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TGF-β Type II Receptor/MCP-5 Axis: at the Crossroad between Joint and Growth Plate Development

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SUMMARY

Despite its clinical significance, the mechanisms of joint morphogenesis are still elusive. Here, we show by combining laser-capture microdissection for RNA sampling with microarray analysis, that the setting in which joint-forming interzone cells develop is distinct from adjacent growth plate chondrocytes and is characterized by down-regulation of chemokines, such as monocyte-chemoattractant protein-5 (MCP-5). Using *in-vivo, ex-vivo* and *in-vitro* approaches, we showed that low levels of interzone-MCP-5 are essential for joint formation and contribute to proper growth plate organization. Mice lacking the TGF-β-type-II-receptor (TβRII) in their limbs (*Tgfbr2*^{Prx1KO}), which lack joint development and fail chondrocyte hypertrophy, showed up-regulation of interzone-MCP-5. *In-vivo* and *ex-vivo* blockade of the sole MCP-5 receptor, CCR2, in *Tgfbr2*^{Prx1KO} led to rescue of joint formation and growth plate maturation; while in control mice determined an acceleration of endochondral growth plate mineralization. Taken together, we characterized the TβRII/MCP-5 axis as an essential crossroad for joint development and endochondral growth.

INTRODUCTION

Synovial joints, which separate adjacent skeletal elements from each other, are important signaling centers that control chondrocyte maturation within the endochondral template (Francis-West et al., 1999). Although their evolutionary, physiological and clinical significance there is limited information on the mechanisms and signaling molecules that lead to joint morphogenesis. Morphologically, the position of each future joint is first

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SUPPLEMENTAL INFORMATIONS

Supplemental information include four Supplemental Figures and Methods and can be found with this article online at .

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delimited by the interzone development, consisting of a region of compact mesenchymal cells between two adjacent growth plates. Interzone cells adopt a nonchondrogenic phenotype, as indicated by the loss of chondrogenic markers such as *Sox-9* and *Collagen 2*, and instead express new sets of genes including *Gdf5, Wnt9a* and *Wnt4* (Hartmann and Tabin, 2001; Pacifici et al., 2006; Storm et al., 1994). There is limited information of the entry mechanism leading to interzone initiation, the gene expression environment in which interzone cells emerge and distinct themselves from adjacent growth plate chondrocytes and how joint morphogenic molecules are interconnected within the joint and with the other skeletogenic components of the developing bone (Dowthwaite et al., 2004; Koyama et al., 2008). Articular cartilage is avascular, and this limits its reparative capacity such that arthritis is the single largest cause of disability in the adult population (CDC, January 12,2007). A greater understanding of joint formation can provide critical insight on the pathogenesis of joint degeneration and to develop novel reparative strategies to treat arthritis.

In adult articular cartilage, inflammatory cytokines critically target the joints causing their degeneration. Monocyte chemoattractant protein-5 (MCP-5, a.k.a. CCL12) belongs to the family of CC chemokines and is the structural and functional homologue of human MCP-1 (Sarafi et al., 1997). MCP-5 binds exclusively to CC-chemokine receptor 2 (CCR2) that is the primary receptor for human MCP-1 (Sarafi et al., 1997; Tsou et al., 2007). MCP-5, as well as MCP-1, is a potent chemoattractant for circulating monocytes to sites of inflammation where they become cytokine-secreting macrophages (Charo and Ransohoff, 2006; Mack et al., 2001; Sarafi et al., 1997). CCR-2 has received major clinical attention in arthritis, as it has been regarded as a therapeutic target to be blocked (Charo and Ransohoff, 2006; Quinones et al., 2005). An increased number of CCR2+ cells is found in the inflamed joints of patients with different forms of arthritis (Nanki et al., 2001; Ruth et al., 2001). CCR-2 is also increased in rodent models of arthritis, although levels change during the different phases of the disease (Szekanecz et al., 2000; Thornton et al., 1999). In addition, increased levels of MCP-1 have been found in patients with osteoarthritis (OA) and rheumatoid arthritis (RA) (De Benedetti et al., 1999; Hayashida et al., 2001). These lines of evidence have led to the development of several antagonists of and antibodies against CCR-2 and MCP-1 (Feria and Diaz-Gonzalez, 2006; Quinones et al., 2005). While the role of inflammatory cytokines such as MCP-5 in the arthritic process appears indisputable, their role in joint development has never been evaluated. A finding of cytokine involvement in joint formation would determine a shift in our current view of joint cells not anymore as passive victims of the disruptive force of cytokines but as active participants in maintaining under control the cytokine effects and therefore joint integrity.

Genetic manipulation of the TGF-β system has revealed critical roles in both joint development and skeletogenesis (Ito et al., 2003; Serra et al., 1997; Spagnoli et al., 2007). TGF-β type II receptor (TβRII) is the only TβR that is capable of binding all the TGF-β isoforms resulting in functional signaling. Mice that are germline-null for the *Tgfbr2* exhibit early embryonic lethality making it impossible to evaluate the role of TGF-β signaling in skeletogenesis (Oshima et al., 1996). In previous studies, by generating the *Tgfbr2-GFP-β-GEO-BAC* imaging (β-Gal and GFP) reporter mouse, we showed that TβRII is highly and specifically expressed in developing joints (Spagnoli et al., 2007). Furthermore, by generating a mouse in which the TβRII signaling is conditionally inactivated in limb buds starting at E9.5 ($Tgfbr2^{Prx1KO}$), we demonstrated that the lack of TGF-β signaling was associated with complete absence of interphalangeal joints as well as with impaired growth plate development (Spagnoli et al., 2007).

In this study, we have found that the joint interzone emerges within the developing limb mesenchyme expressing a gene-expression pattern that is distinct from the adjacent

developing growth plate chondrocytes and is characterized by a down-regulation of chemokines, in particular MCP-5. MCP-5 down-regulation is impaired in the *Tgfbr2*^{Prx1KO} mutants that lack joint development. By using *in-vivo, ex-vivo*, and *in-vitro* approaches we demonstrated that an aberrant presence of MCP-5 within the developing limb is sufficient to impair joint formation and within the developing growth plate, to alter the rate of progression of pre-hypertrophic chondrocytes to hypertrophy and mineralization. The *in-vivo* and *ex-vivo* blockade of the CCR2 signaling (MCP-5 sole receptor) in *Tgfbr2*^{Prx1KO} mice, led to a rescue of their phenotype, restoring joint cavitation and expression of joint morphogenic genes while promoting the proper growth plate maturation. Our findings by demonstrating the essential role of the TβRII/MCP-5 axis in the interplay between the developing articular cartilage and the growth plate, provide evidence of a function for a cytokine during skeletal development and open a novel prospective for evaluating and therapeutically targeting the TβRII/MCP-5 axis in OA.

RESULTS

MCP-5 is differentially expressed in interzone cells and adjacent chondrocytes, and such expression pattern is impaired in *Tgfbr2*^{Prx1KO} mice

In order to investigate the distinct gene expression profile where interzone cells emerge between the forming growth plates, we obtained RNA samples from E14.5 interzone cells and adjacent growth plate chondrocytes of *Tgfr2*$^{flox/flox}$ (normal control, NC) and *Tgfbr2*^{Prx1KO} digits using laser capturing microdissection (LCM). Figure 1A depicts the regions that were subjected to LCM; in the *Tgfbr2*^{Prx1KO}, the presumptive interzone was identified as a bending between two growth plates. After verifying RNA integrity (see Methods), specimens were subjected to microarray analysis. Normalized data were expressed as fold changes of a candidate gene in the interzone sample compared to the expression found in the sample from adjacent growth plate chondrocytes. As initial validation step, we examined the expression of genes that we had previously found to be specifically up-regulated in the interzone compared to adjacent growth plate chondrocytes by in situ hybridization (ISH), immunohistochemistry (IHC) and RT-PCR (Spagnoli et al., 2007). Microarray analysis confirmed that in the NC interzone, *Jagged1*, *Tgfbr2*, *Pdgfrb* and *Wnt9a* were up-regulated respectively 243.9 fold, 12.29 fold, 66.1 fold and 3.43 fold, compared to adjacent growth plate chondrocytes, while such regulation was impaired in the *Tgfbr2*^{Prx1KO} (respectively *Jagged1,* 1.32 fold; *Tgfbr2,* 1.27 fold; *Pdgfrb,* 0.97 fold; *Wnt9a*, 0.99 fold). Next, we performed a pathway analysis using PANTHER (Protein Analysis Through Evolutionary Relationships) Classification System (Celera) to identify functional categories in the joint-forming interzone cells compared to adjacent growth plate chondrocytes. The PANTHER is a unique resource that classifies genes into canonical pathways to predict function (Thomas et al., 2003). As shown in Figure 1B, we found that in the NC interzone cells the “Inflammation mediated by chemokine and cytokine signaling pathway” was extensively down-regulated compared to adjacent chondrocytes; of a total of 337 genes classified in this pathway, 227 were down-regulated more than 2 fold. Within the genes down-regulated, *Mcp-5* was down-regulated ~800 fold and its unique receptor, *Ccr2*, ~11 fold, while such differential expression was impaired in the *Tgfbr2*^{Prx1KO} (Figure 1C). As further validation of the microarrays data, we performed *q*RT-PCR on RNA samples obtained by LCM from interzone and adjacent growth plate chondrocytes from E14.5 NC and *Tgfbr2*^{Prx1KO} as well as ISH and IHC analysis on E14.5 limbs either from NC or *Tgfbr2*^{Prx1KO} mice. As shown in Figure 1D, *q*RT-PCR analyses showed that the expression of *Mcp-5* in NC interzone was more than 900 fold lower than its expression in NC chondrocytes. In contrast, the *Tgfbr2*^{Prx1KO} presumptive interzone expressed levels of MCP-5 that were higher than the NC interzone (more than 130 fold) and were not significantly different from levels found in *Tgfbr2*^{Prx1KO} growth plate chondrocytes. ISH

and IHC studies corroborated these findings (Figure 1E and 1F). Our data show that the joint interzone cells demonstrate a down-regulation of MCP-5 mRNA and protein levels compared to adjacent developing growth plate cells and that such down-regulation is abolished in *Tgfbr2*^{Prx1KO} developing limbs.

To determine MCP-5 expression through joint development we extended IHC analyses of MCP-5 in E12.5; E13.5, E14.5; E16.5 and E18.5 NC (Figure S1). We found that MCP-5 expression started at E13.5 within the developing digits. By E14.5, MCP-5 expression was restricted to the growth plate chondrocytes while was not expressed in the joint at any stage.

Aberrant release of MCP-5 within normal developing limbs alters joint formation, while blockade of CCR2 signaling is able to rescue the joint phenotype of *Tgfbr2*^{Prx1KO} mutants

In order to determine a role for the restricted levels of MCP-5 within the interzone, we used an *ex-vivo* approach in which hydrophobically-modified glycol chitosan (HGC)-nanoparticles loaded with MCP-5 protein were microinjected in the interzone of E14.5 WT dissected autopods (Figure 2A) to lead to an aberrant sustained release of MCP-5 within the developing joints. HGC-nanoparticles are biocompatible and non immunogenic and consist in hydrophilic shells and hydrophobic cores that bind proteins (Hirano, 1999; Li et al., 2010). We have developed a micro-injection unit that allows for precise injection localization and volume accuracy delivery (nanoliters) within distinct regions of developing autopods and reported that proteins are slowly released from HGC without diffusing (~90% in 48h), are biologically active and HGC do not interfere with skeletogenesis, including joint development (Li et al., 2010). Based on these pharmacokinetics studies, HGC-MCP-5 (delivering 1.5 ng of MCP-5 protein) was injected at the joint sites of E14.5 dissected autopods that were cultured for 2 days; un-injected joints from the same embryo were used as control (Figure 2A). IHC for MCP-5 showed that protein was sustainably delivered (after 48 h) within the interzone; Figure 2A shows that MCP-5 was detectable in the interphalangeal joint sections from the 2nd digit implanted with HCG-MCP-5, while it was undetectable in the uninjected control joint interzone of the 5th digit, in which endogenous MCP-5 was restricted to the adjacent growth plate chondrocytes. Compared to un-injected control, the aberrant sustained delivery of MCP-5 from HCG-MCP-5 implant within the developing interzone abolished *Gdf5* expression and altered the interzone *Collagen2* expression pattern. In fact, the interzone of implanted HCG-MCP-5 digits lost the characteristic chondrocyte segmentation and *Collagen2* expressing cells appeared continuously distributed along the entire digit (Figure 2B). Increasing doses (0.75 to 1.5 ng) of implanted MCP-5 within the interzone resulted in gradual impairing of *Gdf5* and *Collagen2* patterns. Similar effects on *Gdf5* and *Collagen2* expressions were found among the 1.5 ng to the 6 ng doses, therefore 1.5 ng was used in all further experiments (data not shown). At least 3 embryos were implanted with each dose and at least 3 sections from each embryo were subjected to ISH analyses.

To determine whether the blockade of CCR2 (the sole MCP5 receptor) would restore interphalangeal joint formation in *Tgfbr2*^{Prx1KO} developing limbs, we used *ex-vivo* as wells as *in-vivo* approaches. We first evaluated CCR2 expression by IHC at different joint developmental time points (E12.5; E13.5, E14.5 and E18.5). CCR-2 expression was first detected within the developing digit at E13.5 and by E14.5 expression was found within the developing interzone and growth plates. This expression pattern was maintained up to E18.5 (Figure S2). To block the CCR2 signaling, we used the HCG-based *ex-vivo* autopod culture technique; in particular, we implanted HCG-MC-21 nanoparticles (delivering 1.5ng of MC-21 antibody) in the presumptive interzone of E14.5 *Tgfbr2*^{Prx1KO} mutants as well as NC littermates. The MC-21 is an anti-mouse CCR-2 monoclonal antibody that has been shown to block the murine CCR-2 with high affinity *in vivo* as well as *in vitro* (Bruhl et al., 2004; Mack et al., 2001). Histological and ISH studies demonstrated that blockade of

MCP-5 signaling was able to restore the joint cavitation and *Gdf5* expression abnormally missing in the mutants. In addition, while in mutant limbs *Sox-9* and *Collagen2* expressing cells were continuously distributed along the digit, in MC-21-implanted mutants they appeared to be restricted to the growth plate chondrocytes, adopting an expression pattern that was similar to controls (Figure 3A). For the *in-vivo* studies, pregnant females potentially carrying *Tgfbr2*^{Prx1KO} embryos were treated orally with 4mg/Kg/day of RS-504393 (a selective CCR2 inhibitor) starting at E11.5 and until either E14.5 or E18.5, when mice were sacrificed. The two time points allowed to determine the need of CCR-2 signaling in joint formation (E14.5) as well as its persistent effect at a later stage (E18.5). As shown in Figure 3B, blockade of MCP-5 signaling in *Tgfbr2*^{Prx1KO} embryos *in-vivo* was sufficient to restore *Gdf5* expression and *Collagen2* segmentation in E14.5 embryos. These effects were maintained in E18.5 *Tgfbr2*^{Prx1KO} embryos resulting in a well-defined joint space in between the growth plates (Figure 3C). Blockade of MCP-5 signaling in NC limbs, both *ex-vivo* and *in-vivo*, did not affect joint maturation and chondrocyte segmentation (Figure 3A–B). Our results indicate that a down-regulation of MCP-5 in developing interzone is needed for proper joint formation, *Gdf5* and *Collagen2* expressions and reveal the critical role for TGF-β signaling in controlling restrained levels of MCP-5 within the developing joints.

MCP-5 acts as a chondrogenic factor on isolated interzone cells. TGF-β inhibits MCP-5 expression on isolated TβRII^{+} cells

We have recently reported that within the interphalangeal joints TβRII expressing cells localize in specific niches such as the ventral and dorsal sites of the interzone (Figure S3) (T. Li, 2011). Reasoning that TβRII expressing cells control the developing environment of the interzone through paracrine and autocrine mechanisms, we relied on the microarray analysis as a source to identify additional genes that encode for membrane-associated proteins that are exclusively expressed in the interzone of NC but not in *Tgfbr2*^{Prx1KO} mice (i.e. *Jagged1* and *Pdgfrb*), to isolate interzone cells from E14.5 autopod mesenchyme. An immunomagnetic selection system was used to isolate Jagged1, TβRII and PDGFRβ positive (Jag^{+}/TβRII^{+}/PDGFR^{+}) cells that accounted for ~1% of the total (~30×10^{6} total cells from 30 embryos). mRNA was collected immediately after cell separation from the Jag^{+}/TβRII^{+}/PDGFR^{+} cell fraction and subjected to RT-PCR. As shown in Figure 4A, Jag^{+}/TβRII^{+}/PDGFR^{+} cells expressed key joint markers, such as *Gdf5*, *Wnt9a*, *Sulf1* and *Collagen1*. With the purpose of understanding the effect of MCP-5 on interzone cells, we cultured the isolated Jag^{+}/TβRII^{+}/PDGFR^{+} fraction in micromass and treated them with or without MCP-5 (20 ng/ml) for 3 days. MCP-5 induced a chondrogenic-like phenotype in the cells that condensate in typical chondrogenic nodules (Figure 4B). In addition, MCP-5 treatment determined a marked decrease in the expressions of *Gdf5*, *Wnt9a* and *Sulf1*, while increasing *Collagen2*, compared with untreated cells (Figure 4C). These *in-vitro* data support our *ex-vivo* and *in-vivo* findings indicating that a down-regulation of MCP-5 is needed in interzone cells to allow a correct expression of key joint markers. The chondrogenic action of MCP-5 can be the consequence of several mechanisms ranging from proliferation to regulation of transcription of chondrogenic gene markers. Future investigations will be needed to dissect such mechanisms.

Hypothesizing that only the TGF-β signaling regulates MCP-5 expression we exclusively isolated TβRII expressing cells (TβRII^{+}) from E14.5 limb mesenchyme of *Tgfbr2-GFP-β-GEO-BAC* transgenic mice. *Tgfbr2-GFP-β-GEO-BAC* mice contain both GFP and LacZ as imaging reporters for *Tgfbr2* expression (Spagnoli et al., 2007). TβRII^{+} cells were FACS sorted using GFP as isolation marker and cultured in micromass for 18h with or without TGF-β (20ng/ml). *q*RT-PCR analyses showed that TGF-β significantly decreased MCP-5

expression compared to untreated controls (Figure 4D). These results support our hypothesis that TGF-β is needed to down-regulate MCP-5 expression.

Aberrant release of MCP-5 within the developing joints of WT mice alters the growth plate organization while blockade of CCR2 signaling in *Tgfbr2*^{Prx1KO} mutants restores the growth plate phenotype

Since lack of joint development in *Tgfbr2*^{Prx1KO} mice was associated with selective perturbative effects on the adjacent growth plate chondrogenesis with deregulated *Ihh* expression and blockage of pre-hypertrophic to hypertrophic chondrocyte progression (Spagnoli et al., 2007), we decided to investigate the contribution of MCP-5 to such derangements. ISH studies of *ex-vivo* limb cultures injected with HGC-MCP-5 showed that the aberrant release of MCP-5 within the interzone of WT mice impaired *Ihh* spatial organization, producing a diffuse pattern of expression along the growth plate, and significantly decreased *Collagen10* expression by hypertrophic chondrocytes (Figure 5). These results indicate that the aberrant presence of MCP-5 within the joint leads to a signal from the joints toward the adjacent growth plate that controls the chondrocyte maturation. It is important to remark that *ex-vivo* cultured limbs progress to chondrocyte maturation. As depicted in Figure S4, histological and ISH analyses showed that E14.5 limbs cultured *ex-vivo* for 2 days progress toward chondrocyte hypertrophy, as indicated by the increase in *Collagen10* expression when compared to E 14.5 uncultured limbs.

To analyze whether the elevated MCP-5 levels found in *Tgfbr2*^{Prx1KO} mutants contributed to the failure of hypertrophic chondrocyte maturation, we analyzed if blockade of MCP-5 signaling in the presumptive interzone of *Tgfbr2*^{Prx1KO} by both *ex-vivo* and *in-vivo* approaches, rescued the growth plate phenotype. Histological and ISH studies demonstrated that both *ex-vivo* HGC-MC21 implant and *in vivo* RS-504383 treatment in *Tgfbr2*^{Prx1KO} embryos rescued the *Ihh* spatial organization and restored *Collagen10* and *Collagen1* expressions to levels similar to the NC (Figure 6A–6B). Notably, in the *ex-vivo* as wells as the *in-vivo* studies, blockade of MCP-5 signaling in NC determined an increase in *Collagen10* and *Collagen1* expressions and a reduction in size of the primary ossification center that appeared to undergo to rapid mineralization (Figures 6A–B). These findings point out that MCP-5 plays an additional role in the growth plate maturation that in this case is independent from the TβRII signaling.

DISCUSSION

Our results demonstrate that within the joint interzone a down-regulation of MCP-5 is critical to drive proper joint and endochondral development and that TβRII signaling is needed in maintaining such down-regulation. Furthermore, we determined that the TβRII-independent expression of MCP-5 within the growth plate plays a role in chondrocyte maturation and hypertrophy. Taken together, these results lead us to propose the model showed in Figure 7. In this model we propose that a crosstalk between the forming interzone and the developing growth plate, mediated by MCP-5 through TβRII-dependent and TβRII-independent mechanisms, is critical for proper joint and skeletal development. Our findings represent a paradigm shift in the joint biology research field that presently perceives joints as the passive victims of inflammatory cytokines that cause their degeneration. Instead, our studies demonstrate that joints hold an active developmental mechanism that is operated by the TβRII signaling to tightly regulate the expression of cytokines such as MCP-5 and such mechanism is essential for joint morphogenesis.

TβRII/MCP-5 axis in joint development

Though no one disputes that interzone is needed for joint formation, there is limited information on the interzone joint-forming cell population and the role that these cells play in the process remains far from clear. The formation of the interzone is specified by an inductive signal in a restricted area of the chondrogenic blastema that leads to loss of Collagen2 expressing cells while joint marker (i.e. GDF-5) expressing cells emerge and segment the adjacent growth plates. It is unclear whether Collagen 2 expressing cells undergo to a selection process (cell death) or whether their transcriptome becomes programmed only toward joint marker expression (Khan et al., 2007). Combining LCM with gene-profiling analyses, we found that the progenitor joint-forming interzone cells develop in a setting that is distinct from the setting of the adjacent growth plate chondrocytes and characterized by a down-regulation of specific cytokines/chemokines, such as MCP-5. Our ex-*vivo* studies demonstrate that this differentially controlled expression of MCP-5 in the interzone versus the adjacent chondrocytes is essential for the proper process of interzone formation leading to expression of key joint markers (such as *Gdf5*), as well as the correct segmentation of adjacent growth plates. Consistently, the aberrant release of MCP-5 within the interzone interfered with its initiation and halted the segmentation process. Furthermore, in our *in-vitro* experiments we demonstrated a direct effect of MCP-5 on isolated interzone cells, characterized by a marked decrease in the expression of key joint markers (*Gdf5*, *Wnt9a* and *Sulf1*) accompanied with a stimulation of *Collagen 2* expression and emergence of a chondrogenic phenotype. Our data support the hypothesis that interzone cells are capable to be committed to either joint forming cells or chondrocytes and MCP-5 plays a critical role in directing their differentiative program.

We have previously reported that the joints of $Tgfbr2^{Prx1KO}$ mutants appear to be arrested in their development about the time when interzone starts to develop; differentiated chondrocytes extend with a continuous pattern across the phalanges lacking interzone cell condensation and *Jagged-1*, *Noggin* and *Gdf-5* expressions (Spagnoli et al., 2007). In the present study, we found that MCP-5 down-regulation was completely impaired in the interzone of $Tgfbr2^{Prx1KO}$ mice indicating that the TβRII signaling is needed to control MCP-5 expression and suggesting that the failure of the mutant's joint formation may be due to the aberrant expression of MCP-5. Our *ex-vivo* and *in-vivo* studies sustained this hypothesis showing that in $Tgfbr2^{Prx1KO}$ mice, the inhibition of MCP-5 activity by blocking its receptor CCR2 restored joint formation and in particular *Gdf-5* expression and chondrocyte segmentation. Furthermore, a direct inhibitory action of TGF-β on MCP-5 expression was found in our *in-vitro* studies by treating $T\beta RII^{+}$ cells (isolated from limb bud mesenchyme) with TGF-β. These findings classify MCP-5 as a key downstream mediator of TβRII signaling in its critical role as a joint-forming initiator. We postulate that interzone cells are programmed by the TβRII signaling to commit to joint forming cells by repressing MCP-5/CCR2 signaling while halting their differentiative program to Collagen 2 expressing chondrocytes.

MCP-5 its human homolog MCP-1 and its receptor CCR-2 have been largely studied in the arthritic process. The current hypothesis is that those MCPs are produced and act primarily through inflammatory cells (mostly monocytes) that are recruited within the arthritic joints (Haringman and Tak, 2004; Tak, 2006). Although there are substantial pharmacological data supporting a role for CCR2 antagonists in improving the course of experimental arthritis, reports with CCR2 knock-out and anti-CCR2 blocking antibody (MC-21) have provided contrasting results. In fact CCR2 knock-out mice are more susceptible to develop arthritis and in experimental arthritis MC-21 treatment improved the disease when given at an early stage while worsened when given later (Bruhl et al., 2004). Clinical studies using different interventions to block the MCP5/CCR-2 axis in patients with RA have showed disappointing results (Quinones et al., 2005). CCR2 is expressed in chondrocytes but its

function is unknown (Borzi et al., 2000; Pulsatelli et al., 1999). Our data provide evidence for a role of the MCP-5/CCR-2 system that targets directly the joint cells. Future studies will be important to determine whether the TβRII signaling regulates MCP-5 expression to maintain articular cartilage integrity in adulthood. In this respect, the OA/RA process could be seen as the failure of an active cell joint population (TβRII cells) that is preserved throughout life to maintain a controlled cytokine environment rather than the result of passive joint cell degeneration due to the influx of inflammatory cells. The lack of CCR2 in the knock-out mice and the timing of the blockage by the MC-21 antibody in the OA studies may have perturbed the TβRII/MCP-5 equilibrium either in the wrong cells or at the wrong time. Consistent with this hypothesis is our observation that TβRII expressing cells localize in specific niches (T. Li, 2011).

TβRII/MCP-5 in endochondral cartilage development

It has been hypothesized that the developing joint acts as signaling center to control the adjacent endochondral template development (Archer et al., 2003). Our *ex-vivo* studies showed that release of MCP-5 within the interzone leads to an impairment of *Ihh* spatial organization and to a decrease of *Collagen10* expression. Notably, this phenotype coincided with the one observed in the $Tgfbr2^{Prx1KO}$ mutants suggesting a role for MCP-5 in the failure of the mutant's chondrocyte progression to hypertrophy. This hypothesis was corroborated by our *ex-vivo* and *in-vivo* experiments: blockade of MCP-5 signaling in $Tgfbr2^{Prx1KO}$ mice rescued *Ihh*, *Collagen10* and *Collagen1* expression patterns. Taken together, these results clearly indicate the need for TβRII signaling in maintaining low levels of MCP-5 in the interzone and therefore to signal, through a still undefined factor, the adjacent growth plate template maturation. Future studies are needed to identify such factor(s); potential candidates could be members of WNT family. The interzone expresses several members of the Wnt/β-catenin system that seem to be involved in joint formation (Hartmann et al., 2000; Hartmann and Tabin, 2001; Luyten et al., 2009; Spater et al., 2006). In addition, Wnt9a and β-catenin regulate *Ihh* expression (Spater et al., 2006). Since in isolated interzone cells we found an inhibitory effect of MCP-5 on *Wnt9a* expression and demonstrated a TβRII-dependent role for MCP-5 in regulating *Ihh* spatial organization *in-vivo*, we speculate *Wnt9a* as the missing ring linking MCP-5 and *Ihh* expressions and foresee future studies in this direction.

An outstanding question is what role, if any, does MCP-5 expressed by chondrocytes within the growth plate play in the endochondral mineralization process? Our data contribute to answer this question. We found that in normal limbs, the inhibition of MCP-5 activity within the growth plate resulted in an increase of *Collagen10* by hypertrophic chondrocytes and, at later stages, *Collagen1* in the ossification center. These findings indicate that, in addition to its indirect action from the joints, MCP-5 within the growth plate affects the growth plate maturation, inhibiting hypertrophy. Since the lack of TβRII signaling in the $Tgfbr2^{Prx1KO}$ mutants does not seem to affect MCP-5 expression in the growth plate, it is likely that this effect of MCP-5 is independent of TβRII signaling. Interestingly, blockade of MCP-5 activity in $Tgfbr2^{Prx1KO}$ mutants, did not lead to an increase in mineralization. One explanation could be that in normal mice, differently from $Tgfbr2^{Prx1KO}$ mutants, blockade of the MCP-5-dependent inhibitory action on chondrocyte maturation could be additive with the MCP-TGF-β-dependent mechanism(s) promoting hypertrophy and ossification. As expected, in control mice no joint derangement was found as consequence of MCP-5 inhibition, being MCP-5 already undetectable in normal joints and indicating that MCP-5 from the growth plate has not effect on joint development.

A model for the TβRII/MCP-5 axis in joint and growth plate development

In Figure 7, we propose a dual role for MCP-5: it regulates cell commitment to either joint forming cells or chondrocytes within the joints and blocks the progression to chondrocyte hypertrophy and mineralization within the growth plate. These MCP-5 actions are TβRII-dependent within the joints while TβRII-independent in the growth plate (Figure 7A). Specifically, TβRII signaling, by blocking MCP-5 expression in the interzone, halts *Collagen2* expression, induces interzone specific markers (i.e. *Gdf5*) and signals to the growth plate in favor of chondrocyte hypertrophy by proper *Ihh* expression. On the other end, MCP-5 within the growth plate through a TβRII-independent mechanism inhibits chondrocyte hypertrophy. The balance between these two opposite signals, by tightly regulating the number of chondrocytes proceeding toward terminal differentiation, is necessary for a proper endochondral ossification process.

The model in Figure 7B shows the TβRII contribution to the MCP-5 actions: in *Tgfbr2*^{Prx1KO} mutants, lack of the TβRII signaling leads to disregulated levels of interzone MCP-5 that, in turn, blocks expression of interzone-specific markers (i.e. *Gdf5*), while increasing *Collagen2* expressing cells, with consequent accumulation of chondrocytes within the joint region. In addition, MCP-5 up-regulation in interzone is accountable for the impairement in *Ihh* spatial organization that alters the number of chondrocytes undergoing hypertrophy and does not counteract the MCP-5-inhibitory effect coming from within the growth plate. As consequence, there is an accumulation of disorganized pre-hypertrophic chondrocytes throughout the growth plate. Blockade of MCP-5 in *Tgfbr2*^{Prx1KO} mutants is sufficient to rescue both their joint phenotype and chondrocyte maturation, indicating in MCP-5 a key regulator for the TGF-β action.

EXPERIMENTAL PROCEDURES

Mouse strains

Tgfbr2^{Prx1KO} mutants were generated as previously reported by crossing *Tgfbr2*$^{flox/flox}$ homozygous females, defined as Normal Control (NC) in the manuscript, with males *Prx-1-Cre* (provided by C. Tabin, Harvard University) (Spagnoli et al., 2007). In the Prx-1-Cre mouse, the Prx-1 limb enhancer drives Cre recombinase expression in limb buds beginning at E9.5 (Logan et al., 2002). Experiments were performed in mice that were C57BL/6 strain for at least 10 generations. *Tgfbr2-GFP-β-GEO-BAC* mice were generated as previously reported (Spagnoli et al., 2007).

For all timed pregnancies, noon of the day when evidence of a vaginal plug was found was considered E0.5. All animal procedures were approved by the animal care committees of Vanderbilt University and University of North Carolina at Chapel Hill.

LCM for RNA sampling and Microarray analysis

We obtained E14.5 autopods from two NC and two *Tgfbr2*^{Prx1KO} littermate embryos. Samples were frozen in OTC and cryosectioned (6μm), then processed with ethanol and xylene, and subjected to LCM. To minimize RNA degradation, the process from sectioning to shipment was performed in less than 20 minutes. We sequentially laser-captured cells in the interzone and the adjacent growth plates from NC and *Tgfbr2*^{Prx1KO} mutants (n=2). In the *Tgfbr2*^{Prx1KO} sections the presumptive interzone was identified as a bending between two growth plates (Figure 1A arrow). Captured specimens were frozen in SuperAmp Lysis Buffer (Miltenyi Biotec) and shipped to Miltenyi Biotec. mRNA was extracted and transcribed into cDNA according to Miltenyi Biotec's undisclosed procedure (SuperAmp Miltenyi Biotec). The average length of cDNA products, measured and analyzed via the Agilent 2100 Bioanalyzer platform and software (Agilent Technologies), ranged from 200–

1000 bp, indicating the integrity of the cDNAs. The purified PCR products (250 ng) were labeled with Cy3-dCTP and hybridized to an Agilent Whole Mouse Genome Oligo Microarrays 4×44K for 17 hours at 65° according to Miltenyi Biotec's undisclosed protocol. Washing, staining and scanning of the microarrays were performed using the Agilent Technologies equipment. Signal intensities from the single-experiment data were normalized by dividing the intensity values by their median. NCBI GEO Accession number GSE37287, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37287. Normalized data were expressed as fold changes of a candidate gene in the interphalangeal interzone sample compared to that obtained in chondrocytes using the Resolver (Rosetta Biosoftware). Pathway analysis was performed using the PANTHER Classification System (Celera) (Thomas et al., 2003). Lists of putative candidate genes with a fold change ± 2 and p-value 0.01 were compared to a reference list (*Mus Musculus*, NCBI) using a binomial test. Microarray data will be accessible through the Gene Expression Omnibus (GEO) with the final version of the manuscript.

Preparation of HGC-MCP-5/MC21 nanoparticles, implantation and *ex-vivo* limb cultures

HGC nanoparticles were prepared as previously reported (Li et al., 2010). Briefly, 25μg of either recombinant mouse MCP-5 (R&D Systems) or MC-21 antibody (anti-CCR2) (donated by M. Mack, Regensburg University) (Mack et al., 2001) were encapsulated into 250μg of HGC nanoparticles. Methylene Blue was added to the mixture to mark the injection site. HGC-protein implantation was performed using a microinjection unit (Picosprizer II) that allowed for precise localization of the injection and volume accuracy (0.0015μl/shot) as previously described (Li et al., 2010). HCG-protein implants (joint site) were performed in WT, NC or *Tgfbr2*^{Prx1KO} by delivering either HGC-MC-21 (1.5 ng, 5-shots, 0.3ng/shot) or increasing doses of HGC-MCP-5 (0.9ng, 1.5ng, 3ng and 6 ng). Limbs were cultured in BGJb medium for 48 hours. HGC-protein implantation was performed within the medial interphalangeal joint of 2nd, 3rd and 4th digit and the 5th uninjected digit of the same limb was used as controls. The forming or presumptive interphalangeal joint was identified as the bending between two adjacent growth plates.

RS-504393 *in-vivo* treatment

Pregnant NC females potentially generating *Tgfbr2*^{Prx1KO} mice, were treated with 4mg/Kg/day of RS-504393 (TOCRIS Bioscience), orally (in drinking water), starting at 11.5 days of gestation, for a total of either 3 (E14.5) or 7 days (E18.5). After euthanasia of the mother, limbs from either E14.5 or E18.5 embryos were dissected and prepared for histological studies. Genotyping was used to confirm the mutant phenotype, RS-504393-treated NC littermates and NC untreated mice were used as controls.

Isolation and culture of Jag^{+}/TβRII^{+}/PDGFR^{+} cells and TβRII^{+} cells

To obtain mesenchyme limb bud cells, the distal tips of E14.5 autopods from either WT or *Tgfbr2-GFP-β-GEO-BAC* embryos were dissected and digested with dispase (1U/ml), as previously reported (Spagnoli et al 2007). To obtain Jag^{+}/TβRII^{+}/PDGFR^{+} cells, WT mesenchyme limb bud cells are subjected to immunoselection using biotinylated TβRII, biotinylated PDGFRB (BAF532 and BAF1042, R&D Systems) and Jagged 1 (sc-6011, Santa Cruz Biothechnolgy) antibodies. Biotinylation of Jagged1 antibody was performed using Lightning-Link Biotin Conjugation Kit (Innova Biosciences) following manufacturer's protocol. Immunoselection was performed in a MACS magnetic system (Miltenyi) as previously described (Longobardi et al 2005). Isolation of TβRII^{+} cells was performed by FACS sorting (MoFlo, Beckman) mesenchyme limb bud cells from *Tgfbr2-GFP-β-GEO-BAC* that contain GFP as gene reporters for *Tgfbr2* (Spagnoli et al., 2007). Micromass cultures were performed as previously reported (Spagnoli et al., 2007). Jag^{+}/

TβRII$^+$/PDGFR$^+$ or TβRII$^+$ cells were treated with ±20ng/ml MCP-5 (for 3 days) or with ±20ng/ml TGF-β (for 18hours), respectively.

Histology, IHC and ISH

Dissected limbs from E14.5 to E18.5 embryos were fixed overnight in 4%PFA at 4°C, paraffin embedded and sectioned at 5μm thickness. Hematoxylin staining was performed following standard protocols.

For IHC, the Vectastain Elite ABC Kit (DakoCytomation) was used. The following primary antibodies were used: anti-MCP-5 (G12) polyclonal (sc-33226, Santa Cruz) and anti-CCR-2 monoclonal (ab32144, Abcam). Histological sections were counterstained with Hematoxylin.

ISH studies were performed as previously reported using DIG-labeled probes (Spagnoli et al., 2007). The MCP-5 plasmid used to generate the MCP-5 probe was provided by Dr Karin E. Peterson (Louisiana State University). After hybridization, probes were detected by incubation with anti-DIG-AP antibody (Roche, Mannheim, Germany, dilution 1:5000).

Images were formatted without using any imaging enhancement.

Quantitative real-time PCR

mRNA from experiments using Jag$^+$/TβRII$^+$/PDGFR$^+$ cells and TβRII$^+$ cells was extracted and reversed transcribed into cDNA by using μMACS mRNA Isolation and cDNA Synthesis kit (Miltenyi Biotec). cDNAs (including from LCM) were analyzed by *q*RT-PCR (MyIQ) using SYBR Green followed by Gene Expression Analysis Software (Bio-Rad). Expression of genes of interest were normalized using either GAPDH or β-actin expressions.

Statistics

Data are presented as mean ±SD. Statistical differences between groups were assessed by one way ANOVA followed by Bonferroni's Multiple Comparison test for all pairwise multiple comparisons, or unpaired Student's *t*-test (GraphPad Prism Software, San Diego, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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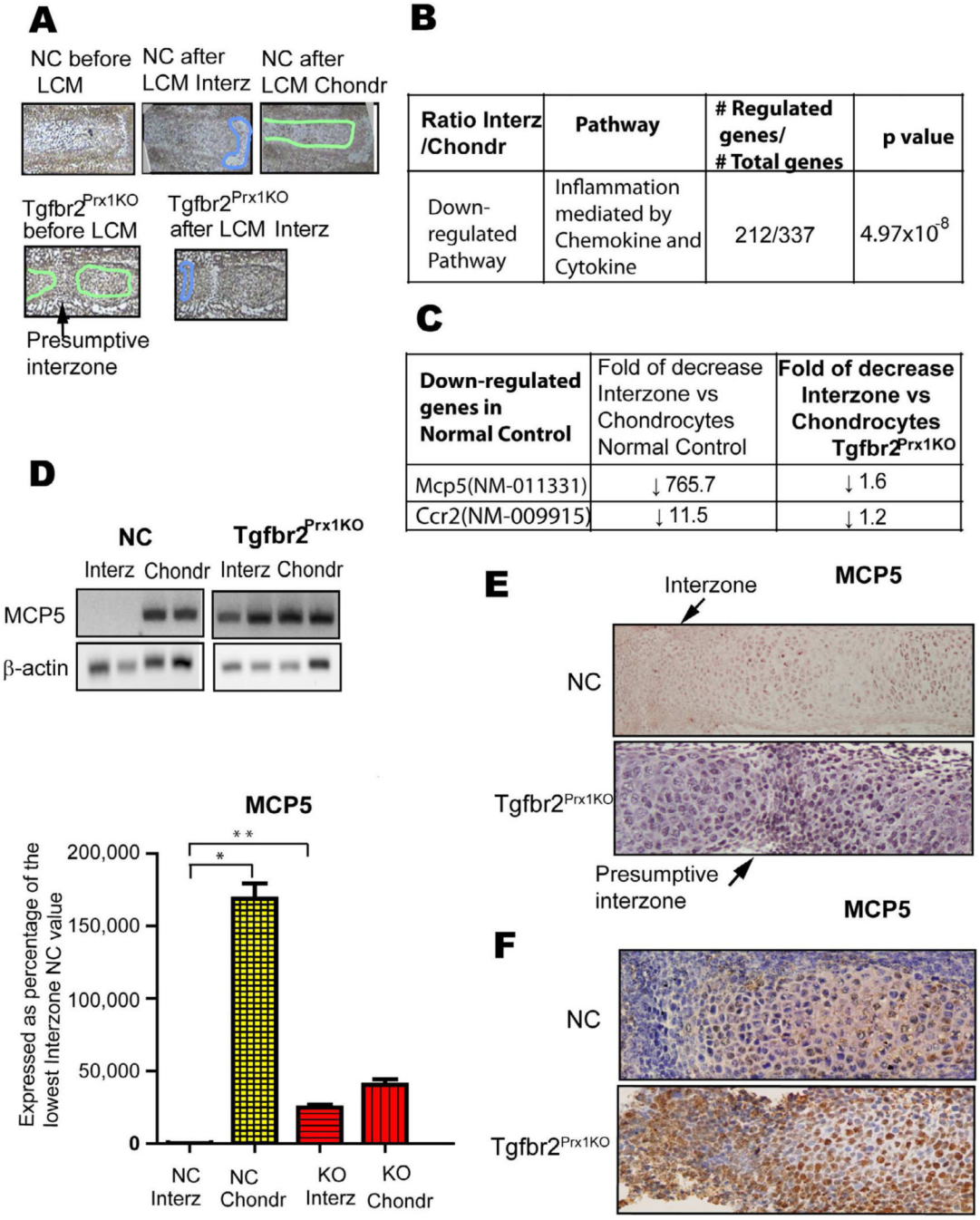
HIGHLIGHTS

1. MCP-5 is down-regulated in joint interzone cells compared to adjacent chondrocytes
2. MCP-5 low expression in interzone is needed for joint and growth plate development
3. TGF-β signaling in limbs is needed to establish a low MCP-5 interzone expression
4. MCP-5 expression in chondrocytes contributes to growth plate development



Ratio Interz /Chondr	Pathway	# Regulated genes/ # Total genes	p value
Down-regulated Pathway	Inflammation mediated by Chemokine and Cytokine	212/337	4.97×10^{-8}

Down-regulated genes in Normal Control	Fold of decrease Interzone vs Chondrocytes Normal Control	Fold of decrease Interzone vs Chondrocytes $Tgfbr2^{Prx1KO}$
Mcp5(NM-011331)	↓765.7	↓1.6
Ccr2(NM-009915)	↓11.5	↓1.2

Figure 1. MCP-5 expression is down-regulated in the interzone of E14.5 NC embryos compared to growth plate chondrocytes; this expression pattern is impaired in *Tgfbr2*^{Prx1KO}

(A) Normal control (NC) and *Tgfbr2*^{Prx1KO} sections before and after LCM of interzone cells (interz) and adjacent growth plate chondrocytes (chondr). (B–C) Microarray and Panther analyses of the cDNAs from LCM showing: (B) the differential regulation of the "Inflammation mediated by chemokines and cytokines" signaling pathway in NC interzone cells related to chondrocytes; (C) differential regulation of *Mcp-5* and its receptor *Ccr2* in the interzone related to chondrocytes of both NC and *Tgfbr2*^{Prx1KO}. (D) qRT-PCR analyses of LCM samples showing expression of *Mcp-5* in the NC interzone, as well as the *Tgfbr2*^{Prx1KO} presumptive interzone, compared to growth plate chondrocytes; experiments

were repeated three times, gel lanes represent two representative experiments; data are represented as mean ±SD, analyzed using one-way ANOVA ($p<0.0001$) followed by Bonferroni's multiple comparison test (*$p<0.0001$; **$p<0.05$). (E–F) Limb buds were dissected from E14.5 NC and $Tgfbr2^{Prx1KO}$ embryos, embedded in paraffin and subjected to (E) ISH and (F) IHC analyses using respectively MCP-5 probe and anti-MCP-5 antibody (proximal side: right). Sections were obtained from at least 4 mutant and control embryos; at least 6 sections for MCP-5 antibody and 8 sections for MCP-5 probe were analyzed. MCP-5 expression pattern was consistent in all the specimens analyzed. The expression pattern of MCP-5 at different joint developmental time points (E12.5, E13.5, E14.5, E16.5 and E18.5) is shown in Figure S1.

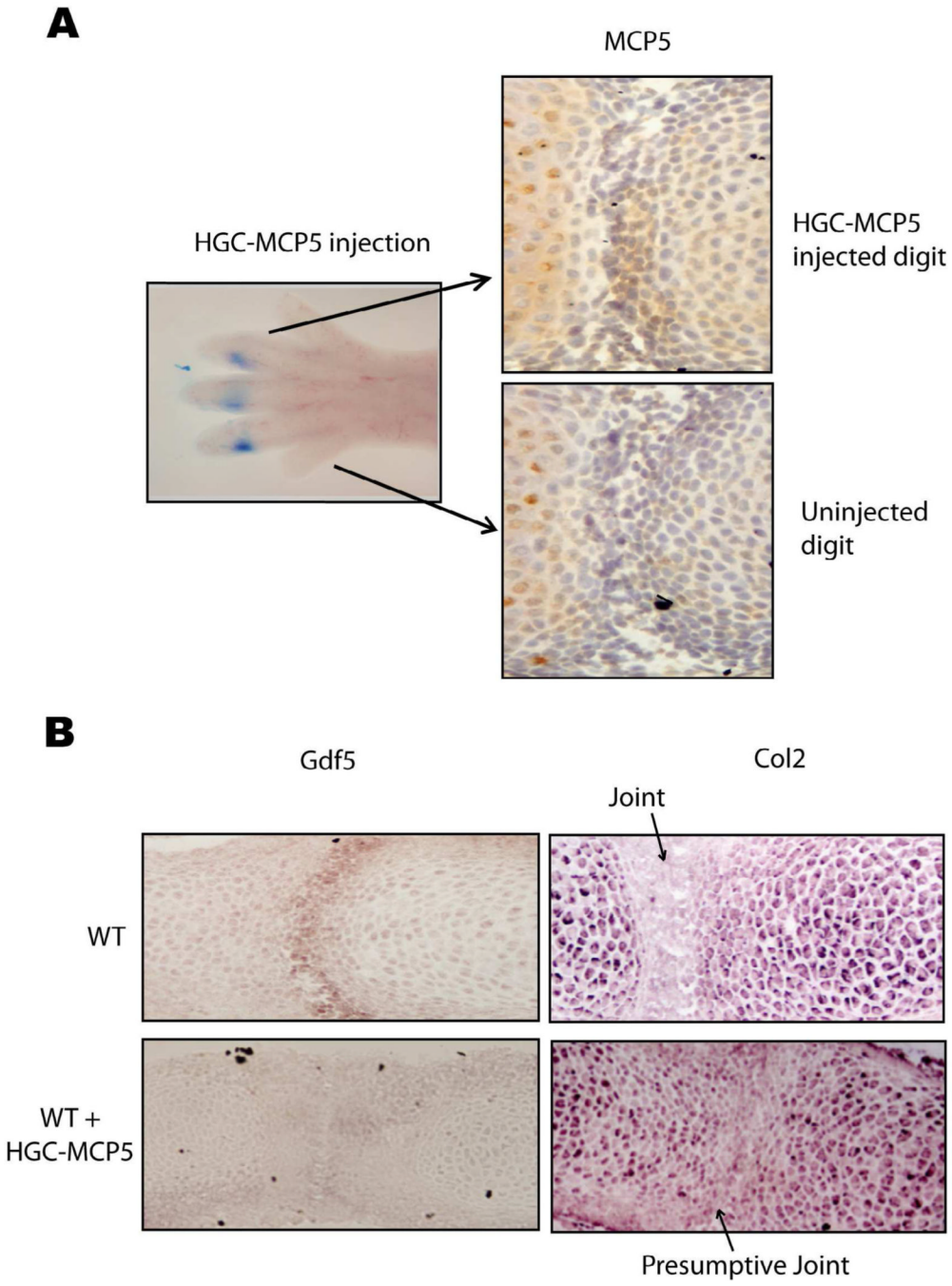


Figure 2. Aberrant expression of *Gdf5* and *Collagen2* in E14.5 WT embryos after HGC-MCP-5 limb implantation and *ex-vivo* culture

(A–B) Recombinant MCP-5 was loaded in HGC-nanoparticle and the HGC-MCP-5 complex (MCP-5, 1.5 ng) was implanted in the 2nd, 3rd and 4th digit (highlighted by Methylene Blue) of dissected E14.5 WT. Injected and uninjected (5th digit) limbs were cultured for 48h, embedded in paraffin and subjected to (A) IHC analyses using MCP-5 antibody and (B) ISH studies using *Gdf5* and Collagen2 (*Col2*) probes (proximal side: right). Eight out of nine WT embryos implanted with HGC-MCP-5 showed an altered phenotype, as described in the Results; analyses were performed in at least 3 sections for each embryo and for each probe.

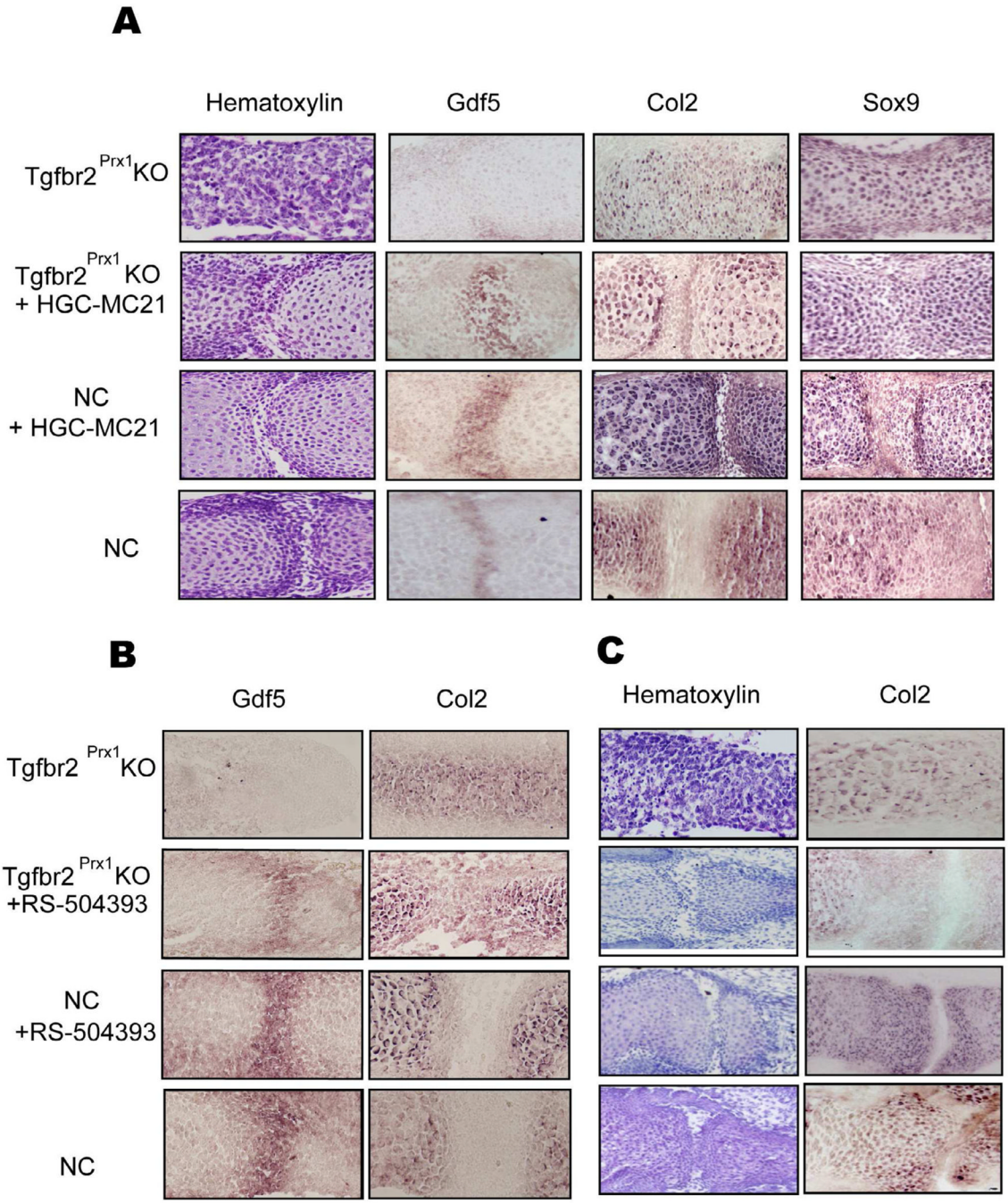


Figure 3. Rescue of *Tgfbr2*^{Prx1KO} joint phenotype by *ex-vivo* HGC-MC-21 limb implantation and RS-504393 *in-vivo* treatment

(A) MC-21 antibody was loaded into HGC-nanoparticle and the resulting HGC-MC-21 complex (MC-21, 1.5 ng) was implanted in the interzone of dissected E14.5 NC and *Tgfbr2*^{Prx1KO} digits. Limbs were cultured for 48h, embedded in paraffin and subjected either to hematoxylin staining or ISH analyses using *Gdf-5*, *Col2* and *Sox9* probes. (B–C) RS-504393 treatments were started at E11.5 on pregnant females potentially carrying *Tgfbr2*^{Prx1KO} embryos and continued until either (B) E14.5 (3 days treatment) or (C) E18.5 (7 days of treatment); embryos were then harvested and genotyped. NC and *Tgfbr2*^{Prx1KO} limbs were dissected, embedded in paraffin and subjected either to hematoxylin staining or

ISH analyses using *Gdf-5* and *Col2* probes (proximal side: left). All the 4 $Tgfbr2^{Prx1KO}$ embryos implanted with HGC-MC-21 showed the phenotype described in the Results; for the RS-504393 treatments, all the 4 $Tgfbr2^{Prx1KO}$ embryos treated for 3 days and all the 9 $Tgfbr2^{Prx1KO}$ embryos treated for 7 days showed the phenotype described in the Results; analyses were performed in at least 3 sections for each embryo and for each probe. The evaluation of CCR2 expression by IHC at different joint developmental time points (E12.5; E13.5, E14.5 and E18.5) is shown in Figure S2.

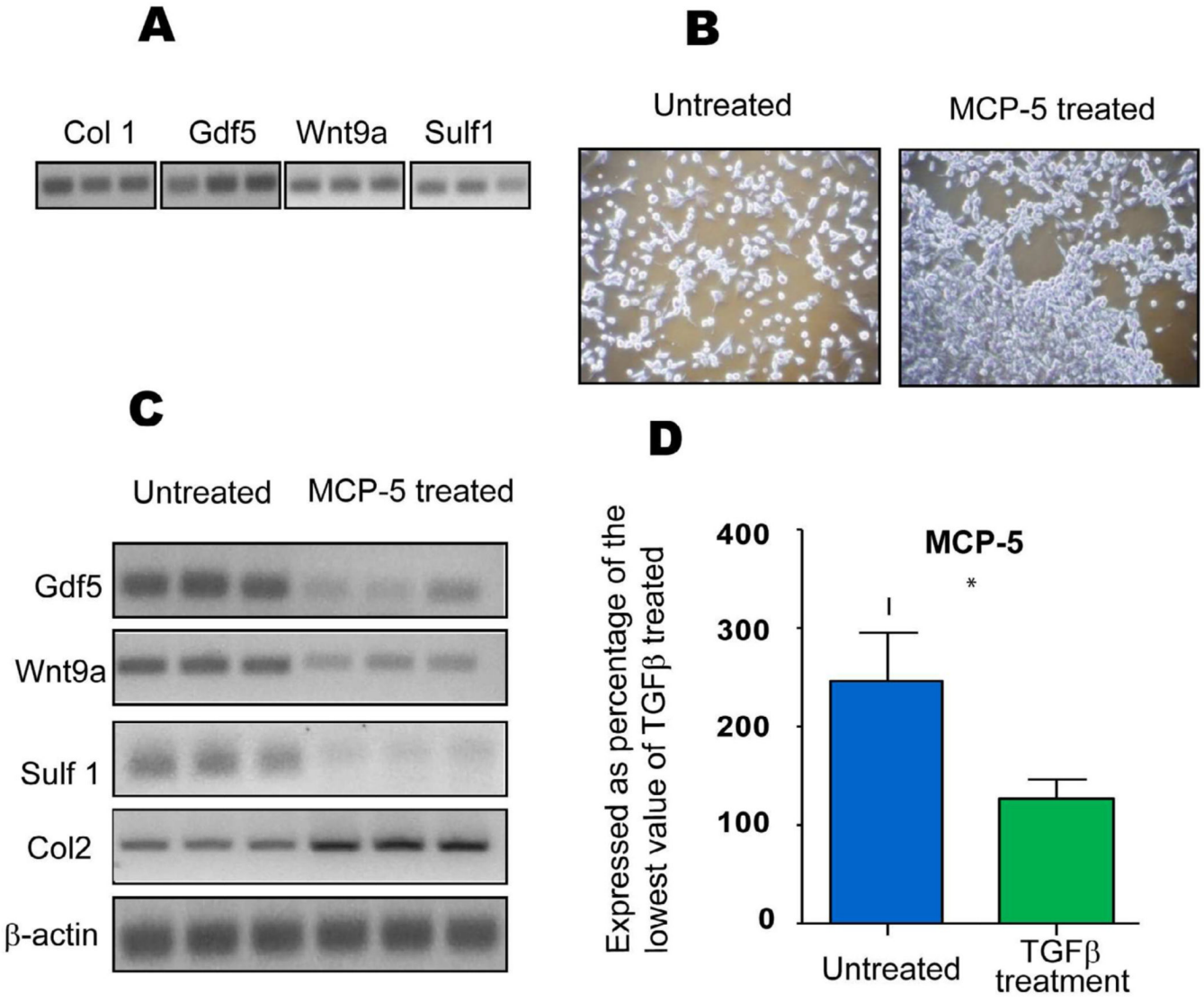


Figure 4. MCP-5 effect on joint interzone cells and relevance of TβRII signaling in regulating its expression *in vitro*

(A-B-C) Jag$^+$/TβRII$^+$/PDGFR$^+$ cells were isolated from E14.5 WT autopods. (A) mRNA was collected after cell separation and subjected to RT-PCR for *Collagen1 (Col1)*, *Gdf5*, *Wnt9a* and *Sulf1*. (B–C) Micromass cultures of isolated Jag$^+$/TβRII$^+$/PDGFR$^+$ were treated ±MCP-5 (20ng/ml) for 3 days and analyzed for morphology (B) and gene expression by RT-PCR for *Gdf5*, *Wnt9a*, *Sulf1* and *Col2*. (D) TβRII$^+$ cells were FACS sorted from limb buds cells isolated from *Tgfbr2-GFP-β-GEO-BAC* digits using GFP as selection marker for TβRII expression. Micromass cultures of TβRII$^+$ cells were treated ±TGF-β (20ng/ml) for 18h and analyzed for MCP-5 expression by *q*RT-PCR (n=3; mean ±SD; *p=0.0425 by one tailed Student's *t*-test). For RT-PCR experiments, gel lanes correspond to different experiments that were repeated at least three times with similar results. The localization of TβRII expressing cells within the interphalangeal joints is visualized in Figure S3.

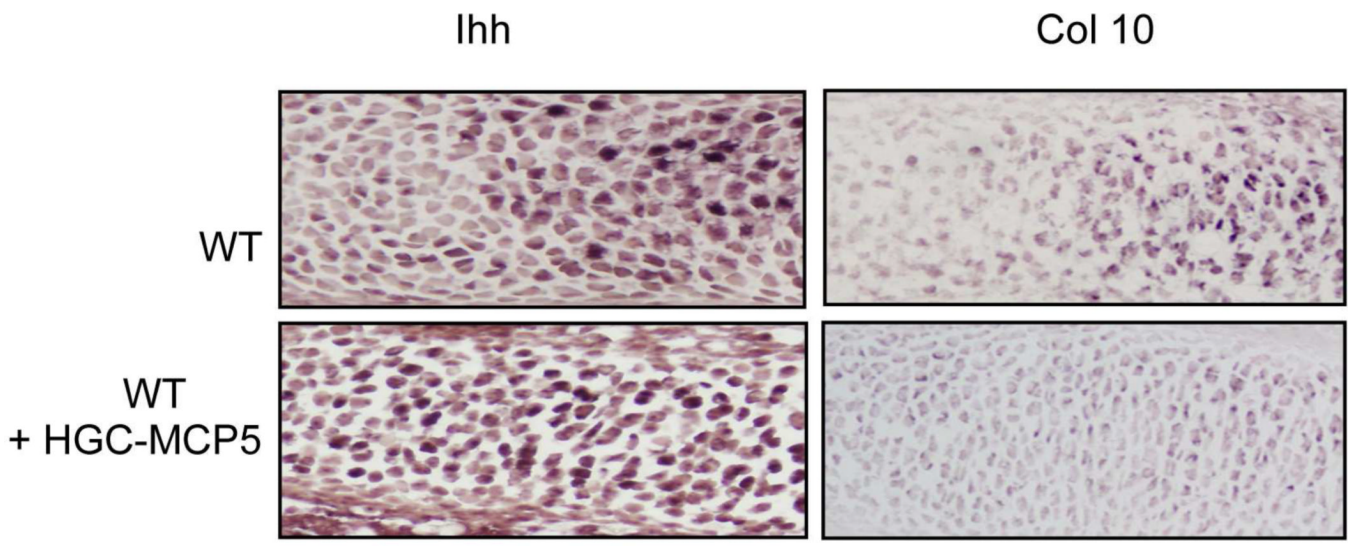


Figure 5. Aberrant expression of *Ihh* and *Collagen10* in E14.5 WT embryos after *ex-vivo* HGC-MCP-5 limb implantation

Recombinant MCP-5 was loaded in HGC-nanoparticle and the resulting HGC-MCP-5 complex (MCP-5, 1.5 ng) was implanted in the interzone of dissected E14.5 WT digits. Limbs were cultured for 48h, embedded in paraffin and subjected to ISH analyses using *Ihh* and *Collagen10* (*Col 10*) probes (proximal side: left). Eight out of nine WT embryos implanted with HGC-MCP-5 showed the altered phenotype; analyses were performed in at least 3 sections for each embryo and for each probe. Figure S4 shows that in limb explants, growth plate chondrocytes are capable to progress to hypertrophy after 2-day *ex-vivo* culture.

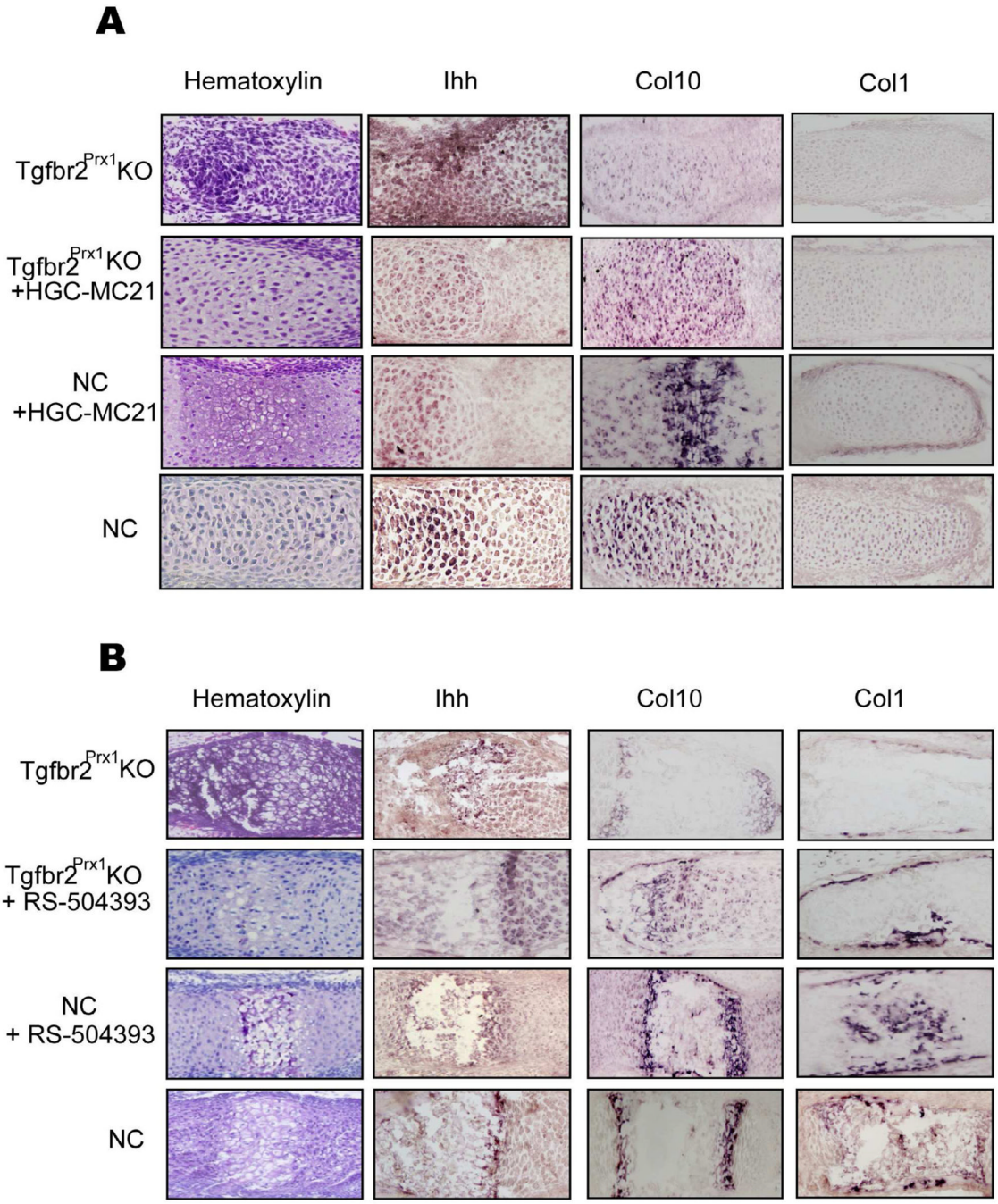


Figure 6. Blockade of CCR-2 signaling, both *ex-vivo* and *in vivo*, rescues the *Tgfbr2*^{Prx1KO} growth plate phenotype and advances ossification in NC growth plates

(A) MC-21 antibody was loaded in HGC-nanoparticle and the HGC-MC-21 complex (MC-21, 1.5 ng) was implanted in the interzone of dissected E14.5 NC and *Tgfbr2*^{Prx1KO} digits. Limbs were cultured for 48h, embedded in paraffin and subjected to hematoxylin staining and ISH analyses using *Ihh*, *Col10* and *Collagen1 (Col1)* probes. (B) RS-504393 treatments were started at E11.5 on pregnant females potentially carrying *Tgfbr2*^{Prx1KO} embryos until E18.5 (7 days of treatment), when embryos were harvested and genotyped. NC and *Tgfbr2*^{Prx1KO} limbs were dissected, embedded in paraffin and subjected either to hematoxylin staining or ISH analyses using *Ihh*, *Col10* and *Col1* probes (proximal side,

left). All of the 4 *Tgfbr2*^{Prx1KO} embryos implanted with HGC-MC-21 and all the 9 *Tgfbr2*^{Prx1KO} embryos treated with RS-504393 showed the phenotype described in the Results; analyses were performed in at least 3 sections for each embryo and for each probe. The evaluation of CCR2 expression by IHC at different joint developmental time points (E12.5; E13.5, E14.5 and E18.5) is shown in Figure S2.

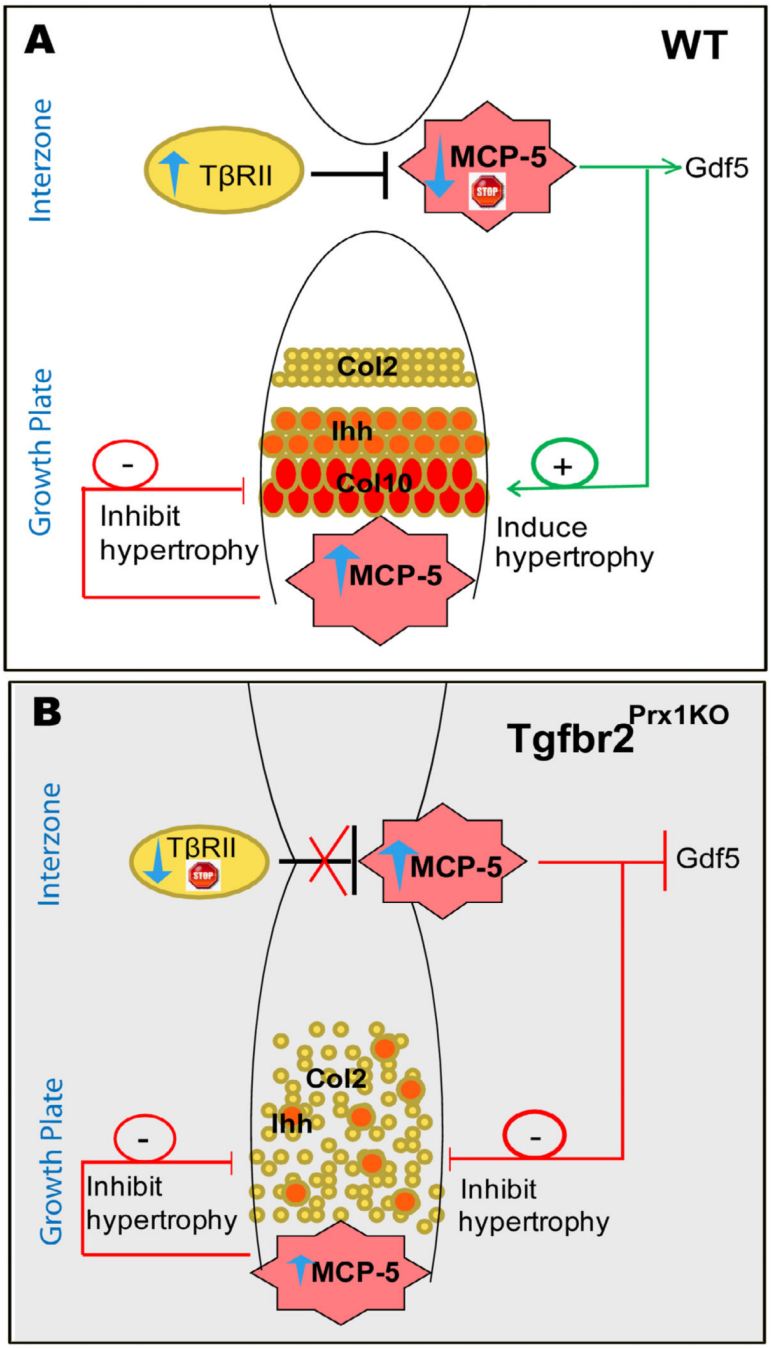


Figure 7. A model for the TβRII^{+}/MCP-5 axis during joint and growth plate development

(A, Wild-type, WT, mice) TβRII down-regulates MCP-5 expression in the interzone, allowing expression of key joint markers (such as *Gdf5*) while inhibiting *Collagen2* expression. In the growth plate, the down-regulation of interzone MCP-5 (TβRII-dependent) signals the correct *Ihh* expression pattern, promoting chondrocyte maturation, while MCP-5 within the growth plate (TβRII-independent) inhibits chondrocyte terminal differentiation. The balance between these two opposite signals is necessary for a proper endochondral ossification process. (B, *Tgfbr2*^{Prx1KO} mutants) Lack of the TβRII signaling leads to up-regulation of interzone MCP-5 with consequent lack of joint formation. Furthermore, MCP-5 abnormal up-regulation in the interzone does not counteract the MCP-5 inhibitory

coming from within the growth plate and enhances *Ihh* altered distribution with consequent impairment in chondrocyte hypertrophy and ossification.