our previous studies, we have characterized STAT-1 as a functional signaling pathway component for the apoptotic action of IGFBP-3 in MCCs (Spagnoli *et al.* 2002, Longobardi *et al.* 2003). We now report that STAT-1 activation is required for the IGFBP-3 antiproliferative action in MCCs and expression of the cell cycle inhibitor p21. STAT-1 recognizes and binds to the palindromic sequence TTCNNGAA (Horvath *et al.* 1995). Such sequences have been identified in the p21 promoter and designed SIE-1, SIE-2 and SIE-3 respectively (Chin *et al.* 1996). In the chondrogenesis process, STAT-1 mediates the antiproliferative and apoptotic effects of fibroblast growth factor receptor-3 (FGFR-3) and an activating mutation of the FGFR-3, such as in thanatophoric dysplasia, is associated with an increase of STAT-1 phosphorylation, p21 expression and cell growth arrest (Su *et al.* 1997). MCC growth arrest and apoptosis play a critical role during the skeletal development process, by regulating MCC number; they determine, for example, the shape of the limbs and define the number of digits (Shum & Nuckolls 2002). We noted that treatment with STAT-1 morpholino antisense oligonucleotide increased des-IGF-I mitogenic response; we hypothesize that STAT-1 may have an inhibitory effect on the IGF-I proliferative effect even in cells untreated with IGFBP-3. Further studies are needed to determine how STAT-1 inhibits IGF-I action independently from IGFBP-3 activation.

We have found that exogenous recombinant non-glycosylated IGFBP-3 from *E. coli* and IGFBP-3 expressed by transfected MCCs decreased cell number with similar effects. Our finding in MCCs differs from reports from previous studies that have shown that glycosylation status of IGFBP-3, although non-essential for IGF binding, influences cell surface binding and antiproliferative responses (Firth & Baxter 1999, Bagnall *et al.* 2003). Different cell surface binding sites and cell types may be responsible for these dissimilarities.

TGF- β effects on cell growth and apoptosis have been shown to correlate with the induction of IGFBP-3 and blockade of IGFBP-3 upregulation by antisense oligonucleotides abrogates the effect of TGF- β on cell growth (Oh *et al.* 1995a, Cohen *et al.* 2000). IGFBP-3 has been also shown to enhance Smad-2 and Smad-3 phosphorylation induced by TGF- β activation of T β RII, and to activate the PAI-1 promoter (Fanayan *et al.* 2000, 2002). Furthermore affinity labeling studies of T β Rs have showed that IGFBP-3 displaced binding of iodinated TGF- β to T β RII and stimulated Smad-2 phosphorylation (Kuemmerle *et al.* 2004). IGFBP-3 has been also shown to bind the latent TGF- β binding protein-1, although the functional significance of this binding remains unclear (Gui & Murphy 2003). In the present study, we have determined that in MCCs IGFBP-3 is not a downstream mediator of TGF- β since IGFBP-3 is not expressed in these cells even in the

presence of TGF- β ; furthermore we have found a novel functional interaction between a specific IGFBP-3 bioactivity and signaling and a TGF- β -induced pathway. In Hs578T breast cancer cell line, expression of a constitutively active Ras protein resulted in the unresponsiveness of the cells to the growth inhibitory effects of IGFBP-3, indicating that activation of MAPK/ERK can inhibit the IGFBP-3 growth inhibitory action (Martin & Baxter 1999). In our study we have identified the signaling mediators of this negative cross-talking.

Several adult tissues including the bone marrow contain mesenchymal stem cells that can become committed progenitors that differentiate into different cell types (Pittenger *et al.* 1999, 2000.). TGF- β is essential to induce the commitment of mesenchymal stem cells into MCCs (Cassiede *et al.* 1996, Mackay *et al.* 1998, Yoo *et al.* 1998, Pittenger *et al.* 1999, 2000, Sekiya *et al.* 2002). We have identified a cross-talk between IGFBP-3 and TGF- β signaling that is critical for regulating MCC growth. Understanding the early growth and differentiation signals of TGF- β and IGFBP-3 in MCCs provides critical information that will contribute to eventual success in the use of MCCs to repair damaged cartilage.

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